IARC MONOGRAPHS

PHARMACEUTICALS

VOLUME 100 A
A REVIEW OF HUMAN CARCINOGENS

IARC MONOGRAPHS
ON THE EVALUATION
OF CARCINOGENIC RISKS
TO HUMANS

International Agency for Research on Cancer
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A REVIEW OF HUMAN CARCINOGENS

This publication represents the views and expert opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, which met in Lyon, 14-21 October 2008

LYON, FRANCE - 2012

IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

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In 1969, the International Agency for Research on Cancer (IARC) initiated a programme on the evaluation of the carcinogenic risk of chemicals to humans involving the production of critically evaluated monographs on individual chemicals. The programme was subsequently expanded to include evaluations of carcinogenic risks associated with exposures to complex mixtures, lifestyle factors and biological and physical agents, as well as those in specific occupations. The objective of the programme is to elaborate and publish in the form of monographs critical reviews of data on carcinogenicity for agents to which humans are known to be exposed and on specific exposure situations; to evaluate these data in terms of human risk with the help of international working groups of experts in chemical carcinogenesis and related fields; and to indicate where additional research efforts are needed. The lists of IARC evaluations are regularly updated and are available on the Internet at http://monographs.iarc.fr/.

This programme has been supported since 1982 by Cooperative Agreement U01 CA33193 with the United States National Cancer Institute, Department of Health and Human Services. Additional support has been provided since 1986 by the Health, Safety and Hygiene at Work Unit of the European Commission Directorate-General for Employment, Social Affairs and Equal Opportunities, and since 1992 by the United States National Institute of Environmental Health Sciences, Department of Health and Human Services. The contents of this volume are solely the responsibility of the Working Group and do not necessarily represent the official views of the U.S. National Cancer Institute, the U.S. National Institute of Environmental Health Sciences, the U.S. Department of Health and Human Services, or the European Commission Directorate-General for Employment, Social Affairs and Equal Opportunities.

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Lorenzo Tomatis (1929-2007)
Founder of the *IARC Monographs* Programme

Lorenzo Tomatis, MD, with other colleagues knowledgeable in primary prevention and environmental carcinogenesis, perceived in the 1960s the growing need to objectively evaluate carcinogenic risks by international groups of experts in chemical carcinogenesis. His vision and determination to provide a reliable source of knowledge and information on environmental and occupational causes of cancer led to his creating the *IARC Monographs* Programme for evaluating cancer risks to humans from exposures to chemicals. The first meeting, held in Geneva in December 1971, resulted in Volume 1 of the IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man [1972], a series known affectionately since as the "orange books". As a champion of chemical carcinogenesis bioassays, Tomatis defined and promoted the applicability and utility of experimental animal findings for identifying carcinogens and for preventing cancers in humans, especially in workers and children, and to eliminate inequalities in judging cancer risks between industrialized and developing countries. Tomatis' foresight, guidance, leadership, and staunch belief in primary prevention continued to influence the *IARC Monographs* as they expanded to encompass personal habits, as well as physical and biological agents. Lorenzo Tomatis had a distinguished career at the Agency, arriving in 1967 and heading the Unit of Chemical Carcinogenesis, before being Director from 1982 to 1993.

Volume 100 of the *IARC Monographs* Series is respectfully dedicated to him.

(photo: Roland Dray)

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NOTE TO THE READER

The term 'carcinogenic risk' in the *IARC Monographs* series is taken to mean that an agent is capable of causing cancer. The *Monographs* evaluate cancer hazards, despite the historical presence of the word 'risks' in the title.

Inclusion of an agent in the *Monographs* does not imply that it is a carcinogen, only that the published data have been examined. Equally, the fact that an agent has not yet been evaluated in a *Monograph* does not mean that it is not carcinogenic. Similarly, identification of cancer sites with *sufficient evidence* or *limited evidence* in humans should not be viewed as precluding the possibility that an agent may cause cancer at other sites.

The evaluations of carcinogenic risk are made by international working groups of independent scientists and are qualitative in nature. No recommendation is given for regulation or legislation.

Anyone who is aware of published data that may alter the evaluation of the carcinogenic risk of an agent to humans is encouraged to make this information available to the Section of IARC Monographs, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France, in order that the agent may be considered for re-evaluation by a future Working Group.

Although every effort is made to prepare the *Monographs* as accurately as possible, mistakes may occur. Readers are requested to communicate any errors to the Section of IARC Monographs, so that corrections can be reported in future volumes.

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[2] Dr Eastmond is receiving research funding from Pfizer (which markets contraceptives that are alternatives to those based on estrogen-progestogen combinations). Until 2006 he received research support from Johnson & Johnson (which markets estrogen-progestogen contraceptives through its Ortho subsidiary).

[3] Dr Jameson in retired from and continues to consult for NIEHS/NTP.

[4] Over the past five years Dr Kaldor has overseen a project funded by a grant from F. Hoffmann-La Roche to his University that led to the establishment of a clinical facility in Cambodia. None of his professional compensation is derived from F. Hoffmann-La Roche. That company also provided a drug for a study funded by the U.S. National Institutes of Health for which Dr Kaldor is the principal investigator. Dr Kaldor also will receive research support from Johnson & Johnson. In addition, Dr Kaldor serves as deputy director for his university's National Centre in HIV Epidemiology and Clinical Research, which receives approximately 10% of its funding from companies that make drugs under consideration at this meeting or that are alternatives to drugs under consideration. Such companies include F. Hoffmann-La Roche, Bristol-Myers Squibb, Pfizer, and Johnson & Johnson.

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[6] Dr Phillips has been approached to consult for Movetis NV (which develops drugs for gastro-intestinal disorders under license from two subsidiaries of Johnson & Johnson, which manufactures estrogen-progestogen contraceptives).

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[7] Dr Titus-Ernstoff received compensation (not exceeding $1000 in total) from the Weinberg Group (a product-defence consulting firm) for writing a review of DES-related health outcomes during 2006-2007. She also contributes (without compensation) to occasional articles in DES-advocacy newsletters.

[8] Dr Tsuda is a non-industry member of the Board of Trustees of the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute, whose member companies are from the chemical, agrochemical, petrochemical, pharmaceutical, biotechnology, and consumer products industries.

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PREAMBLE

The Preamble to the *IARC Monographs* describes the objective and scope of the programme, the scientific principles and procedures used in developing a *Monograph*, the types of evidence considered and the scientific criteria that guide the evaluations. The Preamble should be consulted when reading a *Monograph* or list of evaluations.

A. GENERAL PRINCIPLES AND PROCEDURES

1. Background

Soon after IARC was established in 1965, it received frequent requests for advice on the carcinogenic risk of chemicals, including requests for lists of known and suspected human carcinogens. It was clear that it would not be a simple task to summarize adequately the complexity of the information that was available, and IARC began to consider means of obtaining international expert opinion on this topic. In 1970, the IARC Advisory Committee on Environmental Carcinogenesis recommended '...that a compendium on carcinogenic chemicals be prepared by experts. The biological activity and evaluation of practical importance to public health should be referenced and documented.' The IARC Governing Council adopted a resolution concerning the role of IARC in providing government authorities with expert, independent, scientific opinion on environmental carcinogenesis. As one means to that end, the Governing Council recommended that IARC should prepare monographs on the evaluation of carcinogenic risk of chemicals to man, which became the initial title of the series.

In the succeeding years, the scope of the programme broadened as *Monographs* were developed for groups of related chemicals, complex mixtures, occupational exposures, physical and biological agents and lifestyle factors. In 1988, the phrase 'of chemicals' was dropped from the title, which assumed its present form, *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*.

Through the *Monographs* programme, IARC seeks to identify the causes of human cancer. This is the first step in cancer prevention, which is needed as much today as when IARC was established. The global burden of cancer is high and continues to increase: the annual number of new cases was estimated at 10.1 million in 2000 and is expected to reach 15 million by 2020 (Stewart & Kleihues, 2003). With current trends in demographics and exposure, the cancer burden has been shifting from high-resource countries to low- and medium-resource countries. As a result of *Monographs* evaluations, national health agencies have been able, on scientific grounds, to take measures to reduce human exposure to carcinogens in the workplace and in the environment.

The criteria established in 1971 to evaluate carcinogenic risks to humans were adopted by the Working Groups whose deliberations resulted in the first 16 volumes of the *Monographs* series. Those criteria were subsequently updated by further ad hoc Advisory Groups (IARC, 1977, 1978, 1979, 1982, 1983, 1987, 1988, 1991; Vainio *et al.*, 1992; IARC, 2005, 2006).

The Preamble is primarily a statement of scientific principles, rather than a specification of working procedures. The procedures through which a Working Group implements these principles are not specified in detail. They usually involve operations that have been established as being effective during previous *Monograph* meetings but remain, predominantly, the prerogative of each individual Working Group.

2. Objective and scope

The objective of the programme is to prepare, with the help of international Working Groups of experts, and to publish in the form of *Monographs*, critical reviews and evaluations of evidence on the carcinogenicity of a wide range of human exposures. The *Monographs* represent the first step in carcinogen risk assessment, which involves examination of all relevant information to assess the strength of the available evidence that an agent could alter the age-specific incidence of cancer in humans. The *Monographs* may also indicate where additional research efforts are needed, specifically when data immediately relevant to an evaluation are not available.

In this Preamble, the term 'agent' refers to any entity or circumstance that is subject to evaluation in a *Monograph*. As the scope of the programme has broadened, categories of agents now include specific chemicals, groups of related chemicals, complex mixtures, occupational or environmental exposures, cultural or behavioural practices, biological organisms and physical agents. This list of categories may expand as causation of, and susceptibility to, malignant disease become more fully understood.

A cancer 'hazard' is an agent that is capable of causing cancer under some circumstances, while a cancer 'risk' is an estimate of the carcinogenic effects expected from exposure to a cancer hazard. The *Monographs* are an exercise in evaluating cancer hazards, despite the historical presence of the word 'risks' in the title. The distinction between hazard and risk is important, and the *Monographs* identify cancer hazards even when risks are very low at current exposure levels, because new uses or unforeseen exposures could engender risks that are significantly higher.

In the *Monographs*, an agent is termed 'carcinogenic' if it is capable of increasing the incidence of malignant neoplasms, reducing their latency, or increasing their severity or multiplicity. The induction of benign neoplasms may in some circumstances (see Part B, Section 3a) contribute to the judgement that the agent is carcinogenic. The terms 'neoplasm' and 'tumour' are used interchangeably.

The Preamble continues the previous usage of the phrase 'strength of evidence' as a matter of historical continuity, although it should be understood that *Monographs* evaluations consider studies that support a finding of a cancer hazard as well as studies that do not.

Some epidemiological and experimental studies indicate that different agents may act at different stages in the carcinogenic process, and several different mechanisms may be involved. The aim of the *Monographs* has been, from their inception, to evaluate evidence of carcinogenicity at any stage in the carcinogenesis process, independently of the underlying mechanisms. Information on mechanisms may, however, be used in making the overall evaluation (IARC, 1991; Vainio *et al.*, 1992; IARC, 2005, 2006; see also Part B, Sections 4 and 6). As mechanisms of carcinogenesis are elucidated, IARC convenes international scientific conferences to determine whether a broad-based consensus has emerged

on how specific mechanistic data can be used in an evaluation of human carcinogenicity. The results of such conferences are reported in IARC Scientific Publications, which, as long as they still reflect the current state of scientific knowledge, may guide subsequent Working Groups.

Although the *Monographs* have emphasized hazard identification, important issues may also involve dose–response assessment. In many cases, the same epidemiological and experimental studies used to evaluate a cancer hazard can also be used to estimate a dose–response relationship. A *Monograph* may undertake to estimate dose–response relationships within the range of the available epidemiological data, or it may compare the dose–response information from experimental and epidemiological studies. In some cases, a subsequent publication may be prepared by a separate Working Group with expertise in quantitative dose–response assessment.

The *Monographs* are used by national and international authorities to make risk assessments, formulate decisions concerning preventive measures, provide effective cancer control programmes and decide among alternative options for public health decisions. The evaluations of IARC Working Groups are scientific, qualitative judgements on the evidence for or against carcinogenicity provided by the available data. These evaluations represent only one part of the body of information on which public health decisions may be based. Public health options vary from one situation to another and from country to country and relate to many factors, including different socioeconomic and national priorities. Therefore, no recommendation is given with regard to regulation or legislation, which are the responsibility of individual governments or other international organizations.

3. Selection of agents for review

Agents are selected for review on the basis of two main criteria: (a) there is evidence of human exposure and (b) there is some evidence or suspicion of carcinogenicity. Mixed exposures may occur in occupational and environmental settings and as a result of individual and cultural habits (such as tobacco smoking and dietary practices). Chemical analogues and compounds with biological or physical characteristics similar to those of suspected carcinogens may also be considered, even in the absence of data on a possible carcinogenic effect in humans or experimental animals.

The scientific literature is surveyed for published data relevant to an assessment of carcinogenicity. Ad hoc Advisory Groups convened by IARC in 1984, 1989, 1991, 1993, 1998 and 2003 made recommendations as to which agents should be evaluated in the *Monographs* series. Recent recommendations are available on the *Monographs* programme web site (http://monographs.iarc.fr). IARC may schedule other agents for review as it becomes aware of new scientific information or as national health agencies identify an urgent public health need related to cancer.

As significant new data become available on an agent for which a *Monograph* exists, a re-evaluation may be made at a subsequent meeting, and a new *Monograph* published. In some cases it may be appropriate to review only the data published since a prior evaluation. This can be useful for updating a database, reviewing new data to resolve a previously open question or identifying new tumour sites associated with a carcinogenic agent. Major changes in an evaluation (e.g. a new classification in Group 1 or a determination that a mechanism does not operate in humans, see Part B, Section 6) are more appropriately addressed by a full review.

4. Data for the *Monographs*

Each *Monograph* reviews all pertinent epidemiological studies and cancer bioassays in experimental animals. Those judged inadequate

or irrelevant to the evaluation may be cited but not summarized. If a group of similar studies is not reviewed, the reasons are indicated.

Mechanistic and other relevant data are also reviewed. A *Monograph* does not necessarily cite all the mechanistic literature concerning the agent being evaluated (see Part B, Section 4). Only those data considered by the Working Group to be relevant to making the evaluation are included.

With regard to epidemiological studies, cancer bioassays, and mechanistic and other relevant data, only reports that have been published or accepted for publication in the openly available scientific literature are reviewed. The same publication requirement applies to studies originating from IARC, including meta-analyses or pooled analyses commissioned by IARC in advance of a meeting (see Part B, Section 2c). Data from government agency reports that are publicly available are also considered. Exceptionally, doctoral theses and other material that are in their final form and publicly available may be reviewed.

Exposure data and other information on an agent under consideration are also reviewed. In the sections on chemical and physical properties, on analysis, on production and use and on occurrence, published and unpublished sources of information may be considered.

Inclusion of a study does not imply acceptance of the adequacy of the study design or of the analysis and interpretation of the results, and limitations are clearly outlined in square brackets at the end of each study description (see Part B). The reasons for not giving further consideration to an individual study also are indicated in the square brackets.

5. Meeting participants

Five categories of participant can be present at *Monograph* meetings.

(a) The Working Group

The Working Group is responsible for the critical reviews and evaluations that are developed during the meeting. The tasks of Working Group Members are: (i) to ascertain that all appropriate data have been collected; (ii) to select the data relevant for the evaluation on the basis of scientific merit; (iii) to prepare accurate summaries of the data to enable the reader to follow the reasoning of the Working Group; (iv) to evaluate the results of epidemiological and experimental studies on cancer; (v) to evaluate data relevant to the understanding of mechanisms of carcinogenesis; and (vi) to make an overall evaluation of the carcinogenicity of the exposure to humans. Working Group Members generally have published significant research related to the carcinogenicity of the agents being reviewed, and IARC uses literature searches to identify most experts. Working Group Members are selected on the basis of (a) knowledge and experience and (b) absence of real or apparent conflicts of interests. Consideration is also given to demographic diversity and balance of scientific findings and views.

(b) Invited Specialists

Invited Specialists are experts who also have critical knowledge and experience but have a real or apparent conflict of interests. These experts are invited when necessary to assist in the Working Group by contributing their unique knowledge and experience during subgroup and plenary discussions. They may also contribute text on non-influential issues in the section on exposure, such as a general description of data on production and use (see Part B, Section 1). Invited Specialists do not serve as meeting chair or subgroup chair, draft text that pertains to the description or interpretation of cancer data, or participate in the evaluations.

(c) Representatives of national and international health agencies

Representatives of national and international health agencies often attend meetings because their agencies sponsor the programme or are interested in the subject of a meeting. Representatives do not serve as meeting chair or subgroup chair, draft any part of a *Monograph*, or participate in the evaluations.

(d) Observers with relevant scientific credentials

Observers with relevant scientific credentials may be admitted to a meeting by IARC in limited numbers. Attention will be given to achieving a balance of Observers from constituencies with differing perspectives. They are invited to observe the meeting and should not attempt to influence it. Observers do not serve as meeting chair or subgroup chair, draft any part of a *Monograph*, or participate in the evaluations. At the meeting, the meeting chair and subgroup chairs may grant Observers an opportunity to speak, generally after they have observed a discussion. Observers agree to respect the Guidelines for Observers at *IARC Monographs* meetings (available at http://monographs.iarc.fr).

(e) The IARC Secretariat

The IARC Secretariat consists of scientists who are designated by IARC and who have relevant expertise. They serve as rapporteurs and participate in all discussions. When requested by the meeting chair or subgroup chair, they may also draft text or prepare tables and analyses.

Before an invitation is extended, each potential participant, including the IARC Secretariat, completes the WHO Declaration of Interests to report financial interests, employment and consulting, and individual and institutional research support related to the subject of the meeting. IARC assesses these interests to determine whether there is a conflict that warrants some limitation on participation. The declarations are updated and reviewed again at the opening of the meeting. Interests related to the subject of the meeting are disclosed to the meeting participants and in the published volume (Cogliano *et al.*, 2004).

The names and principal affiliations of participants are available on the *Monographs* programme web site (http://monographs.iarc.fr) approximately two months before each meeting. It is not acceptable for Observers or third parties to contact other participants before a meeting or to lobby them at any time. Meeting participants are asked to report all such contacts to IARC (Cogliano *et al.*, 2005).

All participants are listed, with their principal affiliations, at the beginning of each volume. Each participant who is a Member of a Working Group serves as an individual scientist and not as a representative of any organization, government or industry.

6. Working procedures

A separate Working Group is responsible for developing each volume of *Monographs*. A volume contains one or more *Monographs*, which can cover either a single agent or several related agents. Approximately one year in advance of the meeting of a Working Group, the agents to be reviewed are announced on the *Monographs* programme web site (http://monographs.iarc.fr) and participants are selected by IARC staff in consultation with other experts. Subsequently, relevant biological and epidemiological data are collected by IARC from recognized sources of information on carcinogenesis, including data storage and retrieval systems such as PubMed. Meeting participants who are asked to prepare preliminary working papers for specific sections are expected to supplement the IARC literature searches with their own searches.

For most chemicals and some complex mixtures, the major collection of data and the preparation of working papers for the sections on chemical and physical properties, on analysis, on production and use, and on occurrence are carried out under a separate contract funded by the US National Cancer Institute. Industrial associations, labour unions and other knowledgeable organizations may be asked to provide input to the sections on production and use, although this involvement is not required as a general rule. Information on production and trade is obtained from governmental, trade and market research publications and, in some cases, by direct contact with industries. Separate production data on some agents may not be available for a variety of reasons (e.g. not collected or made public in all producing countries, production is small). Information on uses may be obtained from published sources but is often complemented by direct contact with manufacturers. Efforts are made to supplement this information with data from other national and international sources.

Six months before the meeting, the material obtained is sent to meeting participants to prepare preliminary working papers. The working papers are compiled by IARC staff and sent, before the meeting, to Working Group Members and Invited Specialists for review.

The Working Group meets at IARC for seven to eight days to discuss and finalize the texts and to formulate the evaluations. The objectives of the meeting are peer review and consensus. During the first few days, four subgroups (covering exposure data, cancer in humans, cancer in experimental animals, and mechanistic and other relevant data) review the working papers, develop a joint subgroup draft and write summaries. Care is taken to ensure that each study summary is written or reviewed by someone not associated with the study being considered. During the last few days, the Working Group meets in plenary session to review the subgroup drafts and develop the evaluations. As a result, the entire volume is the joint product of the Working Group, and there are no individually authored sections.

IARC Working Groups strive to achieve a consensus evaluation. Consensus reflects broad agreement among Working Group Members, but not necessarily unanimity. The chair may elect to poll Working Group Members to determine the diversity of scientific opinion on issues where consensus is not readily apparent.

After the meeting, the master copy is verified by consulting the original literature, edited and prepared for publication. The aim is to publish the volume within six months of the Working Group meeting. A summary of the outcome is available on the *Monographs* programme web site soon after the meeting.

B. SCIENTIFIC REVIEW AND EVALUATION

The available studies are summarized by the Working Group, with particular regard to the qualitative aspects discussed below. In general, numerical findings are indicated as they appear in the original report; units are converted when necessary for easier comparison. The Working Group may conduct additional analyses of the published data and use them in their assessment of the evidence; the results of such supplementary analyses are given in square brackets. When an important aspect of a study that directly impinges on its interpretation should be brought to the attention of the reader, a Working Group comment is given in square brackets.

The scope of the *IARC Monographs* programme has expanded beyond chemicals to include complex mixtures, occupational exposures, physical and biological agents, lifestyle factors and other potentially carcinogenic exposures. Over time, the structure of a *Monograph* has evolved to include the following sections:

Exposure data
Studies of cancer in humans
Studies of cancer in experimental animals
Mechanistic and other relevant data
Summary
Evaluation and rationale

In addition, a section of General Remarks at the front of the volume discusses the reasons the agents were scheduled for evaluation and some key issues the Working Group encountered during the meeting.

This part of the Preamble discusses the types of evidence considered and summarized in each section of a *Monograph*, followed by the scientific criteria that guide the evaluations.

1. Exposure data

Each *Monograph* includes general information on the agent: this information may vary substantially between agents and must be adapted accordingly. Also included is information on production and use (when appropriate), methods of analysis and detection, occurrence, and sources and routes of human occupational and environmental exposures. Depending on the agent, regulations and guidelines for use may be presented.

(a) General information on the agent

For chemical agents, sections on chemical and physical data are included: the Chemical Abstracts Service Registry Number, the latest primary name and the IUPAC systematic name are recorded; other synonyms are given, but the list is not necessarily comprehensive. Information on chemical and physical properties that are relevant to identification, occurrence and biological activity is included. A description of technical products of chemicals includes trade names, relevant specifications and available information on composition and impurities. Some of the trade names given may be those of mixtures in which the agent being evaluated is only one of the ingredients.

For biological agents, taxonomy, structure and biology are described, and the degree of variability is indicated. Mode of replication, life cycle, target cells, persistence, latency, host response and clinical disease other than cancer are also presented.

For physical agents that are forms of radiation, energy and range of the radiation are included. For foreign bodies, fibres and respirable particles, size range and relative dimensions are indicated.

For agents such as mixtures, drugs or lifestyle factors, a description of the agent, including its composition, is given.

Whenever appropriate, other information, such as historical perspectives or the description of an industry or habit, may be included.

(b) Analysis and detection

An overview of methods of analysis and detection of the agent is presented, including their sensitivity, specificity and reproducibility. Methods widely used for regulatory purposes are emphasized. Methods for monitoring human exposure are also given. No critical evaluation or recommendation of any method is meant or implied.

(c) Production and use

The dates of first synthesis and of first commercial production of a chemical, mixture or other agent are provided when available; for agents that do not occur naturally, this information may allow a reasonable estimate to be made of the date before which no human exposure to the agent could have occurred. The dates of first reported occurrence of an exposure are also provided when available. In addition, methods of synthesis used in past and present commercial production and different methods of production,

which may give rise to different impurities, are described.

The countries where companies report production of the agent, and the number of companies in each country, are identified. Available data on production, international trade and uses are obtained for representative regions. It should not, however, be inferred that those areas or nations are necessarily the sole or major sources or users of the agent. Some identified uses may not be current or major applications, and the coverage is not necessarily comprehensive. In the case of drugs, mention of their therapeutic uses does not necessarily represent current practice nor does it imply judgement as to their therapeutic efficacy.

(d) Occurrence and exposure

Information on the occurrence of an agent in the environment is obtained from data derived from the monitoring and surveillance of levels in occupational environments, air, water, soil, plants, foods and animal and human tissues. When available, data on the generation, persistence and bioaccumulation of the agent are also included. Such data may be available from national databases.

Data that indicate the extent of past and present human exposure, the sources of exposure, the people most likely to be exposed and the factors that contribute to the exposure are reported. Information is presented on the range of human exposure, including occupational and environmental exposures. This includes relevant findings from both developed and developing countries. Some of these data are not distributed widely and may be available from government reports and other sources. In the case of mixtures, industries, occupations or processes, information is given about all agents known to be present. For processes, industries and occupations, a historical description is also given, noting variations in chemical composition, physical properties and levels of occupational exposure with date and place. For biological agents, the epidemiology of infection is described.

(e) Regulations and guidelines

Statements concerning regulations and guidelines (e.g. occupational exposure limits, maximal levels permitted in foods and water, pesticide registrations) are included, but they may not reflect the most recent situation, since such limits are continuously reviewed and modified. The absence of information on regulatory status for a country should not be taken to imply that that country does not have regulations with regard to the exposure. For biological agents, legislation and control, including vaccination and therapy, are described.

2. Studies of cancer in humans

This section includes all pertinent epidemiological studies (see Part A, Section 4). Studies of biomarkers are included when they are relevant to an evaluation of carcinogenicity to humans.

(a) Types of study considered

Several types of epidemiological study contribute to the assessment of carcinogenicity in humans — cohort studies, case–control studies, correlation (or ecological) studies and intervention studies. Rarely, results from randomized trials may be available. Case reports and case series of cancer in humans may also be reviewed.

Cohort and case–control studies relate individual exposures under study to the occurrence of cancer in individuals and provide an estimate of effect (such as relative risk) as the main measure of association. Intervention studies may provide strong evidence for making causal inferences, as exemplified by cessation of smoking and the subsequent decrease in risk for lung cancer.

In correlation studies, the units of investigation are usually whole populations (e.g. in

particular geographical areas or at particular times), and cancer frequency is related to a summary measure of the exposure of the population to the agent under study. In correlation studies, individual exposure is not documented, which renders this kind of study more prone to confounding. In some circumstances, however, correlation studies may be more informative than analytical study designs (see, for example, the *Monograph* on arsenic in drinking-water; IARC, 2004).

In some instances, case reports and case series have provided important information about the carcinogenicity of an agent. These types of study generally arise from a suspicion, based on clinical experience, that the concurrence of two events — that is, a particular exposure and occurrence of a cancer — has happened rather more frequently than would be expected by chance. Case reports and case series usually lack complete ascertainment of cases in any population, definition or enumeration of the population at risk and estimation of the expected number of cases in the absence of exposure.

The uncertainties that surround the interpretation of case reports, case series and correlation studies make them inadequate, except in rare instances, to form the sole basis for inferring a causal relationship. When taken together with case–control and cohort studies, however, these types of study may add materially to the judgement that a causal relationship exists.

Epidemiological studies of benign neoplasms, presumed preneoplastic lesions and other end-points thought to be relevant to cancer are also reviewed. They may, in some instances, strengthen inferences drawn from studies of cancer itself.

(b) Quality of studies considered

It is necessary to take into account the possible roles of bias, confounding and chance in the interpretation of epidemiological studies. Bias is the effect of factors in study design or execution that lead erroneously to a stronger or weaker association than in fact exists between an agent and disease. Confounding is a form of bias that occurs when the relationship with disease is made to appear stronger or weaker than it truly is as a result of an association between the apparent causal factor and another factor that is associated with either an increase or decrease in the incidence of the disease. The role of chance is related to biological variability and the influence of sample size on the precision of estimates of effect.

In evaluating the extent to which these factors have been minimized in an individual study, consideration is given to several aspects of design and analysis as described in the report of the study. For example, when suspicion of carcinogenicity arises largely from a single small study, careful consideration is given when interpreting subsequent studies that included these data in an enlarged population. Most of these considerations apply equally to case–control, cohort and correlation studies. Lack of clarity of any of these aspects in the reporting of a study can decrease its credibility and the weight given to it in the final evaluation of the exposure.

First, the study population, disease (or diseases) and exposure should have been well defined by the authors. Cases of disease in the study population should have been identified in a way that was independent of the exposure of interest, and exposure should have been assessed in a way that was not related to disease status.

Second, the authors should have taken into account — in the study design and analysis — other variables that can influence the risk of disease and may have been related to the exposure of interest. Potential confounding by such variables should have been dealt with either in the design of the study, such as by matching, or in the analysis, by statistical adjustment. In cohort studies, comparisons with local rates of disease may or may not be more appropriate than those with national rates. Internal comparisons of

frequency of disease among individuals at different levels of exposure are also desirable in cohort studies, since they minimize the potential for confounding related to the difference in risk factors between an external reference group and the study population.

Third, the authors should have reported the basic data on which the conclusions are founded, even if sophisticated statistical analyses were employed. At the very least, they should have given the numbers of exposed and unexposed cases and controls in a case–control study and the numbers of cases observed and expected in a cohort study. Further tabulations by time since exposure began and other temporal factors are also important. In a cohort study, data on all cancer sites and all causes of death should have been given, to reveal the possibility of reporting bias. In a case–control study, the effects of investigated factors other than the exposure of interest should have been reported.

Finally, the statistical methods used to obtain estimates of relative risk, absolute rates of cancer, confidence intervals and significance tests, and to adjust for confounding should have been clearly stated by the authors. These methods have been reviewed for case–control studies (Breslow & Day, 1980) and for cohort studies (Breslow & Day, 1987).

(c) Meta-analyses and pooled analyses

Independent epidemiological studies of the same agent may lead to results that are difficult to interpret. Combined analyses of data from multiple studies are a means of resolving this ambiguity, and well conducted analyses can be considered. There are two types of combined analysis. The first involves combining summary statistics such as relative risks from individual studies (meta-analysis) and the second involves a pooled analysis of the raw data from the individual studies (pooled analysis) (Greenland, 1998).

The advantages of combined analyses are increased precision due to increased sample size and the opportunity to explore potential confounders, interactions and modifying effects that may explain heterogeneity among studies in more detail. A disadvantage of combined analyses is the possible lack of compatibility of data from various studies due to differences in subject recruitment, procedures of data collection, methods of measurement and effects of unmeasured co-variates that may differ among studies. Despite these limitations, well conducted combined analyses may provide a firmer basis than individual studies for drawing conclusions about the potential carcinogenicity of agents.

IARC may commission a meta-analysis or pooled analysis that is pertinent to a particular *Monograph* (see Part A, Section 4). Additionally, as a means of gaining insight from the results of multiple individual studies, ad hoc calculations that combine data from different studies may be conducted by the Working Group during the course of a *Monograph* meeting. The results of such original calculations, which would be specified in the text by presentation in square brackets, might involve updates of previously conducted analyses that incorporate the results of more recent studies or de-novo analyses. Irrespective of the source of data for the meta-analyses and pooled analyses, it is important that the same criteria for data quality be applied as those that would be applied to individual studies and to ensure also that sources of heterogeneity between studies be taken into account.

(d) Temporal effects

Detailed analyses of both relative and absolute risks in relation to temporal variables, such as age at first exposure, time since first exposure, duration of exposure, cumulative exposure, peak exposure (when appropriate) and time since cessation of exposure, are reviewed and summarized when available. Analyses of temporal

relationships may be useful in making causal inferences. In addition, such analyses may suggest whether a carcinogen acts early or late in the process of carcinogenesis, although, at best, they allow only indirect inferences about mechanisms of carcinogenesis.

(e) Use of biomarkers in epidemiological studies

Biomarkers indicate molecular, cellular or other biological changes and are increasingly used in epidemiological studies for various purposes (IARC, 1991; Vainio *et al.*, 1992; Toniolo *et al.*, 1997; Vineis *et al.*, 1999; Buffler *et al.*, 2004). These may include evidence of exposure, of early effects, of cellular, tissue or organism responses, of individual susceptibility or host responses, and inference of a mechanism (see Part B, Section 4b). This is a rapidly evolving field that encompasses developments in genomics, epigenomics and other emerging technologies.

Molecular epidemiological data that identify associations between genetic polymorphisms and interindividual differences in susceptibility to the agent(s) being evaluated may contribute to the identification of carcinogenic hazards to humans. If the polymorphism has been demonstrated experimentally to modify the functional activity of the gene product in a manner that is consistent with increased susceptibility, these data may be useful in making causal inferences. Similarly, molecular epidemiological studies that measure cell functions, enzymes or metabolites that are thought to be the basis of susceptibility may provide evidence that reinforces biological plausibility. It should be noted, however, that when data on genetic susceptibility originate from multiple comparisons that arise from subgroup analyses, this can generate false-positive results and inconsistencies across studies, and such data therefore require careful evaluation. If the known phenotype of a genetic polymorphism can explain the carcinogenic mechanism of the agent being evaluated, data on this phenotype may be useful in making causal inferences.

(f) Criteria for causality

After the quality of individual epidemiological studies of cancer has been summarized and assessed, a judgement is made concerning the strength of evidence that the agent in question is carcinogenic to humans. In making its judgement, the Working Group considers several criteria for causality (Hill, 1965). A strong association (e.g. a large relative risk) is more likely to indicate causality than a weak association, although it is recognized that estimates of effect of small magnitude do not imply lack of causality and may be important if the disease or exposure is common. Associations that are replicated in several studies of the same design or that use different epidemiological approaches or under different circumstances of exposure are more likely to represent a causal relationship than isolated observations from single studies. If there are inconsistent results among investigations, possible reasons are sought (such as differences in exposure), and results of studies that are judged to be of high quality are given more weight than those of studies that are judged to be methodologically less sound.

If the risk increases with the exposure, this is considered to be a strong indication of causality, although the absence of a graded response is not necessarily evidence against a causal relationship. The demonstration of a decline in risk after cessation of or reduction in exposure in individuals or in whole populations also supports a causal interpretation of the findings.

Several scenarios may increase confidence in a causal relationship. On the one hand, an agent may be specific in causing tumours at one site or of one morphological type. On the other, carcinogenicity may be evident through the causation of multiple tumour types. Temporality, precision of estimates of effect, biological plausibility and

coherence of the overall database are considered. Data on biomarkers may be employed in an assessment of the biological plausibility of epidemiological observations.

Although rarely available, results from randomized trials that show different rates of cancer among exposed and unexposed individuals provide particularly strong evidence for causality.

When several epidemiological studies show little or no indication of an association between an exposure and cancer, a judgement may be made that, in the aggregate, they show evidence of lack of carcinogenicity. Such a judgement requires first that the studies meet, to a sufficient degree, the standards of design and analysis described above. Specifically, the possibility that bias, confounding or misclassification of exposure or outcome could explain the observed results should be considered and excluded with reasonable certainty. In addition, all studies that are judged to be methodologically sound should (a) be consistent with an estimate of effect of unity for any observed level of exposure, (b) when considered together, provide a pooled estimate of relative risk that is at or near to unity, and (c) have a narrow confidence interval, due to sufficient population size. Moreover, no individual study nor the pooled results of all the studies should show any consistent tendency that the relative risk of cancer increases with increasing level of exposure. It is important to note that evidence of lack of carcinogenicity obtained from several epidemiological studies can apply only to the type(s) of cancer studied, to the dose levels reported, and to the intervals between first exposure and disease onset observed in these studies. Experience with human cancer indicates that the period from first exposure to the development of clinical cancer is sometimes longer than 20 years; latent periods substantially shorter than 30 years cannot provide evidence for lack of carcinogenicity.

3. Studies of cancer in experimental animals

All known human carcinogens that have been studied adequately for carcinogenicity in experimental animals have produced positive results in one or more animal species (Wilbourn *et al.*, 1986; Tomatis *et al.*, 1989). For several agents (e.g. aflatoxins, diethylstilbestrol, solar radiation, vinyl chloride), carcinogenicity in experimental animals was established or highly suspected before epidemiological studies confirmed their carcinogenicity in humans (Vainio *et al.*, 1995). Although this association cannot establish that all agents that cause cancer in experimental animals also cause cancer in humans, it is biologically plausible that agents for which there is *sufficient evidence of carcinogenicity* in experimental animals (see Part B, Section 6b) also present a carcinogenic hazard to humans. Accordingly, in the absence of additional scientific information, these agents are considered to pose a carcinogenic hazard to humans. Examples of additional scientific information are data that demonstrate that a given agent causes cancer in animals through a species-specific mechanism that does not operate in humans or data that demonstrate that the mechanism in experimental animals also operates in humans (see Part B, Section 6).

Consideration is given to all available long-term studies of cancer in experimental animals with the agent under review (see Part A, Section 4). In all experimental settings, the nature and extent of impurities or contaminants present in the agent being evaluated are given when available. Animal species, strain (including genetic background where applicable), sex, numbers per group, age at start of treatment, route of exposure, dose levels, duration of exposure, survival and information on tumours (incidence, latency, severity or multiplicity of neoplasms or preneoplastic lesions) are reported. Those studies in experimental animals that are judged to be irrelevant to the evaluation or judged to be inadequate

(e.g. too short a duration, too few animals, poor survival; see below) may be omitted. Guidelines for conducting long-term carcinogenicity experiments have been published (e.g. OECD, 2002).

Other studies considered may include: experiments in which the agent was administered in the presence of factors that modify carcinogenic effects (e.g. initiation–promotion studies, co-carcinogenicity studies and studies in genetically modified animals); studies in which the end-point was not cancer but a defined precancerous lesion; experiments on the carcinogenicity of known metabolites and derivatives; and studies of cancer in non-laboratory animals (e.g. livestock and companion animals) exposed to the agent.

For studies of mixtures, consideration is given to the possibility that changes in the physicochemical properties of the individual substances may occur during collection, storage, extraction, concentration and delivery. Another consideration is that chemical and toxicological interactions of components in a mixture may alter dose–response relationships. The relevance to human exposure of the test mixture administered in the animal experiment is also assessed. This may involve consideration of the following aspects of the mixture tested: (i) physical and chemical characteristics, (ii) identified constituents that may indicate the presence of a class of substances and (iii) the results of genetic toxicity and related tests.

The relevance of results obtained with an agent that is analogous (e.g. similar in structure or of a similar virus genus) to that being evaluated is also considered. Such results may provide biological and mechanistic information that is relevant to the understanding of the process of carcinogenesis in humans and may strengthen the biological plausibility that the agent being evaluated is carcinogenic to humans (see Part B, Section 2f).

(a) Qualitative aspects

An assessment of carcinogenicity involves several considerations of qualitative importance, including (i) the experimental conditions under which the test was performed, including route, schedule and duration of exposure, species, strain (including genetic background where applicable), sex, age and duration of follow-up; (ii) the consistency of the results, for example, across species and target organ(s); (iii) the spectrum of neoplastic response, from preneoplastic lesions and benign tumours to malignant neoplasms; and (iv) the possible role of modifying factors.

Considerations of importance in the interpretation and evaluation of a particular study include: (i) how clearly the agent was defined and, in the case of mixtures, how adequately the sample characterization was reported; (ii) whether the dose was monitored adequately, particularly in inhalation experiments; (iii) whether the doses, duration of treatment and route of exposure were appropriate; (iv) whether the survival of treated animals was similar to that of controls; (v) whether there were adequate numbers of animals per group; (vi) whether both male and female animals were used; (vii) whether animals were allocated randomly to groups; (viii) whether the duration of observation was adequate; and (ix) whether the data were reported and analysed adequately.

When benign tumours (a) occur together with and originate from the same cell type as malignant tumours in an organ or tissue in a particular study and (b) appear to represent a stage in the progression to malignancy, they are usually combined in the assessment of tumour incidence (Huff *et al.*, 1989). The occurrence of lesions presumed to be preneoplastic may in certain instances aid in assessing the biological plausibility of any neoplastic response observed. If an agent induces only benign neoplasms that appear to be end-points that do not readily undergo

transition to malignancy, the agent should nevertheless be suspected of being carcinogenic and requires further investigation.

(b) Quantitative aspects

The probability that tumours will occur may depend on the species, sex, strain, genetic background and age of the animal, and on the dose, route, timing and duration of the exposure. Evidence of an increased incidence of neoplasms with increasing levels of exposure strengthens the inference of a causal association between the exposure and the development of neoplasms.

The form of the dose–response relationship can vary widely, depending on the particular agent under study and the target organ. Mechanisms such as induction of DNA damage or inhibition of repair, altered cell division and cell death rates and changes in intercellular communication are important determinants of dose–response relationships for some carcinogens. Since many chemicals require metabolic activation before being converted to their reactive intermediates, both metabolic and toxicokinetic aspects are important in determining the dose–response pattern. Saturation of steps such as absorption, activation, inactivation and elimination may produce nonlinearity in the dose–response relationship (Hoel et al., 1983; Gart *et al.*, 1986), as could saturation of processes such as DNA repair. The dose–response relationship can also be affected by differences in survival among the treatment groups.

(c) Statistical analyses

Factors considered include the adequacy of the information given for each treatment group: (i) number of animals studied and number examined histologically, (ii) number of animals with a given tumour type and (iii) length of survival. The statistical methods used should be clearly stated and should be the generally accepted techniques refined for this purpose (Peto *et al.*, 1980; Gart *et al.*, 1986; Portier & Bailer, 1989; Bieler & Williams, 1993). The choice of the most appropriate statistical method requires consideration of whether or not there are differences in survival among the treatment groups; for example, reduced survival because of non-tumour-related mortality can preclude the occurrence of tumours later in life. When detailed information on survival is not available, comparisons of the proportions of tumour-bearing animals among the effective number of animals (alive at the time the first tumour was discovered) can be useful when significant differences in survival occur before tumours appear. The lethality of the tumour also requires consideration: for rapidly fatal tumours, the time of death provides an indication of the time of tumour onset and can be assessed using life-table methods; non-fatal or incidental tumours that do not affect survival can be assessed using methods such as the Mantel-Haenzel test for changes in tumour prevalence. Because tumour lethality is often difficult to determine, methods such as the Poly-K test that do not require such information can also be used. When results are available on the number and size of tumours seen in experimental animals (e.g. papillomas on mouse skin, liver tumours observed through nuclear magnetic resonance tomography), other more complicated statistical procedures may be needed (Sherman *et al.*, 1994; Dunson *et al.*, 2003).

Formal statistical methods have been developed to incorporate historical control data into the analysis of data from a given experiment. These methods assign an appropriate weight to historical and concurrent controls on the basis of the extent of between-study and within-study variability: less weight is given to historical controls when they show a high degree of variability, and greater weight when they show little variability. It is generally not appropriate to discount a tumour response that is significantly increased compared with concurrent controls by arguing that it falls within the range of historical controls,

particularly when historical controls show high between-study variability and are, thus, of little relevance to the current experiment. In analysing results for uncommon tumours, however, the analysis may be improved by considering historical control data, particularly when between-study variability is low. Historical controls should be selected to resemble the concurrent controls as closely as possible with respect to species, gender and strain, as well as other factors such as basal diet and general laboratory environment, which may affect tumour-response rates in control animals (Haseman *et al.*, 1984; Fung *et al.*, 1996; Greim *et al.*, 2003).

Although meta-analyses and combined analyses are conducted less frequently for animal experiments than for epidemiological studies due to differences in animal strains, they can be useful aids in interpreting animal data when the experimental protocols are sufficiently similar.

4. Mechanistic and other relevant data

Mechanistic and other relevant data may provide evidence of carcinogenicity and also help in assessing the relevance and importance of findings of cancer in animals and in humans. The nature of the mechanistic and other relevant data depends on the biological activity of the agent being considered. The Working Group considers representative studies to give a concise description of the relevant data and issues that they consider to be important; thus, not every available study is cited. Relevant topics may include toxicokinetics, mechanisms of carcinogenesis, susceptible individuals, populations and life-stages, other relevant data and other adverse effects. When data on biomarkers are informative about the mechanisms of carcinogenesis, they are included in this section.

These topics are not mutually exclusive; thus, the same studies may be discussed in more than one subsection. For example, a mutation in a gene that codes for an enzyme that metabolizes the agent under study could be discussed in the subsections on toxicokinetics, mechanisms and individual susceptibility if it also exists as an inherited polymorphism.

(a) Toxicokinetic data

Toxicokinetics refers to the absorption, distribution, metabolism and elimination of agents in humans, experimental animals and, where relevant, cellular systems. Examples of kinetic factors that may affect dose–response relationships include uptake, deposition, biopersistence and half-life in tissues, protein binding, metabolic activation and detoxification. Studies that indicate the metabolic fate of the agent in humans and in experimental animals are summarized briefly, and comparisons of data from humans and animals are made when possible. Comparative information on the relationship between exposure and the dose that reaches the target site may be important for the extrapolation of hazards between species and in clarifying the role of in-vitro findings.

(b) Data on mechanisms of carcinogenesis

To provide focus, the Working Group attempts to identify the possible mechanisms by which the agent may increase the risk of cancer. For each possible mechanism, a representative selection of key data from humans and experimental systems is summarized. Attention is given to gaps in the data and to data that suggests that more than one mechanism may be operating. The relevance of the mechanism to humans is discussed, in particular, when mechanistic data are derived from experimental model systems. Changes in the affected organs, tissues or cells can be divided into three non-exclusive levels as described below.

(i) Changes in physiology

Physiological changes refer to exposure-related modifications to the physiology and/or response of cells, tissues and organs. Examples of potentially adverse physiological changes include mitogenesis, compensatory cell division, escape from apoptosis and/or senescence, presence of inflammation, hyperplasia, metaplasia and/or preneoplasia, angiogenesis, alterations in cellular adhesion, changes in steroidal hormones and changes in immune surveillance.

(ii) Functional changes at the cellular level

Functional changes refer to exposure-related alterations in the signalling pathways used by cells to manage critical processes that are related to increased risk for cancer. Examples of functional changes include modified activities of enzymes involved in the metabolism of xenobiotics, alterations in the expression of key genes that regulate DNA repair, alterations in cyclin-dependent kinases that govern cell cycle progression, changes in the patterns of post-translational modifications of proteins, changes in regulatory factors that alter apoptotic rates, changes in the secretion of factors related to the stimulation of DNA replication and transcription and changes in gap–junction-mediated intercellular communication.

(iii) Changes at the molecular level

Molecular changes refer to exposure-related changes in key cellular structures at the molecular level, including, in particular, genotoxicity. Examples of molecular changes include formation of DNA adducts and DNA strand breaks, mutations in genes, chromosomal aberrations, aneuploidy and changes in DNA methylation patterns. Greater emphasis is given to irreversible effects.

The use of mechanistic data in the identification of a carcinogenic hazard is specific to the mechanism being addressed and is not readily described for every possible level and mechanism discussed above.

Genotoxicity data are discussed here to illustrate the key issues involved in the evaluation of mechanistic data.

Tests for genetic and related effects are described in view of the relevance of gene mutation and chromosomal aberration/aneuploidy to carcinogenesis (Vainio *et al.*, 1992; McGregor *et al.*, 1999). The adequacy of the reporting of sample characterization is considered and, when necessary, commented upon; with regard to complex mixtures, such comments are similar to those described for animal carcinogenicity tests. The available data are interpreted critically according to the end-points detected, which may include DNA damage, gene mutation, sister chromatid exchange, micronucleus formation, chromosomal aberrations and aneuploidy. The concentrations employed are given, and mention is made of whether the use of an exogenous metabolic system in vitro affected the test result. These data are listed in tabular form by phylogenetic classification.

Positive results in tests using prokaryotes, lower eukaryotes, insects, plants and cultured mammalian cells suggest that genetic and related effects could occur in mammals. Results from such tests may also give information on the types of genetic effect produced and on the involvement of metabolic activation. Some end-points described are clearly genetic in nature (e.g. gene mutations), while others are associated with genetic effects (e.g. unscheduled DNA synthesis). In-vitro tests for tumour promotion, cell transformation and gap–junction intercellular communication may be sensitive to changes that are not necessarily the result of genetic alterations but that may have specific relevance to the process of carcinogenesis. Critical appraisals of these tests have been published (Montesano *et al.*, 1986; McGregor *et al.*, 1999).

Genetic or other activity manifest in humans and experimental mammals is regarded to be of

greater relevance than that in other organisms. The demonstration that an agent can induce gene and chromosomal mutations in mammals in vivo indicates that it may have carcinogenic activity. Negative results in tests for mutagenicity in selected tissues from animals treated in vivo provide less weight, partly because they do not exclude the possibility of an effect in tissues other than those examined. Moreover, negative results in short-term tests with genetic end-points cannot be considered to provide evidence that rules out the carcinogenicity of agents that act through other mechanisms (e.g. receptor-mediated effects, cellular toxicity with regenerative cell division, peroxisome proliferation) (Vainio *et al.*, 1992). Factors that may give misleading results in short-term tests have been discussed in detail elsewhere (Montesano *et al.*, 1986; McGregor *et al.*, 1999).

When there is evidence that an agent acts by a specific mechanism that does not involve genotoxicity (e.g. hormonal dysregulation, immune suppression, and formation of calculi and other deposits that cause chronic irritation), that evidence is presented and reviewed critically in the context of rigorous criteria for the operation of that mechanism in carcinogenesis (e.g. Capen *et al.*, 1999).

For biological agents such as viruses, bacteria and parasites, other data relevant to carcinogenicity may include descriptions of the pathology of infection, integration and expression of viruses, and genetic alterations seen in human tumours. Other observations that might comprise cellular and tissue responses to infection, immune response and the presence of tumour markers are also considered.

For physical agents that are forms of radiation, other data relevant to carcinogenicity may include descriptions of damaging effects at the physiological, cellular and molecular level, as for chemical agents, and descriptions of how these effects occur. 'Physical agents' may also be considered to comprise foreign bodies, such as surgical implants of various kinds, and poorly soluble fibres, dusts and particles of various sizes, the pathogenic effects of which are a result of their physical presence in tissues or body cavities. Other relevant data for such materials may include characterization of cellular, tissue and physiological reactions to these materials and descriptions of pathological conditions other than neoplasia with which they may be associated.

(c) Other data relevant to mechanisms

A description is provided of any structure–activity relationships that may be relevant to an evaluation of the carcinogenicity of an agent, the toxicological implications of the physical and chemical properties, and any other data relevant to the evaluation that are not included elsewhere.

High-output data, such as those derived from gene expression microarrays, and high-throughput data, such as those that result from testing hundreds of agents for a single end-point, pose a unique problem for the use of mechanistic data in the evaluation of a carcinogenic hazard. In the case of high-output data, there is the possibility to overinterpret changes in individual end-points (e.g. changes in expression in one gene) without considering the consistency of that finding in the broader context of the other end-points (e.g. other genes with linked transcriptional control). High-output data can be used in assessing mechanisms, but all end-points measured in a single experiment need to be considered in the proper context. For high-throughput data, where the number of observations far exceeds the number of end-points measured, their utility for identifying common mechanisms across multiple agents is enhanced. These data can be used to identify mechanisms that not only seem plausible, but also have a consistent pattern of carcinogenic response across entire classes of related compounds.

(d) Susceptibility data

Individuals, populations and life-stages may have greater or lesser susceptibility to an agent, based on toxicokinetics, mechanisms of carcinogenesis and other factors. Examples of host and genetic factors that affect individual susceptibility include sex, genetic polymorphisms of genes involved in the metabolism of the agent under evaluation, differences in metabolic capacity due to life-stage or the presence of disease, differences in DNA repair capacity, competition for or alteration of metabolic capacity by medications or other chemical exposures, pre-existing hormonal imbalance that is exacerbated by a chemical exposure, a suppressed immune system, periods of higher-than-usual tissue growth or regeneration and genetic polymorphisms that lead to differences in behaviour (e.g. addiction). Such data can substantially increase the strength of the evidence from epidemiological data and enhance the linkage of in-vivo and in-vitro laboratory studies to humans.

(e) Data on other adverse effects

Data on acute, subchronic and chronic adverse effects relevant to the cancer evaluation are summarized. Adverse effects that confirm distribution and biological effects at the sites of tumour development, or alterations in physiology that could lead to tumour development, are emphasized. Effects on reproduction, embryonic and fetal survival and development are summarized briefly. The adequacy of epidemiological studies of reproductive outcome and genetic and related effects in humans is judged by the same criteria as those applied to epidemiological studies of cancer, but fewer details are given.

5. Summary

This section is a summary of data presented in the preceding sections. Summaries can be found on the *Monographs* programme web site (http://monographs.iarc.fr).

(a) Exposure data

Data are summarized, as appropriate, on the basis of elements such as production, use, occurrence and exposure levels in the workplace and environment and measurements in human tissues and body fluids. Quantitative data and time trends are given to compare exposures in different occupations and environmental settings. Exposure to biological agents is described in terms of transmission, prevalence and persistence of infection.

(b) Cancer in humans

Results of epidemiological studies pertinent to an assessment of human carcinogenicity are summarized. When relevant, case reports and correlation studies are also summarized. The target organ(s) or tissue(s) in which an increase in cancer was observed is identified. Dose–response and other quantitative data may be summarized when available.

(c) Cancer in experimental animals

Data relevant to an evaluation of carcinogenicity in animals are summarized. For each animal species, study design and route of administration, it is stated whether an increased incidence, reduced latency, or increased severity or multiplicity of neoplasms or preneoplastic lesions were observed, and the tumour sites are indicated. If the agent produced tumours after prenatal exposure or in single-dose experiments, this is also mentioned. Negative findings, inverse relationships, dose–response and other quantitative data are also summarized.

(d) Mechanistic and other relevant data

Data relevant to the toxicokinetics (absorption, distribution, metabolism, elimination) and

the possible mechanism(s) of carcinogenesis (e.g. genetic toxicity, epigenetic effects) are summarized. In addition, information on susceptible individuals, populations and life-stages is summarized. This section also reports on other toxic effects, including reproductive and developmental effects, as well as additional relevant data that are considered to be important.

6. Evaluation and rationale

Evaluations of the strength of the evidence for carcinogenicity arising from human and experimental animal data are made, using standard terms. The strength of the mechanistic evidence is also characterized.

It is recognized that the criteria for these evaluations, described below, cannot encompass all of the factors that may be relevant to an evaluation of carcinogenicity. In considering all of the relevant scientific data, the Working Group may assign the agent to a higher or lower category than a strict interpretation of these criteria would indicate.

These categories refer only to the strength of the evidence that an exposure is carcinogenic and not to the extent of its carcinogenic activity (potency). A classification may change as new information becomes available.

An evaluation of the degree of evidence is limited to the materials tested, as defined physically, chemically or biologically. When the agents evaluated are considered by the Working Group to be sufficiently closely related, they may be grouped together for the purpose of a single evaluation of the degree of evidence.

(a) Carcinogenicity in humans

The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** The Working Group considers that a causal relationship has been established between exposure to the agent and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence. A statement that there is *sufficient evidence* is followed by a separate sentence that identifies the target organ(s) or tissue(s) where an increased risk of cancer was observed in humans. Identification of a specific target organ or tissue does not preclude the possibility that the agent may cause cancer at other sites.

***Limited evidence of carcinogenicity*:** A positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered by the Working Group to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence.

***Inadequate evidence of carcinogenicity*:** The available studies are of insufficient quality, consistency or statistical power to permit a conclusion regarding the presence or absence of a causal association between exposure and cancer, or no data on cancer in humans are available.

***Evidence suggesting lack of carcinogenicity*:** There are several adequate studies covering the full range of levels of exposure that humans are known to encounter, which are mutually consistent in not showing a positive association between exposure to the agent and any studied cancer at any observed level of exposure. The results from these studies alone or combined should have narrow confidence intervals with an upper limit close to the null value (e.g. a relative risk of 1.0). Bias and confounding should be ruled out with reasonable confidence, and the studies should have an adequate length of follow-up. A conclusion of *evidence suggesting lack of carcinogenicity* is inevitably limited to the cancer sites, conditions and levels of exposure, and length of observation covered by the available studies. In

addition, the possibility of a very small risk at the levels of exposure studied can never be excluded.

In some instances, the above categories may be used to classify the degree of evidence related to carcinogenicity in specific organs or tissues.

When the available epidemiological studies pertain to a mixture, process, occupation or industry, the Working Group seeks to identify the specific agent considered most likely to be responsible for any excess risk. The evaluation is focused as narrowly as the available data on exposure and other aspects permit.

(b) Carcinogenicity in experimental animals

Carcinogenicity in experimental animals can be evaluated using conventional bioassays, bioassays that employ genetically modified animals, and other in-vivo bioassays that focus on one or more of the critical stages of carcinogenesis. In the absence of data from conventional long-term bioassays or from assays with neoplasia as the end-point, consistently positive results in several models that address several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of carcinogenicity in experimental animals.

The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** The Working Group considers that a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well conducted study, ideally conducted under Good Laboratory Practices, can also provide *sufficient evidence*.

A single study in one species and sex might be considered to provide *sufficient evidence of carcinogenicity* when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

***Limited evidence of carcinogenicity*:** The data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

***Inadequate evidence of carcinogenicity*:** The studies cannot be interpreted as showing either the presence or absence of a carcinogenic effect because of major qualitative or quantitative limitations, or no data on cancer in experimental animals are available.

***Evidence suggesting lack of carcinogenicity*:** Adequate studies involving at least two species are available which show that, within the limits of the tests used, the agent is not carcinogenic. A conclusion of *evidence suggesting lack of carcinogenicity* is inevitably limited to the species, tumour sites, age at exposure, and conditions and levels of exposure studied.

(c) Mechanistic and other relevant data

Mechanistic and other evidence judged to be relevant to an evaluation of carcinogenicity and of sufficient importance to affect the overall evaluation is highlighted. This may include data on preneoplastic lesions, tumour pathology, genetic and related effects, structure–activity relationships, metabolism and toxicokinetics,

physicochemical parameters and analogous biological agents.

The strength of the evidence that any carcinogenic effect observed is due to a particular mechanism is evaluated, using terms such as 'weak', 'moderate' or 'strong'. The Working Group then assesses whether that particular mechanism is likely to be operative in humans. The strongest indications that a particular mechanism operates in humans derive from data on humans or biological specimens obtained from exposed humans. The data may be considered to be especially relevant if they show that the agent in question has caused changes in exposed humans that are on the causal pathway to carcinogenesis. Such data may, however, never become available, because it is at least conceivable that certain compounds may be kept from human use solely on the basis of evidence of their toxicity and/or carcinogenicity in experimental systems.

The conclusion that a mechanism operates in experimental animals is strengthened by findings of consistent results in different experimental systems, by the demonstration of biological plausibility and by coherence of the overall database. Strong support can be obtained from studies that challenge the hypothesized mechanism experimentally, by demonstrating that the suppression of key mechanistic processes leads to the suppression of tumour development. The Working Group considers whether multiple mechanisms might contribute to tumour development, whether different mechanisms might operate in different dose ranges, whether separate mechanisms might operate in humans and experimental animals and whether a unique mechanism might operate in a susceptible group. The possible contribution of alternative mechanisms must be considered before concluding that tumours observed in experimental animals are not relevant to humans. An uneven level of experimental support for different mechanisms may reflect that disproportionate resources have been focused on investigating a favoured mechanism.

For complex exposures, including occupational and industrial exposures, the chemical composition and the potential contribution of carcinogens known to be present are considered by the Working Group in its overall evaluation of human carcinogenicity. The Working Group also determines the extent to which the materials tested in experimental systems are related to those to which humans are exposed.

(d) Overall evaluation

Finally, the body of evidence is considered as a whole, to reach an overall evaluation of the carcinogenicity of the agent to humans.

An evaluation may be made for a group of agents that have been evaluated by the Working Group. In addition, when supporting data indicate that other related agents, for which there is no direct evidence of their capacity to induce cancer in humans or in animals, may also be carcinogenic, a statement describing the rationale for this conclusion is added to the evaluation narrative; an additional evaluation may be made for this broader group of agents if the strength of the evidence warrants it.

The agent is described according to the wording of one of the following categories, and the designated group is given. The categorization of an agent is a matter of scientific judgement that reflects the strength of the evidence derived from studies in humans and in experimental animals and from mechanistic and other relevant data.

Group 1: The agent is carcinogenic to humans.

This category is used when there is *sufficient evidence of carcinogenicity* in humans. Exceptionally, an agent may be placed in this category when evidence of carcinogenicity in humans is less than *sufficient* but there is *sufficient evidence of carcinogenicity* in experimental

animals and strong evidence in exposed humans that the agent acts through a relevant mechanism of carcinogenicity.

Group 2.

This category includes agents for which, at one extreme, the degree of evidence of carcinogenicity in humans is almost *sufficient*, as well as those for which, at the other extreme, there are no human data but for which there is evidence of carcinogenicity in experimental animals. Agents are assigned to either Group 2A (*probably carcinogenic to humans*) or Group 2B (*possibly carcinogenic to humans*) on the basis of epidemiological and experimental evidence of carcinogenicity and mechanistic and other relevant data. The terms *probably carcinogenic* and *possibly carcinogenic* have no quantitative significance and are used simply as descriptors of different levels of evidence of human carcinogenicity, with *probably carcinogenic* signifying a higher level of evidence than *possibly carcinogenic*.

Group 2A: The agent is probably carcinogenic to humans.

This category is used when there is *limited evidence of carcinogenicity* in humans and *sufficient evidence of carcinogenicity* in experimental animals. In some cases, an agent may be classified in this category when there is *inadequate evidence of carcinogenicity* in humans and *sufficient evidence of carcinogenicity* in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans. Exceptionally, an agent may be classified in this category solely on the basis of *limited evidence of carcinogenicity* in humans. An agent may be assigned to this category if it clearly belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified in Group 1 or Group 2A.

Group 2B: The agent is possibly carcinogenic to humans.

This category is used for agents for which there is *limited evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals. It may also be used when there is *inadequate evidence of carcinogenicity* in humans but there is *sufficient evidence of carcinogenicity* in experimental animals. In some instances, an agent for which there is *inadequate evidence of carcinogenicity* in humans and less than *sufficient evidence of carcinogenicity* in experimental animals together with supporting evidence from mechanistic and other relevant data may be placed in this group. An agent may be classified in this category solely on the basis of strong evidence from mechanistic and other relevant data.

Group 3: The agent is not classifiable as to its carcinogenicity to humans.

This category is used most commonly for agents for which the evidence of carcinogenicity is *inadequate* in humans and *inadequate* or *limited* in experimental animals.

Exceptionally, agents for which the evidence of carcinogenicity is *inadequate* in humans but *sufficient* in experimental animals may be placed in this category when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans.

Agents that do not fall into any other group are also placed in this category.

An evaluation in Group 3 is not a determination of non-carcinogenicity or overall safety. It often means that further research is needed, especially when exposures are widespread or the cancer data are consistent with differing interpretations.

Group 4: The agent is probably not carcinogenic to humans.

This category is used for agents for which there is *evidence suggesting lack of carcinogenicity*

in humans and in experimental animals. In some instances, agents for which there is *inadequate evidence of carcinogenicity* in humans but *evidence suggesting lack of carcinogenicity* in experimental animals, consistently and strongly supported by a broad range of mechanistic and other relevant data, may be classified in this group.

(e) Rationale

The reasoning that the Working Group used to reach its evaluation is presented and discussed. This section integrates the major findings from studies of cancer in humans, studies of cancer in experimental animals, and mechanistic and other relevant data. It includes concise statements of the principal line(s) of argument that emerged, the conclusions of the Working Group on the strength of the evidence for each group of studies, citations to indicate which studies were pivotal to these conclusions, and an explanation of the reasoning of the Working Group in weighing data and making evaluations. When there are significant differences of scientific interpretation among Working Group Members, a brief summary of the alternative interpretations is provided, together with their scientific rationale and an indication of the relative degree of support for each alternative.

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GENERAL REMARKS

Part A of Volume 100 of the *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* considers all pharmaceutical agents that were first classified as *carcinogenic to humans (Group 1)* in Volumes 1–99.

Volume 100 – General Information

About half of the agents classified in Group 1 were last reviewed more than 20 years ago, before mechanistic studies became prominent in evaluations of carcinogenicity. In addition, more recent epidemiological studies and animal cancer bioassays have demonstrated that many cancer hazards reported in earlier studies were later observed in other organs or through different exposure scenarios. Much can be learned by updating the assessments of agents that are known to cause cancer in humans. Accordingly, IARC has selected *A Review of Human Carcinogens* to be the topic for Volume 100. It is hoped that this volume, by compiling the knowledge accumulated through several decades of cancer research, will stimulate cancer prevention activities worldwide, and will be a valued resource for future research to identify other agents suspected of causing cancer in humans.

Volume 100 was developed by six separate Working Groups:

Pharmaceuticals
Biological agents
Arsenic, metals, fibres, and dusts
Radiation
Personal habits and indoor combustions
Chemical agents and related occupations

Because the scope of Volume 100 is so broad, its *Monographs* are focused on key information. Each *Monograph* presents a description of a carcinogenic agent and how people are exposed, critical overviews of the epidemiological studies and animal cancer bioassays, and a concise review of the toxicokinetic properties of the agent, plausible mechanisms of carcinogenesis, and potentially susceptible populations, and life-stages. Details of the design and results of individual epidemiological studies and animal cancer bioassays are summarized in tables. Short tables that highlight key results appear in the printed version of Volume 100, and more extensive tables that include all studies appear on the website of the *IARC Monographs* programme (http://monographs.iarc.fr). For a few well-established associations (for example, tobacco smoke and human lung cancer), it was impractical to include all studies, even in the website tables. In those instances, the rationale for inclusion or exclusion of sets of studies is given.

Each section of Volume 100 was reviewed by a subgroup of the Working Group with appropriate subject expertise; then all sections of each *Monograph* were discussed together in a plenary session of the full Working Group. As a result, the evaluation statements and other conclusions reflect the views of the Working Group as a whole.

Volume 100 compiles information on tumour sites and mechanisms of carcinogenesis. This information will be used in two scientific publications that may be considered as annexes to this volume. One publication, *Tumour Site Concordance between Humans and Experimental Animals*, will analyse the correspondence of tumour sites among humans and different animal species. It will discuss the predictive value of different animal tumours for cancer in humans, and perhaps identify human tumour sites for which there are no good animal models. Another publication, *Mechanisms Involved in Human Carcinogenesis*, will describe mechanisms known to or likely to cause cancer in humans. Joint consideration of multiple agents that act through similar mechanisms should facilitate the development of a more comprehensive discussion of these mechanisms. Because susceptibility often has its basis in a mechanism, this could also facilitate a more confident and precise description of populations that may be susceptible to agents acting through each mechanism. This publication will also suggest biomarkers that could render future research more informative. In this way, IARC hopes that Volume 100 will serve to improve the design of future cancer studies.

Specific remarks about the review of pharmaceutical agents in this volume

The subgroups on cancer in humans recognized a number of methodological issues complicating the evaluation of some of the studies reviewed, including:

- The widespread use of combination chemotherapy regimens, making it more difficult to isolate the effect of a particular drug.
- Particularly in patients with primary cancers with longer expected survivals, subsequent treatments are commonly given after the initial therapy was not successful, making it more difficult to attribute the development of other malignancies to the initial drug(s) used.
- The variable period of follow-up in patients treated for advanced cancer, many of whom will die in a short period of time due to their primary disease. Hence, the 'real' incidence of second cancers produced by a particular treatment may be underestimated.
- Second cancers can occur either late and sporadically (such as radiation-associated sarcomas, the incidence of which may be increased by concurrent chemotherapy) or have 'peaks' in their time-to-onset (such as alkylating-agent-induced leukaemias, which tend to occur 4–7 years post-exposure with lower incidence before and after). It is therefore critical to assess whether the analytical method appropriately took these different patterns in account.
- Patients who have already developed one cancer may be more prone to the carcinogenic effects of treatment because of inherited polymorphisms in DNA-repair mechanisms or drug-metabolizing enzymes. It is therefore difficult to extrapolate the effect estimates to patients receiving these same drugs for non-malignant disorders such as autoimmune diseases. This is of particular relevance because such individuals often live for decades, and the estimates of carcinogenic potential certainly should influence the choice of treatments. And, because these patients are often treated with a series of regimens in sequence, the same issues arise

as noted above with regard to isolating the effect to a single drug or treatment. Furthermore, some of these diseases (e.g. inflammatory bowel diseases) have an increased 'background' incidence of some cancers.

- The immunosuppression resulting from drugs such as ciclosporin and azathioprine is permissive of the development of new malignancies in transplant recipients. This mechanism should be distinguished from genotoxic effects.

Some anti-neoplastic agents included in this volume have been superseded by newer drugs, and combination therapies. It is important that these new drugs and combinations be evaluated in future volumes. The older agents are included here in order to maintain the historical record of agents known to cause cancer in humans. In addition, because much is often known about the mechanisms through which pharmaceuticals act, the updated *Monographs* in this volume will provide data for subsequent analyses on tumour–site concordance, and on mechanisms involved in human carcinogenesis.

Acute myeloid leukaemia that develops in patients who had been treated with alkylating agents frequently exhibits distinctive characteristics that allow it to be distinguished from acute myeloid leukaemia induced by other agents (such as etoposide or topoisomerase II inhibitors) or that occurs spontaneously. One hallmark of alkylating-agent-induced leukaemia is that it frequently exhibits a clonal loss of either chromosome 5 or 7 or a loss of part of the long arm of one of these chromosomes.

Although the *Monographs* evaluate whether agents can pose a cancer hazard to humans, the Working Group noted that there is evidence that the potency to cause acute myeloid leukaemia varies among the anti-neoplastic agents considered in this volume. In particular, cyclophosphamide, one of the most widely used anti-neoplastic agents, presents a lower risk of leukaemia at therapeutic dose levels than other anti-neoplastic agents that act by an alkylating mechanism.

During the discussion of methoxsalen in combination with ultraviolet radiation, the Working Group noted that some vegetables naturally contain methoxsalen, and that handling such vegetables can result in exposure to methoxsalen. There have been reports of photosensitivity and skin lesions in grocery workers (especially those who frequent tanning salons) and in farmworkers, and this may reflect the possibility of hidden occupational or environmental exposure to methoxsalen in combination with ultraviolet radiation.

The information on sequential estrogen–progestogen contraceptives (classified in Group 1 in Supplement 7, IARC, 1987) is included in the *Monograph* on combined estrogen–progestogen contraceptives, and the evaluation for combined administration is applicable to sequential administration.

Estrogen–progestogen contraceptives have been available for several decades primarily in the form of an oral pill. In consequence, the epidemiological studies followed women who took these contraceptives in oral form. Estrogen–progestogen contraceptives are now being marketed also in the form of a skin patch or a vaginal insert, but it is too soon for cancer studies of these newer methods of administration to have been completed. Because estrogen–progestogen contraceptive skin patches and vaginal inserts use the same types of hormones that are present in oral contraceptives, there is a likelihood that they pose similar cancer hazards.

Although estrogen–progestogen contraceptives and estrogen–progestogen menopausal therapy are discussed in separate *Monographs*, it is important to keep in mind that they use the same types of hormones. The *Monograph* on estrogen–progestogen menopausal therapy discusses recent studies of women who have used both hormonal regimens at different stages of life, and the results are not entirely predictable from looking at the two separately. It will be important to continue to follow

these women in order to understand the effects of prolonged exposure to these exogenous hormones through different stages of life.

Separate sections of this volume are devoted to estrogen-only menopausal therapy, estrogen–progestogen menopausal therapy, combined oral contraceptives, diethylstilbestrol, and tamoxifen. This structure precludes a detailed comparison of the carcinogenicity of these different hormonal products in any single section. Such a comparison can provide information not readily apparent from separate considerations of individual products, and is provided here.

Estrogen products given with and without a progestogen have markedly different carcinogenic or anti-carcinogenic effects, and the same regimens may have markedly different effects in different organs and at different stages of women's lives. Great caution should therefore be exercised in applying observations on the carcinogenic effects of one product on one organ to its possible carcinogenic effect in another organ; and similar caution should be exercised in applying observations on the carcinogenicity of one hormonal product to another, apparently similar, product.

For example, estrogen used without a progestogen by menopausal women clearly increases the risk of endometrial cancer, but estrogen given in combination with at least 21 days of a progestogen each month or cycle does not increase this risk, and may actually reduce the risk of endometrial cancer. Another example of contrasting effects is the increased breast cancer risk associated with menopausal estrogen plus progestogen use, and a much weaker or more delayed increased risk with the use of unopposed estrogen. Unlike in the endometrium, in the breast, progestogens, in the presence of estrogens, stimulate mitotic activity, presumably by activating estrogen receptors. This provides a plausible explanation for the greater risk associated with estrogen–progestogen menopausal treatment than with estrogen treatment alone. Another possible explanation is that estrogen alone tends to be given to women who have had a hysterectomy, whereas estrogen–progestin combination therapy is recommended only for women with an intact uterus. Women who have had a hysterectomy (with or without an oophorectomy) have lower levels of endogenous ovarian hormones than women who have not, and are at lower risk of breast cancer based on very low levels of endogenous estrogens and progestogens.

In epidemiological studies, estimates of relative risk are generally used as measures of the statistical association between an exposure and risk of a disease. Relative risks do not indicate absolute increases or decreases in risk. If the underlying risk in the absence of the exposure of interest is small, then even large relative risks will not indicate a large absolute increase in risk. For example, although the relative risk of breast cancer is increased in current and recent users of oral contraceptives, who tend to be young and therefore at low risk of breast cancer, the absolute increase in risk is very small.

Some *Monographs* in this volume conclude that an agent causes one type of cancer while reducing the risk of another. This does not constitute a recommendation for use in cancer prevention, and it is outside the scope of the *Monographs* to offer medical advice.

Phenacetin, etoposide, and plants containing aristolochic acid

Although the intent of Volume 100 is not to identify new carcinogenic agents, the Working Group was mindful of the statement in the Preamble that "When the available epidemiological studies

pertain to a mixture, process, occupation or industry, the Working Group seeks to identify the specific agent considered most likely to be responsible for any excess risk [part B, section 6(a)]."

During discussion of analgesic mixtures containing phenacetin, the Working Group concluded that phenacetin itself (previously classified in Group 2A in Supplement 7, IARC, 1987) should now be classified in Group 1, noting that the other components of analgesic mixtures containing phenacetin (namely phenazone, aspirin, codeine phosphate, and caffeine) could not explain the increased risks of cancers of the renal pelvis and the ureter.

When reviewing antineoplastic drugs, the Working Group noted that acute myeloid leukaemia induced by alkylating agents, such as busulfan, frequently exhibits clonal loss (partial or total) of either chromosome 5 or 7, thereby distinguishing it from acute myeloid leukaemia induced by topoisomerase II inhibitors, such as etoposide. The latter shows clonal balanced translocations involving the *MLL* gene on chromosome 11 (11q23). Following this line of reasoning, the Working Group classified etoposide itself in Group 1 (previously classified in Group 1 in combination with cisplatin and bleomycin, IARC, 2000).

For different reasons, during discussion of plants of the genus *Aristolochia*, the Working Group concluded that aristolochic acid (previously classified in Group 2A in Volume 82, IARC, 2002) should now be classified in Group 1 based on strong evidence that aristolochic acid-specific DNA adducts and *TP*53 transversions have been found in humans who ingested material from these plant species.

As a result, these three chemical agents have been added to the list of carcinogens classified in Group 1.

The latter evaluation of aristolochic acid in Group 1 shows the promise that mechanistic studies can bring to the identification of carcinogenic hazards. Every reference cited in the section on mechanistic and other relevant data was published after the 2002 *Monograph* on plants of the genus *Aristolochia*. In only six years, these studies were able to convincingly demonstrate that aristolochic acid is the specific agent responsible for the high risk of cancers of the renal pelvis and ureter in people who ingested material from these plant species. It is encouraging to think that other environmental or occupational cancer clusters might be investigated with such speed and resolved with similar confidence.

A summary of the findings of this volume appears in *The Lancet Oncology* (Grosse *et al.*, 2009).

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BUSULFAN

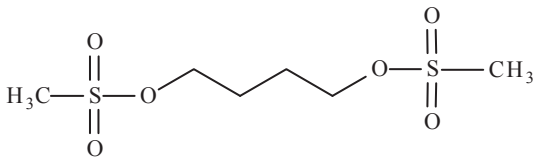
Busulfan was considered by previous IARC Working Groups in 1973 and 1987 (IARC, 1974, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 55-98-1
Chem. Abstr. Name: 1,4-Butanediol, 1,4-dimethanesulfonate-
IUPAC Systematic Name: 4-Methylsulfonyloxybutyl methanesulfonate
Synonyms: 1,4-Bis(methanesulfonoxy)butane; 1,4-bis(methanesulfonyloxy)butane; 1,4-butanediol dimethanesulfonate; 1,4-butanediol dimesylate; busulphan; Busilvex; Busulfex; 1,4-dimethanesulfonoxybutane; 1,4-dimethylsulfonyloxybutane; Myleran
Description: White, crystalline powder (Sweetman, 2008)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_6H_{14}O_6S_2$
Relative molecular mass: 246.3

1.2 Use of the agent

Busulfan is an antineoplastic agent with a cell-cycle nonspecific alkylating action (unlike that of the nitrogen mustards) that has a selective depressant action on the bone marrow. In small doses, it depresses granulocytopoiesis and to a lesser extent thrombocytopoiesis, but has little effect on lymphocytes. With larger doses, severe bone-marrow depression eventually ensues. Information for Section 1.2 is taken from McEvoy (2007), Royal Pharmaceutical Society of Great Britain (2007), Thomson Healthcare (2008), and Sweetman (2008).

1.2.1 Indications

Busulfan is indicated for the palliative treatment of chronic myelogenous (myeloid, myelocytic, granulocytic) leukaemia. It provides symptomatic relief with a reduction in spleen size and a general feeling of well-being. The fall in leukocyte count is usually accompanied by a rise in the haemoglobin concentration. Permanent remission is not induced, and resistance to its beneficial effects gradually develops.

Busulfan has been used in patients with *polycythaemia vera*, and in some patients with myelofibrosis and primary thrombocythaemia.

It has also been used at high doses, orally, and more recently, intravenously, as part of a conditioning regimen to prepare patients for stem-cell transplantation.

1.2.2 Dosage

Busulfan is typically administered orally. The usual adult dose range for remission induction is 4–8 mg, total dose, daily. Dosing on a weight basis is the same for both paediatric and adult patients, approximately 60 µg/kg of body weight or 1.8 mg/m^2 of body surface, daily. This initial dose is continued until the white blood cell count has fallen to between 15000 and 25000 cells/mm^3 (typically 12–20 weeks). Alternatively, the dose of busulfan is halved as the white blood cell count halves. It is stopped earlier if the platelet count falls below 100000 cells/mm^3. Higher doses can be given if the response after 3 weeks is inadequate.

In patients with *polycythaemia vera*, the usual dose is 4–6 mg daily orally, continued for 4–6 weeks with careful monitoring of blood counts. Further courses are given when relapse occurs; alternatively, maintenance therapy may be given at half the dose required for induction. Doses of 2–4 mg daily have been given for essential thrombocythaemia or myelofibrosis.

In conditioning regimens for stem-cell transplantation, busulfan has been given in oral doses of 3.5–4 mg/kg daily in divided doses for 4 days (total dose 14–16 mg/kg), with cyclophosphamide or melphalan, for ablation of the recipient's bone marrow; most centres now use intravenous preparations of busulfan as part of the conditioning regimens. When given by intravenous infusion in a regimen with phenytoin, the usual dose is 0.8 mg/kg ideal body weight every 6 hours for 4 days (total dose, 12.8 mg/kg).

Busulfan is available as a tablet containing 2 mg busulfan for oral administration, and as an injection concentrate for intravenous infusion containing 6 mg/mL (60 mg) busulfan for parenteral administration.

1.2.3 Trends in use

Randomized trials have shown a small survival advantage with treatment with hydroxyurea compared with busulfan, and a major survival advantage with imatinib mesylate. Hence, treatment with busulfan is now used less frequently for patients with chronic myeloid leukaemia.

2. Cancer in Humans

Case reports of chronic myeloid leukaemia patients treated with busulfan who developed various cytological abnormalities have been described, with a few reporting the development of carcinoma (Diamond *et al.*, 1960; Waller, 1960; Gureli *et al.*, 1963; Feingold & Koss, 1969; Becher & Prescher, 1988; Storti *et al.*, 1990; Foa *et al.*, 1993). [The Working Group noted that most patients with chronic myeloid leukaemia are older, and therefore might have been at risk of developing solid tumours independently of exposure to busulfan.]

Long-term follow-up of patients with bronchial carcinoma who were randomized to chemotherapy after pulmonary resection showed that of 69 patients who had been given busulfan and had survived 5 years, four developed acute myeloid leukaemia and a further 15 developed pancytopenia in the following 4 years (Stott *et al.*, 1977). The increase in risk was not related to dose. Among 148 other survivors at 5 years who had not been given busulfan, one case of pancytopenia appeared. No cases of acute myeloid leukaemia developed in patients who received cyclophosphamide. The cases were confined to those who had not received radiation or other cytotoxic agents (Stott *et al.*, 1977).

Patients with myeloproliferative disorders such as *polycythaemia vera* and essential thrombocytosis can potentially develop acute myeloid leukaemia or myelodysplastic syndromes even without specific treatment. The long natural

history of these diseases and the use of a variety of different therapies during an individual patient's clinical course make it difficult to precisely estimate the frequency of conversion to acute myeloid leukaemia, and the contribution of specific treatments. Randomized trials and long-term follow-up cohort studies have shown that this transformation rate is substantially increased in patients treated with alkylating agents that include busulfan and chlorambucil, and radioactive inorganic phosphate (Berk *et al.*, 1981).

In the most recent and largest of such studies, acute myeloid leukaemia developed in 1.3% of 1638 patients with *polycythaemia vera*, with multivariable analyses demonstrating that treatment with busulfan was associated with higher rates of acute myeloid leukaemia (hazard ratio, 8.6 compared to no treatment or treatment with phlebotomy-alone or interferon). A total of 86% of these patients had been followed for < 10 years (Finazzi *et al.*, 2005). A smaller study in 114 patients with essential thrombocytosis showed that three of 14 patients treated with busulfan followed by hydroxyurea developed acute myeloid leukaemia/myelodysplastic syndromes; one additional case was treated with hydroxyurea alone (Finazzi *et al.*, 2000).

3. Cancer in Experimental Animals

Intraperitoneal administration of busulfan to mice did not increase the incidence of tumours in two studies (Stoner *et al.*, 1973; IARC, 1974), but induced lymphomas in two others (Robin *et al.*, 1981; Turton *et al.*, 2006). In a study, intravenous administration of busulfan to mice significantly increased the incidences of thymic and ovarian tumours (Conklin *et al.*, 1965; IARC, 1974). In another study, busulfan in conjunction with X-rays further increased the incidence of thymic lymphomas (IARC, 1974). [The Working Group noted that the increased incidences of thymic lymphomas and ovarian tumours are difficult to interpret in the mouse model.]

Intravenous administration of busulfan to rats for 1 year was reported to induce a variety of tumours in male rats, but the experiments could not be evaluated due to incomplete reporting (Schmähl, 1975; IARC, 1987a). Maternal exposure to busulfan induced uterine adenocarcinoma in the offspring of rats treated with *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (Yoshida *et al.*, 2005).

See Table 3.1.

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

In humans, upon oral administration, busulfan is readily absorbed from the gastrointestinal tract, binds rapidly to plasma proteins (e.g. albumin) and red blood cells, and rapidly disappears from the blood (GlaxoSmithKline, 2004; Sweetman, 2005; Thomson Healthcare, 2008). Busulfan is reported to have a half-life of 2–3 hours (Sweetman, 2005; Thomson Healthcare, 2008). In the liver, it rapidly undergoes both enzymatic and non-enzymatic transformations, primarily through glutathione-mediated processes, to less active, sulfur-containing metabolites (Thomson Healthcare, 2008). Twelve metabolites have been isolated including methanesulfonic acid and 3-hydroxytetrahydrothiophene-1,1-dioxide, two major urinary metabolites (Bishop & Wassom, 1986; GlaxoSmithKline, 2004). In spite of its rapid clearance from the blood and its extensive metabolism, radiolabelled busulfan is excreted relatively slowly, with 25–60% of the radioactivity being excreted, primarily as metabolites, within 48 hours after dosing (McEvoy, 1987; GlaxoSmithKline, 2004).

Table 3.1 Studies of cancer in experimental animals exposed to busulfan (intraperitoneal exposure)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, A/He (M, F) 24 wk Stoner *et al.* (1973)	0, 1.2, 3.0, 6.0 g/kg bw (total dose) 24 times for 8 wk 30/30, 10/10, 10/10, 10/10	Lung: 14/58, 4/18, 1/16, 3/17	NS	85–99% pure
Mouse, BALB/c, XAF1 (M) 50 wk Robin *et al.* (1981)	0, 0.5 mg 4 times over 6 wk 45, 45	Lymphomas: 0/41, 4/35	[$P = 0.0408$]	
Mouse, BALB/c (F) 127 d Turton *et al.* (2006)	0, 8.25, 9.0, 9.75 mg/kg bw 10 times over 21 d 65, 64, 65, 65	Lymphomas: 0/15, 4/14, 1/16, 0/11 (at Day 127 post-dosing)	$P < 0.05$ (8.25 mg/kg)	Mortality: 0/65, 2/64, 3/65, 8/65 [Study not designed as a carcinogenicity study]
Rat, Donryu (F) 15 mo Yoshida *et al.* (2005)	0, 2.5, 5.0 mg/kg bw single oral dose to pregnant females on Day 14 of gestation; 20 mg/kg bw single dose ENNG in uterine horn to offspring at 11 wk Pregnant females: 10, 10, 10; offspring: 27, 24, 24	Uterine adenocarcinomas: 4/16, 6/18, 14/26	$P < 0.05$ (5.0 mg/kg)	

bw, body weight; d, day or days; ENNG, *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine; F, female; M, male; mo, month or months; NS, not significant; wk, week or weeks

4.2 Genotoxic effects

Busulfan is a direct-acting bifunctional alkylating agent that binds to cellular macromolecules including DNA, RNA, and proteins. Consequently, it is capable of producing monoadducts, intrastrand cross-links, and DNA–protein cross-links (Iwamoto *et al.*, 2004; Morales-Ramírez *et al.*, 2006) that are believed to play an important role in its toxic and carcinogenic effects (Bishop & Wassom, 1986; Sanderson & Shield, 1996). Busulfan exhibits an interesting, but poorly understood, selective toxicity for early myeloid precursor cells (Guest & Uetrecht, 2000; Kufe *et al.*, 2003).

Busulfan has been tested for genotoxicity in a variety of assays, both *in vitro* and *in vivo* (Bishop & Wassom, 1986; IARC, 1987b). In-vivo treatment of rodents with busulfan induced dominant lethal mutations, and increased the frequency of chromosomal aberrations or micronuclei in bone-marrow, intestine, embryonic liver, and germ cells (Bishop & Wassom, 1986; IARC, 1987b). In the mouse-specific locus test, increases in mutations were seen in postspermatogonial germ cells (spermatozoa and spermatids), but not in spermatogonia (Ehling & Neuhäuser-Klaus, 1991). Evidence of covalent binding to DNA, RNA, and proteins was also obtained in mice treated *in vivo*. Busulfan induced chromosomal aberrations, sister chromatid exchange, and mutations in human and rodent cells treated *in vitro*. It also induced sex-linked recessive lethal mutations in *Drosophila*, and was mutagenic to bacteria (Bishop & Wassom, 1986; IARC, 1987b).

Patients treated with busulfan for chronic myeloid leukaemia were found to have increased frequencies of sister chromatid exchange and chromosomal aberrations in their peripheral blood lymphocytes (Honeycombe, 1981; Bishop & Wassom, 1986). Haematotoxicity and immunosuppression have also been reported in patients treated with this agent (Bishop & Wassom, 1986).

4.3 Mechanisms of carcinogenesis

Acute myeloid leukaemia that develops in patients who have previously been treated with alkylating agents, such as busulfan, frequently exhibits distinctive characteristics that allow it to be distinguished from acute myeloid leukaemia induced by other agents (such as DNA-topoisomerase II inhibitors) or acute myeloid leukaemia that occurs spontaneously (Pedersen-Bjergaard & Rowley, 1994; Jaffe *et al.*, 2001; Mauritzson *et al.*, 2002; Pedersen-Bjergaard *et al.*, 2006). One of the hallmarks of leukaemias induced by alkylating agents is that they frequently exhibit a clonal loss of either chromosome 5 or 7 (–5, –7) or a loss of part of the long arm of one of these chromosomes (5q–, 7q–). For example, a deletion within the long arm of chromosome 5 involving the bands q23 to q32 is often seen (Jaffe *et al.*, 2001). Leukaemias that have developed in patients treated with busulfan (often in combination with other agents) frequently exhibit these clonal chromosomal changes (Mauritzson *et al.*, 2002).

In addition, mutations in *TP53* are frequently seen in leukaemias with the –5/5q– karyotype, and mutations involving the *AML1* gene as well as mutations in *TP53* and *RAS* are often seen in a subset of leukaemias that exhibit the –7/7q– karyotype (Christiansen *et al.*, 2001, 2005; Pedersen-Bjergaard *et al.*, 2006). These treatment-related acute myeloid leukaemias also frequently exhibit increased methylation of the *p15* promoter (Pedersen-Bjergaard *et al.*, 2006). Although the evidence that busulfan directly induces losses or deletions affecting chromosomes 5 or 7 is limited, this drug has been reported to induce similar types of chromosomal alterations and deletions in a variety of experimental models (see description above), and in the lymphocytes of treated patients (Honeycombe, 1981; Bishop & Wassom, 1986). The detection of elevated levels of chromosomal aberrations in the peripheral blood lymphocytes of patients treated with busulfan is of particular note, as multiple prospective studies have now shown that individuals with increased levels of chromosomal aberrations in these cells are at increased risk of developing cancer (Hagmar *et al.*, 1998, 2004; Liou *et al.*, 1999; Smerhovsky *et al.*, 2001; Boffetta *et al.*, 2007).

4.4 Synthesis

Busulfan is a direct-acting alkylating agent that is carcinogenic via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of busulfan. Busulfan causes acute myeloid leukaemia.

There is *limited evidence* in experimental animals for the carcinogenicity of busulfan.

Busulfan is *carcinogenic to humans (Group 1).*

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CHLORAMBUCIL

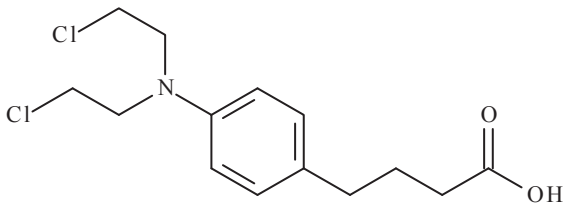
Chlorambucil was considered by previous IARC Working Groups in 1980 and 1987 (IARC, 1981a, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 305-03-3
Chem. Abstr. Name: Benzenebutanoic acid, 4-[bis(2-chloroethyl)amino]-
IUPAC Systematic Name: 4-[4-[Bis(2-chloroethyl)amino]phenyl]butanoic acid
Synonyms: 4-[Bis(2-chloroethyl)amino] benzenebutanoic acid; 4-[*p*-[bis(2-chloroethyl)amino]phenyl]butyric acid; γ-[*p*-bis(2-chloroethyl)aminophenyl] butyric acid; chloraminophene; γ-[*p*-di(2-chloroethyl)-aminophenyl]butyric acid; Leukeran
Description: Flattened needles (O'Neil, 2006); white crystalline powder (European Pharmacopoeia, 1997)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_{14}H_{19}Cl_2NO_2$
Relative molecular mass: 304.2

1.2 Use of the agent

Chlorambucil is an antineoplastic agent derived from chlormethine, and has a similar mode of action. It acts on lymphocytes and to a lesser extent on neutrophils and platelets. Chlorambucil is most effective in those conditions associated with the proliferation of white blood cells, especially lymphocytes. Although formerly widely used in the management of *polycythaemia vera*, it has largely been superseded. Information for Section 1.2 is taken from McEvoy, (2007), Royal Pharmaceutical Society of Great Britain (2007), Thomson Healthcare (2007), and Sweetman (2008).

1.2.1 Indications

Chlorambucil is used in the treatment of chronic lymphocytic leukaemia, Waldenström macroglobulinaemia, indolent non-Hodgkin lymphoma, and in combination with other drugs in patients with Hodgkin lymphoma. Chlorambucil was previously indicated for *polycythaemia vera*.

1.2.2 Dosage

When used as a single-agent antineoplastic drug for the treatment of chronic lymphocytic leukaemia, Waldenström macroglobulinaemia,

and lymphomas, chlorambucil is given orally at initial doses of 100–200 μg/kg body weight daily (usually 4–10 mg once daily), for 3–8 weeks until leukopenia occurs, then reduced to 2–8 mg daily. Lower doses may be given as part of a combination regimen. Alternatively, higher doses of chlorambucil may be given intermittently. For example, in chronic lymphocytic leukaemia, it may be given in an initial single dose of 0.6–1.0 mg/kg body weight, increased by 0.1–0.2 mg/kg body weight at 4-week intervals until control of lymphocytosis and adenopathy is achieved or toxicity occurs. When maximal response is achieved, treatment with chlorambucil is generally stopped rather than continued at lower dose for maintenance therapy. Depending on the magnitude and duration of response, chlorambucil may be restarted when progressive disease is apparent.

Chlorambucil is available as 2 mg tablets.

1.2.3 Trends in use

Chlorambucil is still commonly used as the initial treatment of chronic lymphocytic leukaemia, particularly in older patients. The use of chlorambucil in patients with rheumatoid arthritis has decreased substantially in favour of other immunosuppressive treatments.

2. Cancer in Humans

Many case reports and a few earlier small epidemiological studies of malignancy after therapy with chlorambucil have been described among patients treated for breast cancer, juvenile arthritis, glomerulonephritis, and ovarian cancer (IARC, 1981a, 1987a; Greene *et al.*, 1982; Patapanian *et al.*, 1988; Jones *et al.*, 1996; Asten *et al.*, 1999). [The Working Group noted that though in each study an excess of subsequent malignancy, especially acute myeloid leukaemia is suggested, these reports are difficult to interpret because the cases are few or because they also received radiation or other putative carcinogens.]

More recent studies are presented below.

2.1 Cancers following treatment for various diseases

In a randomized therapy trial of 431 patients with *polycythaemia vera* (Berk *et al.*, 1981), a significant increase in the incidence of acute myeloid leukaemia occurred in patients treated with chlorambucil when compared to phlebotomy or radiotherapy. The excess of acute myeloid leukaemia incidence declined after the first decade after treatment (Najean *et al.*, 1994).

A case–control study compared the relative risk of leukaemia in patients treated with chemotherapy or radiation with patients who only underwent surgery (Kaldor *et al.*, 1990). Approximately 114 cases of leukaemia were identified among 99113 patients with ovarian cancer. All of the alkylating agents assessed, including chlorambucil, cyclophosphamide, thiotepa, treosulfan and melphalan, increased the risk of developing leukaemia. The relative risks attributed to chlorambucil monotherapy were 14 and 23 in the lower and higher dose groups, respectively.

A retrospective analysis compared patients with advanced rheumatoid arthritis treated for a median time of 2 years with either chlorambucil (n = 39) or the antimetabolite 6-mercaptopurine (n = 30) (Patapanian *et al.*, 1988). An increase in the number of skin cancers was observed in the chlorambucil recipients compared to the 6-mercaptopurine recipients that included melanoma and squamous cell carcinoma (8 versus 1, respectively), as well as an increase in acute myeloid leukaemia/myelodysplastic syndromes (AML/MDS) (3 versus 0, respectively).

Some reports have suggested an increased rate of AML/MDS and other cancers in patients with Hodgkin lymphoma treated with variants of the MOPP regimen that include chlorambucil (chlorambucil, vincristine, procarbazine, prednisone [ChlVPP]) (Selby *et al.*, 1990; Swerdlow *et al.*, 1992). However, it is difficult to determine the unique contribution of chlorambucil compared to the effects of the other drugs and the

radiation therapy, which many of these patients also received.

2.2 Cancers following treatment for chronic lymphocytic leukaemia

2.2.1 AML/MDS

The French Cooperative Group on Chronic Lymphocytic Leukaemia conducted two large successive trials that randomized 1535 patients with early-stage chronic lymphocytic leukaemia to observation until disease progression or initial treatment with chlorambucil (Dighiero *et al.*, 1998). Four cases of AML/MDS were reported in the treatment group, and two in the observation group. No information was provided about the nature of treatment these two patients might have received before the onset of acute myeloid leukaemia (no relative risk available). Another retrospective analysis included 389 patients, approximately half of whom were observed, and the others treated with prolonged courses of chlorambucil including maintenance treatment. Four cases of AML/MDS were noted, all in the chlorambucil-treated patients (Callea *et al.*, 2006). [The Working Group noted that all of these patients had also received fludarabine in combination with cyclophosphamide for progressive disease as well.]

In another study assessing initial treatment with chlorambucil compared with fludarabine or the combination of the two drugs, AML/MDS was seen in none of 191 patients treated with chlorambucil alone compared to 1/188 of fludarabine recipients, and 5/142 patients receiving the combination (Morrison *et al.*, 2002). Overall, there does not appear to be a significant increase in AML/MDS in patients with chronic lymphocytic leukaemia treated with chlorambucil alone despite prolonged exposure, older patient age, and long-term patient survival permitting adequate patient follow-up. [The Working Group noted however that because of the number of patients in these studies, it is not possible to exclude a small effect on the relative risk of AML/MDS.]

2.2.2 Epithelial cancers

The initial report of the first large aforementioned observation study suggested an increase in the incidence of epithelial cancers [not further defined] in the chlorambucil recipients (The French Cooperative Group on Chronic Lymphocytic Leukemia, 1990). In contrast, the aggregate data from the two trials mentioned above (Dighiero *et al.*, 1998) and from another observational trial (Cellai *et al.*, 2001) showed no difference between the treated and observation groups, both in the total number of cancers, and in the incidence of skin and lung cancers.

3. Cancer in Experimental Animals

Chlorambucil has been tested for carcinogenicity in mice and rats by intraperitoneal injection, and in male and female mice and female rats by gavage.

Chlorambucil increased the incidence and multiplicity of tumours of the lung and the incidence of tumours of the haematopoietic system in mice (Shimkin *et al.*, 1966; Weisburger *et al.*, 1975; IARC, 1981b), haematopoietic tumours in male rats, and haematopoietic tumours and lymphomas in female rats and mice (Weisburger *et al.*, 1975; IARC, 1981b; Berger *et al.*, 1985; Cavaliere *et al.*, 1990). It induced lung tumours in male and female mice, and mammary gland tumours in female rats and mice (Berger *et al.*, 1985; Cavaliere *et al.*, 1990). It also produced nervous system tumours in rats (Berger *et al.*, 1985).

It had an initiating effect in a two-stage skin carcinogenesis experiment in mice (Salaman & Roe, 1956; IARC, 1981b).

See Table 3.1.

Table 3.1 Studies of cancer in experimental animals exposed to chlorambucil

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, S (NR) 22 wk Salaman & Roe (1956)	Skin 0, 2.7 mg total dose in methanol, administered by skin painting once weekly for 10 wk; croton oil used as promoter (from Week 5 to Week 22) 20 (control), 25	Skin (papillomas): 0/17, 11/19 (58%)	 $P < 0.01$	Purity NR
Mouse, A/J (M, F) 39 wk Shimkin et al. (1966)	i.p. 0, 9.6, 37, 150, 420 mg/kg bw (total dose), 3 ×/wk for 4 wk 45, 60, 60, 60, 173 (male control), 157 (female control)	Lung (adenomas and adenocarcinomas): 43% (0.53 tumours/mouse); 32% (0.42 tumours/mouse); 18/38 (47%, 0.6 tumours/mouse); 48/56 (86%, 1.6 tumours/mouse); 45/47 (96%, 5.1 tumours/mouse); 30/30 (100%, 8.9 tumours/mouse)	 [$P < 0.001$] [$P < 0.001$] [$P < 0.001$]	Purity NR
Mouse, Swiss-Webster (M, F) 15 mo Weisburger et al. (1975)	i.p. 3 mg/kg bw (MTD) or 1.5 mg/kg bw, 3 ×/wk for 6 mo 25/sex/group	Lung: M–22/35 F–20/28 (controls: 10/101 M, 21/153 F) Lymphoma-myeloid leukaemia: M–6/35 F–4/28 (controls: 3/101 M, 3/153 F) Ovary: F–10/28 (controls: 6/153 F)	 $P < 0.001$, $P < 0.001$ $P = 0.004$, $P = 0.012$ $P < 0.001$	Results reported had been combined for the two doses
Rat, Sprague-Dawley (M, F) 15 mo Weisburger et al. (1975)	i.p. 4.5 mg/kg bw (MTD) or 2.2 mg/kg bw, 3 ×/wk for 6 mo 25/sex/group	Haematopoietic/lymphatic system: M–8/33 (control: 2/179 M, 1/181 F)	 $P < 0.001$	Results reported had been combined for the two doses

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague-Dawley (F) Lifetime (3 yr) Berger et al. (1985)	Oral 0, 3, 6, 13.5, 27 mg/kg bw per mo by gavage for 18 mo 30/group, 120 controls	Mammary gland (malignant): 8/120, 2/30, 4/30, 10/30, 5/30 Central & peripheral nervous tissue (malignant): 2/120, 2/30, 1/30, 3/30, 3/30 Haematopoietic & lymphatic tissue: 1/120, 0/30, 4/30, 0/30, 0/30 External auditory canal (malignant): 0/120, 2/30, 0/30, 0/30, 3/30	[$P < 0.001$] (27 mg/kg) [$P < 0.05$] (13.5 and 27 mg/kg) [$P < 0.05$] (6 mg/kg)	Purity > 99% The dose of 3 mg/kg bw was kept constant over all treatment groups. To increase the dose, the frequency of administrations was increased
Mouse, BALB/c (M, F) Lifetime (2 yr) Cavaliere et al. (1990)	Oral 0 or 1 mg/kg bw by gavage 5 ×/wk for 12 wk 53 males, 54 females, 50 (male control), 50 (female control)	Lymphoreticular system: 5/50, 4/50, 7/53, 24/53 Lung (adenomas): 19/50, 7/50, 47/53, 46/54 Mammary gland: 0/50, 2/50, 0/53, 4/54	$P < 0.01$ (F) $P < 0.001$ (M, F) $P < 0.05$ (F)	Purity > 99% Survival was reduced in treated animals of both sexes ($P < 0.001$)

bw, body weight; d, day or days; F, female; i.p., intraperitoneal; M, male; mo, month or months; MTD, maximum tolerated dose; NR, not reported; wk, week or weeks; yr, year or years

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

Chlorambucil is rapidly absorbed following administration to animals as a solution or as an emulsion (Newell *et al.*, 1981; Ganta *et al.*, 2008). It is a highly lipophilic drug but is also a weak acid and can therefore be taken up into cells by passive diffusion. The weak acidic function is ionized to a lesser extent at acidic pH, and therefore favours drug uptake into the relatively neutral intracellular compartment (Parkins *et al.*, 1996; Kozin *et al.*, 2001). Drug accumulation by chronic lymphocytic leukaemia lymphocytes peaks at 30 seconds, while efflux from cells loaded with chlorambucil is almost complete within 30 seconds (Bank *et al.*, 1989). The extracellular pH of tumour tissue is significantly lower than the extracellular pH of normal tissue, and it is expected that this extracellular acidity may enhance the intracellular uptake of chlorambucil by increasing the amount of the free acid (Kozin *et al.*, 2001; Gerweck *et al.*, 2006).

Once inside the cell, chlorambucil reacts as a bifunctional alkylating agent, with common reaction sites including the N^7 position of guanine or adenine, the N^3 position of adenine (the predominant binding site being the N^7 of guanine), and thiol groups of proteins and peptides (Bank, 1992; Barnouin *et al.*, 1998). Reaction with thiol groups of glutathione may lead to export of the conjugate by multidrug resistance proteins, potentially reducing cellular effects (Barnouin *et al.*, 1998). Overexpression of cytosolic glutathione *S*-transferase in some cells leads to increased conjugation to glutathione and consequent removal from the cell, contributing to the resistance of these cells to the effects of chlorambucil (Zhang & Lou, 2003; Zhang *et al.*, 2004). Some cells are also able to cause extensive metabolism of chlorambucil to phenylacetic acid mustard, which is also excreted, again contributing to resistance (Alberts *et al.*, 1980).

4.2 Genotoxic effects

4.2.1 Induction of DNA damage

Reaction of one of the two chloroethyl groups of chlorambucil with the N^7 position of guanine or adenine of double-stranded DNA leads to the formation of mono-adducts. These are repaired rapidly in an error-free fashion by methylguanine methyltransferase (sometimes called alkylguanine alkyltransferase). However, some cells lack this repair activity, usually because of silencing of the corresponding gene, and the unrepaired DNA mono-adduct then forms a complex with mismatch-repair enzymes. The subsequent inhibition of DNA replication can eventually induce DNA breakage (Caporali *et al.*, 2004). The second chloroethyl group of the DNA mono-adduct with chlorambucil can interact with proteins (Loeber *et al.*, 2008) but more importantly, because of its juxtaposition to other bases in the major groove of DNA, it can react with a DNA base to form an interstrand DNA cross-link. This DNA cross-link complex is quite stable (Jiang *et al.*, 1989; Loeber *et al.*, 2008), and its repair requires nucleotide excision repair factors (such as xeroderma pigmentosum complementation group F-excison repair cross-complementing rodent repair deficiency, complementation group, 1–XPF-ERCC1) that act slowly by homologous recombination (Drabløs *et al.*, 2004). The DNA cross-link attracts several binding proteins, probably the BRCA1 and BRCA2 proteins, Fanconi anaemia gene product, and Nijmegen breakage syndrome gene product to form a complex (Wilson *et al.*, 2001). As shown in cultured HeLa cells, addition of chlorambucil prolongs S-phase and induces a corresponding mitotic delay. The magnitude of these effects correlates with the level of DNA cross-links. Treatment of cells in the G_2-phase of the cell cycle does not induce mitotic delay but

does inhibit DNA synthesis in the subsequent cell cycle, and causes a delay in the next mitosis, suggesting that at least some lesions induced by chlorambucil are long-lasting (Roberts, 1975).

4.2.2 Mutational consequences of DNA damage

Chlorambucil has been tested for genotoxicity in several short-term assays *in vitro* and *in vivo*. It has been shown to be mutagenic in bacteria after metabolic activation, to cause gene conversion in yeast, sex-linked recessive mutations in *Drosophila*, mutations in Chinese hamster ovary cells, and clastogenic effects in human lymphocytes *in vitro*, and in animals *in vivo* (IARC, 1987b).

The mutagenicity of chlorambucil has been reported to be related to its ability to form DNA cross-links as well as to transfer an alkyl group to form DNA mono-adducts (Sanderson & Shield, 1996), suggesting that lesions responsible for S-phase and mitotic delays are also responsible for mutagenicity, probably as a consequence of unrepaired DNA damage persisting after DNA replication (Shi & King, 2005). Such mechanisms may involve changes to chromosomes, consistent with observations that chlorambucil can induce sister chromatid exchange, chromosomal aberrations (Speit *et al.*, 1992), and micronuclei (Ashby & Tinwell, 1993; Yaghi *et al.*, 1998). The ability of chlorambucil to induce aneuploidy (Efthimiou *et al.*, 2007) may contribute to its carcinogenicity.

Exposure to chlorambucil increases the frequency of micronucleus induction and chromosomal aberrations in rat bone marrow and spleen *in vivo* (Moore *et al.*, 1995), of mutations at the hypoxanthine-(guanine) phosphoribosyl transferase (*Hprt)* locus in Chinese hamster V79 cells (Speit *et al.*, 1992), of deletions in Chinese hamster (CHO)-AS52 cells (Yaghi *et al.*, 1998), and of gene deletions and translocations in mouse spermatids *in vivo* (Russell *et al.*, 1989; Rinchik *et al.*, 1990).

4.3 Synthesis

Chlorambucil is a direct-acting alkylating agent that is carcinogenic via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of chlorambucil. Chlorambucil causes acute myeloid leukaemia.

There is *sufficient evidence* in experimental animals for the carcinogenicity of chlorambucil.

Chlorambucil is *carcinogenic to humans (Group 1).*

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METHYL-CCNU

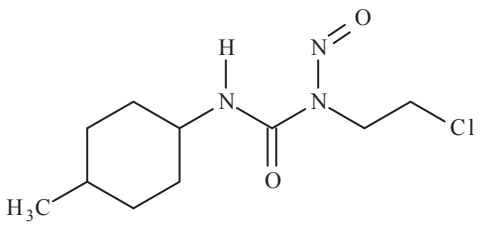
Methyl-CCNU was considered by previous IARC Working Groups in 1980 and 1987 (IARC, 1981, 1987). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 13909-09-6
Chem. Abstr. Name: Urea, *N*-(2-chloroethyl)-*N′*-(4-methylcyclohexyl)-*N*-nitroso-
IUPAC Systematic Name: 1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea
Synonyms: 1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea; 1-(2-chloroethyl)-3-(4-methylcyclohexyl) nitrosourea; methyl-CCNU; *N′*-(4-methylcyclohexyl)-*N*-(2-chloroethyl)-*N*-nitrosourea; semustine
Description: Light yellow powder (NTP, 2005)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_{10}H_{18}ClN_3O_2$
Relative molecular mass: 247.7

1.2 Use of the agent

1.2.1 Indications

Methyl-CCNU is an alkylating agent used alone or in combination with other chemotherapeutic agents to treat several types of cancers, including primary and metastatic brain tumours, Lewis lung tumour, and L1210 leukaemia. It has also been used to treat cancers of the digestive tract, Hodgkin lymphoma, malignant melanoma, and epidermoid carcinoma of the lung (NCI, 1979; US National Institutes of Health, 2000; NTP, 2005).

1.2.2 Dosage

Doses varied depending on the type of cancer and body weight of the individual. The typical oral dose was 125–200 mg/m^2 body surface area, and was repeated every 6 weeks (NTP, 2005). An alternative regimen was reported to be 200–225 mg/m^2 orally every 6–8 weeks (NCI, 1979).

Methyl-CCNU was available as 10, 50, and 100 mg capsules (NCI, 1979).

1.2.3 Trends in use

Methyl-CCNU was used in investigational studies in the 1960s and 1970s; it has never been approved as an antineoplastic drug, and is not listed in any of the standard pharmaceutical references and sources. However, some recently published articles indicate that it has been used in the People's Republic of China to treat various haematopoietic malignancies (Jia *et al.*, 2006; Zhang *et al.*, 2007; Guo *et al.*, 2008).

2. Cancer in Humans

The previous evaluation was based on five studies from the same set of patients described below. No new data were available to the Working Group.

Adjuvant treatment with methyl-CCNU was evaluated in 3633 patients with gastrointestinal cancer treated in nine randomized trials. Among 2067 patients treated with methyl-CCNU, 14 cases of acute myeloid leukaemia occurred, whereas one occurred among 1566 patients treated with other therapies (relative risk, 12.4; 95% confidence interval: 1.7–250). Cumulative (actuarial) risk was 4% at 6 years, and was not affected by concomitant radiotherapy or immunotherapy (Boice *et al.*, 1983). A subsequent report described a strong dose–response relationship, adjusted for survival time, giving a relative risk of almost 40 among patients who had received the highest dose (Boice *et al.*, 1986).

3. Cancer in Experimental Animals

Data on methyl-CCNU were included in a report in which a large number of cancer chemotherapeutic agents were tested for carcinogenicity by intraperitoneal injection in male Sprague-Dawley rats, and male Swiss mice. In rats injected with methyl-CCNU three times weekly for 6 months, the incidence of peritoneal sarcoma increased, and total tumour incidence was reported to increase 1.5-fold over that in controls at 18 months. No increase in tumour incidence was observed in mice (Weisburger, 1977). Intravenous administration of methyl-CCNU to rats was also reported to clearly induce lung tumours (Habs & Schmähl, 1984).

See Table 3.1

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

In humans, following oral administration, methyl-CCNU is well absorbed from the gastrointestinal tract, undergoes rapid chemical decomposition and oxidative metabolism, and is rapidly distributed throughout the body (Sweetman, 2005). Following the oral administration of radiolabelled methyl-CCNU to cancer patients, radioactivity is detected in plasma within 10 minutes, with peak plasma levels attained within 3–6 hours. Radioactivity is slowly eliminated from the plasma, with a half-life of 36 hours reported for the chloroethyl moiety, and two half-lives for the cyclohexyl moiety: an initial half-life of 24 hours, and a subsequent half-life of 72 hours. Approximately 60% of the administered radioactivity is excreted in the urine within 48 hours (Sponzo *et al.*, 1973).

In addition to chemical degradation, methyl-CCNU is metabolized by the cytochrome P450 (CYP) mono-oxygenase system on the cyclohexyl ring carbons, and the 2-chloroethyl side-chain (Reed, 1994). CYP-dependent formation of alkylating metabolites from methyl-CCNU has been observed in studies *in vitro* with rat liver microsomes. The alkylating metabolites bind covalently to DNA and protein (Kramer, 1989; Reed, 1994), and increase mutagenicity in bacteria (Franza *et al.*, 1980). Methyl-CCNU is also metabolized to carbamylating species, but subsequent carbamoylation reactions are not

Table 3.1 Studies of cancer in experimental animals exposed to methyl-CCNU

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague-Dawley (M) 18 mo Weisburger (1977)	i.p. 0, 1.5, 3.0 mg/kg 3 ×/wk for 6 mo 25/group 179 controls	Peritoneal sarcomas: 0/179 (0%), 3/49 (6.1%) Lung: 3/179 (1.1%), 3/49 (6.1%) Mammary gland: 4/179 (2.2%), 3/49 (6.1%) Brain: 2/179 (1.1%), 1/49 (2.0%)	[$P < 0.009$]	Purity NR Dosage groups were combined for each sex Overall increase in malignant tumours: 1.5-fold (53% vs 34%)
Rat, Wistar (M) Lifetime Habs & Schmähl (1984)	i.v. 0, 19, 38, 75, 150 mg/m^2 once every 6 wk 30/group 120 controls	Lung: +, [proven evidence according to the authors]		Purity > 99% (for clinical use)

i.p., intraperitoneal; i.v., intravenously; M, male; mo, month or months; NR, not reported; vs, versus; wk, week or weeks

significantly affected by enzymatic microsomal metabolism (Kramer, 1989; Reed, 1994).

4.2 Genotoxic effects

Methyl-CCNU is a bifunctional antineoplastic agent that undergoes spontaneous chemical decomposition yielding electrophilic compounds. These induce alkylation and carbamoylation of cellular macromolecules, including DNA and protein (Kramer *et al.*, 1986; Kramer, 1989; Reed, 1994). As with other chloroethylnitrosoureas, the majority of the alkylation reactions occur at the N^7 position of guanine, but the critical reaction leading to cytotoxicity is reported to involve alkylation of the O^6 of guanine, which leads to G–C cross-links in DNA (Chu & Sartorelli, 2007). Carbamoylation of proteins is also believed to contribute to the toxicity of methyl-CCNU, and may contribute to carcinogenesis through inhibition of DNA-repair processes (Kramer, 1989; Reed, 1994).

Methyl-CCNU has been tested for genotoxicity in several short-term assays *in vitro* and *in vivo*. The administration of methyl-CCNU *in vivo* results in DNA adducts in the bone marrow, spleen and colon of treated mice (Wheeler *et al.*, 1983), and in the kidney, liver and lung of treated rats (Kramer *et al.*, 1985, 1986). Large increases in the frequency of micronuclei are also seen in the bone-marrow erythrocytes of mice treated with methyl-CCNU (Tinwell & Ashby, 1991; Ashby *et al.*, 1993). Methyl-CCNU induces chromosomal aberrations, micronuclei, sister chromatid exchange, and DNA strand breaks in human or rodent cells *in vitro* (Erickson *et al.*, 1978; Wheeler *et al.*, 1983; Shah *et al.*, 1986; Baumler *et al.*, 1987; Vyas *et al.*, 1988; Tapiero *et al.*, 1989). It also induces mitotic crossing-over in yeast (Ferguson & Turner 1988a, b), and is mutagenic in bacteria (Auletta *et al.*, 1978; Franza *et al.*, 1980; Ashby *et al.*, 1993).

In addition, patients treated with methyl-CCNU (in combination with 5-fluorouracil and vincristine) as cytostatic therapy were found to have increased frequencies of sister chromatid exchange and chromosomal aberrations in their peripheral blood lymphocytes (Gebhart *et al.*, 1980a, b). Myelosuppression has also been reported in patients treated with methyl-CCNU (Young *et al.*, 1973; Breeden *et al.*, 1982).

4.3 Mechanisms of carcinogenesis

Acute myeloid leukaemia that develops in patients who have previously been treated with alkylating agents such as methyl-CCNU frequently exhibits distinctive characteristics that allow it to be distinguished from acute myeloid leukaemia induced by other agents (such as topoisomerase II inhibitors) or acute myeloid leukaemia that occurs spontaneously (Pedersen-Bjergaard & Rowley, 1994; Jaffe *et al.*, 2001; Smith *et al.*, 2003; Pedersen-Bjergaard *et al.*, 2006). One of the hallmarks of leukaemias induced by alkylating agents is that they frequently exhibit a clonal loss of either chromosome 5 or 7 (–5, –7) or a loss of part of the long arm of one of these chromosomes (5q–, 7q–). For example, a deletion within the long arm of chromosome 5 involving the bands q23 to q32 is often seen (Jaffe *et al.*, 2001).

In addition, mutations in *TP53* are frequently seen in leukaemias with the –5/5q– karyotype, and mutations involving the *AML1* gene as well as mutations in *TP53* and *RAS* are also seen in a subset of leukaemias that exhibit the –7/7q– karyotype (Christiansen *et al.*, 2001, 2005; Pedersen-Bjergaard *et al.*, 2006). These treatment-related acute myeloid leukaemias also frequently exhibit increased methylation of the *p15* promoter (Pedersen-Bjergaard *et al.*, 2006). Although methyl-CCNU has not been directly shown to induce losses or deletions affecting chromosomes 5 or 7, this drug has been reported to induce similar types of chromosomal alterations and deletions in a variety of experimental models (see description above), and in the lymphocytes of treated patients (Gebhart *et al.*, 1980a, b). The detection of elevated levels of chromosomal aberrations in the peripheral blood lymphocytes of patients treated with methyl-CCNU is of particular note, as multiple prospective studies have shown that individuals with increased levels of chromosomal aberrations in these cells are at increased risk of developing cancer (Hagmar *et al.*, 1998, 2004; Liou *et al.*, 1999; Smerhovsky *et al.*, 2001; Boffetta *et al.*, 2007).

4.4 Synthesis

Methyl-CCNU is a direct-acting alkylating agent that is carcinogenic via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of methyl-CCNU. Methyl-CCNU causes acute myeloid leukaemia.

There is *limited evidence* in experimental animals for the carcinogenicity of methyl-CCNU.

Methyl-CCNU is *carcinogenic to humans (Group 1)*.

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CYCLOPHOSPHAMIDE

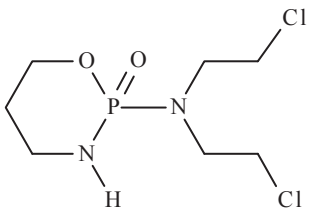
Cyclophosphamide was considered by previous IARC Working Groups in 1980 and 1987 (IARC, 1981, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 50-18-0
Chem. Abstr. Name: 2*H*-1,3,2-Oxazaphosphorin-2-amine, *N*,*N*-bis(2-chloroethyl)tetrahydro-, 2-oxide
IUPAC Systematic Name: *N*,*N*-Bis(2-chloroethyl)-1-oxo-6-oxa-2-aza-1λ^5-phosphacyclohexan-1-amine
Synonyms: 2-[Bis(2-chloroethyl)amino] tetrahydro-2*H*-1,3,2-oxazaphosphorin 2-oxide; bis(2-chloroethyl)phosphoramide cyclic propanolamide ester; *N*,*N*-bis(*β*-chloroethyl)-*N*′,*O*-trimethylenephosphoric acid ester diamide; *N*,*N*-bis(2-chloroethyl)-*N*′,*O*-propylenephosphoric acid ester diamide; Cytoxan; Endoxan; Neosar
Description: Crystalline solid [anhydrous form] (O'Neil, 2006)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_7H_{15}Cl_2N_2O_2P$
Relative molecular mass: 261.1

1.2 Use of the agent

Cyclophosphamide is an antineoplastic agent metabolized to active alkylating metabolites with properties similar to those of chlormethine. It also possesses marked immunosuppressant properties. It is widely used, often in combination with other agents, in the treatment of several malignant diseases. Information for Section 1.2 is taken from McEvoy, (2007), Royal Pharmaceutical Society of Great Britain (2007), and Sweetman (2008).

1.2.1 Indications

Cyclophosphamide is used in the treatment of chronic lymphocytic leukaemia, lymphomas, soft tissue and osteogenic sarcoma, and solid tumours. It is given orally or intravenously. Cyclophosphamide is inactive until metabolized by the liver.

(a) Hodgkin lymphoma

Cyclophosphamide is used in combination regimens (e.g. bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone [known as BEACOPP]) for the treatment of Hodgkin lymphoma.

(b) Non-Hodgkin lymphoma

Cyclophosphamide is used in combination therapy for the treatment of non-Hodgkin lymphoma, including high-grade lymphomas, such as Burkitt lymphoma and lymphoblastic lymphoma, as well as intermediate- and low-grade lymphomas. Cyclophosphamide is commonly used with doxorubicin (hydroxydaunorubicin), vincristine (oncovin), and prednisone (known as the CHOP regimen), with or without other agents, in the treatment of various types of intermediate-grade non-Hodgkin lymphoma. Cyclophosphamide has also been used as a single agent in the treatment of low-grade lymphomas.

(c) Multiple myeloma

Cyclophosphamide is used in combination with prednisone, or as a component of combination chemotherapy (i.e. vincristine, carmustine, melphalan, cyclophosphamide, and prednisone [VBMCP]) for the treatment of multiple myeloma.

(d) Leukaemia

Cyclophosphamide is used commonly for the treatment of chronic lymphocytic (lymphoblastic) leukaemia. Cyclophosphamide is used in combination with busulfan as a conditioning regimen before allogeneic haematopoietic progenitor cell transplantation in patients with chronic myelogenous leukaemia.

Cyclophosphamide is used in the treatment of acute lymphoblastic leukaemia, especially in children. In the treatment of acute myeloid (myelogenous, non-lymphocytic) leukaemia, cyclophosphamide has been used as an additional drug for induction or post-induction therapy.

(e) Cutaneous T-cell lymphoma

Cyclophosphamide is used alone or in combination regimens for the treatment of advanced mycosis fungoides, a form of cutaneous T-cell lymphoma.

(f) Neuroblastoma

Cyclophosphamide alone is used in the treatment of disseminated neuroblastoma. Combination chemotherapy that includes cyclophosphamide is also used for this neoplasm.

(g) Cancer of the ovary

Cyclophosphamide is used in combination chemotherapy (vincristine, actinomycin D, and cyclophosphamide [VAC]) as an alternative regimen for the treatment of ovarian germ-cell tumours.

Cyclophosphamide has been used in combination with a platinum-containing agent for the treatment of advanced (Stage III or IV) epithelial ovarian cancer.

(h) Retinoblastoma

Cyclophosphamide is used in combination therapy for the treatment of retinoblastoma.

(i) Cancer of the breast

Cyclophosphamide is used alone and also in combination therapy for the treatment of breast cancer.

Combination chemotherapy with cyclophosphamide is used as an adjunct to surgery in premenopausal and postmenopausal women

with node-negative or -positive early (TNM Stage I or II) breast cancer. Adjuvant combination chemotherapy that includes cyclophosphamide, methotrexate, and fluorouracil has been used extensively.

Adjuvant combination chemotherapy (e.g. cyclophosphamide, methotrexate, and fluorouracil; cyclophosphamide, adriamycin, and fluorouracil; cyclophosphamide and adriamycin with or without tamoxifen) is used in patients with node-positive early breast cancer (Stage II) in both premenopausal and postmenopausal patients once treatment to control local disease (surgery, with or without radiation therapy) has been undertaken.

In Stage III (locally advanced) breast cancer, combination chemotherapy (with or without hormonal therapy) is used sequentially following surgery and radiation therapy for operable disease or following biopsy and radiation therapy for inoperable disease; commonly employed effective regimens include cyclophosphamide, methotrexate, and fluorouracil; cyclophosphamide, doxorubicin, and fluorouracil; and cyclophosphamide, methotrexate, fluorouracil, and prednisone. These and other regimens also have been used in the treatment of more advanced (Stage IV) and recurrent disease.

(j) Small cell cancer of the lung

Cyclophosphamide is used in combination chemotherapy regimens (e.g. cyclophosphamide, adriamycin, and vincristine [CAV]; cyclophosphamide, adriamycin, and etoposide [CAE]) for the treatment of extensive-stage small cell lung cancer.

(k) Sarcoma

Cyclophosphamide has been used in combination regimens (usually with dactinomycin and vincristine) and as an adjunct to surgery and radiation therapy in the treatment of rhabdomyosarcoma and Ewing sarcoma.

1.2.2 Dosage

Cyclophosphamide is administered orally or by intravenous injection or infusion. Less frequently, the drug has been administered intramuscularly and by intracavitary (e.g. intrapleural, intraperitoneal) injection and direct perfusion.

In patients with no haematological deficiencies receiving cyclophosphamide monotherapy, induction therapy in adults and children is usually initiated with an intravenous cyclophosphamide loading dose of 40–50 mg/kg administered in divided doses over 2–5 days. Other regimens for intravenous administration include 10–15 mg/kg every 7–10 days or 3–5 mg/kg twice weekly.

When cyclophosphamide is administered orally, the usual dose for induction or maintenance therapy is 1–5 mg/kg daily, depending on the tolerance of the patient.

A daily oral dose of 2–3 mg/kg for 60–90 days has been used in children with nephrotic syndrome, and in whom corticosteroids have been unsuccessful. In patients who are to undergo stem-cell transplantation, very high doses of cyclophosphamide such as 60 mg/kg daily for 2 days may be given as part of the conditioning regimen.

Various cyclophosphamide-containing combination chemotherapy regimens have been used in the treatment of breast cancer. One commonly employed regimen for the treatment of early breast cancer includes a cyclophosphamide dosage of 100 mg/m^2 orally on Days 1 through 14 of each cycle combined with intravenous methotrexate at 40 mg/m^2 on Days 1 and 8 of each cycle, and intravenous fluorouracil at 600 mg/m^2 on Days 1 and 8 of each cycle. In patients older than 60 years of age, the initial intravenous methotrexate dosage is reduced to 30 mg/m^2 and the initial intravenous fluorouracil dosage is reduced to 400 mg/m^2. Dosage is also reduced if myelosuppression develops. Cycles

are generally repeated monthly (i.e. allowing a 2-week rest period between cycles) for a total of 6–12 cycles (i.e. 6–12 months of therapy).

Cyclophosphamide is available as 25 and 50 mg tablets for oral administration, and as 200 mg, 500 mg, 1 g, or 2 g vials of powder for reconstitution for parenteral administration.

1.2.3 Trends in use

No information was available to the Working Group.

2. Cancer in Humans

The carcinogenicity of cyclophosphamide in humans was established initially on the basis of a large number of case reports, as well as several epidemiological studies (IARC 1981, 1987a). The interpretation of the epidemiological studies was limited by the small numbers of cases, the difficulty in separating the role of cyclophosphamide from other agents, or both factors.

The most substantial evidence available to previous Working Groups was a Danish study of 602 patients treated "mainly with cyclophosphamide" for non-Hodgkin lymphoma, in which nine cases of acute myeloid leukaemia were observed compared to 0.12 expected (Pedersen-Bjergaard *et al.*, 1985), and a case–control study of leukaemia following ovarian cancer in the former German Democratic Republic where a strong dose–response relationship was observed (Haas *et al.*, 1987). All other studies reported at most three cases of leukaemia or bladder cancer in people who had received cyclophosphamide as the only potentially carcinogenic agent (IARC, 1981; Kinlen, 1985; Greene *et al.*, 1986).

Subsequently, further studies have been published that have provided more detailed information on the carcinogenicity of cyclophosphamide. This review is restricted to epidemiological studies that have used appropriate comparison groups to investigate the role of cyclophosphamide as the cause of specific types of cancer.

There have been several reported cohort studies in which patients treated with cyclophosphamide were followed up, and the occurrence of second cancers investigated. Valagussa *et al.* (1994) followed 2465 women who had received treatment with cyclophosphamide, methotrexate and fluorouracil, a combination in which only cyclophosphamide is considered to have carcinogenic potential in humans. There were three cases of acute myeloid leukaemia observed compared to 1.3 expected, and five cases of bladder cancer compared to 2.1 expected. Statistical significance was not reported but was calculated by the Working Group to be greater than 0.05 for both types of cancer. Smith *et al.* (2003) followed 8563 women who had received cyclophosphamide and doxorubicin as adjuvant therapy for breast cancer and observed 43 cases of acute myeloid leukaemia or myelodysplastic syndromes (AML/MDS). The incidence of AML/MDS overall was seven times higher than expected rates in the general population, and was increased 3-fold in regimens that contained double the cumulative dose of cyclophosphamide.

Several case–control studies have also been reported. For leukaemia, Kaldor *et al.* (1990) investigated 114 cases of a cohort of ovarian cancer patients. The relative risks were, respectively, 2.2 and 4.1 in two increasing dose categories of cyclophosphamide. Neither increase was reported as statistically significant. Travis *et al.* (1994) carried out a study involving 35 cases of leukaemia following non-hodgkin lymphoma, and found that prior treatment with cyclophosphamide was associated with a relative risk of 1.8 that was not statistically significant when comparison was made to treatment with radiotherapy alone. In an investigation by Nandakumar *et al.* (1991) of 97 cases of myeloid leukaemia as second primary cancers, patients receiving cyclophosphamide had a relative risk of 12.6 compared to those treated surgically, and

was substantially higher when prednisone was co-administered with cyclophosphamide. Curtis *et al.* (1992) compared 90 women who developed acute myeloid leukaemia following breast cancer to controls, and found that the risk of leukaemia was 2.6 times greater in those who had received cyclophosphamide, compared to women who had been treated by surgery only.

There have also been two case–control studies of bladder cancer in relation to cyclophosphamide. Kaldor *et al.* (1995) investigated 63 cases of bladder cancer following ovarian cancer, and found that in comparison to surgery alone, the relative risk associated with chemotherapy containing cyclophosphamide as the only potential bladder-cancer-causing agent was 4.2 ($P = 0.025$) in the absence of radiotherapy, and 3.2 ($P = 0.08$) with radiotherapy. Travis *et al.* (1995) studied 31 cases of bladder cancer and 17 cases of kidney cancer as well as matched controls within a cohort of 2-year survivors of non-Hodgkin lymphoma. The relative risk associated with cyclophosphamide treatment was 4.5 ($P < 0.05$) for bladder cancer, and 1.3 for kidney cancer.

2.1 Synthesis

The studies summarized above provide a comprehensive epidemiological basis for identifying cyclosphosphamide as an independent cause of acute myeloid leukaemia and bladder cancer, that fully supports the conclusions drawn from earlier case reports, and more limited studies. Several studies have assessed the risk of all second primary cancers following cyclophosphamide treatment, and some have found rates of occurrence that appear to be elevated, but have not provided evidence for risk of other specific cancer types.

3. Cancer in Experimental Animals

Cyclophosphamide has been tested for carcinogenicity by oral administration to mice and rats, by subcutaneous injection to mice, by topical application to mice, by intravenous injection to rats, by intraperitoneal injection to mice and rats, and by perinatal exposure to mice.

Oral administration of cyclophosphamide resulted in skin tumours in transgenic mice (Yamamoto *et al.*, 1996; Eastin *et al.*, 2001), and in urinary bladder carcinoma, leukaemia, and nervous system tumours in rats (Schmähl & Habs, 1979; Habs & Schmähl, 1983). Subcutaneous injection of cyclophosphamide to mice caused a variety of neoplasms, including mammary gland carcinoma and leukaemia (Schmähl & Osswald, 1970; Walker & Bole, 1971, 1973; Walker & Anver, 1979, 1983; Petru *et al.*, 1989).

Intravenous injection of cyclophosphamide to rats caused both benign and malignant neoplasms (Schmähl, 1967, 1974; Schmähl & Osswald, 1970).

Intraperitoneal administration of cyclophosphamide increased the incidences of lung adenoma and adenocarcinoma, bladder papilloma, and leukaemia in mice (Shimkin *et al.*, 1966; Weisburger *et al.*, 1975; Mahgoub *et al.*, 1999), and mammary gland adenoma and carcinoma in rats (Weisburger *et al.*, 1975).

Administration of cyclophosphamide to newborn mice caused lung and liver adenoma and carcinoma, and Harderian gland adenoma (Kelly *et al.*, 1974; McClain *et al.*, 2001).

See Table 3.1.

Table 3.1 Studies of cancer in experimental animals exposed to cyclophosphamide

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Oral administration				
Mouse, Tg ras H2/CB6F1 & B6C6F1 (M), 9 wk 26 wk Yamamoto et al. (1996)	0, 10, 30 mg/kg bw by gavage (in water, volume NR), twice/wk for 25 wk Initial number/group NR	Tg ras H2/CB6F1: Lung (adenomas)– 0/9, 3/16, 3/27 Multiplicity– 0, 0.19, 0.11 tumours/mouse	[NS][a]	Pharmaceutical grade
		CB6F1: Lung (adenomas)– 0/6, 2/18, 2/20 Multiplicity– 0, 0.11, 0.10 tumours/mouse	[NS][a]	
Mouse, Tg.AC (M, F), 8–9 wk 27 wk Eastin et al. (2001)	0, 10, 30, 60 mg/kg bw by gavage (in water 50% ethanol, volume NR); twice/wk for 26 wk 15/sex/group	Skin tumours (at all sites; histologically confirmed): 5/15, 1/2, 5/5, 5/15 (M); 2/15, 5/11, 11/11, 14/15 (F)	[$P < 0.0001$ for 30 and 60 mg/kg bw doses in female mice][a]	Purity NR; Tg.AC mice are transgenic mice that carry a v-Ha-*ras* oncogene
		Skin tumours (squamous cell papillomas of vulva): 2/15, 4/11, 10/11, 12/15 (F)	[$P \leq 0.0003$ for 30 and 60 mg/kg bw doses in female mice][a]	
		Leukaemia (erythrocytic): 0/15, 0/15, 4/15, 1/15 (F)	$P < 0.05$, for 30 mg/kg bw group	
Rat, Sprague-Dawley (M, F) Lifetime Schmähl & Habs (1979)	0, 0.31, 0.63, 1.25, 2.5 mg/kg bw in drinking-water, 5 ×/wk for life 40/sex/group	Malignant tumours: 4/38, 11/34, 14/36, 15/35, 13/31 (M); 5/34, 11/37, 13/37, 11/33, 9/27 (F)	[$P < 0.05$, for 3 highest doses]	Purity NR
		Urinary bladder (carcinomas): 0/38, 2/34, 2/36, 5/35, 7/31 (M); 0/34, 0/37, 0/37, 0/33, 1/27 (F)	[$P \leq 0.02$ for 2 highest doses in males][a]	
		Lymphoid and haematopoietic tissue (leukaemia): 0/72, 3/71, 6/73, 6/68, 4/58 (M, F)	[$P \leq 0.04$ for 3 highest doses for combined males and females][a]	
		Nervous system (sarcomas): 1/72, 7/71, 5/73, 6/68, 1/58 (M, F)	[$P \leq 0.05$ for 0.31 and 1.25 mg/kg doses for combined males and females][a]	

Table 3.1 (continued)

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague-Dawley (M), 100 d 20 mo Habs & Schmähl (1983)	0, 2.5 mg/kg bw in drinking-water, 5 ×/wk for 20 mo 100/group	Urinary bladder (papillomas or transitional-cell carcinomas): 0/63, 24/80 Nervous system tumours: 1/63, 11/80	[$P < 0.0001$][a] [$P < 0.0076$][a]	Reported as "chemically pure"
Rat, Sprague-Dawley (M), 100[a] Lifetime Schmähl & Habs (1983)	0, 2.5 mg/kg bw in drinking-water, 5 times/wk for life 100/group	Urinary bladder (papillomas): 0/100, 15/100 Urinary bladder (transitional-cell carcinomas): 0/100, 17/100	[$P < 0.0001$][a] [$P < 0.0001$][a]	Purity NR; only data on bladder tumours reported
Subcutaneous injection				
Mouse, NMRI (F) 52 wk Schmähl & Osswald (1970)	0, 26 mg/kg bw/wk (in solvent NR), for 5 wk 50/group	Malignant tumours (primarily mammary carcinomas): 3/46, 28/46	[$P < 0.001$][b]	Purity > 98%
Mouse, New Zealand Black/New Zealand White (F) 64 wk Walker & Bole (1971)	0, 8 mg/kg bw (in saline; volume NR), daily for 64 wk 16, 10	Neoplasms (mainly lymphomas): 0/16, 6/10	$P = 0.00002$	Purity NR
Mouse, New Zealand Black/New Zealand White (M, F) 93 wk Walker & Bole (1973)	0, 1, 8 mg/kg bw (in 100 µL saline), daily for 93 wk 20, 10, 10 per sex	Neoplasms (mainly lymphomas): 2/16, 3/9, 8/9 (M); 1/20, 1/10, 9/9 (F)	$P = 0.003$ for 8 mg/kg bw males; $P < 0.0001$ for 8 mg/kg bw females	Purity NR
Mouse, New Zealand Black/New Zealand White (F) Lifetime Walker & Anver (1979)	0, 5.7, 16 mg/kg bw (in 100 µL saline), daily for life 15, 17, 21	Neoplasms (mainly mammary carcinomas): 0/13, 15/15, 17/19 Mammary carcinomas: 0/13, 5/15, 16/19	[$P < 0.0001$ for 5.7 and 16 mg/kg bw groups][a] [$P \leq 0.03$ for 5.7 and 16 mg/kg bw groups][a]	Purity NR; treatment groups not started simultaneously
Mouse, New Zealand Black/New Zealand White (F), 6 wk Lifetime Walker & Anver (1983)	0, 56 mg/kg bw (in 100 µL saline), weekly for life 15, 22	Neoplasms: 0/13, 17/19	[$P < 0.0001$][a]	Purity NR; groups not started simultaneously; Neoplasms were mainly mammary gland carcinomas, lung adenomas and lymphomas

Table 3.1 (continued)

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, NMRI & AKR (F), 7 wk, Lifetime Petru et al. (1989)	0, 13, 26 mg/kg bw (in saline, volume NR), weekly for life 30/group	Leukaemia (NMRI mice): 2/30, 16/30, 10/30 Leukaemia (AKR mice): 30/30, 25/30, 19/30	$P \leq 0.027$ for 13 & 26 mg/kg bw groups $P \leq 0.006$ for 13 & 26 mg/kg bw groups	Purity NR [negative trend in AKR mice]
Skin application				
Mouse, Tg.AC (M, F), 8–9 wk 27 wk Eastin et al. (2001)	0, 10, 30, 90 mg/kg bw (in 50% ethanol, 3.3 mL/kg bw), 2 ×/wk for 26 wk 15/sex/group	Skin tumours (at site of application): 1/15, 0/15, 2/15, 3/15 (M); 1/15, 0/15, 0/15, 2/15 (F) Skin tumours (at all skin sites): 1/15, 2/15, 3/15, 3/15 (M); 4/15, 3/15, 9/15, 14/15 (F)	[NS][a] [$P = 0.0002$ for 90 mg/kg females][a]	Purity NR; Tg.AC mice are transgenic mice that carry a v-Ha-*ras* oncogene
Intravenous administration				
Rat, BR 46 (M) 23 mo Schmähl (1967)	0, 15 mg/kg bw (vehicle and volume NR), weekly (750 mg/kg bw total dose) 50, 40	Neoplasms (benign and malignant combined): 1/50, 14/26	[$P < 0.001$][b]	Purity > 98%
Rat, BR 46 (M) 23 mo Schmähl & Osswald (1970)	0, 13 mg/kg bw (vehicle and volume NR), weekly for 52 wk 89, 48	Neoplasms: 3/65, 4/36 (benign); 4/65, 6/36 (malignant)	[NS][b]	Purity > 98%
Rat, BR 46 (M) 23 mo Schmähl & Osswald (1970)	0, 33 mg/kg bw (vehicle and volume NR), 5 times every 2 wk 89, 96	Neoplasms: 3/65, 5/66 (benign); 4/65, 16/66 (malignant)	[$P < 0.01$, malignant tumours][b]	Purity > 98%
Rat, Sprague-Dawley (M) 700 d Schmähl, (1974)	0, 13 mg/kg bw (vehicle and volume NR), weekly (670 mg/kg bw total dose) 52, 32	Neoplasms (malignant): 6/52, 14/32	[$P < 0.001$][b]	Purity > 98%
Intraperitoneal administration				
Mouse, dd (M, F) 48 wk Tokuoka (1965)	0 or 5 mg/kg bw (in saline 5 mL/kg), 2 injections/wk for 15 wk 20, 29	Lung (adenomas or carcinomas): 1/20, 3/29 Liver (adenomas): 0/20, 2/29 Testis (interstitial cell tumours): 0/20, 4/29 Mammary gland (carcinomas): 1/20, 3/29	NS NS NS NS	Purity NR

Table 3.1 (continued)

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, A (M, F) 48 wk Tokuoka (1965)	0 or 5 mg/kg bw (in saline 10 mL/kg), 2 injections/wk for 15 wk 16, 25	Lung (adenomas or carcinomas): 2/16, 6/25 Testis (interstitial cell tumours): 0/16, 3/25	NS NS	Purity NR
Mouse, A/J (M, F, equally split) 39 wk Shimkin et al. (1966)	0, 32.2, 129, 516, 1609 µmol/kg bw (total dose; in 200 µL water), 3 injections/wk for 4 wk 360, 30, 30, 30, 30	Lung (adenomas or adenocarcinomas): 107/339, 12/30, 11/26, 20/27, 2/4 (incidence); 0.38, 0.4, 0.6, 1.3, 2.5 (tumours per mouse)	[p < 0.001 (for 516 µmol/kg bw dose, incidence)][b]	Purity NR
Mouse, Swiss-Webster-derived (M, F) 18 mo Weisburger et al. (1975)	0, 12, 25 mg/kg bw (vehicle and volume NR), 3 injections/wk for 6 mo 101, 25, 25 (M) 153, 25, 25 (F)	Lung (adenomas or adenocarcinomas): 10/101, 7/30 (M); 21/153, 10/35 (F) Bladder (papillomas or carcinomas): 3/101 & 4/30 (M)	$P = 0.031$ (M) and $P = 0.027$ (F) (combined 12 & 25 mg/kg bw vs control) $P = 0.048$ (combined 12 & 25 mg/kg bw vs control)	Purity NR; not all control mice were treated with the vehicle
Mouse, 129/Sv & 129/Sv X C57BL/6 *Nf1*$^{+/+}$ & *Nf1*$^{+/-}$(sex NR), 6–10 wk 15 mo Mahgoub et al. (1999)	0 or 100 mg/kg bw/wk (solvent and volume NR) for 6 wk 129/Sv *Nf1*$^{+/+}$: 31 & 5 mice 129/Sv *Nf1*$^{+/-}$: 46 & 12 mice 129/Sv X C57BL/6 *Nf1*$^{+/+}$: 14 & 15 mice 129/Sv X C57Bl/6 *Nf1*$^{+/-}$: 412 & 25 mice	Leukaemia (129/Sv *Nf1*$^{+/+}$): 2/31, 0/5 Leukaemia (129/Sv *Nf1*$^{+/-}$): 8/46, 7/12 Leukaemia (129/Sv X C57BL/6 *Nf1*$^{+/+}$): 0/14, 2/25 Leukaemia (129/Sv X C57BL/6 *Nf1*$^{+/-}$): 0/12, 7/25	 $P = 0.004$	Purity NR
Rat, Sprague-Dawley (M, F) 18 mo Weisburger et al. (1975)	0, 5, 10 mg/kg bw (vehicle and volume NR), 3 injections/wk for 6 mo 179, 25, 25 (M) 181, 25, 28 (F)	Mammary gland (adenomas): 2/105 & 24/53 (F; combined 5 & 10 mg/kg bw) Mammary gland (carcinomas): 13/105 & 9/53 (F; combined 5 & 10 mg/kg bw)	$P = 0.028$ $P = 0.035$	Purity NR; not all control rats were treated with the vehicle

Table 3.1 (continued)

Route Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Perinatal exposure				
Mouse, CD-1 (M, F) 79 wk Kelly et al. (1974)	i.p. injection 0, 0.8, 4.0, 20.0 mg/kg bw (in 10 µL/kg saline), on postnatal Days 1, 3, 6 30/sex/group	Lung (adenomas): 0/28, 2/29, 4/27, 0/21 (M); 1/25, 2/27, 2/28, 3/21 (F)	$P < 0.05$ for 4 mg/kg bw males (life-table analysis)	Purity NR; the 20 mg/kg dose caused marked bw changes and nearly 100% mortality
Mouse, CD-1 (M, F) 1 yr McClain et al. (2001)	Oral 0, 10, 20, 40, 60 mg/kg bw by gavage (100 µL and 200 µL) on postnatal Days 8 & 15 [solvent NR] 48 (control), 24/sex	Liver (adenomas): 2/48, 2/24, 4/24, 6/24, 5/24 (M) Liver (carcinomas): 0/48, 0/24, 1/24, 6/24, 1/24 (M) Lung (adenomas): 3/48, 0/24, 8/24, 12/24, 13/24 (M); 7/48, 3/24, 6/24, 16/24, 13/24 (F) Lung (carcinomas): 0/48, 1/24, 0/24, 6/24, 3/24 (M); 0/48, 1/24, 3/24, 3/24, 0/24 (F) Harderian gland (adenomas): 2/48, 1/24, 1/24, 1/24, 5/24 (F)	[$P < 0.04$ for 40 & 60 mg/kg bw][a] [$P = 0.0009$ for 40 mg/kg bw][a] [$P < 0.005$ for 20, 40, & 60 mg/kg bw (M); 40 & 60 mg/kg bw (F)][a] [$P < 0.03$ for 40 & 60 mg/kg bw (M); 20 & 40 mg/kg bw (F)][a] [$P < 0.04$ for 60 mg/kg bw][a]	Purity NR
Pre and postnatal exposure				
Mouse, BR 46 (M, F) 24 mo Roschlau & Justus (1971)	i.p injection 25 mg/kg bw on gestation Day 14 [solvent and volume NR]. Male and female offspring treated every 2 wk for a total of 30 times Initial number NR	Lung (adenomas): male offspring 4/16, 2/16; female offspring 5/12 & 1/18 Lung (carcinomas): male offspring 0/16, 3/16; female offspring 0/12, 4/18	NS NS	Purity NR

[a] Current Working Group analysis (Fisher Exact test)

[b] Previous Working Group analysis

bw, body weight; d, day or days; F, female; i.p., intraperitoneal; M, male; mo, month or months; NR, not reported; NS, not significant; vs, versus; wk, week or weeks, yr, year or years

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

In most species, cyclophosphamide is rapidly absorbed, metabolized, and excreted. Its metabolic pathway has been studied in several species including mice, rats, hamsters, rabbit, dogs, sheep, and monkeys. Cyclophosphamide is not cytotoxic *per se,* because it requires metabolic activation before it can act as an alkylating agent. Activation takes place predominantly in the liver, although this may occur in other tissues (IARC, 1981).

Cyclophosphamide undergoes metabolism to several intermediates with alkylating activity. The principal metabolites identified are phosphoramide mustard, and acrolein. Phosphoramide mustard can undergo dephosphoramidation to yield nornitrogen mustard, which also has alkylating activity. Metabolites of cyclophosphamide can interact with DNA and proteins, resulting in the formation of adducts. The metabolism of cyclophosphamide and DNA adducts formation are summarized in Fig. 4.1.

A minor pathway results in dechloroethylation and the formation of 2-dechloroethylcyclophosphamide and another alkylating agent, chloroacetaldehyde (Balu *et al.*, 2002).

The other compounds such as 4-ketocyclophosphamide and propionic acid derivative are relatively non-toxic, and are the major urinary metabolites of cyclophosphamide in several species (IARC, 1981).

4.2 Genetic and related effects

4.2.1 Interaction with DNA

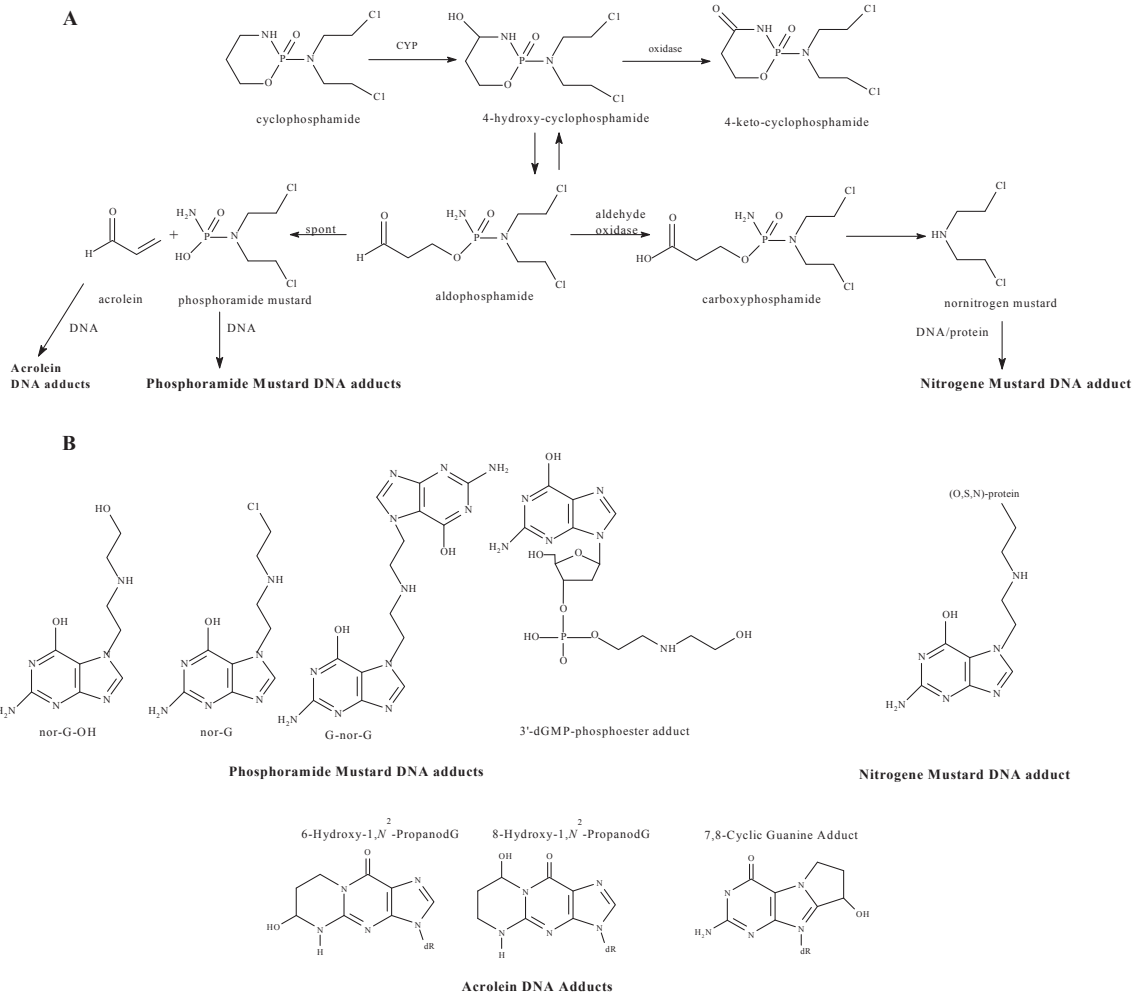
Using 4-hydroperoxycyclophosphamide as an activated form of cyclophosphamide, Mirkes *et al.* (1992) identified by mass spectrometric analysis the formation of the monofunctional adduct *N*-(2-chloroethyl)-*N*-[2-(7-guaninyl) ethyl]amine (nor-G) and the bifunctional adduct *N,N*-bis[2-(7-guaninyl)ethyl]amine (G-nor-G) in rat embryos in in-vitro culture. The monofunctional adduct *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl]amine (nor-G-OH) was detected in bladder tissue of rats injected with [^{3}H] cyclophosphamide (Benson *et al.*, 1988). Using ^{32}P-postlabelling analysis, a phosphotriester was shown to be formed: (1) when phosphoramide mustard was reacted with deoxyguanosine 5′-monophosphate, (2) when cyclophosphamide was incubated with calf thymus DNA in the presence of reconstituted cytochrome P450 (CYP) metabolizing system, and (3) in liver DNA from mice injected intraperitoneally with cyclophosphamide (Maccubbin *et al.*, 1991).

Nornitrogen mustard reacts with guanosine and with guanine bases in DNA to form nor-G initially, but this is converted to a hydroxylated derivative (nor-G-OH), and to a cross-linked (between guanines) adduct (G-nor-G) (Hemminki, 1987). Both monofunctional adducts, but not the cross-linked adduct, were also detected when phosphoramide mustard was reacted with DNA (Cushnir *et al.*, 1990). Acrolein reacts with DNA to form O^{6}-(*n*-propanalyl)guanine, and the product of chloroacetaldehyde reaction with DNA is O^{6}-(ethanalyl)guanine (Balu *et al.*, 2002). Acrolein can produce exocyclic adducts in DNA, including 1,N^{2}-hydroxypropanodeoxyguanosine and 1,N^{6}-hydroxypropanodeoxyadenosine (Chung *et al.*, 1984; Foiles *et al.*, 1990; Smith *et al.*, 1990). The former was detected in acrolein-treated human fibroblasts and in peripheral blood lymphocytes of a dog treated with cyclophosphamide (Wilson *et al.*, 1991).

Nornitrogen mustard also reacts covalently with proteins, and a method for the detection of cysteine-34 residue adducts in human serum albumin has been described (Noort *et al.*, 2002).

The single-cell gel comet assay is used to detect single-strand breaks and other alkali-labile lesions in DNA exposed to cyclophosphamide.

Fig. 4.1 Metabolic pathway of cyclophosphamide



A. Metabolism of cyclophosphamide to phosphoramide mustard, acrolein, and nornitrogen mustard. Cyclophosphamide is metabolized by CYP enzymes to 4-hydroxycyclophosphamide, wich equilibrates with aldophosphamide to spontaneously yield phosphoramide mustard and acrolein. Aldophosphamide is also metabolized by aldehyde oxidase to carboxyphosphamide, which produces nornitrogen mustard. 4-Hydroxy-cyclophosphamide can be oxidized to the inactive 4-keto-cyclophosphamide.
B. Phosphoramide mustard produces multiple monofunctional and bifunctional adducts with guanine, and acrolein forms exocyclic adducts. Nornitrogen mustard forms mono- and bifunctional adducts with guanine.
From Povirk & Shuker (1994), Anderson et al. (1995), Khan et al. (1998)
CYP, cytochrome P450; nor-G, *N*-(2-chloroethyl)-*N*-[2-(7-guaninyl)ethyl]amine; G-nor-G, *N,N*-bis[2-(7-guaninyl)ethyl]amine; nor-G-OH, *N*-(2-hydroxyethyl)-*N*-[2-(7-guaninyl)ethyl] amine; dR, deoxyribose

In vitro studies have demonstrated the comet-forming activity of cyclophosphamide in human hepatoma (Hep G2) cells (Uhl *et al.*, 2000; Yusuf *et al.*, 2000), in primary cultures of rat and human urinary bladder cells (Robbiano *et al.*, 2002), in primary cultures of human leukocytes in the presence of metabolic activation system S9 mix (Hartmann *et al.*, 1995; Hartmann & Speit, 1995; Frenzilli *et al.*, 2000), and in extended-term cultures of human T-lymphocytes, also in the presence of S9 (Andersson *et al.*, 2003). Comet formation was also detected *in vivo* in the urinary bladder mucosa of rats given cyclophosphamide orally (Robbiano *et al.*, 2002), and in peripheral blood cells of patients administered the drug (Hartmann *et al.*, 1995).

4.2.2 Genotoxic effects in humans

There are few reports of DNA-adduct formation by cyclophosphamide in humans. Acrolein-derived DNA adducts, detected by immunochemical methods, were found in blood leukocytes of cancer patients receiving cyclophosphamide (McDiarmid *et al.*, 1991). In another study, mono-adducts and inter-strand cross-links derived from phosphoramide mustard were detected in a single patient administered 1 g/m^2 cyclophosphamide (Souliotis *et al.*, 2003). Increased DNA damage (comet formation) was also observed in the lymphocytes of patients administered cyclophosphamide (Hartmann *et al.*, 1995).

Increased frequencies of several biomarkers of genotoxicity have been observed in the lymphocytes of patients treated with cyclophosphamide, relative to control subjects. These include mutations at the hypoxanthine-(guanine) phosphoribosyl transferase (*HPRT*) locus (Palmer *et al.*, 1986, 1988; Tates *et al.*, 1994; Sanderson *et al.*, 2001), and sister chromatide exchange (Raposa & Várkonyi, 1987; McDiarmid *et al.*, 1990; Sardaş *et al.*, 1994; Mertens *et al.*, 1995; Hartmann *et al.*, 1995).

Other studies reported positive findings for elevated chromosomal aberrations frequencies (Sessink *et al.*, 1994; Rubes *et al.*, 1998; Burgaz *et al.*, 2002), and micronuclei (Yager *et al.*, 1988; Tates *et al.*, 1994; Zúñiga *et al.*, 1996; Burgaz *et al.*, 1999; Rekhadevi *et al.*, 2007) in medical personnel exposed to cyclophosphamide. Increases in frequencies of micronuclei were also detected in buccal cells in some studies (Cavallo *et al.*, 2005; Rekhadevi *et al.*, 2007), but not in another (Burgaz *et al.*, 1999).

4.2.3 Genotoxic effects in experimental systems

(a) Mutagenic effects in vitro

The previous *IARC Monograph* (IARC, 1987b) states that cyclophosphamide induced chromosomal aberrations, sister chromatid exchange, and DNA damage in human cells *in vitro*. It also induced morphological transformation, chromosomal aberrations, sister chromatid exchange, mutation, and unscheduled DNA synthesis (UDS) in rodent cells *in vitro*. It further induced aneuploidy, mutation, recombination, gene conversion, and DNA damage in fungi. It was also reported to act as a mutagen and DNA-damaging agent in bacteria.

The mutagenicity of cyclophosphamide in *Salmonella typhimurium* was enhanced by increased induction of CYPs in S9 liver fractions by a combination of β-naphthoflavone and sodium phenobarbital (Paolini *et al.*, 1991a). Comparison of S9 from liver and kidney of pregnant mice revealed that liver S9 was more effective in activating cyclophosphamide to mutagenic metabolites in *S. typhimurium*, and also in inducing sister chromatid exchange in human peripheral lymphocytes, and Chinese hamster ovary (CHO) cells (Winckler *et al.*, 1987).

In *Saccharomyces cerevisiae*, higher rates of mitotic gene conversion and point mutation by cyclophosphamide were associated with induction of class 2B CYPs in co-cultured epithelial cell

lines from fetal mouse liver (Paolini *et al.*, 1991b). A recombinant plasmid containing a full-length cDNA encoding the rat cytochrome CYP2B1 introduced into *S. cerevisiae* also increased the mutation frequency induced by cyclophosphamide (Black *et al.*, 1989).

CYP2B1 expressed in Chinese hamster V79-derived SD1 cell lines also potentiated cyclophosphamide mutagenesis (6-thioguanine resistance), whereas CYP1A1 expressed in V79-derived XEM_2 cell lines did not (Doehmer *et al.*, 1990, 1992).

Cyclophosphamide was weakly mutagenic (detected by induction of resistance to 6-thioguanine) in differentiated Reuber hepatoma cells H4IIEC3/G^-, but markedly cytotoxic and clastogenic (micronucleus formation) (Roscher & Wiebel, 1988), and also mutagenic in a Chinese hamster epithelial liver cell line (6-thioguanine resistance) (Turchi *et al.*, 1992), and in Chinese hamster lung (CHL) cells in the presence of S9, as measured at microsatellite loci (Kikuno *et al.*, 1995).

Using 4-hydroperoxycyclophosphamide and phosphoradiamidic mustard, the role of different repair enzymes in defining sensitivity was investigated by Andersson *et al.* (1996) in CHO cells. Mutations in excision repair cross-complementing *ERCC1* and *ERCC4* genes caused hypersensitivity to the cyclophosphamide analogues.

Cyclophosphamide induced sister chromatid exchange in mouse primary bone-marrow and spleen cells (Soler-Niedziela *et al.*, 1989), and micronuclei in mouse lymphoma in L5178Y $tk^{+/-}$ cells (Kirsch-Volders *et al.*, 2003), and in parental V79 cells (Kalweit *et al.*, 1999) in the presence of rat liver S9. Of several V79 cell lines engineered to express rat CYPs, increases in micronuclei (Ellard *et al.*, 1991) and sister chromatid exchange (Kulka *et al.*, 1993) were seen in the cells expressing CYP2B1. The rat hepatoma cells lines H4IIEC3/G^- and 2sFou were also susceptible to micronuclei induction by cyclophosphamide (Tafazoli *et al.*, 1995).

Human T-lymphocytes were more susceptible than B-lymphocytes to both chromosomal aberrations and sister chromatid exchange induction by cyclophosphamide in the presence of rat liver S9 (Miller 1991a, b). This difference between T- and B-lymphocytes was not found with mouse cells treated with 4-hydroxycyclophosphamide or phosphoramide mustard (Kwanyuen *et al.*, 1990). In another study (Kugler *et al.*, 1987), rat liver microsomal mix was more effective than rat liver S9 in activating cyclophosphamide to induce chromosomal aberrations. Human lymphocytes from women carrying mutations in the breast cancer susceptibility gene *BRCA1* were more susceptible to micronuclei induction than cells from non-carriers (Trenz *et al.*, 2003). Hep G2 human hepatoma cells were susceptible to sister chromatid exchange and micronuclei induction by cyclophosphamide (Natarajan & Darroudi, 1991) and, in analogous studies, the S9 microsomal fraction of these cells were shown to be capable in activating cyclophosphamide to induce sister chromatid exchange and micronuclei in CHO cells (Darroudi & Natarajan, 1993). Human dental pulp cells formed chromosomal aberrations when exposed to cyclophosphamide in the presence of rat liver S9 (Tsutsui *et al.*, 2006).

In the presence of rat liver S9, cyclophosphamide induced morphological transformation of BALB/3T3 mouse embryonic fibroblast cells (McCarvill *et al.*, 1990).

(b) Mutagenic effects in vivo

The previous *IARC Monograph* (IARC, 1987b) states that cyclophosphamide was found to bind to kidney, liver and lung DNA in mice. It also induced dominant lethality, chromosomal aberrations, micronuclei, sister chromatid exchange, mutations, and DNA damage in rodents *in vivo*. In *Drosophila*, it induced aneuploidy, heritable translocations, and somatic and sex-linked recessive lethal mutations. In patients administered cyclophosphamide, increased incidences of chromosomal aberrations and sister chromatid

exchange in peripheral lymphocytes and bone marrow were observed.

In *Drosophila melanogaster*, cyclophosphamide tested positive for the somatic white-ivory mutation (Batiste-Alentorn *et al.*, 1994), and produced chromosome breaks in spermatocytes (Zijlstra & Vogel, 1989).

Several studies have examined the mutagenic effects of cyclophosphamide in transgenic mice. In MutaMouse, mutation induction was observed in bone marrow (other tissues not studied) (Hoorn *et al.*, 1993). In Big Blue mice, mutation frequencies were elevated in the liver, but not in the testis or spleen in one study (Hoyes *et al.*, 1998), and in another study, in the lung and urinary bladder, but not in the kidney, bone-marrow or splenic T-cells (Gorelick *et al.*, 1999). Another study compared the *lacI* locus in Big Blue mice with the *Hprt* locus in conventional B6C3F1 mice, and cyclophosphamide induced mutations in the endogenous gene in splenic lymphocytes, but not in the transgene (Walker *et al.*, 1999). In rats, cyclophosphamide produced the 'common deletion' mutation in liver mitochondrial DNA, and folic acid supplementation was found to be protective against this damage (Branda *et al.*, 2002).

In two related studies investigating oncogene and tumour-suppressor gene expression in mice, cyclophosphamide was found to induce expression of several genes, including *c-Myc* and *Tp53*, in the spleen and thymus, but not in other tissues (Ember *et al.*, 1995; Ember & Kiss, 1997).

Many studies have investigated the cytogenicity of cyclophosphamide in newts, rodents, dogs, and non-human primates. Results are invariably positive for this compound, and are summarized in Table 4.1.

(c) Mutagenic effects in germ cells

Anderson *et al.* (1995) reviewed the activity of cyclophosphamide in germ cells, and in summary, the germ cell stages that are most sensitive to cyclophosphamide are the postmeiotic stages. Tests for germ-cell damage that examine effects in F_1 progeny in which cyclophosphamide gave positive results include dominant lethality, heritable translocations, specific locus mutations, and malformations. Although cyclophosphamide is not an effective aneugen, it causes structural and numerical chromosomal damage in second meiotic metaphases and first cleavage metaphases, and in F_1 embryos. It is also positive for inducing sister chromatid exchange in germ cells and causes abnormal sperm-head morphology. Most studies have been carried out in mice, but positive results have also been observed in rats and rabbits, e.g. induction of unscheduled DNA synthesis in the testes (reviewed in Anderson *et al.*, 1995), and also in hamsters (Waters & Nolan, 1995).

More recent studies in mice have demonstrated the dominant lethal effects of cyclosphosphamide (Dobrzyńska *et al.*, 1998) as well as intrachromosomal gene conversion and mutation events primarily in meiotic stage cells (Schimenti *et al.*, 1997). In female rats, administration of cyclophosphamide at 16 days of gestation significantly increased nucleolar and synaptonemal complex fragmentation (Cusidó *et al.*, 1995), and in male rats chronic exposure to cyclophosphamide disrupted meiotic events before pachynema during spermatogenesis (Barton *et al.*, 2003).

(d) Modulation of mutagenicity by other agents

A large number of studies have investigated the effects of agents in modulating the genotoxicity of cyclophosphamide, and are summarized in Table 4.2.

4.3 Mechanisms of carcinogenesis

All of the available evidence indicates that cyclophosphamide exerts its carcinogenic activity via a genotoxic mechanism (McCarroll *et al.*, 2008). The metabolite widely thought to be responsible for the antitumour activity

Table 4.1 Positive cytogenicity studies of cyclophosphamide in newts, rodents, dogs, and non-human primates

Species	Cytogenetic end-point investigated	Additional considerations	Reference
Mouse	SCE	Bone-marrow cells. Reduction in frequency with increasing numbers of cell division	Morales-Ramírez et al. (1990)
Mouse	SCE	Bone-marrow cells. A comparison of wild and laboratory mice	Huang et al. (1990)
Mouse	MN	Bladder epithelial cells	Konopacka (1994)
Mouse	CA	Bone-marrow cells. Effects of malnutrition and alcohol	Terreros et al. (1995)
Mouse	MN	Peripheral blood reticulocytes and PCE in bone marrow	Hatanaka et al. (1992)
Mouse	MN	Splenocytes	Benning et al. (1992)
Mouse	MN	Bone-marrow PCE. Comparison of i.p. and p.o. administration	Wakata et al. (1989)
Mouse	MN	7 organs compared (bone marrow, forestomach, stomach, small intestine, large intestine, urinary bladder, lung)	Sycheva (2001)
Mouse	Intrachromosomal recombination	Spleen cells Transgenic mouse model with *lacZ* transgenic expression depending on somatic interchromosomal inversion	Sykes et al. (1998)
Mouse	MN	PCE in adult bone-marrow cells and fetal liver cells. Male, female, pregnant female, and fetal mice compared	Harper et al. (1989)
Mouse	MN SCE	Transplacental exposure; fetal liver cells	Porter & Singh (1988)
Mouse	MN CA	Bone-marrow and peripheral blood cells (CA) and peripheral blood erythrocytes (MN). Chronic ingestion of cyclosphosphamide; results positive for MN, negative for CA	Director et al. (1998)
Mouse	MN	Bone-marrow cells. In-vivo/in-vitro assay	Odagiri et al. (1994)
Mouse	CA SCE	Bone-marrow and spleen cells. In-vivo/in-vitro assay vs in-vivo assay	Krishna et al. (1987)
Mouse	SCE	Bone-marrow and spleen cells. In-vivo/in-vitro assay vs in-vivo assay	Krishna et al. (1988)
Rat	CA	Liver cells of neonates exposed *in utero*	Saxena & Singh (1998)
Rat	CA	Bone-marrow cells. Comparison in liver cells before and after partial hepatectomy of treated rats	Rossi et al. (1987)
Rat	CA SCE	Bone-marrow cells. Regenerating hepatocytes (SCE)	Masuda et al. (1990)
Rat	MN	Peripheral blood reticulocytes and bone-marrow cells comparison	Hayashi et al. (1992)
Mouse	MN	Bone-marrow PCE (positive), hepatocytes (negative)	Parton & Garriott (1997)
Rat	MN	Bone-marrow cells and peripheral blood reticulocytes. 14 rat strains compared	Hamada et al., (2001)
Rat	MN	Bone-marrow cells and peripheral blood reticulocytes. Effect of ageing studied	Hamada et al. (2003)
Rat	MN	Pre-estrous vaginal cells	Zúñiga-González et al. (2003)

Table 4.1 (continued)

Species	Cytogenetic end-point investigated	Additional considerations	Reference
Rat	CA MN	Bone-marrow cells. Simultaneous evaluation of two end-points in the same animal	Krishna et al. (1991)
Rat	MN	Bone-marrow, spleen, peripheral blood cells	Abramsson-Zetterberg et al. (1999)
Rat	MN	Embryos, treatment during pre-implantation period	Giavini et al. (1990)
Rat	MN CA	Bone-marrow and spleen cells. In-vivo/in-vitro assay	Moore et al. (1995)
Newt	MN	Larvae exposed to agent. Red blood cells	Fernandez et al. (1989)
Mouse, Chinese hamster	CA SCE	Bone-marrow cells Comparison of different routes of administration	Jenderny et al. (1988)
Rat, mouse	MN SCE Sperm morphology	Bone-marrow cells (MN). Splenocytes (SCE). Rats more susceptible than mice	Simula & Priestly (1992)
Rat, mouse, Chinese hamster	MN SCE	Bone-marrow cells. Species comparison Susceptibility ranked into the order rat > mouse > Chinese hamster	Madle et al. (1986)
Mouse, rat, Chinese hamster, Armenian hamster, guinea-pig	CA	Bone-marrow cells. Interspecies comparison Susceptibility ranked into the order guinea-pig > rat > mouse > Chinese hamster > Armenian hamster	Nersessian et al. (1992)
Dog (beagle)	MN	Peripheral blood reticulocytes and bone-marrow cells comparison	Harper et al. (2007)
Monkey	MN	Peripheral blood reticulocytes and bone-marrow cells comparison	Hotchkiss et al. (2008)
Marmoset	MN	Peripheral blood erythrocytes	Zúñiga-González et al. (2005)

CA, chromosomal aberrations; i.p., intraperitoneal; MN, micronuclei; PCE, polychromatic erythrocytes; p.o., per oral; SCE, sister chromatid exchange; vs, versus

Table 4.2 Studies of modulation of cyclophosphamide genotoxicity *in vivo* and *in vitro*

Agent	Experimental system	End-point measured	Effect	Reference
Retinol Retinoic acid	CHEL cells *in vitro*	SCE	Inhibitory	Cozzi et al. (1990)
Apigenin	Human lymphocytes + S9 *in vitro*	SCE CA	Inhibitory	Siddique et al. (2008)
β-carotene Retinal α-tocopherol Riboflavin	Human lymphocytes + S9 *in vitro*	SCE	Inhibitory	Edenharder et al. (1998)
Vitamin C	Human lymphocytes *in vitro*	SCE	Enhancing	Edenharder et al. (1998)
Vitamin K_1	Human lymphocytes *in vitro*	SCE	Inhibitory or enhancing (dependent on timing)	Edenharder et al. (1998)
Melatonin	CHO cells + S9 *in vitro*	SCE CA	Inhibitory	De Salvia et al. (1999)
Melatonin	CHO cells + S9 *in vitro*	Comet formation (DNA damage)	Inhibitory	Musatov et al. (1998)
O^6-alkylguanine-DNA alkyltransferase (AGT)	CHO cells *in vitro*	*Hprt* mutation	Inhibitory	Cai et al. (1999)
Buthionine sulfoximine	V79 cells and CHO +S9 *in vitro*	SCE	Enhancing	Köberle & Speit (1990)
Prostaglandin E_2	Mouse lymphoid L1210 leukaemia cells *in vivo*	SCE	Enhancing	Mourelatos et al. (1995)
Garlic extract	Swiss albino mice *in vivo*	CA (bone-marrow cells)	Inhibitory	Shukla & Taneja (2002)
Indole-3-carbinol	Swiss albino mice *in vivo*	CA (bone-marrow cells)	Inhibitory	Shukla et al. (2004)
Ascorbic acid	Pregnant CBA/CaH mice *in vivo*	CA SCE (pre-implantation embryos)	Inhibitory (SCE no effect)	Kola et al. (1989)
Ascorbic acid	Pregnant NMRI Kisslegg mice *in vivo*	CA SCE (pre-implantation embryos)	Inhibitory (SCE no effect)	Vogel & Spielmann (1989)
β-glucan	Male CD-1 mice *in vivo*	CA (bone-marrow and spermatogonial cells)	Inhibitory	Tohamy et al. (2003)

Table 4.2 (continued)

Agent	Experimental system	End-point measured	Effect	Reference
Nafenopin	Male Wistar rats *in vivo*	CA MN	Enhancing CA in bone marrow and MN in hepatocytes. Inhibitory on MN in bone marrow	Voskoboinik et al. (1997)
Prostaglandin E_2	BALB/c mice inoculated with Ehrlich ascites tumour cells *in vivo*	SCE (Ehrlich ascites tumour cells)	Inhibitory	Mourelatos et al. (1993)
Ginsenoside Rh_2	Male C57BL/6 mice *in vivo*	MN (bone-marrow cells) Comet formation (DNA damage) (white blood cells)	Inhibitory	Wang et al. (2006)
Verapamil	Male BALB/c and C57BL/6 mice *in vivo*	CA (bone-marrow cells)	Enhancing	Nesterova et al. (1999)
Citrus extract	Male BALB/c mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Hosseinimehr & Karami (2005a)
Captopril	Male NMRI mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Hosseinimehr & Karami (2005b)
Spirulina fusiformis	Male Swiss albino mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Premkumar et al. (2001a)
Saffron (*Crocus sativus* L.)	Male Swiss albino mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Premkumar et al. (2001b)
Melatonin and its derivatives	Male albino mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Elmegeed et al. (2008)
Vitamin C	Male Swiss albino mice *in vivo*	MN (bone-marrow cells)	Inhibitory	Ghaskadbi et al. (1992)
Malaria infection	Female C57BL/6 mice	MN (bone-marrow cells)	Inhibitory	Poça et al. (2008)
Lipoic acid	Male Wistar rats *in vivo*	MN (bone-marrow cells and peripheral blood cells)	Inhibitory	Selvakumar et al. (2006)
Folic acid	Newborn Wistar rats (fetal exposure) *in vivo*	MN (peripheral blood erythrocytes)	Inhibitory	Gómez-Meda et al. (2004)
Taenia taeniformis infection	Sprague-Dawley rats	MN (peripheral blood erythrocytes)	Enhancing	Montero et al. (2003)
O^6-methylguanine-DNA methyltransferase	C57BL/6 wild type and *Mgmt*$^{-/-}$ mice	*Hprt* mutation (splenic lymphocytes)	Inhibitory (non-significant)	Hansen et al. (2007)

CA, chromosomal aberrations; CHEL, Chinese hamster epithelial liver; CHO, Chinese hamster ovary; Hprt, hypoxanthine(guanine)phosphoribosyl transferase; MN, micronuclei; SCE, sister chromatid exchange

of cyclophosphamide is the phosphoramide mustard (Povirk & Shuker, 1994). This metabolite is also generally considered to be the most genotoxic, but the contribution of acrolein, which is highly toxic, to the genotoxic activity of cyclophosphamide is less clear.

It is well established that the treatment of cancer patients with cyclophosphamide results in inflammation of the urinary bladder (haemorrhagic cystitis), which is not seen with other alkylating agents (Forni *et al.*, 1964; Liedberg *et al.*, 1970). In rats, cyclophosphamide treatment resulted in cystitis as well (Crocitto *et al.*, 1996), and in mice, mutagenic activity has been detected in urine following cyclophosphamide treatment (Te *et al.*, 1997). The ultimate alkylating metabolite of cyclophosphamide, phosphoramide mustard, is metabolized but was not shown to cause cytotoxicity and had minimal morphological effects on the mouse bladder, but an intermediate in the formation of the acrolein metabolite, diethylcyclophosphamide administered by intraperitoneal injection, caused severe cystitis in male rats, and less extensive inflammation in female rats (Cox, 1979). Acrolein administered to rats by intraperitoneal injections increased urothelial cell proliferation (Sakata *et al.*, 1989). Acrolein is the only metabolite of cyclophosphamide that is known to be both reactive and cytotoxic (IARC, 1995). Collectively, these data indicate that acrolein is the likely causative agent in cyclophosphamide-induced cystitis. Cystitis is an established condition associated with the development of both squamous cell and urothelial bladder cancers (Michaud, 2007). However, intraperitoneal injections of acrolein by itself only induced bladder hyperplasia, not cancer (Cohen *et al.*, 1992), and oral administration studies in mice and rats did not result in carcinogenic effects (IARC, 1995). Thus it is plausible that acrolein-induced cystitis plays a promoting role in cyclophosphamide bladder tumorigenesis that is initiated by other cyclophosphamide metabolites.

The protective effect of O^6-alkylguanine-DNA alkyltransferase (AGT) against cyclophosphamide mutagenicity (*Hprt* mutations) (Cai *et al.*, 1999), and cytotoxicity (Friedman *et al.*, 1999) in CHO cells implies some involvement of acrolein-derived DNA damage. However, mice deficient in this protein (called O^6-methylguanine-DNA methyl transferase [MGMT] in this study) were less susceptible to cyclophosphamide tumorigenesis, not more (Nagasubramanian *et al.*, 2008). Studies of sister chromatid exchange induced in human lymphocytes by acrolein and phosphoramide mustard suggest that phosphoramide mustard is the more potent genotoxic agent (Wilmer *et al.*, 1990). Furthermore, analysis of *TP53* mutations in cyclophosphamide-associated human bladder cancers suggests that the mutations detected are characteristic of DNA damage caused by phosphoramide mustard, rather than by acrolein (Khan *et al.*, 1998).

4.4 Synthesis

Cyclophosphamide, after its bioactivation to alkylating metabolites, is carcinogenic via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of cyclophosphamide. Cyclophosphamide causes cancer of the bladder, and acute myeloid leukaemia.

There is *sufficient evidence* in experimental animals for the carcinogenicity of cyclophosphamide.

Cyclophosphamide is *carcinogenic to humans (Group 1)*.

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ETOPOSIDE IN COMBINATION WITH CISPLATIN AND BLEOMYCIN

Etoposide, cisplatin, and bleomycin were considered by a previous IARC Working Group in 1999 (IARC, 2000). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

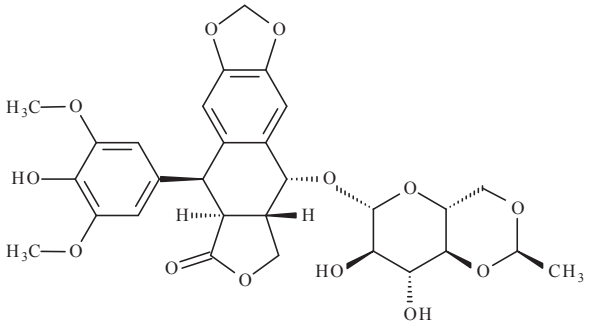
1. Exposure Data

1.1 Identification of the agent

1.1.1 Etoposide

Chem. Abstr. Serv. Reg. No.: 33419-42-0
Chem. Abstr. Name: Furo[3′,4′:6,7] naphtho[2,3-*d*]-1,3-dioxol-6(5a*H*)-one, 9-[4,6-*O*-(1*R*)-ethylidene-β-D-glucopyranosyl] oxy]-5,8,8a,9-tetrahydro-5-(4-hydroxy-3,5-dimethoxyphenyl)-, (5*R*,5a*R*,8a*R*,9*S*)-
IUPAC Systematic Name: Furo[3′,4′:6,7] naphtho[2,3-*d*]-1,3-dioxol-6(5a*H*)-one, 9-[(4,6-*O*-ethylidene-β-D-glucopyranosyl) oxy]-5,8,8a,9-tetrahydro-5-(4-hydroxy-3,5-dimethoxyphenyl)
Synonyms: Celltop; Etopophos; Eposin; furo[3′,4′:6,7]naphtho[2,3-*d*]-1,3-dioxol-6(5a*H*)-one, 9-[(4,6-*O*-ethylidene-β-D-glucopyranosyl) oxy]-5,8,8a,9-tetrahydro-5-(4-hydroxy-3,5-dimethoxyphenyl)-, [5*R*-[5a,5ab,8aa,9b(*R**)]]-; 4′-demethyl-1-*O*-[4,6-*O*-(ethylidene)-β-D-glucopyranosyl]epipodophyllotoxin; 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); 4′-demethylepipodophyllotoxin ethylidene-β-D-glucopyranoside; Toposar; Vepesid(e); pyrano[3,2-*d*]-1,3-dioxin, furo[3′,4′:6,7] naphtho[2,3-*d*]-1,3-dioxol-6(5a*H*)-one deriv.
Description: White to yellow-brown crystalline powder; white to off-white crystalline powder [phosphate salt] (McEvoy, 2007)

(a) Structural and molecular formulae, and relative molecular mass

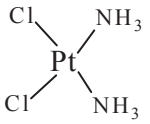


$C_{29}H_{32}O_{13}$
Relative molecular mass: 588.6

1.1.2 Cisplatin

Chem. Abstr. Serv. Reg. No.: 15663-27-1
Chem. Abstr. Name: Platinum, diamminedichloro-, (SP-4–2)-
IUPAC Systematic Name: Azane; dichloroplatinum
Description: Yellow to orange crystalline powder (McEvoy, 2007)

(a) Structural and molecular formulae, and relative molecular mass

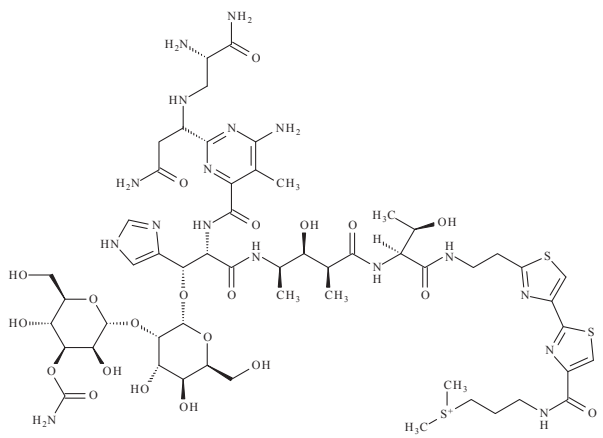


$Cl_2H_6N_2Pt$
Relative molecular mass: 300.0

1.1.3 Bleomycin

Chem. Abstr. Serv. Reg. No.: 11056-06-7
Chem. Abstr. Name: Bleomycin
IUPAC Systematic Name: Bleomycin
Description: Cream-coloured, amorphous powder [sulfate salt] (McEvoy, 2007)

(a) Structural and molecular formulae, and relative molecular mass



$C_{55}H_{84}N_{17}O_{21}S_3$
Relative molecular mass: 1415.6

1.2 Use of the agents

Information for Section 1.2 is taken from McEvoy (2007), Royal Pharmaceutical Society of Great Britain (2007), and Sweetman (2008).

1.2.1 Etoposide

(a) Indications

Etoposide is a semisynthetic derivative of podophyllotoxin with antineoplastic properties; it interferes with the activity of topoisomerase II, thus inhibiting DNA synthesis, and is most active against cells in the late S- and G_2-phases of the cell cycle. It is used, usually in combination with other antineoplastics, in the treatment of tumours of the testis, small cell cancer of the lung, and in acute leukaemias.

(i) Testicular neoplasms

Etoposide or etoposide phosphate may be used intravenously as a component of various chemotherapeutic regimens for the treatment of refractory testicular tumours in patients who have already received appropriate surgery, chemotherapy, and radiation therapy. Etoposide alone can be used in the treatment of disseminated non-seminomatous testicular carcinoma (Stage III), and in patients whose disease is refractory to cisplatin-containing combination chemotherapy. Cisplatin-containing combination chemotherapy regimens are used as initial therapy in patients with Stage III or unresectable Stage II non-seminomatous testicular carcinoma. For the initial treatment of advanced non-seminomatous testicular carcinoma, regimens containing cisplatin and bleomycin, in combination with etoposide, are used.

(ii) Cancer of the lung

Etoposide has been widely used for the treatment of lung cancer. Etoposide is used intravenously (either as etoposide or etoposide phosphate) in combination chemotherapy regimens for the treatment of small cell lung

carcinoma; etoposide also has been used orally, either alone or as a component of combination therapy for this cancer. Furthermore, etoposide has been used in conjunction with a platinum agent (i.e. cisplatin or carboplatin) and ifosfamide with mesna.

Etoposide is also used as part of first- or second-line combination chemotherapy regimens for the treatment of non-Hodgkin lymphoma, Hodgkin lymphoma, acute myeloid leukaemia, a variety of paediatric solid tumours, acute lymphocytic leukaemia, and in high-dose conditioning programmes before haematopoietic stem-cell transplantation.

(b) Dosage

Etoposide is administered orally and by slow intravenous infusion. Etoposide phosphate is administered by intravenous infusion.

The usual intravenous dose of etoposide ranges from 50–120 mg/m^2 daily for 5 days. Alternatively, 100 mg/m^2 has been given on alternate days to a total of 300 mg/m^2. The usual oral dose of etoposide is 100–240 mg/m^2 daily for 5 consecutive days. Courses may be repeated after 3–4 weeks.

Etoposide is available as a 50 mg liquid-filled capsule and as 100, 150, 200, 250, 500 mg and 1 g (20 mg/mL) solutions for injection concentrates and for intravenous infusion. Etoposide phosphate is available as 500 mg and 1 g (of etoposide) solutions for injection, and 100 mg (of etoposide) solutions for injection and intravenous infusion.

(c) Trends in use

No information was available to the Working Group.

1.2.2 Cisplatin

(a) Indications

The antineoplastic cisplatin is a platinum-containing complex that may act similarly to the alkylating agents. Its antineoplastic actions are cell-cycle non-specific and are dependent upon its *cis* configuration; they appear to be related to its hydrolysis in the body to form reactive hydrated species. Although it causes immunosuppression, stimulation of the host immune response against the tumour has been suggested as contributing to cisplatin's antineoplastic action.

Cisplatin is used in the treatment of tumours of the testis, usually as a major component of combination chemotherapy regimens, and particularly with bleomycin and etoposide (BEP), or with bleomycin and a vinca alkaloid. It is also used to treat metastatic ovarian tumours, cervical tumours, lung cancer, advanced bladder cancer, and squamous cell carcinoma of the head and neck.

(b) Dosage

Lower doses are generally used for combination chemotherapy regimens than in single agent therapy; 20 mg/m^2 or more is given once every 3–4 weeks. A dose of 20 mg/m^2 daily for 5 days every 3–4 weeks has been used in combination chemotherapy for the treatment of testicular tumours.

Various analogues of cisplatin have been developed or investigated including those with fewer adverse effects (e.g. carboplatin, nedaplatin), an altered spectrum of activity (oxaliplatin), or activity on oral dosage (satraplatin).

Various adjustments to the administration of cisplatin have been suggested in an attempt to improve its effectiveness while reducing its toxicity.

Toxicity is reported to be reduced when cisplatin is given by continuous intra-arterial or intravenous infusion. It has also been suggested that giving cisplatin in the evening rather than in the morning results in less damage to renal function, apparently because of circadian variations in urine production. However, another study found that morning, rather than evening, doses of cisplatin resulted in less renal damage.

(c) Trends in use

No information was available to the Working Group.

1.2.3 Bleomycin

(a) Indications

Bleomycin is an antineoplastic antibiotic that binds to DNA and causes strand scissions, and is probably most effective in the G_2- and M-phases of the cell cycle. It is used to treat malignant disease particularly squamous cell carcinomas, including those of the cervix and external genitalia, oesophagus, skin, and head and neck; Hodgkin lymphoma and other lymphomas; malignant neoplasms of the testis and malignant effusions. It has also been tried in other malignancies, including carcinoma of the bladder, lung, and thyroid, and some sarcomas, including Kaposi sarcoma.

Bleomycin is often used with other antineoplastics, with etoposide and cisplatin (BEP) in testicular tumours, and with doxorubicin, vinblastine, and dacarbazine (ABVD) for Hodgkin lymphoma. Bleomycin is given as the sulfate by either the intramuscular, intravenous, or subcutaneous route. It may also be given intraarterially or instilled intrapleurally or intraperitoneally. Bleomycin hydrochloride has also been given parenterally for malignant neoplasms, and bleomycin sulfate has been applied topically for the local treatment of skin tumours.

(b) Dosage

Doses are calculated in terms of the base, and are given in units, but the units used for preparations in the United Kingdom, which were formerly equivalent to those of the United States Pharmacopoeia (USP), are now international units equivalent to those of the European Pharmacopoeia. Because 1000 international units is equivalent to 1 USP unit, the United Kingdom doses now appear to be a thousand times greater than those previously in use, or than those in use in the Unites States of America, and care is recommended in evaluating the literature.

In the United Kingdom, the licensed dose as a single agent for squamous cell or testicular tumours is 15000 international units (15 USP units) three times a week, or 30000 international units twice a week, by intramuscular or intravenous injection, although in practice, treatment of malignancy will generally be with combination regimens. This may be repeated, at usual intervals of 3–4 weeks, up to a total cumulative dose of 500000 international units. The dose and total cumulative dose should be reduced in those over 60 years of age (see below). Continuous intravenous infusion at a rate of 15000 international units per 24 hours for up to 10 days or 30000 international units per 24 hours for up to 5 days may also be used. In patients with lymphoma, a dose of 15000 international units once or twice weekly by intramuscular injection has been suggested, to a total dose of 225000 international units. Dosage should be reduced in combination regimens if necessary. In the treatment of malignant effusions, a solution of 60000 international units in 100 mL of 0.9% sodium chloride may be instilled into the affected serous cavity. Treatment may be repeated as necessary up to a total cumulative dose of 500000 international units depending on the patient's age.

In the USA, licensed doses for lymphomas as well as squamous cell and testicular neoplasms are 250–500 international units/kg (0.25–0.5 USP units/kg), or 10000–20000 international units/m^2 (10–20 USP units/m^2), given once or twice weekly. In view of the risk of an anaphylactoid reaction, it has been suggested that patients with lymphomas should receive two test doses of 2000 international units (2 USP units) or less initially. In patients with Hodgkin lymphoma, once a 50% response has been achieved it may be maintained with 1000 international units (1 USP unit) of bleomycin daily, or 5000 international units (5 USP units) weekly.

In the United Kingdom, licensed product information suggests that a total dose of 500000 international units (500 USP units) should not be exceeded. Total cumulative dose should not exceed 300000 international units in patients aged 60–69 years, 200000 international units in those aged 70–79 years, and 100000 international units in those aged 80 years and over; the weekly dose should be no more than 60000, 30000, and 15000 international units, respectively. In the USA, the maximum total dose is 400000 international units (400 USP units); it is generally agreed that patients receiving 400000 international units or more are at increased risk of pulmonary toxicity.

Dosage should be reduced in patients with renal impairment.

(c) Trends in use

No information was available to the Working Group.

2. Cancer in Humans

A previous *IARC Monograph* (IARC, 2000) reviewed studies of cancer in humans following exposure to etoposide, and concluded that the combination of etoposide, bleomycin and cisplatin was carcinogenic, causing acute myeloid leukaemia in humans; but was unable to draw a separate conclusion about the carcinogenicity of etoposide alone (Table 2.1).

In the current evaluation, a large number of cohort studies published in the 1990s were re-assessed (Ratain *et al.*, 1987; Pedersen-Bjergaard *et al.*, 1991; Pui *et al.*, 1991; Curtis *et al.*, 1992; Bajorin *et al.*, 1993; Bokemeyer & Schmoll, 1993; Nichols *et al.*, 1993; Smith *et al.*, 1993, 1999; Sugita *et al.*, 1993; Winick *et al.*, 1993; Haupt *et al.*, 1994, 1997; Heyn *et al.*, 1994; Bokemeyer *et al.*, 1995; Boshoff *et al.*, 1995; Parkin *et al.*, 1997; Duffner *et al.*, 1998; Kollmannsberger *et al.*, 1998; Yagita *et al.*, 1998). A cumulative total of over 50 cases of acute myeloid leukaemia or myelodysplatic syndromes were reported in patients who had received etoposide for the treatment of a range of malignant diseases. These studies generally reported substantial increases (of the order of 10- to 100-fold) in the incidence of leukaemia compared to general population rates, as well as increases in leukaemia incidence with higher intensity administration of etoposide. [The Working Group noted that interpretation of many of these studies was limited by the difficulty in distinguishing the role of etoposide from that of other potentially leukaemogenic agents that had been received by the patients in the studies. However, in several studies, other agents involved in treatment of participating patients were recognized as being non-leukaemogenic.]

In an Italian study of patients treated for Langerhans cell histiocytosis (Haupt *et al.*, 1994, 1997), a large increased risk of acute myeloid leukaemia, based on five cases, was found after treatment with etoposide alone, or in combination with agents that are not recognized to cause leukaemia.

In cohort studies of germ-cell tumours in men, treatment with etoposide, cisplatin and bleomycin was associated with an increased risk for acute myeloid leukaemia (Pedersen-Bjergaard *et al.*, 1991; Bajorin *et al.*, 1993; Bokemeyer & Schmoll, 1993; Nichols *et al.*, 1993; Bokemeyer *et al.*, 1995; Boshoff *et al.*, 1995; Kollmannsberger *et al.*, 1998). On the basis of the data from these studies, the Working Group estimated a relative risk for acute myeloid leukaemia approximately 40 times greater than that of the general population.

A large increased risk for acute myeloid leukaemia was also found in one cohort study of lung cancer patients treated with etoposide, cisplatin and vindesine (Ratain *et al.*, 1987).

Table 2.2 shows results from a case–control study investigating 61 cases of leukaemia or myelodysplastic syndromes following solid

Table 2.1 Cohort studies of the risk for secondary acute myeloid leukaemia or myelodysplastic syndromes after treatment of germ-cell tumours with etoposide-containing regimens

Reference Study location	Study Population	Cumulative dose of etoposide (mg/m²)	No. of patients	Additional chemotherapy or radiotherapy	No. of observed cases	Follow-up period (yr)	Relative risk (95%CI) for AML or MDS	Cumulative incidence (95%CI) for AML or MDS	Comments
Pedersen-Bjergaard et al. (1991) Denmark	212 men diagnosed in 1979–89	1800–3600		Cisplatin, bleomycin	5 (4 AML, 1 MDS)	5.7	336 (92–861) (AML)	4.7% (SE, 2.3) at 5.7 yr (AML + MDS) 11% (SE, 5.0) at 5.7 yr (AML)	
		1800–2000	130		0				
		2000–3600	82		5				
Bajorin et al. (1993) New York, USA	340 men diagnosed in 1982–90	800–5000		Cisplatin, cyclophosphamide	2 (AML)	≥ 5	NR	< 1% at 5 yr for 1 AML who received etoposide only	1 case also received cyclophosphamide
Bokemeyer & Schmoll (1993) Germany	293 men diagnosed in 1970–90	≤ 2000	221	Cisplatin, bleomycin, vinblastine, anthracyclines, dactinomycin, ifosfamide	3 (1 ALL, 2 solid tumours)	Median, 5.1	2.3 (0.1–13)	1.0% (0.0–2.2) at 5 yr	SMR for etoposide-treated patients
		> 2000	72		0				
Nichols et al. (1993) Indiana, USA	538 men diagnosed in 1982–91	1500–2000		Cisplatin, bleomycin, ifosfamide	2 (AML)	Median, 4.9	66 (8–238)	NR [< 1% at 5 yr for AML]	3 cases observed in another group [size unknown] of patients treated with 2000 mg/m² (n=2) and 4400 mg/m² (n=1) etoposide
Bokemeyer et al. (1995) Germany	128 men diagnosed in 1983–93	Median cumulative dose:			1 (AML)	Median, 4.5	[30–35] (NS)	0.8% (0–2.3) at 4.5 yr	Etoposide-treated patients; possible overlap with study by Bokemeyer & Schmoll (1993)
		3750	22	Cisplatin, bleomycin, ifosfamide	1 (AML)	6.1			
		3800	50	Cisplatin, bleomycin	0	5.2			
		3800	41	Cisplatin, ifosfamide	0	3.4			
		5300	15	Carboplatin, ifosfamide, autologous stem-cell rescue	0	2.3			

Table 2.1 (continued)

Reference Study location	Study Population	Cumulative dose of etoposide (mg/m²)	No. of patients	Additional chemotherapy or radiotherapy	No. of observed cases	Follow-up period (yr)	Relative risk (95%CI) for AML or MDS	Cumulative incidence (95%CI) for AML or MDS	Comments
Boshoff et al. (1995) United Kingdom	679 men diagnosed in 1979–92	720–5000		Vincristine, methotrexate, cisplatin, bleomycin, actinomycin D, cyclophosphamide, vinblastine, carboplatin	6 AML, 4 solid tumours	Median, 5.7; 2 (n = 541); > 5 (n = 331)	150 (55–326) based on 3 cases who received 7-agent regimen	NR	Mediastinal germ cell-cancer patients included in patient population but none of them developed secondary tumour
		≤ 2000	636		4 (AML)				
		> 2000	25		2 (AML)				
Kollmannsberger et al. (1998) Germany, France	302 men, 15–55-yr old diagnosed in 1986–96	2400–14000			4 AML, 2 MDS in mediastinal germ-cell cancer patients	Median, 4.3	**SIR**. 160 (44–411)	1.3% (0.4–3.4) at 4.3 yr	Mediastinal germ-cell cancer patients included in patient population
		First-line therapy, 2400–6000	141	Cisplatin, ifosfamide, autologous stem-cell support	2 (AML)	3.5			161 patients included after failing first-line therapy
		2400–14000	161	Cisplatin, cyclophosphamide, ifosfamide, carboplatin, autologous stem-cell support	2 (AML)	4.8–5.6			

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CI, confidence interval; MDS, myelodysplastic syndromes; NR, not reported; NS, not significant; SE, standard error; SIR, standardized incidence ratio; SMR, standardized morbidity ratio; yr, year or years

Table 2.2 Case–control study of secondary leukaemia and treatment of primary tumour with etoposide-containing regimen

Reference, study location and period	Characteristics of cases	Characteristics of controls	Exposure assess-ment	Organ site (ICD code)	Exposure categories	No. of exposed cases	Relative risk (95%CI)	Adjustment for potential con-founders	Comments
Le Deley et al. (2003) France 1980–99	61 patients with leukaemia or MDS following first malignancy after 1980	196 controls patients with cancer but no secondary leukaemia matched by age, sex, year of diagnosis of first cancer and minimum survival	Medical records	Leukaemia	< 1.2 g/m^2 etoposide and < 170 mg/m^2 anthracyclines	8	1.0 (ref)	Type of first tumour, dose of anthracyclines	Exposure to etoposide alone not provided
					More in one or the other category	42	7 (2.6–19)		

CI, confidence interval; MDS, myelodysplastic syndromes; ref, reference

tumours among children in France (Le Deley *et al.*, 2003). The risk for leukaemia increased strongly with cumulative dose of etoposide in multivariate analyses.

3. Cancer in Experimental Animals

As per the previous IARC evaluation of etoposide (IARC, 2000), a single study was evaluated. The incidence of leukaemia was not increased in wild-type (*Nf1*$^{+/+}$) and heterozygous (*Nf1*$^{+/-}$) neurofibromatosis-1 (*Nf1*) gene knockout 129/Sv mice treated by gastric intubation for 6 weeks with 100 mg/kg body weight/week etoposide (Mahgoub *et al.*, 1999).

Bleomycin and cisplatin were each evaluated in previous *IARC monographs*. In animals, there was *limited evidence* for the carcinogenicity of bleomycin in animals, and *sufficient evidence* for the carcinogenicity of cisplatin (IARC, 1987a).

4. Other Relevant Data

Etoposide, cisplatin and bleomycin have very different profiles in terms of cellular uptake and generation of potentially cytotoxic lesions, but all three have actions that result in DNA damage leading to carcinogenicity.

4.1 Absorption, distribution, metabolism, and excretion

The general pharmacology of etoposide has been reviewed in Hande (1992). It is highly protein-bound in plasma with a free plasma fraction of approximately 6%, and its uptake into cells, which occurs by passive diffusion, is relatively slow (Tannock *et al.*, 2002). In contrast, efflux can be driven by active outward transport mechanisms such as P-glycoprotein and members of the MRP (multidrug resistance protein) family (Brock *et al.*, 1995). The main intracellular target-binding proteins for etoposide are topoisomerase IIα and IIβ, and it is likely that the IIα isoenzyme is the more biologically important target (Errington *et al.*, 1999). Studies using V79 cells have indicated that the distribution of topoisomerase IIα is highly dependent on its phosphorylation, and that phosphorylation promotes its location to the nucleus (Oloumi *et al.*, 2000).

After intravenous administration, cisplatin is bound to plasma proteins, with the protein-bound drug thought to be biologically inactive (Johnsson *et al.*, 1998), while the free drug is transported both in and out of cells by cupric ion transporters (Safaei & Howell, 2005). Inside the cell, cisplatin can react with protein sulfhydryl groups but is not sufficiently chemically reactive to react directly with DNA. Reaction with sulfhydryl groups of glutathione, however, makes it susceptible to the multidrug resistance transporter MRP2, which may also determine its intracellular concentration (Borst *et al.*, 2000).

Bleomycin comprises a mixture of chemical entities that are strongly bound in biological fluids to divalent ions such as copper. It appears to be transported into the cell by high-affinity L-carnitine transporters (Aouida *et al.*, 2004), but does not seem to be a substrate for P-glycoprotein-mediated efflux (Kang *et al.*, 2000).

4.2 Mechanisms of carcinogenesis

4.2.1 Induction of DNA damage

Etoposide, once bound to topoisomerase IIα, does not impede the ability of this enzyme to form double-stranded breaks but does impede the religation of DNA (Osheroff, 1989), leading to the formation of a stabilized DNA–topoisomerase II complex. This complex can generate double-stranded DNA breaks. In addition, collision of advancing DNA replication fork with etoposide–topoisomerase complexes can

lead to the formation of double-stranded DNA breaks (Baldwin & Osheroff, 2005; Tanaka *et al.*, 2007).

Cisplatin, under the low chloride ionic environment within the cell, reacts with water and the resulting monohydrate form reacts with DNA, predominantly at the N^7 position of guanine (Go & Adjei, 1999; Wang & Lippard, 2005; Bell *et al.*, 2006). The remaining chloro ligand is also replaced by water and leads to reaction with a second purine; the most common complexes are d(GpG) and d(ApG) intrastrand complexes, with a smaller proportion of interstrand complex formation. The formation of a complex bends the double helix (Takahara *et al.*, 1995), and promotes the binding of a variety of proteins containing high-mobility group domains (Huang *et al.*, 1994). Subsequent events in human cells are still not completely clear.

Bleomycin, once inside the cell, binds to guanosine–cytosine-rich portions of DNA by partial intercalation of the bithiazole ring. A portion of the molecule binds to divalent metals including iron, the active ligand, and copper, an inactive ligand. Molecular oxygen is then converted to reactive oxygen species in an iron-catalysed reduction, which generate several DNA lesions (Burger, 1998). One type of lesion is a DNA double-strand break, while another is a DNA lesion, which upon DNA replication can lead to a double-strand DNA break. Bleomycin is less cytotoxic to cells that are in the G_1-phase of the cell cycle (Mirabelli & Crooke, 1981).

4.2.2 Mutational consequences of DNA damage

Interference with the ability of DNA polymerase to synthesize a cDNA strand, which is a function of all three of these drugs, is thought to lead to several effects including mutations, sister chromatid exchange, and chromosomal aberrations (Kaufmann, 1989). Each of these individual drugs (etoposide, cisplatin and bleomycin) induces sister chromatic exchange and aneuploidy (IARC, 1987b, 2000; Pommier *et al.*, 1988; Chibber & Ord, 1989; Au *et al.*, 2001; De Mas *et al.*, 2001; Cantero *et al.*, 2006). Of the three drugs, the strongest evidence for drug-induced cancer is provided by etoposide, which induces monocytic and myelomonocytic leukaemia through a specific chromosomal translocation (Kudo *et al.*, 1998), and this may be a general response to topoisomerase II poisons (Ferguson & Baguley, 1996).

Acute myeloid leukaemia develops in patients previously treated with epipodophyllotoxin-type topoisomerase II inhibitors such as etoposide and teniposide, and frequently exhibits distinctive characteristics that allow it to be distinguished from acute myeloid leukaemia induced by other agents (such as alkylating agents) or acute myeloid leukaemia that occurs spontaneously (Pedersen-Bjergaard & Rowley, 1994; Pedersen-Bjergaard *et al.*, 2006). The induced leukaemias are typically classified as the monocytic or myelomonocytic subtypes, have short latency periods of 2–3 years, and frequently exhibit balanced translocations involving the myeloid–lymphoid or mixed lineage leukaemia (*MLL*) gene (also known as acute lymphoblastic leukaemia-1 (*ALL-1*), human trithorax (*HRX*), and human homologue of *Drosophila* trithorax gene (*HTRX-1*)) located on the long arm of chromosome 11 (11q23). *MLL* encodes a transcription factor that plays a role in the regulation of haematopoietic development (Fidanza *et al.*, 1996; Hess *et al.*, 1997). Recent studies have shown that the four most common *MLL* translocation partner genes (*AF4*, *AF9*, *ENL*, and *AF10*) encode nuclear proteins that are part of a network involved in the methylation of lysine 79 of histone H3 proteins (H3K79 methylation) (Meyer *et al.*, 2006), indicating an important role for this pathway in induced leukaemias.

Approximately 85% of treatment-related leukaemia patients who exhibit 11q23 translocations have previously been treated with

topoisomerase-II-inhibiting drugs, primarily etoposide or anthracyclines (doxorubicin, daunorubicin) (Bloomfield *et al.*, 2002; Mauritzson *et al.*, 2002). Etoposide has also been shown to induce breakages, rearrangements, and translocations within the *MLL* gene in experimental systems (e.g., mouse embryonic stem cells and in haematopoietic $CD34^+$ cells in culture, including human long-term repopulating haematopoietic stem cells) (Blanco *et al.*, 2004; Libura *et al.*, 2005, 2008; Sung *et al.*, 2006). This provides strong evidence of a causal link between etoposide exposure and *MLL* translocations in a crucial target cell for leukaemogenesis (Allan & Travis, 2005). In addition, topoisomerase II recognition sites are located close to the breakpoints in many of the treatment-related leukaemias seen in patients, providing additional evidence for the role of topoisomerase II in the formation of the translocations (Allan & Travis, 2005).

The ability of various *MLL* chimeric genes formed through translocation to transform mouse haematopoietic cells has been demonstrated by several investigators (Corral *et al.*, 1996; Dobson *et al.*, 1999; Lavau *et al.*, 2000; So *et al.*, 2003; Wang *et al.*, 2005). Upon expression of the chimeric gene or infusion of gene-expressing cells, the mice exhibited altered haematopoiesis, which progressed to more serious myeloproliferative disorders that mimicked the corresponding human disease. In most of these studies, the mice developed frank leukaemias (Corral *et al.*, 1996; Dobson *et al.*, 1999; Lavau *et al.*, 2000; Forster *et al.*, 2003; So *et al.*, 2003). However in one study (Wang *et al.*, 2005), treatment with a mutagenic agent such as γ-radiation or *N*-ethyl-*N*-nitrosourea was necessary for the manifestation of leukaemia.

4.3 Synthesis

Combined therapy with bleomycin, etoposide and cisplatin, a common form of chemotherapy for testicular germ-cell malignancies, has led not only to a large number of long-term survivors but also to a significant proportion of patients with secondary malignancies. Mechanistic studies of these three drugs have demonstrated that each is genotoxic, with evidence of induction of DNA damage, chromosomal aberrations, and aneuploidy. Etoposide is distinguished from the other two drugs by its ability to induce chromosomal translocations affecting the *MLL* gene, which are often seen in patients that develop therapy-related acute myeloid leukaemia.

Etoposide in combination with cisplatin and bleomycin is carcinogenic via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of etoposide in combination with cisplatin and bleomycin. Etoposide in combination with cisplatin and bleomycin causes acute myeloid leukaemia.

There is *limited evidence* in humans for the carcinogenicity of etoposide alone.

There is *inadequate evidence* in experimental animals for the carcinogenicity of etoposide alone.

No data were available to the Working Group for the carcinogenicity of etoposide in combination with cisplatin and bleomycin in experimental animals.

Etoposide in combination with cisplatin and bleomycin is *carcinogenic to humans (Group 1).*

Etoposide is *carcinogenic to humans (Group 1).*

In making the overall evaluation of etoposide alone, the Working Group took into consideration the following:

- The acute myeloid leukaemias induced by drugs, including etoposide, that target topoisomerase II exhibit distinctive characteristics (i.e. morphology, latency, and karyotypes) that

allow them to be distinguished from leukaemias induced by alkylating agents.

• The high frequency of 11q23 translocations in the leukaemias associated with etoposide treatment and the localization of the breaks within the *MLL* gene, a gene involved in haematopoiesis.

• The clustering of the breakpoints within the *MLL* gene in the leukaemias induced by drugs that target topoisomerase II, and the presence of topoisomerase II recognition sites near these breakpoints.

• The ability of etoposide to induce breakages, rearrangements, and translocations within the *MLL* gene in model systems including long-term repopulating human haematopoietic stem cells, an important target cell for leukaemogenesis.

• The ability of the chimeric *MLL* genes resulting from 11q23 translocations to alter haematopoiesis, and to induce leukaemias in mice.

• The observations that bleomycin and cisplatin exert their genotoxic effects through mechanisms not involving inhibition of topoisomerase II.

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MELPHALAN

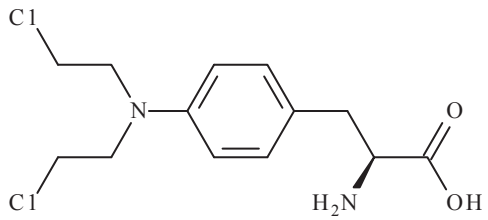
Melphalan was considered by previous IARC Working Groups in 1974 and 1987 (IARC, 1975, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 148-82-3; 3223-07-2 [hydrochloride]
Chem. Abstr. Name: L-Phenylalanine, 4-[bis(2-chloroethyl)amino]-
IUPAC Systematic Name: (2*S*)-2-Amino-3-[4-[bis(2-chloroethyl)amino]phenyl] propanoic acid
Synonyms: Alanine nitrogen mustard; Alkeran; melphalan; L-PAM; L-phenylalanine mustard; L-phenylalanine mustard hydrochloride; phenylalanine mustard; L-sarcolysine
Description: Off-white to buff powder with a faint odour (Sweetman, 2008)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_{13}H_{18}Cl_2N_2O_2$
Relative molecular mass: 305.2

1.2 Use of the agent

Information for Section 1.2 is taken from McEvoy (2007), Royal Pharmaceutical Society of Great Britain (2007), Thomson Healthcare (2007), and Sweetman (2008).

1.2.1 Indications

Melphalan is an antineoplastic agent that acts as a bifunctional alkylating agent. It is used in the treatment of multiple myeloma, advanced ovarian adenocarcinoma, early and advanced breast cancer, childhood neuroblastoma, and *polycythaemia vera*. Melphalan is also used for regional arterial perfusion in localized malignant melanoma, and localized soft-tissue sarcoma of the extremities.

1.2.2 Dosage

Melphalan is usually given orally as a single daily dose or in divided doses; it is also given intravenously as the hydrochloride.

The oral dose administered to treat multiple myeloma may vary according to the regimen. Typical dose regimens include 150 µg/kg daily for 4–7 days; 250 µg/kg daily for 4 days; or 6 mg daily for 2–3 weeks. Melphalan is usually combined with corticosteroids. Courses are followed by a rest period of up to 6 weeks to

allow recovery of haematological function, and are then repeated, or maintenance therapy may be instituted, usually with a daily dose of 1–3 mg, or up to 50 µg/kg.

Similar oral dose regimens (150–200 µg/kg daily for 5 days, repeated every 4–8 weeks) have been used to treat ovarian adenocarcinoma and advanced breast cancer, although with the development of newer agents, melphalan is now used infrequently for the treatment of these tumours. The dose administered by mouth to treat *polycythaemia vera* is initially 6–10 mg daily, reduced after 5–7 days to 2–4 mg daily until satisfactory response, then further reduced to 2–6 mg per week for maintenance.

Melphalan has also been given intravenously; a single dose of 1 mg/kg, repeated in 4 weeks if the platelet and neutrophil counts permit, has been used to treat ovarian adenocarcinoma. In multiple myeloma, melphalan has been administered as a single agent intravenously at a dose of 400 µg/kg or 16 mg/m^2, infused over 15–20 minutes; the first four doses are given at 2-week intervals, and further doses are given at 4-week intervals depending on toxicity. High-dose melphalan has been given intravenously in some malignancies: doses of 100–240 mg/m^2 have been used for neuroblastoma, and 100–200 mg/m^2 in multiple myeloma, generally followed by autologous stem-cell transplantation. Melphalan may be given by local arterial perfusion in the management of melanoma and soft-tissue sarcoma. A typical dose range for upper extremity perfusions is 0.6–1 mg/kg, whereas for lower extremity perfusions, dose ranges of 0.8–1.5 mg/kg (in melanoma) or 1–1.4 mg/kg (in sarcoma) are typically used.

Melphalan is available as 2 mg tablets for oral administration, and as a 50 mg (melphalan hydrochloride) solution for injection for parenteral administration.

Melphalan is also used intravenously as part of isolated hyperthermic limb perfusions for patients with malignant melanoma in transit and limb sarcomas.

1.2.3 Trends in use

The availability of several newer chemotherapeutic and biological therapies has substantially reduced the use of melphalan in patients with solid tumours, whereas the use of high-dose intravenous melphalan followed by autologous stem-cell infusion has increased following randomized studies that demonstrated a disease-free, and in some studies, an overall survival advantage in patients with multiple myeloma.

2. Cancer in Humans

Epidemiological studies of patients with ovarian carcinoma (Reimer *et al.*, 1977; Einhorn *et al.*, 1982; Greene *et al.*, 1982, 1986; Kaldor *et al.*, 1990), multiple myeloma (Gonzalez *et al.*, 1977; Law & Blom, 1977; Bergsagel *et al.*, 1979; Dent *et al.*, 2000) or breast cancer (Fisher *et al.*, 1985; Curtis *et al.*, 1992) have consistently shown very large excesses of acute myeloid leukaemia in the decade following therapy with melphalan.

Since then, a large number of epidemiological studies have contributed to the weight of evidence for the carcinogenicity of melphalan, in particular, two large case–control studies detailed below.

A case–control study conducted in a cohort of 82700 women who received adjuvant chemotherapy for breast cancer, and who were followed up for at least 18 months after completion of treatment, identified 90 cases of leukaemia, of which 80 were acute myeloid leukaemia/myelodysplatic syndromes. The relative risk (RR) of developing acute myeloid leukaemia was highest in patients who received alkylating agents with radiation treatment (RR, 17.4) compared to those who were treated with alkylating agents (RR, 10) or radiation treatment alone (RR, 2.4). The relative risk was 10-fold greater for patients who received

melphalan (RR, 31.4) compared to those who received cyclophosphamide (Curtis *et al.*, 1992).

A case–control study compared the relative risk of leukaemia in patients treated with chemotherapy or radiation to patients who only underwent surgery (Kaldor *et al.*, 1990). Approximately 90 cases [The Working Group noted that older nomenclature was used, and it was difficult to precisely assess the diagnosis in some patients] of acute myeloid leukaemia or myelodysplatic syndromes were identified among ~99000 patients with ovarian cancer. All of the alkylating agents (including chlorambucil, cyclophosphamide, thiotepa, treosulfan, and melphalan) increased the risk of developing leukaemia in a dose-dependent manner. Relative risks for melphalan ranged from 12–23 depending on the total dose used.

3. Cancer in Experimental Animals

Melphalan has been tested in mice by oral, intraperitoneal, and dermal application; in rats by intraperitoneal injection, and in monkeys by oral administration (Table 3.1).

In mice, the administration of melphalan produced forestomach papillomas, lymphosarcomas, and skin and lung tumours (IARC, 1975, 1987a; Satoh *et al.*, 1993; Eastin *et al.*, 2001). In rats, melphalan caused mammary gland tumours, and peritoneal sarcomas (IARC, 1975, 1987a). Results in monkeys were inconclusive (Thorgeirsson *et al.*, 1994; Schoeffner & Thorgeirsson, 2000).

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

In humans, following oral administration, melphalan is absorbed from the gastrointestinal tract with a wide range in bioavailability (range 25–89%, mean 56%) (Sweetman, 2008). It also exhibits considerable variability with respect to the time of its appearance in the plasma (range ~0–6 hours), and in the peak plasma concentration achieved (range 70–4000 ng/mL, depending upon the dose) (GlaxoSmithKline, 2007). This variability may be due to incomplete intestinal absorption, variable first-pass metabolism, and/or rapid hydrolysis. Upon absorption, approximately 60–90% of plasma melphalan is bound to plasma proteins such as albumin, and to a lesser degree, α_1-acid glycoprotein, with approximately 30% being bound irreversibly (GlaxoSmithKline, 2007). Melphalan does not undergo metabolic activation and is inactivated in the plasma, primarily by non-enzymatic hydrolysis to monohydroxymelphalan and dihydroxymelphalan. Apart from these hydrolysis products, no other melphalan metabolites have been detected in humans (GlaxoSmithKline, 2007). Melphalan enters cells through active transport, mostly by the high-affinity L-amino acid transport system, which carries glutamine and leucine (Nieto & Vaughan, 2004).

As a consequence of its inconsistent absorption, melphalan can also exhibit considerable variability in its elimination. For example, when given intravenously, melphalan exhibited a fairly consistent half-life (14–40 minutes in a study of ten patients given a 20 mg/m^2 dose) but was absorbed variably, and found to have a half-life of 36–552 minutes in a study of 13 patients administered a 0.6 mg/m^2 oral dose (Hall & Tilby, 1992). About 10% of the drug is excreted unchanged in the urine within 24 hours, and about 30% of administered melphalan (including metabolites) is excreted in the urine within 9 days of oral administration (McEvoy, 2007; Sweetman, 2008). Approximately 20–50% of the dose is eliminated via faeces (McEvoy, 2007).

Table 3.1 Studies of cancer in experimental animals exposed to melphalan

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, S (NR) 22 wk Roe & Salaman (1956)	Skin 0, 1.44, 3 mg total dose administered by skin painting once weekly for 10 wk 60, 25, 25	Skin (papillomas): 5/53, 2/19, 4/22 Lung (adenomas): 10/17 (1.7/mouse), 13/18 (3.6/mouse), 11/13 (5.1/mouse)	[$P < 0.05$] for 3 mg	Croton oil used as promoter (experimental Weeks 5–22)
Mouse, A/J (M, F) 39 wk Shimkin et al. (1966)	i.p. 0, 0.27, 1.07, 4.27, 17.1 mg/kg bw total dose 3 times/wk for 4 wk 1400, 60, 60, 60, 60	Lung: 307/777, 23/58, 37/56, 43/56, 40/41 Tumours/mouse : 0.5, 0.6, 1.0, 2.1, 4.0	[$P < 0.0001$] for 1.07, 4.27 and 17.1 mg/kg bw	
Mouse, Swiss (M, F) 18 mo Weisburger et al. (1975)	i.p. 0.75, 1.5 mg/kg bw 3 times/wk for 6 mo 25 (M) + 25 (F)	Lung: M–11/44 F–10/23 Lymphosarcomas: M–13/44	 $P = 0.012$ $P = 0.001$ $P < 0.001$	Dosage groups were combined for each sex; 254 mice served as untreated controls
Mouse, Tg.AC (M, F) 27 wk Eastin et al. (2001)	Oral gavage 0, 0.25, 1.0 & 4.0 mg/kg bw/wk for 26 wk 15/sex/group	Forestomach (squamous cell papillomas): M–8/14, 14/15, 15/15, 14/15; F–12/15, 14/14, 13/14, 13/14	[$P \leq 0.03$] for 0.25, 1.0, 4.0 mg/kg bw doses in male mice	
Mouse, Tg.AC (M, F) 27 wk Eastin et al. (2001)	Topical application (in methanol, 3.3 mL/kg bw), 0, 0.25, 1.0, 4.0 mg/kg bw/wk for 26 wk 15/sex/group	Skin tumours (in area of the vulva): F–1/15, 3/15, 7/15, 12/15	[$P \leq 0.02$] for 1.0 & 4.0 mg/kg bw in female mice	
Mouse, A/J (F) 24 wk Satoh et al. (1993)	i.p. 0, 4.0 µmol/kg bw/d, 3 d/wk 12, 13	Lung (adenomas): 4/12 & 13/13 (incidence) Tumours per mouse: 0.42 ± 0.67, 12.15 ± 1.68	[$P = 0.0005$] (incidence) [$P < 0.001$] for multiplicity	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, (F) 17 mo Presnov & Iushkov (1964)	i.p 0, 10 mg/kg bw, single application 40, 60	Mammary gland (fibroadenomas): 0/30, 9/33		A mixture of melphalan and medpalan (D isomer of melphalan) was used
Rat, Sprague-Dawley CD (M, F) 18 mo Weisburger et al. (1975)	i.p. 0.9, 1.8 mg/kg bw 3 times/wk for 6 mo 25/sex	Peritoneum (sarcomas): M–11/20 F–10/23	 $P < 0.001$ $P < 0.001$	Dosage groups were combined for each sex; 360 rats served as untreated controls
Monkey, (NR) 16 yr Thorgeirsson et al. (1994), Schoeffner & Thorgeirsson (2000)	Nasogastric tube (in DMSO, volume NR) 0.1 mg/kg bw/5 d/wk	Malignant tumours: 5/12		Incidence in control animals could not be determined

bw, body weight; d, day or days; DMSO, dimethylsulfoxide; F, female; i.p., intraperitoneal; M, male; mo, month or months; NR, not reported; w, week or weeks; yr, year or years

4.2 Mechanisms of carcinogenesis

4.2.1 Induction of DNA damage

Melphalan is a direct-acting, bifunctional alkylating agent that binds to cellular macromolecules (Osborne *et al.*, 1995). As a phenylalanine derivative of nitrogen mustard, it is capable of producing a variety of DNA adducts including mono-adducts at the N^7 of guanine and the N^3 of adenine as well as interstrand cross-links, and pre-mutagenic lesions that are believed to play a critical role in its toxic and carcinogenic effects (Povirk & Shuker, 1994; Lawley & Phillips, 1996; GlaxoSmithKline, 2007).

Melphalan has been shown to bind to DNA, RNA, and protein in cells *in vitro* (Tilby *et al.*, 1987, 1995; Povirk & Shuker, 1994; Osborne *et al.*, 1995), and DNA-binding has been seen in rats treated *in vivo* (Van den Driessche *et al.*, 2004a, b). The formation of DNA cross-links *in vitro* and *in vivo* has also been reported based on the measurement of specific adducts (Osborne & Lawley, 1993) or by changes in DNA migration in DNA strand breakage or electrophoretic assays (Ringborg *et al.*, 1990; Popp *et al.*, 1992; Souliotis *et al.*, 2003, 2006; Cordelli *et al.*, 2004; Dimopoulos *et al.*, 2005).

4.2.2 Mutational consequences of DNA damage

Melphalan has been tested for genotoxicity in an assortment of short-term assays, both *in vitro* and *in vivo*. Increased frequencies of dominant lethal mutations, chromosomal aberrations, micronuclei, and DNA strand breaks have been observed in several studies following treatment of rodents with melphalan (IARC, 1987b; Shelby *et al.*, 1989; Generoso *et al.*, 1995; Morita *et al.*, 1997; Tsuda *et al.*, 2000; Sgura *et al.*, 2005, 2008; Ranaldi *et al.*, 2007). In the mouse-specific locus mutation and heritable translocation tests, increases in mutations were seen in both spermatagonial as well as postspermatogonial germ cells (Russell *et al.*, 1992a, b; Generoso *et al.*, 1995; Witt & Bishop, 1996). The observed mutations originated primarily from large deletions in the postspermatogonial cells whereas other types of mutagenic mechanisms predominated in the spermatogonial cells (Witt & Bishop, 1996).

Melphalan also induced chromosomal aberrations, sister chromatid exchange, micronuclei, mutations at the *HPRT* gene, and DNA damage in human cells *in vitro* (IARC, 1987b; Mamuris *et al.*, 1989a, b; Sanderson *et al.*, 1991; Routledge *et al.*, 1992; Sorsa *et al.*, 1992; Vock *et al.*, 1999; Efthimiou *et al.*, 2007; Escobar *et al.*, 2007). It also induced transformation of C3H 10T1/2 and other cells (IARC, 1987b; Miller *et al.*, 1994; Kowalski *et al.*, 2001). In cultured rodent cells, it induced chromosomal aberrations, sister chromatid exchange, gene mutations, and DNA damage (IARC, 1987b; Austin *et al.*, 1992; Preuss *et al.*, 1996; Allan *et al.*, 1998). In addition, it induced aneuploidy and sex-linked recessive lethal mutations in *Drosophila*, and mutation in bacteria (IARC, 1987b).

Increased frequencies of chromosomal aberrations and sister chromatid exchange occurring in the peripheral blood lymphocytes have been reported in multiple studies of patients treated therapeutically with melphalan (IARC, 1987b; Raposa & Várkonyi, 1987; Mamuris *et al.*, 1989b, 1990; Popp *et al.*, 1992; Amiel *et al.*, 2004). In addition, DNA-binding (DNA mono-adducts and interstrand cross-links) to *TP53* and *N-RAS*, two important cancer-related genes, has been seen in the peripheral blood cells of patients administered melphalan (Souliotis *et al.*, 2003, 2006; Dimopoulos *et al.*, 2005, 2007). Decreased migration of DNA in strand breakage assays indicative of DNA cross-link formation has been observed in melphalan-treated cancer patients (Popp *et al.*, 1992; Spanswick *et al.*, 2002). Haematotoxicity and immunosuppression have also been reported in patients treated with this anticancer agent (Goldfrank *et al.*, 2002; GlaxoSmithKline, 2007).

Acute myeloid leukaemia that develops in patients previously treated with alkylating agents such as melphalan frequently exhibits distinctive characteristics that allow it to be distinguished from acute myeloid leukaemia induced by other agents (such as topoisomerase II inhibitors) or acute myeloid leukaemia that occurs spontaneously (Rödjer *et al.*, 1990; Pedersen-Bjergaard & Rowley, 1994; Jaffe et al, 2001; Pedersen-Bjergaard *et al.*, 2006). One of the hallmarks of leukaemias induced by alkylating agents is that they frequently exhibit a clonal loss of either chromosome 5 or 7 (–5, –7) or a loss of part of the long arm of one of these chromosomes (5q–, 7q–). For example, a deletion within the long arm of chromosome 5 involving the bands q23 to q32 is often seen (Jaffe *et al.*, 2001). Leukaemias developed in patients treated with melphalan (often in combination with other agents) frequently exhibit these clonal chromosomal changes (Rödjer *et al.*, 1990).

In addition, mutations in *TP53* are frequently seen in leukaemias with the –5/5q– karyotype, and mutations involving the *AML1* gene as well as mutations in *TP53* and *RAS* are often seen in a subset of leukaemias that exhibit the –7/7q– karyotype (Christiansen *et al.*, 2001, 2005; Pedersen-Bjergaard *et al.*, 2006). These treatment-related acute myeloid leukaemias also frequently exhibit increased methylation of the *p15* promoter (Pedersen-Bjergaard *et al.*, 2006). While there is some evidence that melphalan may directly induce damage targeting chromosomes 5 or 7 (Mamuris *et al.*, 1989a, b, 1990; Amiel *et al.*, 2004), this drug has also been reported to induce nonspecific chromosomal alterations in a variety of experimental models, and in the lymphocytes of treated patients (described above). The detection of elevated levels of chromosomal aberrations in the peripheral blood lymphocytes of melphalan-treated patients is of particular note, as multiple prospective studies have now shown that individuals with increased levels of chromosomal aberrations in these cells are at increased risk of developing cancer later in life (Hagmar *et al.*, 1998, 2004; Liou *et al.*, 1999; Smerhovsky *et al.*, 2001).

4.3 Synthesis

Melphalan is a direct-acting alkylating agent that is carcinogenic via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of melphalan. Melphalan causes acute myeloid leukaemia.

There is *sufficient evidence* in experimental animals for the carcinogenicity of melphalan.

Melphalan is *carcinogenic to humans (Group 1).*

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MOPP

MOPP was considered by a previous IARC Working Group in 1987 (IARC, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

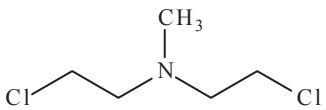
1.1.1 MOPP

MOPP is a combination chemotherapy regimen, the acronym being derived from the first initials of its constituents (as they were referred to at the time it was developed, i.e. mechlorethamine, oncovin, procarbazine, and prednisone).
Chem. Abstr. Serv. Reg. No.: 113803-47-7
Chem. Abstr. Name: Vincaleukoblastine, 22-oxo-, mixture with 2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine hydrochloride, 17,21-dihydroxypregna-1,4-diene-3,11,20-trione and *N*-(1-methylethyl)-4-[(2-methylhydrazino) methyl]benzamide
Synonyms: Vincristine-prednisone-nitrogen mustard-procarbazine mixture

1.1.2 Chlormethine

Chem. Abstr. Serv. Reg. No.: 51-75-2; 55-86-7 [hydrochloride]
Chem. Abstr. Name: Ethanamine, 2-chloro-*N*-(2-chloroethyl)-*N*-methyl-
IUPAC Systematic Name: 2-Chloro-*N*-(2-chloroethyl)-*N*-methylethanamine
Synonyms: Bis(β-chloroethyl)methylamine; bis(2-chloroethyl)methylamine; *N*,*N*-bis(2-chloroethyl)methylamine; Caryolysine; chloramine; chlorethazine; di(2-chloroethyl)methylamine; *N*,*N*-di(chloroethyl) methylamine; mechlorethamine; methyl-β,β-dichlorodiethylamine; methylbis(β-chloroethyl)amine; methylbis(2-chloroethyl)amine; methyldi(2-chloroethyl)amine; *N*-methyl-2,2′-dichlorodiethylamine; *N*-methylbis(β-chloroethyl)amine; *N*-methylbis(2-chloroethyl)amine; Mustargen; nitrogen mustard
Description: hygroscopic, light yellow brown crystalline powder [hydrochloride salt] (McEvoy, 2007); also reported as white or almost white crystalline powder [hydrochloride salt] (Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass

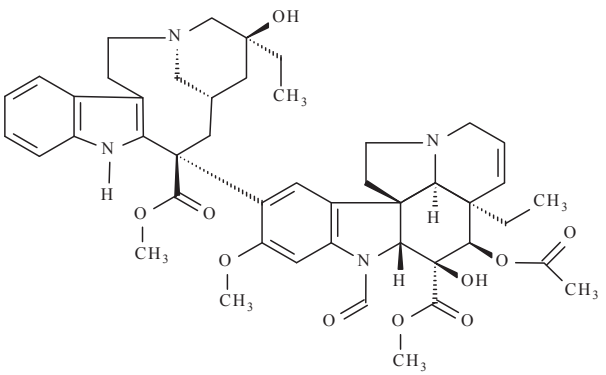


$C_5H_{11}Cl_2N$
Relative molecular mass: 156.1

1.1.3 Vincristine

Chem. Abstr. Serv. Reg. No.: 57-22-7; 2068-78-2 [sulfate salt]
Chem. Abstr. Name: Vincaleukoblastine, 22-oxo
IUPAC Systematic Name: Leurocristine
Synonyms: LCR; leucristine; Oncovin; VCR; Vincasar PFS; (+)-vincristine
Description: white, off-white, or slightly yellow, hygroscopic, amorphous or crystalline powder [sulfate salt] (McEvoy, 2007; Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass

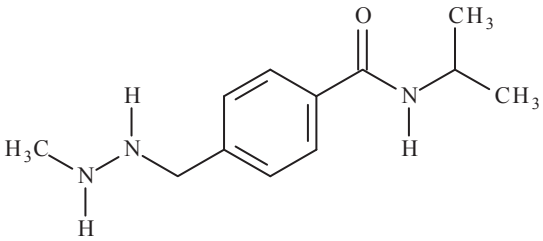


$C_{46}H_{56}N_4O_{10}$
Relative molecular mass: 825.0

1.1.4 Procarbazine

Chem. Abstr. Serv. Reg. No.: 671-16-9; 366-70-1 [hydrochloride]
Chem. Abstr. Name: Benzamide, *N*-(1-methylethyl)-4-[(2-methylhydrazino) methyl]-
IUPAC Systematic Name: 4-[(2-Methylhydrazinyl) methyl]-*N*-propan-2-ylbenzamide
Synonyms: Benzamide, *N*-(1-methylethyl)-4-[(2-methylhydrazino)methyl]-; 1-methyl-2-[*p*-(isopropylcarbamoyl) benzyl]hydrazine; 4-[(2-methylhydrazino)methyl]-*N*-isopropylbenzamide; *p*-(2-methylhydrazinomethyl)-*N*-isopropylbenzamide; *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide.
Description: white to pale yellow, crystalline powder with a slight odour [hydrochloride salt] (McEvoy, 2007)

(a) Structural and molecular formulae, and relative molecular mass

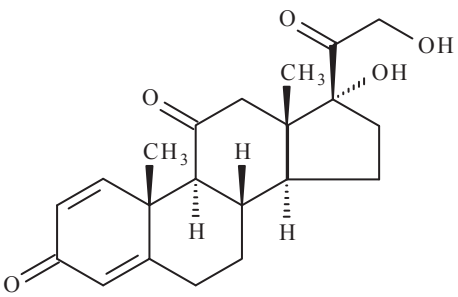


$C_{12}H_{19}N_3O$
Relative molecular mass: 221.3

1.1.5 Prednisone

Chem. Abstr. Serv. Reg. No.: 53-03-2
Chem. Abstr. Name: Pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-
IUPAC Systematic Name: (8*S*,9*S*,10*R*,13*S*,14*S*,17*R*)-17-Hydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-6,7,8,9,12,14,15,16-octahydrocyclopenta[*a*] phenanthrene-3,11-dione
Synonyms: 1,2-Dehydrocortisone; 17,21-dihydroxypregna-1,4-diene-3,11,20-trione; 17α,21-dihydroxy-1,4-pregnadiene-3,11,20-trione
Description: white to practically white, odourless, crystalline powder (McEvoy, 2007; Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass



$C_{21}H_{26}O_5$

Relative molecular mass: 358.4

1.2 Use of the combination

Information for Section 1.2 is taken from Fischer et al. (2003), McEvoy (2007), Skeel (2007), and Sweetman (2008).

1.2.1 Indications

MOPP, developed in the mid-1960s, was the first combination chemotherapy regimen for treating Hodgkin lymphoma. It has been used alone or in combination (in part or total) with ABVD [adriamycin, bleomycin, vinblastine, dacarbazine] chemotherapy.

1.2.2 Dosage

In the treatment of advanced Hodgkin lymphoma, the usual dose of chlormethine hydrochloride in the MOPP regimen is 6 mg/m^2 given intravenously on Days 1 and 8 of a 28-day cycle. In subsequent cycles of MOPP therapy, dose reductions are sometimes considered depending on the depth and duration of treatment-induced neutropenia or thrombocytopenia.

The usual recommended adult dose of vincristine sulfate is 1.4 mg/m^2 on Days 1 and 8 of a 28-day cycle, often limiting the dose to 2 mg in adults, and the usual paediatric dose is 1.5–2 mg/m^2. For children weighing 10 kg or less, the recommended therapy is initiated at 0.05 mg/kg once weekly. The dose of procarbazine is 100 mg/m^2 daily on Days 1 to 14 of a 28-day cycle. The dose of prednisone is 40 mg/m^2 daily on Days 1 to 14, on Cycles 1 and 4 only. The MOPP regimen is repeated every 28 days for a total of 4–8 courses depending on the response to treatment, and on the initial stage of Hodgkin lymphoma.

Chlormethine (as the hydrochloride) is available as a 10 mg injection solution for intravenous administration. Vincristine (as the sulfate) is available as a 1 mg/mL (1 and 2 mg) injection solution for intravenous-only administration. Procarbazine (as the hydrochloride) is available as a 50 mg (procarbazine equivalent) capsule for oral administration. Prednisone is available as 1, 2.5, 5, 10, 20, and 50 mg tablets for oral administration, and as 5 mg/5 mL or 5 mg/mL solutions for oral administration.

1.2.3 Trends in use

ABVD has supplanted MOPP as the regimen of choice for the initial treatment of Hodgkin lymphoma. MOPP is sometimes used if patients fail to respond or relapse after ABVD treatment, although other regimens have been developed that are used when autologous stem-cell transplantation is considered as salvage therapy.

2. Cancer in Humans

2.1 Acute myeloid leukaemia

MOPP was one of the first attempts at combining several active agents in the treatment of cancer and, until about 15 years ago, was the most common regimen used for the treatment of Hodgkin lymphoma (Devita *et al.*, 1970). In 1972, roughly 5 years after the introduction of intensive combined chemotherapy for Hodgkin disease, the first report of subsequent acute

myeloid leukaemia appeared (Arseneau *et al.*, 1972). Since then, multiple centres and collaborative treatment groups in Europe and North America have performed studies that led to the conclusion that MOPP treatment was causally associated with subsequent acute myeloid leukaemia, and myelodysplastic syndromes (Baccarani *et al.*, 1980; Brusamolino *et al.*, 1982; Coltman & Dixon, 1982; Glicksman *et al.*, 1982; Boivin *et al.*, 1984; IARC, 1987a). Summary estimates of the relative risk of acute myeloid leukaemia after MOPP chemotherapy relative to a healthy population have been calculated to vary from 9 (Jacquillat *et al.*, 1983) through 40 (Bergsagel *et al.*, 1982; Henry-Amar, 1983) to well over 100 (Glicksman *et al.*, 1982; Bartolucci *et al.*, 1983; Boivin *et al.*, 1984). The rates of acute myeloid leukaemia/myelodysplastic syndromes were highest in patients receiving combined modality therapy in which MOPP was combined with irradiation.

[The Working Group noted that observed variations in both relative and actuarial risks are probably due to differences in methodology, treatment, and patient populations. Furthermore, although cases of acute myeloid leukaemia can occur at any time after initial treatment, there is a sharp peak in incidence approximately 4–7 years after treatment with alkylating-agent-based chemotherapy with much lower rates both before and afterwards (Blayney *et al.*, 1987).]

[The Working Group noted that the use of multi-agent chemotherapy regimens makes the identification of risk from individual constituents very difficult, although the focus in the MOPP regimen has been on the nitrogen mustard (Henry-Amar *et al.*, 1989) and procarbazine because there is little evidence of leukaemogenesis from vinca alkaloids or corticosteroids.]

Randomized trials have shown that ABVD produced a lower incidence of acute myeloid leukaemia in ABVD recipients than in MOPP recipients (Santoro *et al.*, 1987; Cimino *et al.*, 1991; Maurizi Enrici *et al.*, 1997; Cellai *et al.*, 2001; Duggan *et al.*, 2003) thereby providing further indirect evidence that MOPP is involved in the higher rates of acute myeloid leukaemia observed in the earlier studies, rather than the underlying Hodgkin lymphoma.

2.2 Cancer of the lung

A nested case–control study of 222 cases of lung cancer occurring among 19046 patients treated for Hodgkin lymphoma resulted in a relative risk of 4.2 (95%CI: 2.1–8.8; adjusted for smoking history) for patients treated with alkylating agents in general, and 5.0 (95%CI: 2.1–13.6) for those treated with MOPP specifically, with increasing risks with higher cumulative doses of both chlormethine and procarbazine (Travis *et al.*, 2002). Two smaller case–control studies of lung cancer in patients with Hodgkin lymphoma concurred with this evidence (Kaldor *et al.*, 1992; Swerdlow *et al.*, 2001).

See Table 2.1 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-07-Table2.1.pdf

2.3 Other sites

As noted in the earlier *IARC Monograph* (IARC, 1987a) and supported by more recent publications, the development of other tumours, including non-Hodgkin lymphoma, breast cancer (Kaldor *et al.*, 1987), sarcoma, melanoma (Tucker *et al.*, 1985), malignancies of the central nervous system, and carcinomas of the thyroid and gastrointestinal system have also been reported to increase after treatment for Hodgkin lymphoma (Boivin *et al.*, 1984; Cellai *et al.*, 2001). [The Working Group noted that estimates in these studies were generally based on few outcomes, and are difficult to interpret. In addition, soft tissue sarcomas, as well as cancers of the lung, breast, and thyroid occur predominantly in areas of prior irradiation, thus

making it even more complicated to estimate the contribution of MOPP in the development of these cancers, which can develop decades after successful completion of therapy for Hodgkin lymphoma.]

3. Cancer in Experimental Animals

No data were available to the Working Group.

4. Other Relevant Data

Each of the drugs used in the MOPP combined chemotherapy has a unique or complementary mechanism of action, and has been previously evaluated by IARC (IARC, 1987b).

Chlormethine was considered by IARC Working Groups in 1975 and 1987 (IARC, 1975, 1987b).

In humans, following its in-vivo administration, chlormethine is rapidly converted into an ethylene immonium ion which reacts with the guanine residues in adjacent strands of DNA as well as with thiol groups of proteins (Boyland, 1946; Verly, 1964). It is usually cleared from the blood in a few minutes. A very small proportion is excreted unchanged in the urine (Sweetman, 2008). An intravenous administration of chlormethine to dogs cleared rapidly from the blood, with 0.01% found in the urine, low levels found in the tissues, the highest concentration being in the bone marrow (Ishidate, 1959; Mellett & Woods, 1960). After intravenous injection of $^{14}CH_3$-chlormethine to mice, significant levels of the radioactivity were observed in the brain, spinal cord, lungs, and submaxillary glands (Tubaro & Bulgini, 1968). In rats, 16% of an injected dose of chlormethine was found present in the spleen, lung, kidney, liver and blood, and 17% was excreted in the urine (Obrecht *et al.*, 1964).

Chlormethine is a bifunctional alkylating agent that binds to DNA, forming mono-adducts primarily at the N^7 position of guanine, and interstrand and intrastrand cross-links (Povirk & Shuker, 1994). As indicated in earlier *IARC Monographs* (IARC, 1987b), chlormethine induced dominant lethal mutations and micronuclei in the bone-marrow cells of mice exposed *in vivo,* and alkylated DNA of ascites cells in experimental animals treated *in vivo*. It induced chromosomal aberrations, sister chromatid exchange, and unscheduled DNA synthesis in human cells *in vitro*. In rodent cells *in vitro*, it induced sister chromatid exchange, chromosomal aberrations and DNA damage; studies on the induction of mutation were inconclusive. It transformed mouse C3H 10T1/2 cells. Chlormethine induced aneuploidy and somatic mutation and recombination in *Drosophila*, chromosomal aberrations in plants, mitotic recombination and mutation in fungi, and mutation and DNA damage in fungi. It was also reported in one study to induce chromosomal aberrations in lymphocytes of treated patients.

Vincristine sulfate was considered by IARC Working Groups in 1981 and 1987 (IARC, 1981, 1987b).

After intravenous injection in humans, vincristine is extensively protein-bound and is reported to be concentrated in blood platelets. It is cleared rapidly from the blood, metabolized in the liver, and excreted primarily in the bile, about 70–80% of a dose is found in faeces, as unchanged drug and metabolites, while 10–20% appears in the urine over a 72-hour period (Bender *et al.*, 1977; Jackson *et al.*, 1978). The terminal half-life may range from 19–155 hours. Vincristine does not appear to cross the blood–brain barrier (Sweetman, 2008).

After intravenous or intraperitoneal injection of vincristine to different animal species, it is cleared from the blood and distributed to most tissues (lung, liver, kidney, pancreas, spleen, and brain) (Castle *et al.*, 1976; El Dareer *et al.*, 1977a;

Jackson *et al.*, 1980). It is excreted in urine and faeces as unchanged drug and as metabolites in approximately the same proportions. In mice and monkeys, a very low concentration of vincristine appears to cross the blood–brain barrier (El Dareer *et al.*, 1977a; Jackson *et al.*, 1980).

Vincristine sulfate is a Vinca alkaloid that interferes with microtubule assembly and spindle formation and consequently blocks the replication of cells during mitosis. As indicated in earlier *IARC Monographs* (IARC, 1987b), it induced micronuclei in the bone-marrow cells of mice and hamsters treated *in vivo*. It induced aneuploidy in and transformation of Syrian hamster embryo cells, but did not transform mouse C3H 10T1/2 cells. It did not induce structural chromosomal aberrations, sister chromatid exchange or unscheduled DNA synthesis in rodent cells *in vitro*. It induced mutation in mouse lymphoma cells but not in other rodent cells. It did not induce sex-linked recessive lethal mutations in *Drosophila*, and was not mutagenic to bacteria.

Procarbazine (hydrochloride) was considered by IARC Working Groups in 1981 and 1987 (IARC, 1981, 1987b).

In humans, procarbazine is given orally as it is absorbed readily from the gastrointestinal tract. The plasma half-life is approximately 7 minutes (Raaflaub & Schwartz, 1965). It is rapidly metabolized to azo derivatives and hydrogen peroxide (mainly in the liver and the kidneys), and only about 5% is excreted unchanged in the urine (Oliverio, 1973). Using ^{14}C-radiolabelled procarbazine, 70% was shown to appear in urine within 24 hours, less than 5% as the unchanged parent compound, and the remainder predominantly as *N*-isopropylterephthalamic acid. Between 10–20% of the drug is exhaled as carbon dioxide and methane via the lungs. Faecal excretion is negligible (Bollag, 1965; Schwartz *et al.*, 1967). After intravenous injection in humans and in dogs, the drug and its metabolites cross the blood–brain barrier, and diffuses in the cerebrospinal fluid within a short time, which may account for some of its central nervous toxicity (Oliverio, 1973).

In animals, after oral administration, procarbazine is also readily absorbed from the gut. Its plasma half-life in dogs and rats is 12 and 24 minutes, respectively (Raaflaub & Schwartz, 1965; Reed, 1975). In rodents and dogs, the main urinary metabolite is *N*-isopropylterephthalamic acid (Oliverio *et al.*, 1964). Methane and carbon dioxide can be exhaled after intraperitoneal injection to rats suggesting that the metabolism of procarbazine proceeds via formation of methylhydrazine (Dost & Reed, 1967). Procarbazine and its metabolite monomethylhydrazine are demethylated by rat hepatic enzymes, forming the azo and azoxy metabolites (Baggiolini & Bickel, 1966). In rats, *N*-isopropyl-*para*-toluamide and methane were also found as metabolites (Weinkam & Shiba, 1978).

Procarbazine is a methylhydrazine derivative metabolized to reactive intermediates that decompose to produce a methyl diazonium cation, which methylates DNA, and is believed to be responsible for its toxic and carcinogenic effects (Kufe *et al.*, 2006). As summarized in earlier *IARC Monographs* (IARC, 1987b), procarbazine gave positive results for germ-cell mutation in the mouse-specific locus test, and caused mutation in the mouse spot test. It induced micronuclei and structural chromosomal aberrations in mice treated *in vivo*, but conflicting results were obtained in tests for dominant lethal mutations and negative results in the heritable translocation test. It induced sister chromatid exchange in mice and Chinese hamsters, and caused DNA damage in rodents treated *in vivo*. Procarbazine did not transform Syrian hamster embryo cells. It induced mutation but not sister chromatid exchange in rodent cells *in vitro*. It induced aneuploidy, dominant lethal mutations, sex-linked recessive lethal mutations and somatic mutation and recombination in *Drosophila*, but did not cause heritable translocations. It induced

mutation, gene conversion and mitotic recombination in fungi. Conflicting results were obtained for mutation in bacteria, both *in vitro* and in host-mediated assays; it induced DNA damage in bacteria.

Prednisone was considered by IARC Working Groups in 1981 and 1987 (IARC, 1981, 1987b).

Prednisone is readily absorbed from the gastrointestinal tract in animals and humans. Prednisone and prednisolone, its active metabolite, have been detected in serum within 1 hour after prednisone administration by different routes in different species (Colburn *et al.*, 1976; El Dareer *et al.*, 1977b).

Prednisone is converted to prednisolone, its biologically active form, after the reduction of the 11-oxo to the 11-β-hydroxyl group catalysed by the enzyme 11-β-hydroxydehydrogenase (Jenkins & Sampson, 1967), and to several other metabolites (El Dareer *et al.*, 1975, 1977b). Metabolism takes place primarily in the liver. After intravenous administration of prednisone to a monkey, the unchanged drug and prednisolone were distributed to most of the tissues with the highest concentrations in the kidneys for prednisone, and in the liver for prednisolone (El Dareer *et al.*, 1977b).

In humans, orally administered prednisone produces lower circulating concentrations of prednisolone than prednisolone itself given by the same route (Tse & Welling, 1979), with considerable intra- and intersubject variation (Hsueh *et al.*, 1979).

Prednisone is bound to serum proteins (albumin and corticosteroid-binding globulin) in humans and animals (Lang & Stevens, 1970; Feldman *et al.*, 1972; Pickup, 1979).

In humans, prednisone is excreted in urine with a greater level after intravenous administration than after oral dosage (Hsueh *et al.*, 1979). The corresponding 20 β-alcohols such as 20-dihydroprednisolone are present in smaller amounts (Gray *et al.*, 1956). Other uncommon metabolites are mentioned in Bush & Mahesh (1964).

Prednisone is a synthetic glucocorticoid with multiple modes of action, and produces a range of anti-inflammatory and immunosuppressive effects (Sweetman, 2008). In the earlier *IARC Monographs* (IARC, 1987b), it was reported that there were no data available on the genetic and related effects of prednisone in humans. It was also indicated that prednisone did not induce chromosomal aberrations in bone-marrow cells of rats treated *in vivo*, and was not mutagenic to bacteria.

In addition to studies on the individual drugs, there have been several investigations into the genotoxic effects of this drug combination. For example, Clare *et al.* (1982) investigated the ability of the MOPP drug combination to induce sister chromatid exchange in peripheral blood lymphocytes exposed in culture, and determined that the resulting dose-related increase in sister chromatid exchange was very similar to that seen with chlormethine alone. In another series of studies, Goldstein (1984, 1987, 1987–1988) tested MOPP as well as three of its components (chlormethine, vincristine, and procarbazine) individually and in combination for their ability to induce dominant lethal mutations using an in-vitro assay following the in-vivo treatment of mice. Significant increases were reported for MOPP, chlormethine, and procarbazine (Goldstein, 1984; 1987–1988). In a follow-up study, significant increases in mutation were seen with chlormethine alone and in the two- and three-drug combinations that contained chlormethine, leading to the conclusion that the observed increases were primarily due to chlormethine (Goldstein, 1987). Similarly, the mutagenic and genotoxic effects of the drug combination have been investigated in cancer patients undergoing treatment (Sen *et al.*, 1990; Caggana *et al.*, 1991; Brandriff *et al.*, 1994; Abdallah *et al.*, 1995; Zheng *et al.*, 2000; Bilban-Jakopin & Bilban, 2001; Mkacher *et al.*, 2003). In

most of the reports, the patients were also being treated with ionizing radiation or other antineoplastic drugs so that when effects were seen, it was not possible to determine which effects or portion of the effects were due to the MOPP treatment. However, in many of these reports, there were individuals or groups of patients who had only received MOPP and who showed elevated frequencies of mutation, sister chromatid exchange or chromosomal aberrations in their peripheral blood lymphocytes or sperm (Brown *et al.*, 1988; Sen *et al.*, 1990; Caggana *et al.*, 1991; Brandriff *et al.*, 1994; Zheng *et al.*, 2000). While considerable variability in response was seen, these reports are largely consistent with the in-vitro and animal results, indicating that the drug combination is mutagenic and genotoxic. Myelotoxicity is also commonly seen in patients treated with this combination of drugs (Benjamin *et al.*, 1976).

Acute myeloid leukaemia that develops in patients that have previously been treated with alkylating agents such as chlormethine and procarbazine frequently exhibits distinctive characteristics that allow it to be distinguished from acute myeloid leukaemia induced by other agents (such as topoisomerase II inhibitors) or acute myeloid leukaemia that occurs spontaneously (Pedersen-Bjergaard & Rowley, 1994; Jaffe *et al.*, 2001; Mauritzson *et al.*, 2002; Pedersen-Bjergaard *et al.*, 2006). One of the hallmarks of leukaemias induced by alkylating agents is that they frequently exhibit a clonal loss of either chromosome 5 or 7 (–5, –7) or a loss of part of the long arm of one of these chromosomes (5q–, 7q–). For example, a deletion within the long arm of chromosome 5 involving the bands q23 to q32 is often seen (Jaffe *et al.*, 2001). Leukaemias that have developed in patients treated with MOPP (often in combination with other agents) have been reported to exhibit these clonal chromosomal changes (Christiansen *et al.*, 2001; Hayani *et al.*, 1992).

In addition, mutations in *TP53* are frequently seen in leukaemias with the –5/5q– karyotype, and mutations involving the *AML1* gene as well as mutations in *TP53* and *RAS* are often seen in a subset of leukaemias that exhibit the –7/7q– karyotype (Christiansen *et al.*, 2001, 2005; Pedersen-Bjergaard *et al.*, 2006). These treatment-related acute myeloid leukaemias also frequently exhibit increased methylation of the *p15* promoter (Pedersen-Bjergaard *et al.*, 2006). Although the evidence that MOPP directly induces losses or deletions affecting chromosomes 5 or 7 is limited, the individual drugs have been reported to induce similar types of chromosomal alterations in a variety of experimental models as described above.

4.1 Synthesis

The MOPP combination as well as individual components, except for prednisone, are genotoxic, and induce cancer via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of MOPP. MOPP causes cancer of the lung, and acute myeloid leukaemia.

No data were available to the Working Group for the carcinogenicity of MOPP in experimental animals.

MOPP is *carcinogenic to humans (Group 1).*

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TAMOXIFEN

Tamoxifen was considered by a previous IARC Working Group in 1996 (IARC, 1996). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

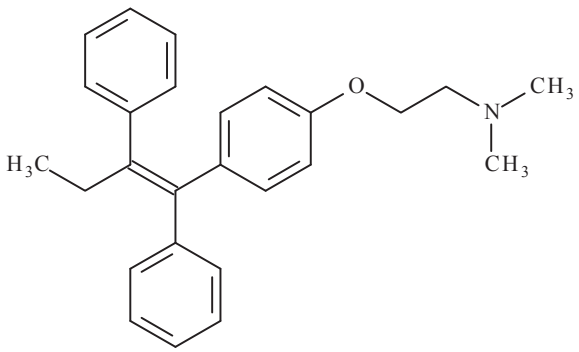
1. Exposure Data

1.1 Identification of the agent

1.1.1 Tamoxifen

Chem. Abstr. Serv. Reg. No.: 10540-29-1
Chem. Abstr. Name: (*Z*)-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-*N*,*N*-dimethylethanamine
IUPAC Systematic Name: 2-[4-[(*Z*)-1,2-Diphenylbut-1-enyl]phenoxy]-*N*,*N*-dimethylethanamine
Synonyms: 1-*p*-*β*-Dimethylaminoethoxyphenyl-*trans*-1,2-diphenylbut-1-ene; (*Z*)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]ethyldimethylamine
Description: Crystalline solid (O'Neil, 2006)

(a) Structural and molecular formulae, and relative molecular mass

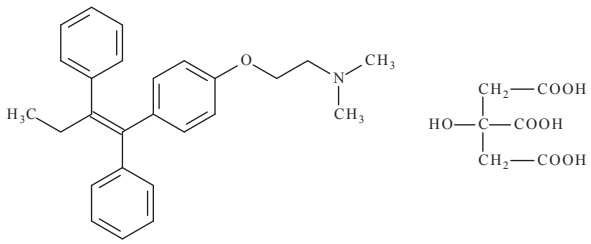


$C_{26}H_{29}NO$
Relative molecular mass: 371.52

1.1.2 Tamoxifen citrate

Chem. Abstr. Serv. Reg. No.: 54965-24-1
Chem. Abstr. Name: (*Z*)-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-*N*,*N*-dimethylethanamine, 2-hydroxy-1,2,3-propanetricarboxylate (1:1)
IUPAC Systematic Name: 2-[4-[(*Z*)-1,2-Diphenylbut-1-enyl]phenoxy]-*N*,*N*-dimethylethylamine; 2-hydroxypropane-1,2,3-tricarboxylic acid
Synonyms: Kessar; Nolvadex; Soltamox; tamoxifen citrate; *Z*-tamoxifen citrate
Description: Fine, white, odourless crystalline powder (O'Neil, 2006)

(a) Structural and molecular formulae, and relative molecular mass



$C_{26}H_{29}NO.C_6H_8O_7$

Relative molecular mass: 563.64

1.2 Use of the agent

Information for Section 1.2 is taken from IARC (1996), AstraZeneca Pharmaceuticals LP (2005), Royal Pharmaceutical Society of Great Britain (2007), and Thomson Healthcare (2007).

1.2.1 Indications

Tamoxifen has been available since the early 1970s for the first-line treatment of metastatic breast cancer in postmenopausal women. Tamoxifen has also been used as adjuvant therapy for treatment of postmenopausal, node-positive women with positive estrogen-receptor or progesterone-receptor levels and, since the early 1990s, for the treatment of postmenopausal node-negative women with positive estrogen-receptor or progesterone-receptor levels. In the late 1980s and early 1990s, it was also widely used in treating postmenopausal receptor-negative women (IARC, 1996; Wolff & Abeloff, 2002; Albain, 2004).

In women with ductal carcinoma *in situ* following breast surgery and radiation therapy, tamoxifen is used to reduce the risk of invasive breast cancer.

Tamoxifen has been considered as a chemopreventive agent to reduce the incidence of breast cancer in women at high risk of breast cancer.

Tamoxifen has been used as the first-line therapy for hormone-responsive male breast cancer, and is also used as adjuvant therapy for estrogen receptor- or progesterone receptor-positive male breast cancer.

Tamoxifen has also been used for anovulatory infertility.

1.2.2 Dosage

Tamoxifen is available as 10 mg and 20 mg tablets (each tablet contains 15.2 mg and 30.4 mg, respectively, of tamoxifen citrate and as an oral solution (each 5 mL solution contains 15.2 mg tamoxifen citrate, equivalent to 10 mg tamoxifen).

(a) Cancer of the breast

(i) Metastatic breast cancer

For the treatment of metastatic breast cancer in women, the usual dosage of tamoxifen is 20–40 mg daily, typically starting with the 20 mg dose. Dosages exceeding 20 mg daily are typically given in divided doses (morning and evening). A 20 mg oral dose is administered as 10 mL of the oral solution.

(ii) Adjuvant therapy of breast cancer

When tamoxifen is used as an adjunct to surgery and radiation therapy in the treatment of breast cancer, the usual dosage of the drug is 20–40 mg daily. Dosages exceeding 20 mg daily typically are given in divided doses (morning and evening). The optimum duration of adjuvant tamoxifen therapy has not been established, but therapy for about 5 years has become the norm.

When tamoxifen is used in combination with chemotherapy as an adjunct to surgery in the treatment of breast cancer in postmenopausal women or in women 50 years of age or older who have positive axillary lymph nodes, the usual dosage of the drug is 10 mg twice daily. The optimum duration of adjuvant tamoxifen therapy has not been established.

(iii) Ductal carcinoma in situ (DCIS)

In women with DCIS following breast surgery and radiation therapy, tamoxifen is used for a recommended duration of 5 years to reduce the risk of invasive breast cancer.

(iv) Chemoprevention in women at high risk of breast cancer

For reduction in the incidence of breast cancer in women at high risk, the recommended dosage of tamoxifen is 20 mg daily given for 5 years.

(v) Male breast cancer

For the treatment of advanced (metastatic) breast cancer in men, the usual dosage of tamoxifen is 20–40 mg daily. Tamoxifen alone or in combination with radiation therapy was also used as an adjunct to surgery in the treatment of breast cancer in men at a dosage of 20 mg daily, usually for 1–2 years.

(b) Other uses

For anovulatory infertility, 20 mg of tamoxifen is administered daily on Days 2, 3, 4, and 5 of the cycle; if necessary, the daily dose may be increased to 40 mg and then 80 mg for subsequent courses; if menstrual cycles are irregular, the initial course may be started on any day, with subsequent course starting 45 days later or on Day 2 of cycle if menstruation occurs.

1.2.3 Trends in use

Although tamoxifen citrate is still available as a breast cancer treatment, it has largely been replaced by other treatments (AstraZeneca PLC, 2004, 2007).

2. Cancer in Humans

2.1 Cancer of the endometrium

In the sections that follow, the most important studies considered in the previous *IARC Monograph* (IARC, 1996) are included. Case reports are not considered.

Detection bias may pertain to both cohort studies and randomized clinical trials as tamoxifen is known to increase the frequency of symptoms such as vaginal bleeding or discharge, which may lead to gynaecological evaluation. In addition, tamoxifen is known to induce benign gynaecological changes such as endometrial hyperplasia and polyps. Other changes include poorly defined thickening of the endometrium that may be revealed by ultrasound examination.

The longer survival of tamoxifen-treated patients may lead to greater duration of follow-up in which second cancers may occur. The appropriate methods of statistical analysis in this context are life table analyses or analyses of rates based on person–years at risk.

2.1.1 Cohort studies

Of 11 cohort studies of women with breast cancer, three were based on data from the SEER (Surveillance, Epidemiology and End Results) Program in the United States of America (Curtis *et al.*, 1996, 2004; Newcomb *et al.*, 1999), all included substantial numbers of cases of endometrial cancer, and all found significant elevations of risk for endometrial cancer. Specific histological types were evaluated in the Curtis *et al.* (2004) study. The relative risk was higher for malignant mixed mullerian tumours (MMMTs) than for endometrial adenocarcinomas, although the excess absolute risk was smaller—an additional 1.4 versus 8.4 cancers per 10000 women per year, respectively. [The Working Group noted that the Curtis *et al.* (2004) study is an extension of Curtis *et al.* (1996). It is probable that there is some

overlap with the cohort reported by Newcomb *et al.* (1999), but the authors do not discuss this nor cite the paper by Newcomb *et al.* (1999). In these studies, the absence of hysterectomy could not be confirmed, nor were individual records of tamoxifen use available. Misclassification of hormonal treatment in the studies may have led to an underestimation of the difference in risk for cancer of the uterine corpus between the groups.]

Of the remaining cohort studies of women with breast cancer, one was a nested case–control study (Cook *et al.*, 1995) in which tamoxifen use was more common in the controls (31% versus 26%). Another small cohort study (Katase *et al.*, 1998) found no increase in risk of endometrial cancer in those treated with tamoxifen. Three of the other cohort studies found a positive but non-significant elevation of the risk of endometrial cancer (Matsuyama *et al.*, 2000; Ursic-Vrscaj *et al.*, 2001; Yamazawa *et al.*, 2006), though the numbers of endometrial cancer cases in these studies were small. Of the two remaining cohort studies, one (Bouchardy *et al.*, 2002) found significantly elevated risks of uterine cancer with tamoxifen use, and the other (Lavie *et al.*, 2008), borderline increases in risk. There was one additional cohort study, based on women at high risk of breast cancer, with a significantly increased risk of endometrial cancer following tamoxifen use (Beiner *et al.*, 2007).

See Table 2.1 available online at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-08-Table2.1.pdf.

2.1.2 Case–control studies

The case–control studies considered by the Working Group were those that compared tamoxifen use in women with breast cancer who did (cases) or did not (controls) subsequently develop endometrial cancer. A fundamental requirement for the controls is that they were at risk of developing endometrial cancer (i.e. they had an intact uterus). Determinants of risk for endometrial cancer are confounding factors in the studies discussed below only to the extent that they influence the likelihood of tamoxifen prescription. As in any case–control study, information and selection bias may also be present. Finally, the possibility that endometrial cancer was diagnosed preferentially in women who had received tamoxifen constitutes a potential bias that is considered in the introductory remarks to Section 2.1.

All of the seven case–control studies found elevations in risk of endometrial cancer following tamoxifen use. Three of the studies found significant elevations of risk following tamoxifen use (Mignotte *et al.*, 1998; Bergman *et al.*, 2000; Swerdlow & Jones, 2005). In two (Bergman *et al.*, 2000; Swerdlow & Jones, 2005), greater (significant) increases in risk followed durations of use of tamoxifen of 5 years or more. The study of Chu *et al.* (2007) was conducted to examine whether a genetic variant of the *CYP3A4* gene, *CYP3A4*1B*, influences endometrial cancer risk–alone or when associated with tamoxifen exposure, as tamoxifen is metabolized by various cytochrome P450 (CYP) enzymes, but predominantly by CYP3A4. This resulted in the finding of an increase in risk in women who carried the *CYP3A4*1B* allele following treatment with tamoxifen.

[Although several potential confounders were not systematically addressed in most of the case–control studies, the Working Group considered that these were unlikely to have had a major effect on the reported relative risks.]

See Table 2.2 available online at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-08-Table2.2.pdf.

2.1.3 Randomized trials

In most of the randomized trials of breast cancer treatment, small numbers of endometrial cancers were reported. In two of the largest trials, however, there was a strong and statistically significant association between risk of

endometrial cancer and use of tamoxifen (Fisher *et al.*, 1996; Rutqvist & Johansson, 2007). Data from the large majority of the trials individually cited in IARC (1996) were included in the meta-analyses described below. Therefore, these trials are not further discussed here.

All of the reports show statistically significant elevations of the risk of endometrial cancer except in the trial of Delozier *et al.* (2000), in which all participants received tamoxifen for at least 2 years. The pooled analysis of The Early Breast Cancer Trialists' Collaborative Group (1998) was the largest of these analyses. Information was collected in 1995, which was analysed centrally. The incidence of endometrial cancer had approximately quadrupled in trials of 5 years of tamoxifen use (although the number of cases was small, and these ratios were not significantly different from the findings at 2 years). Mortality from endometrial cancer was also significantly increased in those who received tamoxifen, 27 versus 5 deaths in controls, for a 10-year risk per 1000 of 1.7 versus 0.4. Two of the reports (Braithwaite *et al.*, 2003; Cuzick *et al.*, 2003) included findings from chemoprevention trials using tamoxifen as well as treatment trials. [The Working Group noted that there is some overlap in these reports with some of the treatment trial data included in The Early Breast Cancer Trialists' Collaborative Group (1998) overview analysis, but the extent is unknown.] The 7-year follow-up data from the National Surgical Adjuvant Breast and Bowel Project chemoprevention trial confirmed the earlier reported excess of endometrial cancer in those who received tamoxifen (20 mg/day) (Fisher *et al.*, 2005).

See Table 2.3 available online at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-08-Table2.3.pdf.

2.2 Contralateral breast cancer

Although for some of the smaller trials of breast cancer treatment there seemed to be little difference in the number of contralateral breast cancer in tamoxifen-treated women compared with controls, for the larger trials, there was a substantially and significantly reduced risk for contralateral breast cancer in tamoxifen-treated women compared with controls. In a combined analysis of nearly all trials published in 1992 with data available to 1990, there was a significant reduction of 39% in contralateral breast cancer in the tamoxifen-treated groups (IARC, 1996), confirmed by subsequent overview analyses. In the Cuzick *et al.* (2003) overview analysis of nine treatment trials, the benefit was restricted to reduction in estrogen-receptor-positive cancers.

See Table 2.4 available online at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-08-Table2.4.pdf.

Four cohort studies reported on contralateral breast cancer. In three (Cook *et al.*, 1995; Curtis *et al.*, 1996; Newcomb *et al.*, 1999), the risk of contralateral breast cancer was reduced in the tamoxifen-treated women, compared with women with no reported tamoxifen use. However, Matsuyama *et al.* (2000) reported no risk reduction in their cohort study from Japan.

See Table 2.5 available online at http://monographs.iarc.fr/ENG/Monographs/vol100A/100-A-08-Table2.5.pdf.

2.3 Chemoprevention of cancer of the breast

Four trials of chemoprevention have been conducted with one being stopped because the desired end-point was reached earlier than anticipated (Fisher *et al.*, 2005). The latest data available from this trial are summarized in Table 2.6 (available online at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-08-Table2.6.pdf), together with the results of a

meta-analysis combining earlier data from this trial with that available from the three other trials (Cuzick *et al.*, 2003). It is apparent that there is a significant reduction in the incidence of breast cancer in the women who received tamoxifen. As for contralateral breast cancer, the benefit was restricted to reduction in estrogen-receptor-positive cancers.

2.4 Gastrointestinal cancers

Although an excess of gastrointestinal cancer was reported following an early combined analysis of three Scandinavian trials, this was not confirmed by other studies reported to 1996 (IARC, 1996). Data on gastrointestinal cancer was reported in five cohort studies (Curtis *et al.*, 1996; Newcomb *et al.*, 1999; Matsuyama *et al.*, 2000; Srinivasan *et al.*, 2005; Chandanos *et al.*, 2006), with only one providing data on all gastrointestinal sites combined (Curtis *et al.*, 1996). Three studies provided data on oesophageal cancer, four on stomach cancer, and four on colon or colorectal cancer. None of the studies reported significant excess risks for oesophageal, stomach or colorectal cancer in comparisons between breast cancer patients treated with tamoxifen and those who did not receive tamoxifen. However, Newcomb *et al.* (1999) reported a borderline significant positive association between hormonal therapy use and colorectal cancer in the period 5 or more years after diagnosis, while Matsuyama *et al.* (2000) and Chandanos *et al.* (2006) reported significant excesses of stomach cancer in comparison to the general population. [The Working Group thought that such comparisons are biased, and although comparisons between subjects with breast cancer treated and not treated with tamoxifen may also not be entirely valid as discussed above, they are preferable to comparisons with the general population.]

In The Early Breast Cancer Trialists' Collaborative Group (1998) analysis, tamoxifen had no apparent effect on the incidence of colorectal cancer. However, in the Braithwaite *et al.* (2003) meta-analysis, tamoxifen was associated with significantly increased risks of gastrointestinal cancers (reported in 16 trials), with a relative risk of 1.31 (95% CI: 1.01–1.69).

See Table 2.7 available online at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-08-Table2.7.pdf.

2.5 Cancer of the ovary

Five cohort studies (Cook *et al.*, 1995; Curtis *et al.*, 1996; Newcomb *et al.*, 1999; Matsuyama *et al.*, 2000; Ursic Vrscaj *et al.*, 2001), and two case–control studies (Metcalfe *et al.*, 2005; Swerdlow & Jones, 2007) evaluated the role of tamoxifen therapy in women with breast cancer in relation to the risk of subsequent ovarian cancer. No study showed any indications of increased risk, though the numbers of cases in some of the cohort studies were very small.

See Table 2.8 available online at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-08-Table2.8.pdf and Table 2.9 available online at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-08-Table2.9.pdf.

2.6 Synthesis

In summary, the potential effect of tamoxifen in increasing the risk of endometrial cancer among women with breast cancer has been reported in nine cohort studies, four case–control studies, five randomized controlled treatment trials, and one major chemoprevention trial; the majority of these in meta-analyses, though there were four separate reports from individual trials. The data from the observational studies and randomized controlled trials are largely consistent in showing that tamoxifen, whether given as adjuvant therapy for women

with breast cancer or for chemoprevention in women at high risk for breast cancer, increases the risk for endometrial cancer. Also, there is evidence that the use of tamoxifen in the treatment of breast cancer significantly reduces the incidence of contralateral breast cancer. The use of tamoxifen, when given for chemoprevention, reduces the incidence of estrogen-receptor-positive breast cancers.

Finally, there is some indication that the risk of various forms of gastrointestinal cancer may be increased in tamoxifen-treated patients, however the data are not consistent.

3. Cancer in Experimental Animals

Tamoxifen has been tested for carcinogenicity by oral and subcutaneous administration to adult and infant mice and rats, and by transplacental exposure to mice.

3.1 Oral administration

3.1.1 Mouse

In mice treated orally, tamoxifen increased the incidence of benign ovarian and benign testicular tumours in one study (Tucker *et al.*, 1984), but did not increase the tumour incidence in two other studies (Carthew *et al.*, 1996a; Martin *et al.*, 1997).

3.1.2 Rat

Rats dosed orally with tamoxifen had an increased incidence of benign and malignant liver cell tumours in multiple studies (Greaves *et al.*, 1993; Hard *et al.*, 1993; Hirsimäki *et al.*, 1993; Williams *et al.*, 1993, 1997; Ahotupa *et al.*, 1994; Hasmann *et al.*, 1994; Carthew *et al.*, 1995b; Dragan *et al.*, 1995; Kärki *et al.*, 2000; Carthew *et al.*, 2001; Kasahara *et al.*, 2003). When given at a lower dose level, tamoxifen decreased the incidence of benign and malignant mammary gland tumours in female rats, and benign pituitary tumours in both sexes (Maltoni *et al.*, 1997).

See Table 3.1.

3.2 Subcutaneous administration

3.2.1 Mouse

In mice treated subcutaneously, tamoxifen decreased the incidence of mammary tumours in multiple studies (Jordan *et al.*, 1990, 1991). One study using a transgenic mouse model susceptible to spontaneous mammary tumours resulted in an increased incidence of malignant mammary tumours (Jones *et al.*, 2005).

3.2.2 Rat

Female rats administered the tamoxifen metabolite 4-hydroxytamoxifen subcutaneously had a decreased incidence of benign and malignant mammary tumours in one study (Sauvez *et al.*, 1999).

See Table 3.2.

3.3 Perinatal administration

3.3.1 Mouse

Tamoxifen given orally or subcutaneously to female neonatal mice increased the incidence of uterine tumours in one study (without an obvious dose–response) (Newbold *et al.*, 1997), but had no effect upon urogenital tumours in three other studies (Green *et al.*, 2005; Waalkes *et al.*, 2006a; Razvi *et al.*, 2007). Male mice exposed transplacentally to arsenite and treated neonatally with tamoxifen had a decreased incidence of benign and malignant lung tumours in one study (Waalkes *et al.*, 2006b). One study in which tamoxifen was administered transplacentally to mice was negative (Diwan *et al.*, 1997).

See Table 3.3.

Table 3.1 Studies of cancer in experimental animals exposed to tamoxifen (oral exposure)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, Alderley Park Strain 1 (M, F) 15 mo Tucker et al. (1984)	0, 5, 50 mg/kg bw/d for 3 mo by gastric intubation, then in the diet for 12 mo 25/sex/group	Testis (benign interstitial cell tumours): 0/25, 2/25 (8%), 21/25 (84%) Ovary (granulosa cell adenomas): 0/25, 9/25 (36%), 9/25 (36%)	$P < 0.0001$ for 50 mg/kg dose[a] $P = 0.0008$ for 5 & 50 mg/kg doses[a]	Purity NR; age NR
Mouse, B6C3F1 (F) 24 mo Carthew et al. (1996a)	0 or 420 mg/kg diet for 8 wk, then 140 mg/kg diet for 22 mo 30/group	*At 3, 6, and 9 mo:* Uterus (tumours)–0/5, 0/5 *At 24 mo:* Uterus (tumours)–0/15, 0/15		Purity NR; only the uterus examined
Mouse, B6C3F1 (F) 24 mo Martin et al. (1997)	0 or 420 mg/kg diet for 8 wk, then 140 mg/kg diet for 22 mo 48/group	Liver (adenomas)–0/15, 2/15 (13%)	NS	Purity > 98%; complete histopathology
Rat, Alderley Wistar-derived (M, F) 107 wk Greaves et al. (1993)	0, 5, 20, 35 mg/kg bw/d for 2 yr by gastric intubation (suspension in 0.5% hydroxypropyl methyl cellulose in 0.1% polysorbate 80; 5 mL/kg) M: 102, 51, 51, 51 F: 104, 52, 52, 52	Liver (hepatocellular adenomas): M–1/102 (1%), 8/51 (16%), 11/51 (22%), 8/51 (16%) F–1/104 (1%), 2/52 (4%), 6/52 (12%), 9/52 (17%) Liver (hepatocellular carcinomas): M–1/102 (1%), 8/51 (17%), 34/51 (67%), 34/51 (67%) F–0/104, 6/52 (12%), 37/52 (71%), 37/52 (71%) Liver (hepato/cholangiocellular carcinomas): M–0/102, 0/51, 2/51 (4%), 5/51 (10%) F–0/104, 0/52, 4/52 (8%), 5/52 (10%)	$P < 0.0001$ for trend for each sex $P < 0.0001$ for trend for each sex $P < 0.0001$ for trend for each sex	Purity NR
Rat, Sprague-Dawley (Crl:CD(BR) (F) 15 mo Hard et al. (1993)	0, 11.3, 22.6 mg/kg bw/d for 12 mo by gastric intubation (suspension in 0.5% carboxymethyl cellulose; 5 mL/kg) 57, 84, 75/group	*At 12 mo:* Liver (adenomas)–0/18, 21/36 (58%), 24/24 (100%) Liver (carcinomas)–0/18, 16/36 (44%), 24/24 (100%) *At 15 mo:* Liver (adenomas)–0/13, 13/21 (62%), 9/9 (100%) Liver (carcinomas)–0/13, 13/21 (62%), 8/9 (89%)	$P < 0.001$ for 11.3, 22.6 mg/kg $P < 0.001$ for 11.3, 22.6 mg/kg $P < 0.001$ for 11.3, 22.6 mg/kg $P < 0.001$ for 11.3, 22.6 mg/kg	Purity = 99%

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague-Dawley (F) 15 mo Hirsimäki et al. (1993)	0, 11.3, 45 mg/kg bw/d for 12 mo by gastric intubation (suspension in 0.5% carboxymethyl cellulose, volume NR) 20/group (5 rats/group killed after 6 and 12 mo)	*At 12 mo:* Liver (hepatocellular carcinomas)–0/5, 0/5, 3/5 (60%) *At 15 mo:* Liver (hepatocellular carcinomas)–0/8, 1/8 (12%), 6/6 (100%)	 *P* = 0.003 for 45 mg/kg group[a]	Purity 99%; small number of animals at each time point
Rat, Sprague-Dawley (Crl:CD(BR) (F), age NR 15 mo Williams et al. (1993)	0, 2.8 (0.56 mg/mL), 11.3 (2.6 mg/mL), 45.2 (9.04 mg/mL) mg/kg bw/d for 12 mo by gastric intubation (suspension in 0.5% carboxymethyl cellulose) 55–57, 57 controls (10 rats/group killed after 6 or 12 mo; 5 rats/group killed after 7 mo)	*At 6 mo:* Liver (adenomas)–0/10, NR, 0/10, 5/7 (71%) Liver (carcinomas)–0/10, NR, 0/10, 2/7 (29%) *At 7 mo*: Liver (adenomas)–0/5, NR, 0/5, 3/4 (75%) Liver (carcinomas)–0/5, NR, 0/5, 3/4 (75%) *At 12 mo:* Liver (adenomas)–0/10, 0/10, 5/10 (50%), 2/4 (50%) Liver (carcinomas)–0/10, 0/10, 1/10 (10%), 3/4 (75%) *At 15 mo:* Liver (adenomas)–0/9, 0/22, 5/11 (45%), NR Liver (carcinomas)–0/9, 0/22, 5/11 (45%), NR	 *P* = 0.003 for 45.2 mg/kg group[a] NS[a] *P* = 0.05 for 45.2 mg/kg group[a] *P* = 0.05 for 45.2 mg/kg group[a] *P* = 0.02 for 11.3 mg/kg group[a] *P* = 0.01 for 45.2 mg/kg group[a] *P* = 0.03 for 11.3 mg/kg group[a] *P* = 0.03 for 11.3 mg/kg group[a]	Purity NR; small number of animals at each time point; age NR

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague-Dawley (F) 15 mo Ahotupa et al. (1994)	0, 11.3, 45 mg/kg bw/d for 12 mo by gastric intubation (suspension in 0.5% carboxymethyl cellulose, volume NR) 5/group	*At 12 mo:* Liver (hepatocellular caracinomas)–0/5, 0/5, 4/5 (80%) *At 15 mo:* Liver (hepatocellular caracinomas)–0/5, 0/5, 5/5 (100%)	$P = 0.02$ at 45 mg/kg dose[a] $P = 0.004$ at 45 mg/kg dose[a]	Purity > 99%; small number of animals at each time point
Rat, (Strain, NR) (M, F) 24 mo Hasmann et al. (1994)	0 (diet only), 36 mg/kg bw/d for 24 mo 50/group/sex, 0 (placebo)	Liver (hepatocellular adenomas): M–8/50 (16%), 7/49 (14%), 50/50 (100%) F–2/50 (4%), 1/50 (2%), 25/50 (50%) Liver (hepatocellular carcinomas): M–0/50, 0/49, 49/50 (98%) F–0/50, 0/50, 50/50 (100%) Liver (cholangiomas): M–0/50, 0/49, 8/50 (16%) F–0/50, 0/50, 17/50 (34%)	$P < 0.0001$ for both sexes vs both control groups[a] $P < 0.0001$ for both sexes vs both control groups[a] $P \leq 0.003$ for both sexes vs both control groups[a]	Purity NR; very few experimental details. Vehicle unspecified
Rat, F344/Tox; Wistar (LAC-P), LEW Ola (Lewis) (F) 20 mo Carthew et al. (1995a, 1996b)	0 or 420 ppm in diet for 180 d 11 mo (Wistar & Lewis) or 20 mo (Fischer)5–10/group	*At 6 mo:* Liver (tumours): Fischer–0/5, 0/5 Wistar–0/5, 3/5 (60%) Lewis–0/5, 1/5 (20%) *At 11 mo:* Liver (hepatocellular carcinomas): Wistar–10/10 (100%) Lewis–10/10 (100%) *At 20 mo:* Liver (hepatocellular carcinomas)–Fischer 10/10 (100%)	 NS[a] NS[a] NS[a]	Purity > 98%; incidence in control rats at 11 and 20 mo not indicated

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Wistar (Alderley Park; TOX-P) (F) 20 mo Carthew et al. (1995b)	0 or 420 ppm for 3 mo in the diet, 36/group	*At 12 mo:* Liver (tumours)–0/6, 0/6 *At 20 mo:* Liver (adenomas and carcinomas)–0/15, 5/15 (33%)	$P = 0.02$[a]	Purity NR; only liver examined
Rat, Fischer (F) 18 mo Dragan et al. (1995)	0, 250, 500 mg/kg diet 15, 22 controls	*At 18 mo*: Liver (hepatocellular carcinomas)–0/22, 1/15 (6.7%), 8/15 (53%)	$P = 0.0002$ for 500 mg/kg group[a]	Purity NR; rats received a 70% partial hepatectomy 2 wk before being placed on tamoxifen diet; age NR
Rat, Sprague-Dawley (F), age NR 104 wk Mäntylä et al. (1996)	0, 11.3, 45 mg/kg bw/d by gastric intubation (solvent and volume NR) for 13, 20, 26, or 52 wk 25–104, 109 controls	Endometrium (squamous cell carcinomas): 0/109, 0/25, 2/104 (2%)	NS[a]	Purity > 99%; there was a lack of study details; age NR
Rat, Wistar (Alderley Park; TOX-P) (F) 20 mo Carthew et al. (1996a)	0 or 420 mg/kg diet for 3 mo 48, 50 controls	*At 3, 6, and 9 mo:* Uterus (tumours)–0/6, 0/6 *At 20 mo:* Uterus (deciduomas)–0/26, 2/24 (8%) Uterus (haemangiomas)–0/26, 1/24 (4%) Uterus (leiomyomas)–0/26, 1/24 (4%)		Purity NR; only the uterus examined
Rat, Sprague-Dawley [Crl:CD(BR)] and F344 (F) 36 wk Williams et al. (1997)	0 or 40 mg/kg bw/day by gastric intubation (suspension in 0.5% carboxymethyl cellulose, 8 mg/mL) for 36 wk 26, 22 controls	Liver (hepatocellular adenomas): Sprague-Dawley–0/10, 3/4 (75%) Fischer–0/10, 1/10 (10%) Liver (hepatocellular carcinomas): Sprague-Dawley–0/10, 3/4 (75%) Fischer–0/10, 0/10	$P = 0.01$ for Sprague-Dawley[a] $P = 0.01$ for Sprague-Dawley[a]	Purity 99%; used tamoxifen citrate; small number of animals

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague-Dawley (M, F) 159 wk Maltoni et al. (1997)	0 or 3.3 mg/kg bw/day by gastric intubation (suspension in water, volume NR), 6 d per wk for 159 wk 100/sex/group	Mammary gland (fibroadenomas): M–1/100, 6/100 F–37/100, 0/100	NS[a], $P < 0.0001$[a]	Purity NR
		Mammary gland (adenocarcinomas): M–0/100, 2/100 F–8/100, 0/100	NS[a], $P = 0.003$[a]	
		Pituitary gland (adenomas): M–11/100, 5/100 F–16/100, 2/100	NS[a], $P = 0.0004$[a]	
		Adrenal gland (medullary pheochromocytomas): M–19/100, 16/100 F–17/100, 12/100	NS[a], NS[a]	
		Liver (adenomas or carcinomas): M–0/100, 3/100 F–0/100, 4/100	NS[a], NS[a]	
		Pancreas (islet cell adenomas or adenocarcinomas): M–8/100, 1/100 F–1/100, 0/100	$P = 0.02$[a]	
		Testes (Leydig cell tumours): M–4/100, 0/100	NS[a]	
Rat, Sprague-Dawley (F) 154 wk Maltoni et al. (1997)	0 or 3.3 mg/kg bw/day by gastric intubation (suspension in water, volume NR) for 8 d (consecutive) every 8 wk 150/group	Mammary gland (fibroadenomas): 68/150 (28%), 42/150 (45%)	$P = 0.0009$[a]	Purity NR
		Mammary gland (adenocarcinomas): 15/150 (10%), 5/150 (3%)	NS[a]	
		Pituitary gland (adenomas): 45/150 (15%), 22/150 (30%)	$P = 0.001$[a]	
		Adrenal gland (medullary pheochromocytomafibromas): 18/150 (12%), 8/150 (5%)	$P = 0.03$[a]	
		Liver (carcinomas): 1/150 (1%), 0/15	NS[a]	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague-Dawley (F) 87 wk Maltoni et al. (1997)	0 or 3.3 mg/kg bw/day by gastric intubation (suspension in water, volume NR), 6 d per wk for 40 wk 139/group	Mammary gland (fibroadenomas): 65/139 (46%), 48/139 (35%) Mammary gland (adenocarcinomas): 12/139 (9%), 4/139 (3%) Pituitary gland (adenomas): 44/139 (32%), 27/139 (19%) Adrenal gland (medullary pheochromocytomafibromas): 15/139 (11%), 13/139 (9%)	$P = 0.03$[a] $P = 0.02$[a] $P = 0.01$[a] NS[a]	Purity NR; age, 56 wk
Rat, Sprague-Dawley (F) 15 mo Kärki et al. (2000)	0 or 45 mg/kg bw/day by gastric intubation (suspension in 0.5% carboxymethyl cellulose, volume NR) for 12 mo 15/group	*At 6 mo:* Liver (adenomas)–0/5, 1/5 (20%) *At 12 mo:* Liver (adenomas)–0/5, 1/5 (20%) *At 12 mo:* Liver (hepatocellular carcinomas)–0/5, 4/5 (80%) *At 15 mo:* Liver (hepatocellular carcinomas)–0/5, 5/5 (100%)	$P = 0.02$[a] $P = 0.004$[a]	Purity > 99%; used tamoxifen citrate; only the liver examined; small number of animals at each time point
Rat, Wistar (Han) (F) 34 mo Carthew et al. (2001)	420 ppm in diet for 0, 1, 4, 8, or 12 wk 36/group	Liver cancer: 0/36; 0/36; 2/36 (5%); 3/36 (8%); 11/36 (30%)	$P = 0.0002$ for 12-week exposure	Purity NR; only the liver examined; histopathology conducted, but tumour type NR
Rat, Sprague-Dawley (F) 52 wk Kasahara et al. (2003)	0 or 20 mg/kg bw/day by gastric intubation (suspension in 0.5% carboxymethyl cellulose, volume NR) for 52 wk 14/group	Liver (adenomas): 0/14, 2/14 (14%) Liver (carcinomas): 0/14, 11/14 (78%)	$P < 0.0001$[a]	Purity NR; used tamoxifen citrate; only liver examined

[a] Working Group analysis (1-tailed Fisher exact test)

bw, body weight; d, day or days; F, female; M, male; mo, month or months; NR, not reported; NS, not significant; vs, versus; wk, week or weeks; yr, year or years

Table 3.2 Studies of cancer in experimental animals exposed to tamoxifen (subcutaneous administration)

Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, C3H/OUJ (F) 15 mo Jordan et al. (1990)	Silastic implant of tamoxifen (0 or 28 mg; 125 µg/d drug released over 6 mo) - Intact mice: 11–15; 11 controls. Implanted 2 or 5 wk after pregnancy/weaning cycle. Killed after 1, 2, 3, 4, 8 or 16 wk - Ovariectomized mice: 11–15, 22 controls. Implanted 1 or 4 wk after ovariectomy. Killed after 1, 2, 3, 4, 8, 16 or 24 wk	Mammary gland (tumours): Intact controls–11/11 (100%) Ovariectomized controls–12/22 (55%) Intact mice + tamoxifen–3/11 (27%); 7/15 (46%) Ovariectomized mice+ tamoxifen–5/11 (45%); 3/7 (43%)	$P < 0.001$ vs intact control for each of the tamoxifen groups	Purity NR; ovariectomy performed 1 wk after pups were weaned; no histopathology
Mouse, CH3/OUJ (F) 24 mo Jordan et al. (1991)	Experiment 1: 11/group Group 1: intact control Group 2: ovariectomized control Group 3: intact tamoxifen Group 4: ovariectomized tamoxifen (0 or ~28 mg; 2 or 5 wk after pregnancy/weaning cycle over 9 mo)	Mammary gland (tumours): 11/11 (100%) ≥ 10/11 (≥ 90%) 5/11 (45%) 5/11 (45%)	$P = 0.006$ (intact tamoxifen vs intact control); $P \leq 0.03$ (ovariectomized tamoxifen vs ovariectomized control)	Purity NR; ovariectomy performed 1 wk after pups were weaned; no histopathology; only mammary gland and uterus examined
	Experiment 2: 30/group Group 1: intact control Group 2: ovariectomized control Group 3: intact tamoxifen Group 4: ovariectomized tamoxifen (0 or ~56 mg at 3 and 9 mo of age, over 17 mo)	Mammary gland (tumours): 30/30 (100%) 15/30 (50%) 5/30 (17%) 6/30 (20%)	$P < 0.003$ (intact and ovariectomized tamoxifen vs controls)	Purity NR; ovariectomy performed at 2.5 mo of age; no histopathology; only mammary gland examined

Table 3.2 (continued)

Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Jordan et al. (1991) (contd)	Experiment 3: 11/group	Mammary gland (tumours):		Purity NR; no histopathology; only mammary gland examined
	Group 1: intact control	~10/11 (91%)		
	Group 2: intact tamoxifen (0 or ~42 mg at 3 and 9 mo of age, over 12 mo)	~4/11 (36%)	$P < 0.02$	
	Experiment 4: 20/group	Mammary gland (tumours):	$P < 0.001$ (for all tamoxifen treatments vs control)	Purity NR; no histopathology; only mammary gland examined
	Group 1: control	20/20 (100%)		
	Group 2: ~14 mg over 3 mo	~4/20 (~20%)		
	Group 3: ~28 mg over 6 mo	~4/20 (~20%)		
	Group 4: ~56 mg over 12 mo (implant received at 3 and 9 mo of age, replaced every 6 mo) Duration of Experiment 4, 16 mo	~5/20 (~25%)		
Mouse, Brca1$^{Co/Co}$MMTV-Cre/p53± (F) 8 mo Jones et al. (2005)	0 or 25 mg tamoxifen, over 6 mo 31, 28 controls	Mammary gland (adenocarcinomas): 8/22 (36%), 16/25 (64%)	$P < 0.05$ (incidence); $P = 0.039$ (time-to-tumour)	Purity NR
Rat, Sprague-Dawley (F) 22 mo Karim et al. (2003)	0 or 200 µg/d tamoxifen for 60 d; then 20 µg/d for the remainder of the experiment 6, 10 controls	Ovary (tumours): at 12 mo, 0/1, 0/2; at 17 mo, 0/3, 0/3; at 22 mo, 0/4, 0/1		Purity NR; histopathology on genital tract and ovaries only; control rats did not appear to receive implants; small number of animals at each time point
4-Hydroxytamoxifen				
Rat, Sprague-Dawley (F) 101 wk Sauvez et al. (1999)	0, 0, 20, 140, 1 000 µg/kg/day 4-hydroxytamoxifen (in ethanol and water (65:35), volume NR) 50/group	Liver (hepatocellular adenomas): 0/50, 0/50, 0/50, 3/50 (6%) 1/50 (2%)	NS	Purity NR; complete histopathology
		Mammary gland (benign and malignant): 31/50; 21/49; 11/50, 0/50, 0/49	$P < 0.05$ vs control (for all treated groups)[a]	

[a] Working Group analysis (1-tailed Fisher Exact test)

bw, body weight; d, day or days; F, female; mo, month or months; NR, not reported; NS, not significant; wk, week or weeks

Table 3.3 Studies of cancer in experimental animals exposed to tamoxifen (perinatal exposure)

Species, strain (sex), age Duration Reference	Dosing regimen Route Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, CD-1 [Crl:CD-1(ICR) BR] (F), Day 1 of life 17 mo Newbold et al. (1997)	0, 1, 2, 5, 10, 25, 50 µg/pup/d on Days 1–5 of life by subcutaneous injection (in corn oil, volume NR) Initial number of mice NR	Uterus (adenocarcinomas): 0/12, 4/21 (19%), 3/16 (19%), 0/11, 7/14 (50%), 1/11 (9%), 0/11	$P = 0.005$ for 10 µg dose group[a]	Purity NR; only reproductive tract examined
Mouse, CD-1 (F), Day 2 of life 36 mo Green et al. (2005)	0, 1 mg/kg bw/day on Days 2–5 of life by gastric intubation (mixture of peanut oil, lecithin, condensed milk (2:0.2:3); 5 µL/g bw) 88, 97 controls	Uterus (tumours) at interim sacrifices at 1.5, 3, 6, 9, and 12 mo: 0/4, 0/4 Uterus (tumours) at 36 mo: 0/77, 0/68		Purity NR
Mouse, CD-1 (F), Day 1 of life 90 wk Waalkes et al. (2006a)	Transplacental exposure to arsenite (85 ppm from 8–18 of gestation) of 0, 10 µg/pup/day on Days 1–5 of life by subcutaneous injection (in corn oil, volume NR) 35/group (female offspring)	Urogenital (adenomas or carcinomas): 0/33, 4/35 (11%)	NS	Purity > 99%
Mouse, CD-1 (F), Day 1 of life 18 mo Razvi et al. (2007)	Experiment 1: 0, 10 µg/pup/d on Days 1–5 of life by subcutaneous injection (in 5 µl peanut oil). Killed at 1.5, 3, 6, 12 and 18 mo 30/group Experiment 2: 5, 10, 25 or 50 µg/pup/d on Days 1–5 of life by subcutaneous injection (in 5 µl peanut oil) 35/group. Killed at 3, 6, 12 and 18 mo	Experiment 1 Uterus (adenocarcinomas) at 1.5, 3, 6 and 12 mo: 0/4, 0/4 Experiment 2 Uterus (adenocarcinomas) at 3, 6, and 12 mo: 0/4, 0/4 Experiments 1 and 2 Uterus (adenocarcinomas) at 18 mo: 0/20, 0/15, 0/20, 0/17, 0/16		Purity 99%; complete necropsy, histopathology on reproductive tract only
Mouse, CD-1 (M), Day 1 of life 90 wk Waalkes et al. (2006b)	Transplacental exposure to arsenite (85 ppm from Days 8–18 of gestation) 0, 10 µg/pup/day on Days 1–5 of life by subcutaneous injection (in corn oil, volume NR) 35/group (male offspring)	Liver (adenoma or carcinomas): 2/35 (6%), 0/30 Lung (adenomas or adenocarcinomas): 14/35 (40%), 2/30 (7%)	NS $P < 0.05$	Purity ≥ 99%

Table 3.3 (continued)

Species, strain (sex), age Duration Reference	Dosing regimen Route Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, CD-1 (F), 8–10 wk 78 wk Diwan et al. (1997)	0, 5 or 7.5 mg/kg bw by gastric intubation (in tricaprylin, volume NR), Days 12–18 of gestation. Killed at 12, 24, 52 and 78 wk 10 (dams)/group	*At 12 wk:* Uterus (deciduomas)–0/10, 0/10, 1/10 (10%) *At 24 wk:* Uterus (deciduomas)–0/10, 0/10, 2/10 (20%) *At 52 wk:* Uterus (leimyomas)–0/15, 0/15, 1/15 (67%) *At 53–78 wk*: Uterus (leimyomas)–0/24, 1/23 (4%), 3/22 (14%) Uterus (leimyosarcomas)–0/24, 0/23, 1/22 (4%) Ovary (granulosa tumours)–0/24, 0/23, 3/22 (17%)		Purity NR
Rat, Wistar (Han) (F), Day 2 of life 35 mo Carthew et al. (2000)	0, 1 mg/kg bw/d by gastric intubation (in a mixture of peanut oil, lecithin, and condensed milk (2:0.2:3)) on Days 2–5 after birth 78, 72 controls	*At 3, 6, 9, and 12 mo:* Reproductive tract (tumours)–0/6, 0/6 *At 24–35 mo:* Endometrium (adenocarcinomas)–3/48 (6%),13/54 (24%) Uterus (adenosquamous carcinomas)–0/48, 1/54 (2%) Vagina/cervix (squamous cell carcinomas)–0/48, 5/54 (9%)	$P = 0.01$ $P = 0.04$	Purity NR; only reproductive tract examined
Rat, Sprague-Dawley (M, F), Day 1 of life 15 mo Karlsson (2006)	0, 14 mg/kg bw/d by subcutaneous injection (in 4 µL/g bw of an aqueous mixture of 133 mM NaCl, 2.59% polyethylene glycol 3 000, 0.173% polysorbate 80, 0.99 mM propyl parahydroxybenzoate, 10.3 mM methyl parahydroxybenzoate, and 7% ethanol) on Days 1–5 of life M: 6, 8 controls F: 15, 6 controls	Reproductive tract tumours: M–0/8, 0/5 F–0/6, 0/15		Purity > 99.5%; small number of animals

[a] Working Group analysis (1-tailed Fisher exact test)

bw, body weight; d, day or days; F, female; M, male; mo, month or months; NR, not reported; NS, not significant; wk, week or weeks

3.3.2 Rat

The administration of tamoxifen to female neonatal rats caused an increase in reproductive tract tumours in one study (Carthew *et al.*, 2000), but no effect in another study of shorter duration with fewer animals (Karlsson, 2006).

3.4 Administration with known carcinogens

In several studies in both male and female rats, tamoxifen enhanced the hepatocarcinogenicity of previously administered *N*,*N*-diethylnitrosamine (IARC, 1996). In one study in rats, tamoxifen enhanced the development of *N*-nitrosodiethylamine-induced kidney tumours (Dragan *et al.*, 1995). In another study, the administration of tamoxifen to pregnant rats increased mammary gland tumours in offspring subsequently treated with 7,12-dimethylbenz[*a*]anthracene (Halakivi-Clarke *et al.*, 2000).

See Table 3.4.

3.5 Synthesis

Oral administration of tamoxifen increased the incidence of testicular tumours in one study in mice and malignant liver cell tumours in multiple studies in rats. At lower dose level, tamoxifen decreased the incidence of benign and malignant mammary gland tumours in female rats, and pituitary tumours in both sexes.

Subcutaneous administration of tamoxifen decreased the incidence of mammary tumours in multiple studies in mice. One study using a transgenic mouse model showed an increased incidence in mammary tumours.

Perinatal exposure to tamoxifen increased the incidence of reproductive tract tumours in mice and rats.

4. Other Relevant Data

In the previous *IARC Monograph* (IARC, 1996), tamoxifen was found to increase liver tumour incidence in rats. The available evidence indicated that tamoxifen is both a genotoxic carcinogen and a tumour promoter in rat liver, and that humans are likely to be less susceptible to the genotoxicity of the drug. It was suggested that tissue-specific effects of tamoxifen binding to the estrogen receptor on gene expression might be involved in the ability of tamoxifen to increase or decrease tumour risk. The pertinent mechanistic data that appeared since this review are summarized below.

4.1 Absorption, distribution, metabolism, and excretion

(a) Humans

Tamoxifen is well absorbed after oral administration, and appears to be more than 99% bound to plasma proteins (mostly to albumin) (Lien *et al.*, 1989). The absorption of tamoxifen shows a wide interindividual variation, which is probably due to differences in liver metabolism and differences in absorption in the gastrointestinal tract. In rats, mice, dogs and rhesus monkeys, most of the dosed material appears in the faeces, but bile-duct cannulation experiments with dogs and rats showed also a biliary excretion (Fromson *et al.*, 1973a).

The pharmacokinetics of tamoxifen appear to be biphasic, with a distribution phase of 7–14 hours, and an elimination phase of about 7 days (Fromson *et al.*, 1973b). The elimination half-life of *N*-desmethyltamoxifen is around 7 days, and 4-hydroxytamoxifen has a shorter half-life than tamoxifen (Buckley & Goa, 1989).

Several metabolites have been identified in the urine and plasma of human breast cancer patients (IARC, 1996). Metabolites detected in plasma

Table 3.4 Studies of cancer in experimental animals exposed to tamoxifen and known carcinogens

Species, strain (sex), age Duration Reference	Dosing regimen Route Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Fischer (F) 18 mo Dragan et al. (1995)	0, 250, 500 mg/kg diet by oral administration (following a single dose of 10 mg *N*-nitrosodiethylamine/kg body weight in trioctanoin (route and volume NR)). Killed at 6 or 18 mo 8–18; 19 controls	Liver (hepatocellular carcinomas): 2/17 (12%), 11/18 (61%), 8/8 (100%) Kidney (renal cell carcinomas): 0/19, 0/18, 2/8 (25%)	$P \leq 0.003$ for each group[a]; $P = 0.008$ for trend	Purity NR; rats received a 70% partial hepatectomy 2 wk before being placed on tamoxifen diet. *N*-nitrosodiethylamine administered 24 h after partial hepatectomy; age, NR
Rat, Sprague-Dawley (F) 18 wk Halakivi-Clarke et al. (2000)	0 or 20 µg tamoxifen by subcutaneous injection (in 50 µl of 2% dimethylsulfoxide in peanut oil) to pregnant rats on gestation Days 15–20. Female offspring treated at 45 days of age by gavage with 10 mg 7,12-dimethylbenz[*a*] anthracene in 1 mL peanut oil 22, 24 controls (offspring)	Mammary gland (adenocarcinomas): 50%, 95%	$P < 0.001$	Purity NR; histopathology limited to mammary gland on representative animals

[a] Working Group analysis (1-tailed Fisher Exact test)

bw, body weight; mo, month or months; NR, not reported; wk, week or weeks

include tamoxifen, *N*-desmethyltamoxifen, and tamoxifen-*N*-oxide; and in urine, glucuronides of four hydroxylated metabolites (4-hydroxytamoxifen, 4-hydroxy-*N*-desmethyltamoxifen, dihydroxytamoxifen and another monohydroxy- (possibly α-hydroxy) *N*-desmethyltamoxifen) (Poon *et al.*, 1993). In another study, seven metabolites were identified in plasma (*N*-didesmethyltamoxifen, α-hydroxytamoxifen, 4-hydroxytamoxifen, tamoxifen-*N*-oxide, α-hydroxy-*N*-desmethyltamoxifen, 4-hydroxy-*N*-desmethyltamoxifen, and 4-hydroxytamoxifen-*N*-oxide) (Poon *et al.*, 1995).

In biopsy and autopsy samples obtained from patients with breast cancer treated with tamoxifen, levels of tamoxifen and its metabolites (*N*-desmethyl-, *N*-didesmethyl-, 4-hydroxy- and 4-hydroxy-*N*-desmethyl-) were 10- to 60-fold higher in tissues (liver, lung, pancreas, brain, adipose) than in serum, with particularly high levels in liver and lung tissues. Similarly, pancreatic tumours, and brain metastases from breast cancer were found to contain high levels of the drug. Specimen of skin and bone tissue also contained tamoxifen and some metabolites (Lien *et al.*, 1991). Furthermore, tamoxifen, 4-hydroxytamoxifen and *N*-desmethyltamoxifen were also found in postmortem and biopsy analyses of liver from tamoxifen-treated patients (Martin *et al.*, 1995).

CYP3A4 and, to a lesser extent, isoforms 2D6, 2B6, 3A5, 2C9, and 2C19 mediate the conversion of tamoxifen to α-hydroxytamoxifen (Notley *et al.*, 2005), whereas hydroxysteroid sulfotransferase 2A1 (SULT2A1) catalyses the formation of sulfate ester from α-hydroxytamoxifen (Apak & Duffel, 2004). A recent study (Singh *et al.*, 2008) demonstrated the expression of genes encoding the enzymes CYP3A4 and SULT2A1 involved in the bioactivation of tamoxifen in the human endometrium. It has been reported that women carrying CYP3A4*1B, a variant of CYP3A4, are at increased risk for tamoxifen-induced endometrial cancer (Chu *et al.*, 2007).

(b) Experimental systems

In experimental animals, concentrations of tamoxifen and its metabolites are 8- to 70-fold higher in tissues (brain, adipose, liver, heart, lung, kidney, uterus, testis) than in serum. The highest levels are found in lung and liver tissue, with substantial amounts found in kidney and adipose tissue (Lien *et al.*, 1991).

Tamoxifen can be metabolized *in vitro* by both microsomal cytochrome P450 and flavin mono-oxygenase pathways to intermediates that bind irreversibly to microsomal proteins (Mani & Kupfer, 1991). Incubation of tamoxifen with rat liver microsomes results in the formation of three major polar metabolites (*N*-oxide, *N*-desmethyl and 4-hydroxy derivatives) (Mani *et al.*, 1993, 1994). Peroxidases may also metabolize tamoxifen to a reactive intermediate that binds covalently to protein (Davies *et al.*, 1995), and to DNA (Pathak *et al.*, 1995).

In both human liver homogenate and human hepatic G2 cell line treated with a mixture of tamoxifen and its deuterated analogues, the following metabolites were detected: α-hydroxytamoxifen, 4-hydroxytamoxifen, *N*-desmethyltamoxifen, and tamoxifen *N*-oxide. In the liver homogenate, *N*-didesmethyltamoxifen was also detected (Poon *et al.*, 1995).

When primary cultures of human, rat and mouse hepatocytes were incubated with tamoxifen (10 μM) for 18–24 hours, the concentration of α-hydroxytamoxifen in the medium was 50-fold lower in the human cultures than in the rat and mouse cultures (Phillips *et al.*, 1996a).

4.2 Genetic and related effects

4.2.1 Direct genotoxicity

(a) DNA adducts

(i) Humans

Tamoxifen–DNA adducts have not been detected in human liver *in vivo* (IARC, 1996), and the low level of DNA covalent-binding by α-hydroxytamoxifen in cultured human hepatocytes (Phillips *et al.*, 1996a) probably reflects the intrinsic chemical reactivity of α-hydroxytamoxifen rather than enzymatic activation, as this metabolite is a poor substrate for human sulfotransferases (Glatt *et al.*, 1998a; Shibutani *et al.*, 1998a). Moreover, the glucuronidation pathway predominates in incubations of α-hydroxytamoxifen with human liver microsomes (Boocock *et al.*, 2000), which presumably leads to detoxification.

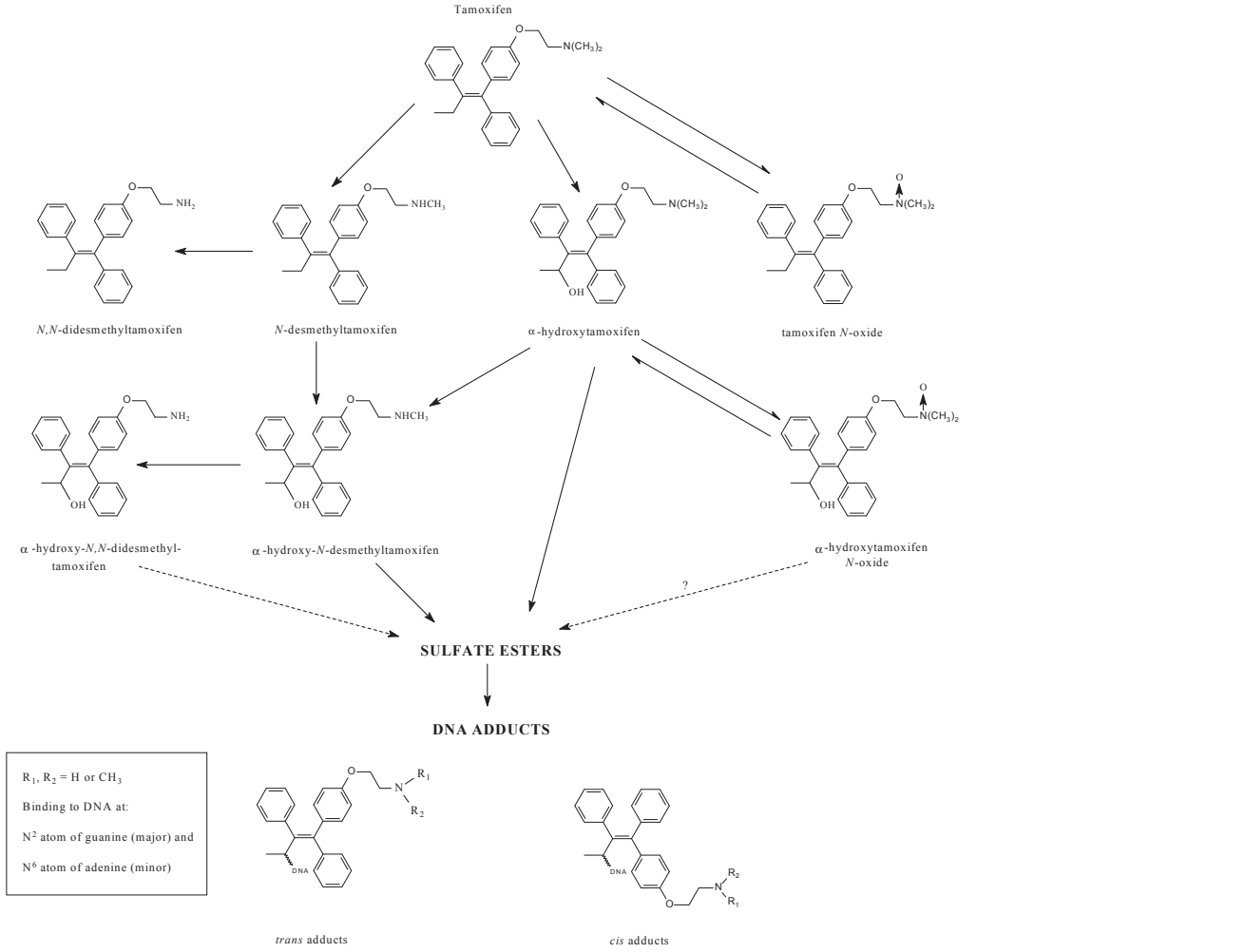
There are conflicting results on the formation of tamoxifen–DNA adducts in humans. The evidence for (Hemminki *et al.*, 1996; Shibutani *et al.*, 1999, 2000a; Martin *et al.*, 2003) and against (Carmichael *et al.*, 1996, 1999; Beland *et al.*, 2004a) such adducts in the human endometrium *in vivo* has been reported by several groups. This is also the case in studies on the formation of tamoxifen–DNA adducts from incubation of tamoxifen with human endometrium explants, with positive (Sharma *et al.*, 2003) and negative (Beland *et al.*, 2004b) findings being reported in samples from the same origin. Some studies reported the presence of such adducts in white blood cells from tamoxifen-treated patients (Hemminki *et al.*, 1997; Umemoto *et al.*, 2004), while others reported negative results (Phillips *et al.*, 1996b; Bartsch *et al.*, 2000). With the exception of the study by Martin *et al.* (2003), who used accelerator mass spectrometry, most studies used ^{32}P-postlabelling for adduct detection. Beland *et al.* (2004a, b) used HPLC coupled with tandem mass spectrometry, which can provide unequivocal structural characterization.

A recent study has reported the presence of (*E*)-α-(deoxyguanosin-N^2-yl)tamoxifen (dG-Tam) at levels of 1–7 adducts/10^9 nucleotides in enzymatically hydrolysed colorectal DNA from 3/10 women administered a single dose of 20 mg ^{14}C-labelled tamoxifen approximately 18 hours before colon resection surgery. The detection methodology involved HPLC coupled with accelerator mass spectrometry, and the identification was based upon comparison with an authentic adduct standard. All colon samples had detectable levels of CYP3A4 (Brown *et al.*, 2007).

(ii) Experimental systems

DNA adducts have been detected at dose-dependent levels in rat liver following administration of tamoxifen (IARC, 1996), and some of its metabolites, such as *N*-desmethyltamoxifen, α-hydroxytamoxifen, and α-hydroxy-*N*-desmethyltamoxifen (Brown *et al.*, 1998, 1999; Martin *et al.*, 1998; Phillips *et al.*, 1999, 2005; Gamboa da Costa *et al.*, 2000, 2001; White *et al.*, 2001). A quantitatively minor phase I pathway that leads to the metabolic activation of tamoxifen to DNA-binding electrophiles in rat liver is catalysed by CYP3A enzymes. This involves hydroxylation at the allylic (α) carbon of tamoxifen (Kim *et al.*, 2003) and *N*-desmethyltamoxifen, which is then followed by phase II conjugation. Although acetyltransferases have been proposed as mediators in the activation of α-hydroxylated tamoxifen metabolites, the most convincing evidence indicates that activation occurs through sulfotransferase-mediated sulfation, specifically by the STA2 isoform of hydroxysteroid sulfotransferase (Davis *et al.*, 1998, 2000; Glatt *et al.*, 1998a, b; Shibutani *et al.*, 1998a, b; Kim *et al.*, 2005; Phillips *et al.*, 2005). In addition, a parallel adduct formation pathway involving *N*-demethylation, as well as α-hydroxylation and *O*-sulfonation occurs (Fig. 4.1). The *N*-demethylation of tamoxifen is also mediated by the CYP3A subfamily (IARC, 1996). In-vitro reactions conducted with either

Figure 4.1 Proposed pathways of activation of tamoxifen in rat liver.



Compiled by the Working Group

the synthetic model esters, α-acetoxytamoxifen and α-acetoxy-*N*-desmethyltamoxifen (Osborne *et al.*, 1996; Dasaradhi & Shibutani, 1997; Kitagawa *et al.*, 2000) or the corresponding synthetic sulfates (Dasaradhi & Shibutani, 1997; Gamboa da Costa *et al.*, 2000) have led to the identification of the major DNA adducts as (*E*)-α-(deoxyguanosin-N^2-yl)tamoxifen (dG-Tam) and (*E*)-α-(deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen (dG-desMe-Tam), which exist as mixtures of epimers at the allylic carbon. Minor adducts from these reactions include the *Z* diastereomers from dG-Tam and dG-desMe-Tam (Dasaradhi & Shibutani, 1997; Osborne *et al.*, 1997; Kitagawa *et al.*, 2000), and a deoxyadenosine-tamoxifen adduct, linked through the amino group of adenine (Osborne *et al.*, 1997). Comparison with characterized synthetic standards has confirmed that dG-Tam and dG-desMeTam are the major adducts formed in rat liver following treatment with tamoxifen regardless of the rat strain, the route of administration, or the length of exposure (Osborne *et al.*, 1996; Rajaniemi *et al.*, 1998, 1999; Brown *et al.*, 1999; Phillips *et al.*, 1999; Firozi *et al.*, 2000; Gamboa da Costa *et al.*, 2000). Interestingly, the *R* enantiomers of α-hydroxytamoxifen (Osborne *et al.*, 2001) and α-hydroxy-*N*-desmethyltamoxifen (Osborne *et al.*, 2004) have much higher binding affinity in rat hepatocytes than the corresponding *S* isomers, presumably as a result of better affinity of the *R* enantiomers for the sulfotransferases.

Although a significant level of the didesmethylated analogue of dG-Tam and dG-desMeTam was detected in rat liver following administration of the putative metabolite, α-hydroxy-*N*,*N*-didesmethyltamoxifen, the low extent of binding obtained upon dosage with *N*,*N*-didesmethyltamoxifen indicates that metabolic activation to α-hydroxy-*N*,*N*-didesmethyltamoxifen is a minor pathway in the rat (Gamboa da Costa *et al.*, 2003). Likewise, metabolism via 4-hydroxytamoxifen does not seem to be a significant pathway to DNA-adduct formation in the rat (Beland *et al.*, 1999; Osborne *et al.*, 1999; Kim *et al.*, 2006a), despite the fact that the metabolite can be activated enzymatically to products covalently bound to DNA in cell-free or subcellular systems (Pathak *et al.*, 1995, 1996), and both its oxidation products, 4-hydroxytamoxifen quinone methide (Marques & Beland, 1997) and α-4-dihydroxytamoxifen (Hardcastle *et al.*, 1998) give DNA adducts upon reaction with DNA *in vitro*. An additional minor pathway to DNA adduct formation in rat liver has been reported to proceed via α-hydroxytamoxifen *N*-oxide, again involving binding at the α carbon through the exocyclic nitrogen of deoxyguanosine (Umemoto *et al.*, 1999, 2001).

While tamoxifen–DNA adducts are consistently detected in rat liver, most studies have not detected DNA adducts in the uterus and other extrahepatic tissues from rats administered tamoxifen or tamoxifen metabolites (Li *et al.*, 1997; Brown *et al.*, 1998; Beland *et al.*, 1999; Carthew *et al.*, 2000; Gamboa da Costa *et al.*, 2001; Phillips *et al.*, 2005). However, one study, which involved the use of accelerator mass spectrometry, reported that [^{14}C]tamoxifen binds to DNA in the liver, intestine, reproductive tract, spleen, lung, and kidney of rats dosed orally (White *et al.*, 1997). However, this methodology does not provide any structural information. [The Working Group noted that it was not clear whether the measured radioactivity corresponded exclusively to tamoxifen covalently bound to DNA.]

Tamoxifen also forms DNA adducts in mouse liver, though the levels are typically lower than in the rat (IARC, 1996). In addition, chronic exposure does not lead to accumulation of DNA adducts, which, combined with the absence of tamoxifen-induced cell proliferation, may account for the lack of hepatic carcinogenicity in the mouse, as opposed to the rat (Martin *et al.*, 1997). Similarly to what is found in the rat, the major DNA adducts in mouse liver are dG-Tam and dG-desMeTam; although

still minor, the adduct diastereomers derived from α-hydroxytamoxifen *N*-oxide make up a higher proportion in the mouse than in the rat (Umemoto *et al.*, 2000, 2001). The presence of DNA adducts in mouse extrahepatic tissues, including the uterus, has not been investigated.

Low levels of combined dG-Tam and dG-desMeTam were detected by different methods in the liver, brain cortex, kidney, ovary, and uterus of a group of three female cynomolgus monkeys dosed with a daily regimen of 2 mg tamoxifen/kg body weight for 30 days (Schild *et al.*, 2003; Shibutani *et al.*, 2003). These studies have shown that tamoxifen DNA adducts can be formed in extrahepatic tissues of non-human primates.

(b) Additional genotoxic effects

Tamoxifen induces micronuclei in metabolically proficient human cells and causes aneuploidy and chromosomal aberrations in rat liver (IARC, 1996; Styles *et al.*, 1997). Moreover, both tamoxifen and α-hydroxytamoxifen cause mutations in the *lacI* reporter gene and the *cII* gene in the livers of Big Blue transgenic rats (Davies *et al.*, 1996, 1997, 1999; Styles *et al.*, 2001; Chen *et al.*, 2002; Gamboa da Costa *et al.*, 2002) although α-hydroxytamoxifen causes significantly higher mutant frequencies than does tamoxifen, the mutational spectra induced by the two compounds are very similar in both genes, with the predominant mutations being G→T transversions. Mutations are not observed in extrahepatic tissues, including the uterus, which is in agreement with the general lack of detection of DNA adducts in rat tissues other than the liver. Consistent with the mutation profile in rat liver, when single-stranded shuttle vectors containing each of the four dG-Tam diastereomers were transfected into simian kidney (COS7) cells, the prevalent mutations were, in all instances, G→T transversions (Terashima *et al.*, 1999). Likewise, when α-hydroxytamoxifen was tested in V79-rHSTa cells, a mammalian cell line with stable expression of rat hydroxysteroid sulfotransferase A (STA2), mutations at the *Hprt* gene were mainly GC→TA transversions, although single G:C base-pair deletions and partial/complete exon skippings were also observed, almost exclusively at guanines on the non-transcribed strand (Yadollahi-Farsani *et al.*, 2002). Additionally, both 4-hydroxytamoxifen quinone methide and the model ester, α-acetoxytamoxifen, are promutagenic using adducted pSP189 plasmid DNA containing the *supF* gene transfected into cultured human fibroblasts and kidney cells (McLuckie *et al.*, 2002, 2005). Experiments involving the use of site-specific modified oligonucleotides as templates in primer extension reactions with several mammalian DNA polymerases indicate that all four dG-Tam diastereomers have high miscoding potential (G→T mutation) (Shibutani & Dasaradhi, 1997; Yasui *et al.*, 2006). These adducts undergo nucleotide excision repair *in vitro* (Shibutani *et al.*, 2000b). A comparative study in excision-repair-deficient (XPC knockout) and wild-type mice indicated that they have similar removal rates in both strains, which indicates that hepatic tamoxifen DNA-adducts are not efficiently repaired by this pathway (Kim *et al.*, 2006b).

A study of the DNA-damaging potential of tamoxifen in normal human peripheral blood lymphocytes and MCF-7 breast cancer cells using the comet assay reported evidence for the presence of free radicals, which might account, in part, for the genotoxicity of tamoxifen under the experimental conditions presumably due to incomplete repair of double-strand breaks (Wozniak *et al.*, 2007).

Both tamoxifen and its β-chlorinated analogue toremifene, which has a much lower potential for DNA-adduct formation (Gamboa da Costa *et al.*, 2007), are associated with endometrial K-*ras* codon 12 mutations (Wallén *et al.*, 2005), although a different study concluded that toremifene has a much lower potential than tamoxifen for K-*ras* mutation induction in the

human endometrium (Hachisuga *et al.*, 2005). [The Working Group noted that mutations in *TP53* and *K-RAS* are low-frequency lesions in the common form of endometrial cancer and even those mutations appear late in the course of tumour development (Sherman, 2000).]

4.2.2 Estrogen-receptor-mediated mechanism

Experimental evidence increasingly supports the importance of estrogen-receptor-mediated gene regulation as the mechanism responsible for the differential action of tamoxifen in distinct tissues (Wu *et al.*, 2005). Selective estrogen-receptor modulators such as tamoxifen have tissue-specific estrogenic activity. Tamoxifen is an estrogen-receptor antagonist in the breast but an estrogen-receptor agonist in the bone and uterus. The two main forms of the estrogen receptor, estrogen receptor-α and estrogen receptor-β, have different tissue expression profiles. The uterus predominantly expresses estrogen receptor-α but the observation of increased cell proliferation and excessive response to estrogen in estrogen-receptor-β-knockout mice has suggested that estrogen receptor-β could modulate estrogen receptor-α in the uterus, and have an antiproliferative role (Lecce *et al.*, 2001). Tamoxifen stimulates proliferation of the human endometrial epithelium (Mourits *et al.*, 2002). Tamoxifen-liganded estrogen receptors associate with multiple co-activator proteins, which together determine tamoxifen binding and transactivation activity (Shang, 2006). Tamoxifen regulates gene transcription in epithelial cells from type I endometrial carcinomas (Wu *et al.*, 2005), and transcriptional responses have been identified in epithelial cells but not in stromal cells (Pole *et al.*, 2004). There is also evidence that the genes targeted by tamoxifen differ from those targeted by estrogen (Pole *et al.*, 2005).

4.3 Synthesis

There is strong evidence that in rat liver, tamoxifen is a genotoxic carcinogen through a pathway involving α-hydroxylation, sulfation of the α-hydroxy metabolite, and subsequent DNA-adduct formation.

Evidence for the role of this pathway in induction of human endometrial tumours is less compelling; rather, the data suggest that the carcinogenicity of tamoxifen is associated with an estrogen-receptor-dependent pathway.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of tamoxifen. Tamoxifen causes cancer of the endometrium.

For cancer of the female breast, there is *evidence suggesting lack of carcinogenicity*. An inverse relationship has been established between exposure to tamoxifen and cancer of the female breast.

There is *sufficient evidence* in experimental animals for the carcinogenicity of tamoxifen.

Tamoxifen is *carcinogenic to humans (Group 1)*.

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THIOTEPA

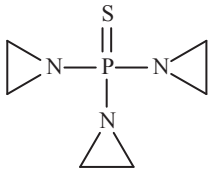
Thiotepa was considered by a previous IARC Working Group in 1989 (IARC, 1990). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 52-24-4
Chem. Abstr. Name: Aziridine, 1,1′,1″-phosphinothioylidynetris-
IUPAC Systematic Name: *tris*(Aziridin-1-yl)-sulfanylideneλ^5phosphane
Synonyms: Phosphorothioic acid triethylenetriamide; phosphorothioic triamide, *N*,*N*′,*N*″-tri-1,2-ethanediyl-; thiophosphamide; thiophosphoramide, *N*,*N*′,*N*″-tri-1,2-ethanediyl-; Thioplex; thiotriethylenephosphoramide; *N*,*N*′,*N*″-triethylenethiophosphoramide; tri(ethyleneimino)thiophosphoramide; tri-1-aziridinylphosphine sulfide; triaziridinylphosphine sulfide; triethylenethiophosphoramide; triethylenethiophosphorotriamide; tris(1-aziridinyl)phosphine sulfide; tris(aziridinyl)phosphine sulfide
Description: Fine, white, crystalline flakes with a faint odour (McEvoy, 2007)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_6H_{12}N_3PS$
Relative molecular mass: 189.22

1.2 Use of the agent

Information for Section 1.2 is taken from McEvoy (2007), Royal Pharmaceutical Society of Great Britain (2007), and Sweetman (2008).

1.2.1 Indications

Thiotepa has been used intravesically for the treatment of residual tumours and as adjuvant therapy for prophylaxis of superficial bladder cancer. Thiotepa has also been used parenterally in the palliative treatment of adenocarcinoma of the breast and ovary. Thiotepa may be used by intracavitary injection to control pleural, pericardial, or peritoneal effusions caused by metastatic tumours.

1.2.2 Dosage

Thiotepa may be administered by intravenous, intramuscular, intrapleural, intraperitoneal, intrapericardial or intratumour injection, or by intravesical instillation.

Thiotepa may be given rapidly intravenously in doses of 0.3–0.4 mg/kg at intervals of 1–4 weeks. The drug has also been given intravenously in doses of 0.2 mg/kg or 6 mg/m^2 daily for 4 or 5 days at intervals of 2–4 weeks. Thiotepa has also been given intramuscularly in doses of 15–30 mg in various schedules.

The usual intracavitary dose of thiotepa is 0.6–0.8 mg/kg at intervals of at least 1 week, although a dose of 15–30 mg has been used intrapericardially.

For the treatment of superficial bladder tumours, the dose of thiotepa generally ranges from 30–60 mg, instilled by catheter in saline directly into the bladder. The usual course of treatment is once a week for 4 weeks. Single doses of 90 mg in 100 mL of sterile water have also been used prophylactically following local resection.

For malignant effusions, doses of up to 60 mg of thiotepa in 20–60 mL of sterile water may be instilled after aspiration; in the USA, the licensed dose is 0.6–0.8 mg/kg, a dose similar to that suggested for injection directly into tumours.

Thiotepa is available as 15 and 30 mg solutions for parenteral administration.

1.2.3 Trends in use

Although thiotepa has largely been replaced by the nitrogen mustards, it still has specific uses, particularly as a component of experimental high-dose chemotherapy regimens.

2. Cancer in Humans

Several cases of leukaemia following treatment with thiotepa alone have been reported. As was the case in the previous *IARC Monograph* (IARC, 1990), only one analytical study focused specifically on the cancer risk of thiotepa in humans (Kaldor *et al.*, 1990). This study, which used a case–control methodology within a cohort of women treated for ovarian cancer, found a strong association between the risk for leukaemia and treatment with thiotepa with a relative risk of 8.3 in the lower dose group (n = 4), and 9.7 in the higher dose group (n = 5).

3. Cancer in Experimental Animals

Thiotepa was tested for carcinogenicity by intraperitoneal administration in mice and rats, and by intravenous administration in male rats (Table 3.1).

It increased the incidence of lung tumours and malignant lymphomas in mice of each sex. In rats, intraperitoneal administration increased the incidence of lymphohaematopoietic malignancies in males and of uterine adenocarcinomas and mammary carcinomas in females. Squamous cell carcinomas of the skin or ear were also induced in both sexes. Intravenous administration to male rats induced tumours at a variety of sites (IARC, 1990).

Since the previous *IARC Monograph* (IARC, 1990), a study with CB6F1-TgH*ras*2 transgenic (*ras*H2) mice was performed during an interlaboratory validation study. Thiotepa was intraperitoneally administered to two groups of 15 male and 15 female *ras*H2 mice, 7–9 weeks of age, at doses of 1 and 2 mg/kg bw, 3 times per week for 24 weeks. Two similar groups of wild-type mice were also treated, and two groups of ten male and ten female *ras*H2 and wild-type mice served as vehicle controls. Forestomach papillomas, lung

Table 3.1 Studies of cancer in experimental animals exposed to thiotepa

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, A/He (M, F) 24 wk Stoner et al. (1973)	i.ep. 0 (untreated), 0, 19, 47, 94 (total dose) mg/kg bw (total 12 doses) 3 ×/wk for 4 wk 100, 160, 20, 20, 20	Lung: 18/94, 48/154, 11/20, 10/19, 16/20	$P < 0.05$ (47 mg/kg bw) $P < 0.001$ (94 mg/kg bw)	95–99% pure Control groups were either untreated or treated with vehicle i.ep. 3 ×/wk for 8 wk (total 24 doses)
Mouse, B6C3F1 (M, F) 86 wk NCI (1978)	i.ep. 0, 0, 1.15, 2.3 mg/kg bw 3 ×/wk for 52 wk 15, 15, 35, 35 15, 15, 35, 35	Malignant lymphomas: M–1/18[a], 1/8, 2/24, 26/28 F–0/29[a], 0/14, 5/26, 32/32	$P < 0.001$ (2.3 mg/kg) $P < 0.001$ (2.3 mg/kg)	98 ± 1% pure
Rat, Sprague-Dawley (M, F) 86 wk NCI (1978)	i.ep. 0, 0.7, 1.4, 2.8 mg/kg bw 3 ×/wk for 52 wk 20+20, 39, 35, 35 20+20, 31, 35, 35	Myeloid leukaemia / malignant lymphomas: M–0/29[a] vs 6/34 (0.7 mg/kg) M–0/30[a] vs 6/16 (1.4 mg/kg) Skin or ear (squamous cell carcinomas): M–0/29[a] vs 7/33 (0.7 mg/kg) M–0/30[a] vs 3/13 (1.4 mg/kg) Uterine (adenocarcinomas): F–0/28[a], 7/21 (1.4 mg/kg) Mammary gland (adenocarcinomas): F–1/28[a], 8/24 (1.4 mg/kg) Skin (squamous cell carcinomas): F–0/28[a], 8/21 (1.4 mg/kg)	 $P = 0.020$ $P = 0.001$ $P = 0.009$ $P = 0.023$ $P = 0.001$ $P = 0.006$ $P < 0.001$	98 ± 1% pure Analyses of the incidence in the high dose groups are not included, due to low survival for both sexes
Rat, BR 46 (M) 52 wk Schmähl & Osswald (1970), Schmähl (1975)	i.v. 0, 1 mg/kg bw, weekly for 52 wk 89, 48	Malignant tumours[b]: 4/65, 9/30 Benign tumours: 3/65, 5/30	$P < 0.01$	> 98% pure

[a] pooled control group
[b] tumours of various origin
bw, body weight; F, female; i.ep., intraepithelial; i.v., intravenous; M, male; vs, versus; wk, week or weeks

adenomas, and thymic lymphomas were induced in both treated *ras*H2 and wild-type mice. Lung adenocarcinomas were observed only in treated *ras*H2 mice. There was a higher incidence of forestomach papillomas in male *ras*H2 mice treated with 2 mg/kg thiotepa than in the corresponding wild-type mice and *ras*H2 controls. The increase in the incidence of forestomach papillomas was dose-dependent in *ras*H2 mice (Yamamoto *et al.*, 1998a, b). [The Working Group noted the limited reporting of the study, i.e., no tumour incidences were provided.]

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

In humans, intravenous injection of thiotepa results in a peak blood concentration of thiotepa within 5 minutes, and after intraperitoneal administration, the peak plasma concentration is reached within 25 minutes. Distribution of thiotepa is rapid and is followed by fast elimination from the plasma compartment, with a half-life of 1–3 hours. Triethylenephosphoramide (TEPA), a metabolite of thiotepa, is detected in plasma 5–10 minutes after intravenous injection, and persists longer in plasma with a half-life of 3–21 hours. Both thiotepa and TEPA penetrate the cerebrospinal fluid (IARC, 1990; Maanen *et al.*, 2000).

The urinary excretion of unchanged thiotepa is approximately 0.1–1.5% of the total administered thiotepa, and that of TEPA, 0.2–25%. Thiotepa can form conjugates with glutathione, and can be excreted as a thiotepa-mercapturic acid conjugate in the urine (Maanen *et al.*, 2000).

Following thiotepa administration to rodents (intraperitoneal or intravenous injection), thiotepa is distributed rapidly to different organs, with most available for metabolism in the liver (IARC, 1990; Maanen *et al.*, 2000). Many metabolic studies of thiotepa in various species (rat, dog, rabbit, and humans) have resulted in the identification of TEPA as the major metabolite of thiotepa. The metabolism of thiotepa to TEPA is mediated by hepatic cytochrome P450 (CYP) (Teicher *et al.*, 1989; Hagen *et al.*, 1991; Chang *et al.*, 1995).

Plasma elimination of thiotepa after intravenous administration to mice follows a two-compartment model. The half-life is 0.21 minutes for the first phase, and 9.62 minutes for the second. The major urinary metabolite in rats, rabbits, and dogs following intravenous injection of ^{32}P-thiotepa is TEPA. However, most of the radioactivity in mouse urine is recovered as inorganic phosphate. TEPA is largely excreted unchanged after administration to rats, and 5–30% is converted to phosphate (IARC, 1990; Maanen *et al.*, 2000).

4.2 Genotoxic effects

4.2.1 Interaction with DNA

Both thiotepa and TEPA are alkylating agents. As alkylating agents, these compounds are potentially trifunctional. The principal site of reaction in DNA is the N^7 position of guanine. Hydrolysis of thiotepa and TEPA produces aziridine (ethyleneimine), a reactive monofunctional alkylating agent, that reacts to form 7-(2-aminoethyl)deoxyguanosine in DNA, which is an unstable adducted base that leads to depurinated sites (Musser *et al.*, 1992). An aminoethyl-N^3-adenine adduct is also formed (Andrievsky *et al.*, 1991; Musser *et al.*, 1992). Thiotepa can also act as a bifunctional alkylating agent, forming interstrand cross-links between guanine bases (at the N^7 position) of DNA (Maanen *et al.*, 2000).

In common with other alkylating agents, therapeutic cytotoxicity is accompanied by mutagenic damage (Sanderson & Shield, 1996). Thiotepa cytotoxicity is attenuated by DNA

repair, principally base-excision repair (Limp-Foster & Kelley, 2000; Kobune *et al.*, 2001; Xu *et al.*, 2001), and inhibition of DNA-repair processes enhances cytotoxicity (Frankfurt, 1991; Frankfurt *et al.*, 1993). However, lymphoblastoid cell lines derived from patients with Fanconi anaemia are hypersensitive to thiotepa (but not TEPA), implying the formation of interstrand cross-links (Cohen *et al.*, 1991). Cells that are defective in p53 are also more sensitive to thiotepa (Seo *et al.*, 2002).

4.2.2 Mutagenic effects

(a) Mutagenicity in vitro

In the previous *IARC Monograph* (IARC, 1990), it was reported that the compound induced gene mutations in *Salmonella typhimurium* and *Aspergillus nidulans*, and chromosomal aberrations and sister chromatid exchange in root meristem cells of *Vicia faba*. It also induced gene mutations, unscheduled DNA synthesis, micronuclei, sister chromatid exchange, and chromosomal aberrations in mammalian cells *in vitro*. It also induced morphological transformation of mouse cells. One study reported that thiotepa did not induce significant levels of DNA damage in rat or human testicular cells at up to 1000 μM (Bjørge *et al.*, 1996), measured as single-strand breaks and alkali-labile sites by alkaline elution.

(b) Mutagenicity in vivo

Studies of mutant frequencies in the endogenous hypoxanthine(guanine)phosphoribosyl transferase *(Hprt)* and the transgenic *LacI* gene of Big Blue rats have found that thiotepa induced more mutations in *Hprt* than in *LacI* (Chen *et al.*, 1998). The most common mutation was GC→TA transversions. *Hprt* mutations in lymphocytes were also analysed in Fischer 344 rats treated with thiotepa or TEPA, where GC→TA transversions were also the most common mutations observed (Casciano *et al.*, 1999; Chen *et al.*, 1999).

In the previous *IARC Monograph* (IARC, 1990), thiotepa induced micronuclei in the bone marrow of rats and mice, chromosomal aberrations in mouse bone-marrow and liver cells, and in peripheral lymphocytes of rhesus monkeys and rabbits. It also caused sister chromatid exchange in mouse bone marrow *in vivo*. Increased frequencies of chromosomal aberrations were observed in peripheral lymphocytes of patients receiving thiotepa therapy.

Subsequent studies have reported that thiotepa induces chromosomal aberrations in bone-marrow cells of Armenian hamsters (*Cricetulua migratorius*), although at a lower frequency than in other rodents (Nersessian, 1994).

When administered to rhesus monkeys (*Macaca mulatta*) by bolus injection, thiotepa was more cytotoxic (chromosomal aberrations in bone marrow) than when the same dose was given by continuous infusion over 96 hours (Rao *et al.*, 2005). The induction of chromosomal aberrations and sister chromatid exchange in rhesus monkeys by intravenous injection of thiotepa led to an increase of the number of both sister chromatid exchange and chromosomal aberrations 14 hours after injection after which these levels began to fall. Sister chromatid exchange frequency reached control levels after 1 month, whereas chromosomal aberration frequency remained elevated after 6 months (Kuzin *et al.*, 1989). The dietary antimutagens chlorophyllin, β-carotene and α-linolenic acid inhibited thiotepa-induced chromosomal aberrations in Chinese hamsters by up to 85% (Renner, 1990).

Thiotepa was reported previously to induce chromosomal aberrations in germ cells, sperm abnormalities, and dominant lethal mutation in mice *in vivo* (IARC, 1990). In a subsequent study, thiotepa was reported to give similar yields of dominant lethal mutations in different strains of mice (Lyon & Glenister, 1991), in contrast to earlier reports showing differences among strains (Surkova & Malashenko, 1975, 1977). Subsequent studies have also reported that thiotepa produced

very low yields of translocations in mouse stem cells (De Luca *et al.*, 1990).

Thiotepa induced chromosomal aberrations in the reproductive cells of the female yellow fever mosquito (*Aedes aegypti*) (Puttaraju, 1994).

4.3 Synthesis

Thiotepa is an alkylating agent that is carcinogenic via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of thiotepa. Thiotepa causes leukaemia.

There is *sufficient evidence* in experimental animals for the carcinogenicity of thiotepa.

Thiotepa is *carcinogenic to humans (Group 1)*.

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TREOSULFAN

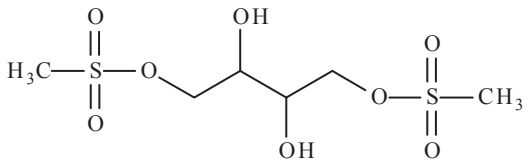
Treosulfan was considered by previous IARC Working Groups in 1980 and 1987 (IARC, 1981, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 299-75-2
Chem. Abstr. Name: 1,2,3,4-Butanetetrol, 1,4-dimethanesulfonate, (2*S*, 3*S*)-
IUPAC Systematic Name: [(2*S*,3*S*)-2,3-Dihydroxy-4-methylsulfonyloxybutyl]methanesulfonate
Synonyms: 1,2,3,4-Butanetetrol, 1,4-dimethanesulfonate, [*S*-(*R**,*R**)]-; dihydroxybusulfan; dihydroxymyleran; Ovastat; (2*S*,3*S*)-threitol 1,4-bismethanesulfonate; L-threitol 1,4-bis(methanesulfonate)
Description: White, odourless, crystalline powder (IARC, 1981)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_6H_{14}O_8S_2$
Relative molecular mass: 278.3

1.2 Use of the agent

Treosulfan is a prodrug of a bifunctional alkylating cytotoxic agent (Scheulen *et al.*, 2000; Sweetman, 2008).

1.2.1 Indications

Treosulfan is used to treat ovarian cancer (Royal Pharmaceutical Society of Great Britain, 2007). In addition, preclinical and clinical activity have been demonstrated against some other solid tumours, and haematological malignancies (Scheulen *et al.*, 2000). It has also been used for bone-marrow ablation before stem-cell transplantation, and to treat malignant melanoma, and breast cancer.

1.2.2 Dosage

Treosulfan is given orally or by intravenous or intraperitoneal administration. Treosulfan is available as 1 g and 5 g powders for reconstitution for injection or as a 250 mg capsule (Royal Pharmaceutical Society of Great Britain, 2007).

1.2.3 Trends in use

Treosulfan is commercially available in Europe for the treatment of ovarian cancer. In the USA, treosulfan is under clinical development and, at the time of writing, had not yet received approval from the US Food and Drug Administration (FDA) (Anakena, 2008). In April 2011, the US National Cancer Institute Clinical Trials database listed 15 active clinical trials using treosulfan, alone or in combination, in the treatment regimens (NCI, 2011). Treosulfan is listed in the FDA's orphan drug database (FDA, 2008).

2. Cancer in Humans

The first evaluation of treosulfan as a carcinogen (IARC, 1981) was based on the earlier results of the Danish cohort described below.

Two epidemiological studies have focused on the risk of leukaemia following treatment with treosulfan. In a cohort of 553 Danish patients with ovarian cancer treated only with treosulfan and followed for 9 years (over 1700 person–years) after treatment, 13 patients developed acute myeloid leukaemia, mostly within 5 years after the start of chemotherapy. The relative risk of acute myeloid leukaemia was in excess of 100, and there was a significant correlation between cumulative dose of treosulfan and risk of leukaemia (Pedersen-Bjergaard *et al.*, 1985). In an international case–control study of women treated for ovarian cancer, Kaldor *et al.* (1990) found that the relative risk was 3.6 in the group treated with the lowest dose of treosulfan, and 33.0 within the highest dose group. [The Working Group noted that there may have been an overlap between the two studies, as the case–control study included Denmark, and covered a similar time period as the Danish cohort study.]

3. Cancer in Experimental Animals

No data were available to the Working Group.

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

Treosulfan is a prodrug that is converted non-enzymatically first to a mono-epoxide – (2*S*,3*S*)-1,2-epoxy-3,4-butanediol-4-methanesulfonate – and then to a diepoxide – L-diepoxybutane, which is also a metabolite of butadiene – under physiological conditions. Such conversions are assumed to account for the alkylating and therapeutic activities of treosulfan. After oral and intravenous administration of treosulfan to humans, the parent drug is found in the serum at a higher concentration after the intravenous dosing, and about 15% of unchanged drug is excreted in urine (Hilger *et al.*, 2000)

4.2 Genotoxic effects

4.2.1 Interaction with DNA

As a bifunctional alkylating agent, treosulfan alkylates DNA and creates interstrand cross-links in cell-free systems (plasmid DNA), and in intact cells (Hartley *et al.*, 1999), preferentially at guanine residues. Prior to any short-term tests for genotoxicity, treosulfan was predicted to be active based on its structure (Shelby, 1988).

4.2.2 Mutagenicity in vitro

Treosulfan is mutagenic in *Salmonella typhimurium* strains TA100 and TA1535 in the absence of metabolic activation, as is diepoxybutane without external activation (Zeiger & Pagano, 1989). These strains detect base-pair substitutions at G:C base pairs. Treosulfan is

not mutagenic to TA102, which is sensitive to base-pair substitutions at A:T (Abu-Shakra *et al.*, 2000).

Treosulfan is mutagenic in Chinese hamster ovary cells, at the guanine phosphorybosyl transferase *(Gpt)* locus (Zhu & Zeiger, 1993); diepoxybutane is also mutagenic, but at a lower concentration.

4.2.3 Mutagenicity in vivo

Earlier literature contains reports that treosulfan induced chromosomal aberrations in several plant species, including *Allium cepa* (onion), *Hordeum sativum* (barley), *Nigella damascena* (love-in-a-mist), and *Vicia faba* (vetch), but did not produce chlorophyll mutations in *Arabidopsis thaliana* (thale cress) (IARC, 1981, 1987b). Subsequently, in an in-vivo study, treosulfan gave positive results in a mouse bone-marrow micronucleus assay (Shelby *et al.*, 1989), inducing an approximately 20-fold increase in the frequency of micronucleated polychromatic erythrocytes. In another study, treosulfan induced micronuclei in mouse bone-marrow, and peripheral blood cells (Gulati *et al.*, 1990).

4.3 Synthesis

Treosulfan is carcinogenic via a genotoxic mechanism.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of treosulfan. Treosulfan causes acute myeloid leukaemia.

No data were available to the Working Group for the carcinogenicity of treosulfan in experimental animals.

Treosulfan is *carcinogenic to humans (Group 1).*

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DIETHYLSTILBESTROL

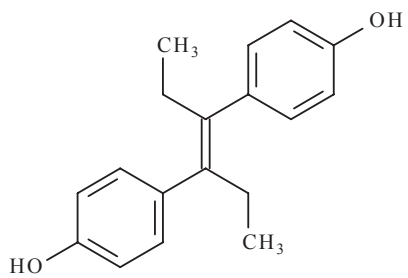
Diethylstilbestrol was considered by previous IARC Working Groups in 1978 and 1987 (IARC, 1979a, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 56–53–1
Chem. Abstr. Name: Phenol, 4,4′-[(1*E*)-1,2-diethyl-1,2-ethenediyl]bis-
IUPAC Systematic Name: 4-[(*E*)-4-(4-Hydroxyphenyl)hex-en-3-yl]phenol
Synonyms: (*E*)-3,4-Bis(4-hydroxyphenyl)-3-hexene; (*E*)-4,4′-(1,2-diethyl-1,2-ethenediyl)bisphenol; (*E*)-diethylstilbestrol; α,α′-diethyl-4,4′-stilbenediol; α,α′-diethylstilbenediol; 4,4′-dihydroxy-α,β-diethylstilbene; 4,4′-dihydroxydiethylstilbene; phenol, 4,4′-(1,2-diethyl-1,2-ethenediyl)bis-, (*E*)-; 4,4′-stilbenediol, α,α′-diethyl-, *trans*-;
Description: white, odourless, crystalline powder (McEvoy, 2007)

(a) Structural and molecular formulae, and relative molecular mass



$C_{18}H_{20}O_2$
Relative molecular mass: 268.35

1.2 Use of the agent

Information for Section 1.2 is taken from IARC (1979a), McEvoy (2007), Royal Pharmaceutical Society of Great Britain (2007), and Sweetman (2008).

1.2.1 Indications

Diethylstilbestrol is a synthetic non-steroidal estrogen that was historically widely used to prevent potential miscarriages by stimulating the synthesis of estrogen and progesterone in the placenta (in the United States of America, especially from the 1940s to the 1970s)

(Rogers & Kavlock, 2008). It was also used for the treatment of symptoms arising during the menopause and following ovariectomy, and for senile (atrophic) vaginitis and vulvar dystrophy. Diethylstilbestrol was used as a postcoital emergency contraceptive ('morning-after pill'). It has also been used for the prevention of postpartum breast engorgement, for dysfunctional menstrual cycles, and for the treatment of female hypogonadism.

Diethylstilbestrol is now rarely used to treat prostate cancer because of its side-effects. It is occasionally used in postmenopausal women with breast cancer.

Diethylstilbestrol was also used as a livestock growth stimulant.

1.2.2 Dosage

Historically, diethylstilbestrol was used for the treatment of symptoms arising during the menopause (climacteric) and following ovariectomy in an oral daily dose of 0.1–0.5 mg in a cyclic regimen. For senile vaginitis and vulvar dystrophy, it was given in an oral daily dose of 1 mg, or, for vulvar dystrophies and atrophic vaginitis, in suppository form in a daily dose of up to 1 mg. As a postcoital emergency contraceptive ('morning-after pill'), it was given as an oral dose of 25 mg twice a day for 5 days starting within 72 hours of insemination. An oral dose of 5 mg 1–3 times per day for a total of 30 mg was typically given in combination with methyltestosterone for the prevention of postpartum breast engorgement. For dysfunctional uterine bleeding, diethylstilbestrol was given in an oral dose of 5 mg 3–5 times per day until bleeding stopped. It was also used for the treatment of female hypogonadism, in an oral dose of 1 mg per day (IARC, 1979a; McEvoy, 2007).

The typical dosage of diethylstilbestrol is 10–20 mg daily to treat breast cancer in postmenopausal women, and 1–3 mg daily to treat prostate cancer. Diethylstilbestrol has also been given to treat prostate cancer in the form of its diphosphate salts (Fosfestrol).

When used as pessaries in the short-term management of menopausal atrophic vaginitis, the daily dose was 1 mg (Royal Pharmaceutical Society of Great Britain, 2007; Sweetman, 2008).

Diethylstilbestrol is available as 1 mg and 5 mg tablets for oral administration in several countries (Royal Pharmaceutical Society of Great Britain, 2007).

Diethylstilbestrol is no longer commercially available in the USA (McEvoy, 2007).

1.2.3 Trends in use

Most reports about diethylstilbestrol use are from the USA. The number of women exposed prenatally to diethylstilbestrol worldwide is unknown. An estimated 5 to 10 million US citizens received diethylstilbestrol during pregnancy or were exposed to the drug *in utero* from the 1940s to the 1970s (Giusti *et al.*, 1995).

A review of 51000 pregnancy records at 12 hospitals in the USA during 1959–65 showed geographic and temporal variation in the percentage of pregnant women exposed: 1.5% of pregnancies at the Boston Lying-In Hospital, and 0.8% at the Children's Hospital in Buffalo were exposed to diethylstilbestrol; at the remaining ten hospitals, 0.06% of pregnant women were exposed (Heinonen, 1973). At the Mayo Clinic during 1943–59, 2–19% (mean, 7%) of pregnancies per year were exposed (Lanier *et al.*, 1973).

The peak years of diethylstilbestrol use in the USA varied from 1946–50 at the Mayo Clinic, 1952–53 at the General Hospital in Boston, and 1964 at the Gundersen Hospital in Wisconsin (Nash *et al.*, 1983). Over 40% of the women in the US National Cooperative Diethylstilbestrol Adenosis (DESAD) cohort were exposed during the early 1950s (1950–55) (Herbst & Anderson, 1990). Among cases of clear cell adenocarcinoma (CCA) of the cervix and vagina recorded in the Central Netherlands Registry, born

during 1947–73, the median year of birth was 1960 (Hanselaar *et al.*, 1997). In the Registry for Research on Hormonal Transplacental Carcinogenesis, which registers cases of CCA of the vagina and cervix in the USA, Australia, Canada, Mexico and Europe, most of the exposed women from the USA were born during 1948–65 (Herbst, 1981; Melnick *et al.*, 1987).

Diethylstilbestrol doses varied by hospital. Based on the record review at 12 hospitals in the USA, the highest doses were administered at the Boston Lying-in, where 65% of treated pregnant women received total doses higher than 10 g, up to 46.6 g, for a duration of up to 9 months. At all the other hospitals, most women (74%) received < 0.1 g (Heinonen, 1973). Data available from the DESAD project indicate that median doses were 3650 mg (range 6–62100 mg) for women identified through the record review, whereas the median dose exceeded 4000 mg for women who entered the cohort through referral (self or physician), more of whom were affected by diethylstilbestrol-related tissue changes (O'Brien *et al.*, 1979). Diethylstilbestrol doses may have varied over time, but this has not been reported.

The use of diethylstilbestrol and other estrogens during pregnancy is now proscribed in many countries (Anon, 2008), and diethylstilbestrol use is no longer widespread for other indications.

Until the 1970s, it was common practice to stimulate the fattening of beef cattle and chickens by mixing small amounts of diethylstilbestrol into the animal feed or by implanting pellets of diethylstilbestrol under the skin of the ears of the animals. In the early 1970s, concern over trace amounts of the hormone in meat led to bans on the use of diethylstilbestrol as a livestock growth stimulant (Anon, 2008).

2. Cancer in Humans

The previous *IARC Monograph* (IARC, 1987a) states that there is sufficient evidence of a causal association between CCA of the vagina/cervix and prenatal exposure to diethylstilbestrol. That *Monograph* also cited clear evidence of an increased risk of testicular cancer in prenatally diethylstilbestrol-exposed male offspring, an association that is now uncertain due to the publication of recent studies. The association between diethylstilbestrol administered during pregnancy and breast cancer was considered established, but the latent period remained uncertain. Evidence was mixed for an association between diethylstilbestrol exposure during pregnancy and cancer of the uterus, cervix, and ovary. Finally, the *IARC Monograph* states that there is sufficient evidence of a causal relationship between uterine cancer and use of diethylstilbestrol as hormonal therapy for menopausal symptoms.

The studies cited in this review represent key historical reports relevant to the association between diethylstilbestrol and human cancer. Only studies of key cancer end-points published since the most recent *IARC Monograph* in 1987 are shown in the tables, available online.

2.1 Women exposed to diethylstilbestrol during pregnancy

2.1.1 Breast cancer incidence

Historically, nearly all of the studies assessing diethylstilbestrol in relation to invasive breast cancer incidence or mortality involve the retrospective and/or prospective follow-up of women with verified exposure to diethylstilbestrol during pregnancy. The results of some early studies suggested modestly increased risk, with relative risks (RR) ranging from 1.37 to 1.47

(Clark & Portier, 1979; Greenberg *et al.*, 1984; Hadjimichael *et al.*, 1984). However, a standardized incidence ratio (SIR) of 2.21 was reported from the Dieckmann clinical trial cohort (Hubby *et al.*, 1981), despite null results from an earlier analysis of the same cohort (Bibbo *et al.*, 1978). Historically, null results were also reported from a small US cohort (eight cases) (Brian *et al.*, 1980), and two small cohorts arising from separate clinical trials in London, the United Kingdom (four and 13 cases, respectively) (Beral & Colwell, 1981; Vessey *et al.*, 1983).

Two reports published since the previous *IARC Monograph* are consistent with a modest association between diethylstilbestrol exposure during pregnancy and breast cancer incidence (see Table 2.1 available et http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-11-Table2.1.pdf). The first of these (Colton *et al.*, 1993) was based on further follow-up of the Women's Health Study (WHS) (Greenberg *et al.*, 1984). The WHS cohort was originally assembled at three US medical centres (Mary Hitchcock Memorial Hospital in Hanover; Boston Lying-in Hospital in Boston; Mayo Clinic in Rochester) and a private practice in Portland (Greenberg *et al.*, 1984). At all participating WHS centres, diethylstilbestrol exposure (or lack of exposure) during pregnancy was based on a review of obstetrics records during 1940–60. Although exact diethylstilbestrol doses administered to women in the WHS are largely unknown, they are believed to have been relatively low. In the 1989 WHS follow-up, health outcomes, including breast cancer diagnosis and mortality, were retrospectively and prospectively ascertained in 2864 exposed and 2760 unexposed women. The data produced a relative risk of 1.35 for breast cancer risk based on 185 exposed and 140 unexposed cases (Colton *et al.*, 1993), whereas the earlier study reported a relative risk of 1.47 (Greenberg *et al.*, 1984).

The second report was based on the US National Cancer Institute (NCI) Combined Cohort Study, which in 1994 combined and extended follow-up of the WHS cohort (by 5 years), and the Dieckmann clinical trial cohort (by 14 years). The Dieckmann clinical trial was conducted in 1951–52 (Dieckmann *et al.*, 1953) to assess the efficacy of diethylstilbestrol for preventing adverse pregnancy outcomes. Administered diethylstilbestrol doses were high, with a cumulative dose of 11–12 g (Bibbo *et al.*, 1978). The combined WHS and Dieckmann cohorts produced a modestly elevated relative risk of 1.25 for breast cancer (Titus-Ernstoff *et al.*, 2001).

Based on data from the Dieckmann clinical trial cohort (Hubby *et al.*, 1981) and the NCI Combined Cohort Study (Titus-Ernstoff *et al.*, 2001), the influence of diethylstilbestrol on breast cancer risk did not differ according to family history of breast cancer, reproductive history, prior breast diseases, or oral contraceptive use. Although the first follow-up of the Dieckmann clinical trial cohort suggested breast cancer occurred sooner after trial participation in the diethylstilbestrol-exposed women (Bibbo *et al.*, 1978), this was not seen in the subsequent follow-up (Hubby *et al.*, 1981), in the WHS cohort (Greenberg *et al.*, 1984; Colton *et al.*, 1993), in the NCI Combined Cohort Study (Titus-Ernstoff *et al.*, 2001), or the Connecticut study (Hadjimichael *et al.*, 1984). In both the NCI Combined Cohort Study (Titus-Ernstoff *et al.*, 2001) and the Connecticut study (Hadjimichael *et al.*, 1984), the elevated risk associated with diethylstilbestrol was not apparent 40 or more years after exposure.

Data from the WHS (Greenberg *et al.*, 1984) and the Dieckmann clinical trial cohort (Bibbo *et al.*, 1978; Hubby *et al.*, 1981) did not show systematic differences in breast tumour size, histology or stage at diagnosis for the diethylstilbestrol-exposed and -unexposed women. No differences between exposed and unexposed women with regard to breast self-examination or mammography screening were noted in follow-up data from the WHS (Colton *et al.*, 1993).

[The Working Group noted it seemed unlikely the increased risk in diethylstilbestrol-exposed women was due to an increased surveillance of exposed women or to confounding by lifestyle factors.]

Historically, a few studies have suggested an association between exposure to diethylstilbestrol during pregnancy and an increased risk of breast cancer mortality; these include an analysis based on the first follow-up report of women in the Dieckmann clinical trial (RR, 2.89; 95%CI: 0.99–8.47) (Clark & Portier, 1979), and a study in Connecticut (RR, 1.89; 95%CI: 0.47–7.56) (Hadjimichael *et al.*, 1984). More recent studies are consistent with a modest association, including an analysis of fatal breast cancer in a large American Cancer Society (ACS) cohort of gravid women (RR, 1.34; 95%CI: 1.06–1.69) (Calle *et al.*, 1996), the second follow-up of women in the WHS (RR, 1.27; 95%CI: 0.84–1.91) (Colton *et al.*, 1993), and the NCI Combined Cohort Study, which for this analysis combined and extended the follow-up of the WHS women by 8 years and the Dieckmann women by 17 years (hazard ratio [HR] 1.38; 95%CI: 1.03–1.85) (Titus-Ernstoff *et al.*, 2006a). Similarly to the NCI study of breast cancer incidence (Titus-Ernstoff *et al.*, 2001), the ACS study showed that risk of breast cancer mortality did not differ by family history of breast cancer, reproductive history, or hormone use; also, the elevated risk was no longer evident 40 or more years after exposure (Calle *et al.*, 1996).

In summary, evidence from large, recent cohort studies suggests a modest association between diethylstilbestrol exposure during pregnancy and increased breast cancer incidence and mortality. Notably, these associations were apparent in women participating in the Dieckmann clinical trial cohort, minimizing the possibility of distortion due to confounding by the clinical indication for diethylstilbestrol use. The increased risk of breast cancer mortality also argues against an artefactual association stemming from the heightened surveillance of diethylstilbestrol-exposed women.

Diethylstilbestrol was also prescribed for the treatment of menopausal symptoms, but the use of diethylstilbestrol in menopause has not been assessed systematically in relation to breast cancer risk, and the association is unclear.

2.1.2 Other cancer sites

An early study suggested a relationship between the use of diethylstilbestrol to treat gonadal dysgenesis and an increased risk of endometrial cancer in young women (Cutler *et al.*, 1972). An increased risk of endometrial cancer was also reported in association with the use of diethylstilbestrol to treat symptoms of menopause (Antunes *et al.*, 1979).

Two follow-up studies indicated (Hoover *et al.*, 1977) or suggested (Hadjimichael *et al.*, 1984) an increased risk of ovarian cancer among women exposed to diethylstilbestrol during pregnancy, but the number of exposed cases was small. Similarly, early attempts to assess the risk of cervical and other cancers were limited by small case numbers (Hadjimichael *et al.*, 1984). The large and more recent NCI Combined Cohort study did not show an association between diethylstilbestrol exposure during pregnancy and the incidence of cancer of the endometrium, ovary, or cervix (Titus-Ernstoff *et al.*, 2001).

Although relative risks were elevated for brain and lymphatic cancers in the Connecticut study (Hadjimichael *et al.*, 1984) and for stomach cancer in the NCI Combined Cohort Study (Titus-Ernstoff *et al.*, 2001), confidence intervals were wide. A recent report from the large ACS study showed no association between diethylstilbestrol taken during pregnancy and pancreatic cancer mortality (1959 deaths in 387981 women) (Teras *et al.*, 2005). The NCI Combined Cohort study did not find associations between diethylstilbestrol exposure during pregnancy and death due to cancers other than breast cancer (Titus-Ernstoff *et al.*, 2006a).

2.2 Women exposed *in utero*

2.2.1 Clear cell adenocarcinoma of the vagina and cervix

Substantial evidence indicates that women exposed *in utero* to diethylstilbestrol have a markedly increased risk of CCA of the vagina and cervix. The earliest report, published in 1970, described seven cases of adenocarcinoma (six CCA) in women of ages 15–22 years who had been exposed prenatally to diethylstilbestrol (Herbst & Scully, 1970). The following year, a case–control study based on these seven cases plus an additional case (eight cases) and 32 matched controls showed a strong statistical association between prenatal diethylstilbestrol exposure and risk of vaginal CCA based on seven exposed cases and zero exposed controls ($P < 0.00001$) (Herbst *et al.*, 1971). A second case–control study published the same year, involving five cases identified through the New York State Cancer Registry and eight matched controls, also supported an association between prenatal exposure to synthetic estrogens and vaginal CCA based on five exposed cases and zero exposed controls (Greenwald *et al.*, 1971). The strength of this evidence was based primarily on the rarity of CCA, particularly in young women, and on the high proportion of cases that were exposed to a medication that was used relatively infrequently. Based on these reports, the FDA issued a bulletin against prescribing diethylstilbestrol during pregnancy in late 1971 (Anon, 1972).

Additional evidence published in 1972 established a link between prenatal diethylstilbestrol exposure and CCA. That study identified seven cases of CCA occurring in girls aged 7–19 years; of the four mothers who were successfully contacted, three reported diethylstilbestrol use during the first trimester of pregnancy and one reported taking a hormone of unknown type for vaginal bleeding (Noller *et al.*, 1972). A study of the California Tumor Registry during 1950–69 showed an increase of vaginal tumours in girls aged 10–19 years (Linden & Henderson, 1972). Subsequent case series, two of which were based in California, supported the link between prenatal diethylstilbestrol exposure and CCA at both sites (Henderson *et al.*, 1973; Hill, 1973).

The only follow-up study of prenatal diethylstilbestrol exposure in relation to risk of CCA is the NCI Combined Cohort Study, which combined pre-existing US cohorts with verified diethylstilbestrol exposure (or lack of exposure), including:

- daughters of women who participated in the Dieckmann clinical trial (Dieckmann *et al.*, 1953),
- daughters of women enrolled in the WHS (Greenberg *et al.*, 1984),
- daughters of women treated with diethylstilbestrol at a Boston infertility clinic and their unexposed sisters (the Horne cohort), and
- more than 5000 women (including more than 4000 exposed) who were initially identified through medical records or referral (self or physician), and enrolled during the 1970s in the multicentre DESAD study (Labarthe *et al.*, 1978).

Follow-up of the NCI Combined Cohort through 1994 ascertained three diethylstilbestrol-exposed cases of vaginal CCA, producing an SIR of 40.7 (95%CI: 13.1–136.2). Continued follow-up through 2001 ascertained an additional exposed case of cervical CCA, producing an SIR of 39 (95%CI: 15–104) (see Table 2.2 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-11-Table2.2.pdf), and indicating a cumulative risk of 1.6 per 1000 of CCA of the vagina/cervix from birth through 39 years of age (Troisi *et al.*, 2007).

An early study comparing internationally ascertained diethylstilbestrol-exposed CCA cases, recorded in the Registry for Research on Transplacental Carcinogenesis at the University of Chicago, to diethylstilbestrol-exposed non-cases

in the DESAD study suggested that CCA risk is influenced by early gestational exposure, but not by dose. Evidence was unclear for an influence of prior miscarriage (Herbst *et al.*, 1986). Another University of Chicago registry-based study published since the previous *IARC Monograph* found that maternal vaginal bleeding during pregnancy was not associated with case status, reducing the likelihood that pregnancy complications confounded the association between diethylstilbestrol and CCA (Sharp & Cole, 1990). The same study also found that CCA occurring in diethylstilbestrol-exposed women was associated with earlier gestational exposure and with greater body weight and greater height at ages 14–15 years (Sharp & Cole, 1991). [The Working Group noted that, possibly, greater body weight and height in the early teenage years was a proxy for early puberty, which may have increased the time at risk.] A recent study comparing diethylstilbestrol-exposed CCA cases to diethylstilbestrol-controls did not identify postnatal factors that influenced the risk of this developing cancer (Palmer *et al.*, 2000).

Vaginal adenosis is an established, although non-obligatory, precursor of CCA that affects between 34–88% of diethylstilbestrol-exposed women (Antonioli & Burke, 1975; Bibbo *et al.*, 1975; Herbst *et al.*, 1975; Kaufman & Adam, 1978; O'Brien *et al.*, 1979) and fewer than 4% of unexposed women (Bibbo *et al.*, 1975; Herbst *et al.*, 1975). The lower prevalence (34–35%) was found in diethylstilbestrol-exposed women who were identified through a medical record review (Herbst *et al.*, 1975; Robboy *et al.*, 1979); also, in these studies, tissues were biopsied only when changes were seen upon clinical examination or colposcopy. The higher prevalence (88%) was reported in women many of whom had been referred for study because of other diethylstilbestrol-related vaginal anomalies (Antonioli & Burke, 1975). Several studies suggested the likelihood of vaginal epithelial changes, including adenosis, is greater in women who received higher diethylstilbestrol doses (O'Brien *et al.*, 1979), women of young ages (aged 13–26 years in Mattingly & Stafl, 1976), and women who were exposed early in gestation (defined variously as before Weeks 16, 19 or 20, or during the first trimester) (Herbst *et al.*, 1975; Mattingly & Stafl, 1976; Kaufman & Adam, 1978; O'Brien *et al.*, 1979). A decreasing prevalence with age has been seen in case series (Kaufman *et al.*, 1982), in the DESAD study (Robboy *et al.*, 1981), and in prospective follow-up studies of diethylstilbestrol-exposed women, suggesting possible regression (Burke *et al.*, 1981; Noller *et al.*, 1983). Although most women affected by adenosis do not develop CCA, adenosis is present in up to 100% of vaginal CCA (Herbst *et al.*, 1972; Herbst *et al.*, 1974; Robboy *et al.*, 1984a).

2.2.2 Squamous neoplasia of the cervix

Around the time of puberty, the outer cervical epithelium undergoes a transition from the original columnar epithelium to squamous epithelium. The area affected by this change (squamous metaplasia), known as the cervical transformation zone (squamo-columnar junction), is at increased risk of malignancy. Early clinical series suggested the extended transformation zone associated with prenatal diethylstilbestrol exposure might increase susceptibility for squamous neoplasia/dysplasia in these women (Stafl & Mattingly, 1974; Fetherston, 1975; Fowler *et al.*, 1981). A study comparing diethylstilbestrol-exposed and -unexposed women showed a higher percent of dysplastic squamous cells in the exposed (11%) than in the unexposed (7%) based on cytology; the prevalence was greater (27%) in exposed women with pathologically confirmed adenosis (Herbst *et al.*, 1975). In a subsequent study of 280 women exposed to diethylstilbestrol in the first trimester, 82% were affected by adenosis and nearly all (96%) of these had abnormal colposcopic findings (Mattingly & Stafl, 1976).

The baseline examination of the DESAD study women who were identified through medical record review did not find elevated rates of squamous dysplasia in the diethylstilbestrol-exposed group (Robboy *et al.*, 1981), but the 7-year follow-up of 1488 (744 exposed) women noted higher rates of cervical squamous cell dysplasia and carcinoma *in situ* in the diethylstilbestrol-exposed compared to the unexposed women (15.7 versus 7.9 cases per 1000 person–years) based on cytology or biopsy (Robboy *et al.*, 1984b). The difference between exposed and unexposed was more apparent when the analyses were confined to cases identified through biopsy (as opposed to cytology) (5.0 versus 0.4 cases per 1000 person–years) (Robboy *et al.*, 1984b). [The Working Group noted that studies relying on selective biopsy may exaggerate the association between prenatal diethylstilbestrol exposure and risk of cervical neoplasia.] A recent analysis of the NCI Combined Cohort Study showed a doubling of the risk of high-grade intraepithelial neoplasia (squamous cell dysplasia) in the women exposed prenatally to diethylstilbestrol compared to the unexposed; the risk appeared to be higher for those with intrauterine exposure within 7 weeks of the last menstrual period (RR, 2.8; 95%CI: 1.4–5.5) (Hatch *et al.*, 2001). There were not enough confirmed cases of invasive cervical cancer for a meaningful analysis.

A study of 5421 questionnaire respondents (representing 41% of 13350 queried) who had been enrolled previously in the Netherlands Diethylstilbestrol Information Centre (NDIC), in which prenatal diethylstilbestrol exposure was validated using medical records, found evidence of a 5-fold risk (prevalence ratio [PrR]: 5.4; 95%CI: 2.8–9.5) of confirmed non-clear-cell-adenocarcinoma cervical cancer in comparison to the number of cases expected based on age and calendar year rates derived from a cancer registry (Verloop *et al.*, 2000). [The Working Group noted that because a low proportion of women returned their questionnaires, participation bias may have inflated the PrR.]

2.2.3 Cancer of the breast

A study in the Netherlands based on 5421 questionnaires returned to the NDIC found a modestly elevated risk of breast cancer for diethylstilbestrol-exposed women, but the confidence intervals were wide (PrR, 1.5; 95%CI: 0.7–2.9) (Verloop *et al.*, 2000). Findings based on the 1994 and 2001 follow-up of the NCI Combined Cohort Study did not show an overall increase of breast cancer rates in prenatally exposed women (Hatch *et al.*, 1998; Troisi *et al.*, 2007) (see Table 2.3 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-11-Table2.3.pdf). Relative risks from the two reports were 1.18 (95%CI: 0.56–2.49) (Hatch *et al.*, 1998) and 1.35 (95%CI: 0.85–2.10) (Troisi *et al.*, 2007). A more detailed analysis of the 2001 follow-up data gave an incidence rate ratio (IRR) of 2.05 (95%CI: 1.12–3.76) in women aged 40 years or more, and 0.57 (95%CI: 0.24–1.34) in women aged less than 40 years. The data also showed an elevated risk for women aged 50 years or more (IRR, 3.85; 95%CI: 1.06–14.0) (Palmer *et al.*, 2006). [The Working Group noted that women aged 50 years or more contributed 3% of the person–years in these analyses.] While speculative, women approaching the age of 50 years in this cohort would have been exposed during the peak years (1952–53 for the Dieckmann clinical trial and DESAD cohort members), which might have involved higher doses. If the association is real, the increased risk in older women might reflect higher exposure rather than age-related risk. In the same study, risk appeared to be elevated for older women with high (versus low) diethylstilbestrol exposure classified using known dose (38%) or assumed dose based on geographic region. There was no evidence that the risk in women aged 40 years or more was influenced by the timing of gestational exposure, which was known for 75%

of the exposed subjects. Also, there was no indication of effect modification by known breast cancer risk factors. Diethylstilbestrol exposure did not influence the receptor status of the breast tumour or lymph node involvement, but the association was evident in women with larger tumours (≥ 2 cm), arguing against screening bias (Palmer *et al.*, 2006).

2.2.4 Other sites

The study based on the NDIC produced a prevalence ratio of 2.9 (95%CI: 0.8–7.5) based on four cases of ovarian cancer observed in women prenatally exposed to diethylstilbestrol (1.36 cases expected) (Verloop *et al.*, 2000). The NCI Combined Cohort Study, however, showed no evidence of an association between prenatal diethylstilbestrol exposure and ovarian cancer in the 1994 or 2001 follow-up (Hatch *et al.*, 1998; Troisi *et al.*, 2007). The SIR was 0.88 (95%CI: 0.44–1.80) based on eight cases in the exposed at the time of the 2001 follow-up (Troisi *et al.*, 2007).

Based on one case, the NDIC study suggested an association between prenatal diethylstilbestrol exposure and cancer of the vulva (PrR, 8.8; 95%CI: 0.2–49.0) but confidence intervals were wide (Verloop *et al.*, 2000).

The NCI Combined Cohort Study found no evidence of an association between prenatal diethylstilbestrol exposure and endometrial cancer (SIR, 1.04; 95%CI: 0.52–2.10) based on eight cases in the exposed (Troisi *et al.*, 2007).

The NCI Combined Cohort Study suggested possible increases of lymphoma, lung and brain/nervous system cancers in prenatally exposed women, but the estimates were imprecise and compatible with chance (Troisi *et al.*, 2007). Sites for which there was no indication of increased risk included the thyroid and colorectum (Troisi *et al.*, 2007).

Based on the present studies of women, there is scant evidence to support an association between prenatal exposure to diethylstilbestrol and tumours other than the established relationship with CCA affecting the cervix and vagina.

2.3 Men exposed to diethylstilbestrol

2.3.1 Men exposed through cancer therapy

Early case reports of breast cancer occurring in prostate cancer patients treated with diethylstilbestrol implied a possible link; however, the extent to which some of these tumours represented metastatic prostate cancer is uncertain (Bülow *et al.*, 1973).

2.3.2 Men exposed in utero

(a) Cancer of the testes

Several studies have examined prenatal diethylstilbestrol exposure in relation to testicular cancer, but findings have been inconsistent. Because the diethylstilbestrol-exposed men now have passed the age of highest risk for testicular cancer, the question of an association is likely to remain unanswered.

Based on the findings from several case–control studies examining this relationship, most of which relied completely (Henderson *et al.*, 1979; Schottenfeld *et al.*, 1980; Depue *et al.*, 1983; Brown *et al.*, 1986) or partly (Moss *et al.*, 1986) on self-reported hormone use, the previous *IARC Monograph* concluded there is sufficient evidence of a relationship between prenatal diethylstilbestrol exposure and testicular cancer. Three of the contributing studies found possible evidence of an association (Henderson *et al.*, 1979; Schottenfeld *et al.*, 1980; Depue *et al.*, 1983) and two did not (Brown *et al.*, 1986; Moss *et al.*, 1986). Of the three studies that found possible evidence, the association was not of statistical significance in two (Henderson *et al.*, 1979; Schottenfeld *et al.*, 1980). The strongest association arose from a study in California that assessed hormone use during the first

trimester of pregnancy with a relative risk of 8.00 (95%CI: 1.3–4.9); 2/9 case mothers (and none of the control mothers) specified using diethylstilbestrol (Depue *et al.*, 1983). Data from some studies showed (Brown *et al.*, 1986) or suggested (Schottenfeld *et al.*, 1980) an increased risk for the sons of women who had experienced spotting or bleeding during the index pregnancy, a possible marker for diethylstilbestrol use not recalled by the mother. Four of the contributing studies relied partly (Schottenfeld *et al.*, 1980) or entirely (Henderson *et al.*, 1979; Depue *et al.*, 1983; Moss *et al.*, 1986) on neighbourhood controls. [The Working Group noted both of these approaches may have resulted in overmatching and attenuation of a possible relationship between prenatal diethylstilbestrol exposure and risk of testicular cancer.] It is also possible the mothers' reporting was inaccurate, in part because of the amount of time that had passed since the pregnancy and in part because women of the diethylstilbestrol era were not always given complete information about their medical care. [The Working Group noted that errors of recall or recall bias may have influenced the results of these studies.]

Early cohort studies of men exposed *in utero* to diethylstilbestrol also have been largely inconclusive. No testicular cancer cases were identified in the sons of women exposed to high doses of diethylstilbestrol through participation in the Dieckmann clinical trial (11–12 g) (Gill *et al.*, 1979), or a clinical trial involving diabetic women in the UK (mean of 17.9 g) (Beral & Colwell, 1980), although both cohorts were small. One case of fatal teratoma was ascertained in the 138 exposed (no cases in the unexposed) sons of women who participated in a separate high-dose (mean of 11.5 g) clinical trial at the University College Hospital in London (Vessey *et al.*, 1983).

Two studies have been published since the previous *IARC Monograph*. The first study, a case–control design, matched controls to cases by an obstetrician (Gershman & Stolley, 1988) (see Table 2.4 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-11-Table2.4.pdf). The source of diethylstilbestrol exposure status was unclear, but apparently was not based on the medical record. The analysis did not show an association between prenatal diethylstilbestrol exposure and testicular cancer. The NCI Combined Cohort Study assessed 2759 (1365 exposed, 1394 unexposed) sons born to women in the WHS study, the Dieckmann clinical trial, and the Horne cohort, as well as sons identified through the Mayo Clinic with retrospective follow-up for an average of 16.9 years (1978–94) (Strohsnitter *et al.*, 2001) (see Table 2.5 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-11-Table2.5.pdf). For all participants, diethylstilbestrol exposure (or lack of exposure) was verified by the medical or clinical trial record. In this study, the SIR for prenatally exposed men was 2.04 (95%CI: 0.82–4.20) based on seven cases observed in the exposed and 3.4 expected. The relative risk was 3.05 (95%CI: 0.65–22.0) in the internal comparison (two unexposed cases). None of the cases in the NCI Combined Cohort study arose from the Dieckmann clinical trial cohort in which women were consistently given high doses of diethylstilbestrol (cumulative dose of 11–12 g) during the first trimester, although the subcohort was small in size (205 exposed, 187 unexposed). All of the elevated risk was due to an excess of exposed cases arising in the Mayo cohort (five cases in 660 exposed, one case in 592 unexposed). Among those for whom diethylstilbestrol dose was known, the mothers of cases and noncases received 12.5 and 10 mg/day, respectively, doses that are lower than those received by the Dieckmann clinical trial or Horne cohorts (Strohsnitter *et al.*, 2001). The relative risk was unchanged when the analyses were confined to 138 men whose mothers were given diethylstilbestrol during the first trimester of pregnancy but increased to 5.91 (95%CI: 1.05–46.1) after excluding from the analysis men who were

exposed prenatally to both diethylstilbestrol and progestogen.

Cryptorchidism increases the risk for testicular cancer (Sarma *et al.*, 2006). An increased prevalence of cryptorchidism was not seen in the exposed men in either of the two small cohort studies involving the sons of women who received high doses through participation in separate clinical trials in the UK (a mean of 17.9 g in Beral & Colwell, 1980; mean of 11.5 g in Vessey *et al.*, 1983). However, an increased prevalence of cryptorchidism (17/308 exposed versus 1/307 unexposed; $P < 0.005$) was seen in the sons of women exposed to high doses of diethylstilbestrol through participation in the Dieckmann clinical trial (Gill *et al.*, 1979), suggesting a possible pathway linking diethylstilbestrol and testicular cancer (no cases were noted). In the case–control study that addressed this connection, only 1/22 testicular cancer cases affected by cryptorchidism was also exposed to diethylstilbestrol (Schottenfeld *et al.*, 1980).

(b) Other sites

In the NCI Combined Cohort Study, findings were suggestive for bone and thyroid cancer, but estimates were imprecise.

2.4 Offspring (third generation) of women who were exposed to diethylstilbestrol *in utero*

2.4.1 Third-generation women

Follow-up of the prenatally exposed and unexposed second-generation women participating in the NCI Combined Cohort in 1994, 1997, and 2001 included inquiries about cancers occurring in their offspring (Titus-Ernstoff *et al.*, 2008; see Table 2.6 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-11-Table2.6.pdf). Based on the mothers' unconfirmed reports, two cases of ovarian cancer occurred (diagnoses at ages 7 and 20 years) in the 2539 daughters of prenatally exposed women. The SIR in the exposed was 5.3 (95%CI: 1.3–21) based on 0.38 cases expected. No cases were reported in the 1423 unexposed third-generation daughters.

In 2001, the NCI Combined Cohort Study initiated a follow-up study of the adult daughters of women who either had or had not been exposed to diethylstilbestrol *in utero* (Titus-Ernstoff *et al.*, 2008). The results of the baseline survey, which enrolled 793 third-generation women (463 exposed, 330 unexposed), confirmed two cases of ovarian cancer in exposed women (diagnosis ages of 20 and 22 years), including one of the cases that had been reported by the mother. No cases of ovarian cancer were observed in the daughters of women who were not exposed to diethylstilbestrol *in utero*. The SIR was 14.68 (95%CI: 3.67–58.71) based on 0.14 expected cases. Because only half of the second-generation women had allowed contact with their daughters, participation bias was a possible explanation for this finding. However, the SIR remained elevated (6.6; 95%CI: 1.7–26) when based on all adult daughters of prenatally exposed women, regardless of whether they participated in the third-generation study (0.30 cases expected).

Only one study involved clinical examinations of third-generation women (Kaufman & Adam, 2002). Most of the mothers had a history of diethylstilbestrol-related changes, but no vaginal or cervical anomalies were noted upon colposcopic examination of 28 third-generation daughters. Although the study was based on small numbers and did not include hysterosalpingography, the absence of anomalies is inconsistent with the high prevalence of diethylstilbestrol-related vaginal epithelial changes affecting prenatally exposed women.

2.4.2 Third-generation men

In the NCI Combined Cohort Study and based on the mothers' reports, the SIR provided no evidence of increased cancer risk in men born to women exposed prenatally to diethylstilbestrol.

2.5 Synthesis

Diethylstilbestrol is associated with cancer of the breast in women who were exposed while pregnant. Diethylstilbestrol also causes CCA in the vagina and cervix of women who were exposed *in utero*. Finally, a positive association has been observed between exposure to diethylstilbestrol and cancer of the endometrium, and between in-utero exposure to diethylstilbestrol and squamous cell carcinoma of the cervix, and cancer of the testis.

3. Cancer in Experimental Animals

3.1 Oral administration

3.1.1 Mouse

Dietary exposure of diethylstilbestrol induced tumours in many sites, such as the ovary, endometrium and cervix of the uterus, and mesothelioma (origin not indicated) (Greenman *et al.*, 1986). Mammary adenocarcinoma incidence was increased in C3H/HeN-MTV+ female mice (Greenman *et al.*, 1987). Dietary diethylstilbestrol induced thyroid follicular cell adenoma in C57BL/6 mice (Greenman *et al.*, 1990).

Diethylstilbestrol was considered negative in the oral studies in Tg.AC mouse, which is one of the models selected for examination by topical application of either mutagenic or non-mutagenic carcinogens with papilloma formation at the site of application (Eastin *et al.*, 2001). Effect of dietary diethylstilbestrol was studied in $p53^{\pm}$ mice. Interstitial cell hyperplasia and tumours were observed in the testis, and pituitary hyperplasia and adenomas were observed in females; however, the incidences of these lesions were not statistically significant (Storer *et al.*, 2001). When diethylstilbestrol was given to CB6F1-rasH2 transgenic mice, benign tumours and hyperplasia of the Leydig cells in the testes were noted. The incidence of Leydig cell tumours in the rasH2 males at high dose was significantly higher than in vehicle control males (4/15 versus 0/15; $P < 0.05$) (Usui *et al.*, 2001). Carcinogenicity of dietary diethylstilbestrol was investigated in two mouse knockout models, the Xpa homozygous knockout, and the combined Xpa homozygous and p53 heterozygous knockout. The incidence of osteosarcoma and testicular interstitial cell adenomas was higher in male Xpa/p53 mice. One Xpa male had osteosarcoma, which was not observed in wild-type mice. Xpa mice were no more sensitive than wild-type mice for compounds like diethylstilbestrol. The Xpa/p53 mouse model nevertheless showed an increased susceptibility to diethylstilbestrol in inducing osteosarcoma and testicular cell adenoma in males (McAnulty & Skydsgaard, 2005).

See Table 3.1.

3.2 Subcutaneous and/or intramuscular administration

3.2.1 Mouse

The effects of diethylstilbestrol on urethane-induced lung carcinogenesis were assessed in the mouse. Results indicate that diethylstilbestrol promotes lung carcinogenesis (Jiang *et al.*, 2000).

See Table 3.2.

Table 3.1 Studies of cancer in experimental animals exposed to diethylstilbestrol (oral exposure)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse C3H/HeN-MTV (F) 133 wk Greenman *et al.* (1986)	640 ppb DES in diet from 3, 5, 7 or 9 wk of age until 133 wk of age DES-free diet for controls 192/group, 96 controls	Ovary (granulosa cell tumours): Control–6/75 (8%) DES–0/181; 1/180 (0.5%); 3/183 (2%); 0/183		Age at the start of DES treatment was a major factor in susceptibility of mice to mammary carcinogenesis of C3H/HeN-MTV female mice
		Ovary (tubular adenomas): Control–16/75 (21%) DES–1/181 (1%); 24/180 (13%); 44/183 (24%); 59/183[a] (32%)	[$P < 0.0001$][a]	
		Pituitary gland (adenomas): Control–1/67 (1%) DES–8/173 (5%); 10/180 (6%); 16/180 (9%); 21/179[b] (12%)	[$P < 0.0065$][b]	
		Uterus (endometrial carcinomas): Control–(0/77) DES–0/182; 6/189 (3%); 9/191[c] (5%); 8/192 (4%)	[$P < 0.0449$][c]	
		Uterus (cervical carcinomas): Control–(0/77) DES–0/182; 5/189 (3%); 13/191[d] (7%); 9/192[e] (5%)	[$P < 0.05$][d,e]	
		Mesothelioma:[1] Control–(0/77) DES–0/182; 13/189 (7%); 28/191 (15%); 29/192[f] (15%)	[$P < 0.0001$][f]	
		Mammary gland (adenocarcinomas): Control–(0/73) DES–0/182; 0 189; 4/185 (2%); 3/182 (2%)		
Mouse C3H/HeN-MTV (F) 61 wk Greenman *et al.* (1987)	320 ppb DES in diet for 4, 8, 26 and 140 wk 640 ppb DES in diet for 4, 8, 26, and 140 wk DES-free diet for controls 48–72, 312 controls	Mammary (adenocarcinomas): Control–234/295 (79%) 320 ppb–59/71 (83%); 60/72 (83%); 69/72[a] (96%); 68/72[a] (94%) 640 ppb–45/48 (94%); 46/48[a] (96%); 46/48[a] (96%); 47/48[a] (98%)	[$P < 0.05$][a]	
Mouse C57BL/6 (M, F) 153 wk (M), 143 wk (F) Greenman *et al.* (1990)	DES 0, 5, 10, 20, 40, 160, 320, 640 ppb DES-free diet for controls 72/sex/group	Thyroid (follicular cell adenomas): M–2/48 (4%); 0/51; 1/47 (2%); 3/48 (6%); 6/51 (11%); 27/51 (50%); 3/58 (5%); 0/43	$P < 0.001$ (negative trend)	
		F–13/64 (20%); 11/56 (20%); 10/51 (20%); 10/55 (18%); 16/61 (26%); 14/48 (29%); 4/60 (7%); 0/50	$P < 0.001$ (negative trend)	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse Tg.AC[2] (M, F) 27 wk Eastin *et al.* (2001)	DES 0, 30, 240, or 480 µg/kg bw in corn oil i.g. twice weekly for 26 wk then once during Week 27; control corn oil 15/sex/group	No effect on the incidences of either forestomach papillomas or skin tumours in either sex		No table available because of negative data
Mouse p53± transgenic (M, F) 26 wk Storer *et al.* (2001)	M– p53± (0, 50, 250 ppm), wild type (0, 50, 250 ppm) F– p53± (0, 500, 1000 ppm), wild type (0, 500, 1000 ppm) 15/sex/group	Testis (interstitial cell tumours): M–p53± 0/15; 0/15; 2/15 (13%) M–wild type 0/15; 0/13; 0/15 Pituitary gland (adenomas): F–p53± 0/15; 2/15 (13%); 2/15 (13%) F–wild type 0/15; 0/15; 0/14	Incidences of interstitial cell and pituitary tumours were not statistically significant	
Mouse CB6F1-rasH2 transgenic (M, F) 26 wk Usui *et al.* (2001)	15/sex/group M– rasH2 (0, 0.1, 0.3, 1.0 ppm), wild type (0, 0.1, 0.3, 1.0 ppm) F– rasH2 (0, 0.1, 0.3, 1.0 ppm), wild type (0, 0.1, 0.3, 1.0 ppm)	Leydig cell (tumours): M–rasH2 0/15; 0/14; 0/15; 4/15[a] (27%) M–wild type 0/15; 0/15; 1/15 (7%); 2/15 (14%) Lung (adenomas): F–rasH2 1/14 (7%); 0/14; 4/14 (28%); 2/14 (14%) F–wild type NR	$P < 0.05$[a]	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse Xpa/p53[4] (M, F) 39 wk McAnulty & Skydsgaard (2005)	M– Xpa (0, 100, 300, 1500 ppb), wild type (0, 1500 ppb) Xpa/p53 (0, 1500 ppb) F– Xpa (0, 100, 300, 1500 ppb), wild type (0, 1500 ppb) Xpa/p53 (0, 1500 ppb) 15/sex/group	Osteosarcomas: M–Xpa 0/15; 0/14; 0/13; 1/6 (17%) M–wild type 0/15; 0/15 M–Xpa/p53 0/13; 5/6[a] (83%) Testis (interstitial cell adenomas): M–Xpa 0/15; 0/14; 3/13 (23%); 1/6 (17%) M–wild type 0/15; 1/15 (7%) M–Xpa/p53 0/13; 4/6[b] (67%) Osteosarcomas: F–Xpa 0/15; 0/15; 0/15; 0/2 F–wild type 0/15; 0/15 F–Xpa/p53 0/13; 2/2 (100%) Mammary (carcinomas): F–Xpa 0/15; 0/15; 0/15; 0/2 F–wild type 0/15; 0/15 F–Xpa/p53 1/13 (8%); 0/2	 $P < 0.05$[a] $P < 0.05$[b]	

[1] Origin was not described.
[2] The Tg.AC genetically engineered mouse, carrying a v-Ha-ras oncogene fused to the promoter of the gamma-globin gene.
[3] CB6F1-rasH2 transgenic mice with human c-Ha-ras proto-oncogene
[4] Xpa homozygous and p53 heterozygous deficient condition
d, day or days; DES, diethylstilbestrol; F, female; i.g., intragastric; M, male; NR, not reported; wk, week or weeks

Table 3.2 Studies of cancer in experimental animals exposed to diethylstilbestrol (intramuscular injection)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse Kunming (F) 18 wk Jiang *et al.* (2000)	Single i.p. injection of U in saline (50 mg/kg) + DES, i.m. injections one wk later 5 or 50 mg/kg bw once every wk for 18 wk Control: saline and DMSO + saline 26–28, 58 controls	Lung (macroscopic tumours): U alone–(9/27 (33%), 0.69 ± 1.04) U + DES 5 mg–(17/28[a] (61%), 1.80 ± 1.79[b]) U + DES 50 mg–(20/26[b] (77%), 3.81 ± 2.83[b]) Lung (malignant tumours):[1] U alone–(5/27) (18%) U + DES 5 mg–(9/28) (32%) U + DES 50 mg–(17/26[b]) (65%)	$P < 0.05$[a, b], $P < 0.01$ vs U alone group, respectively	DES is a promoter of lung carcinogenesis Age at start NR, animal weight 17–20 g

[1] Malignant tumours were combinations of adenocarcinoma, papillocarcinoma (author's translation), and mixed type cancer
DES, diethylstilbestrol; i.m., intramuscular; i.p., intraperitoneal; NR, not reported; U, urethane; vs, versus; wk, week or weeks

3.3 Subcutaneous implantation

3.3.1 Rat

Diethylstilbestrol pellets were implanted in lactating Wistar-MS rats after irradiation (260 cGy). A significantly higher incidence of mammary tumours was observed in the 260 cGy plus diethylstilbestrol group compared with the 260 cGy-alone group. The latency period was shortest in the diethylstilbestrol-treated group irradiated during the late lactation period. Diethylstilbestrol treatment alone in virgin rats, without irradiation ($n = 20$), did not produce any tumours (Suzuki *et al.*, 1994).

Implanted diethylstilbestrol silastic tubes induced significantly larger and highly haemorrhagic pituitary tumours in female F344 rats but not in Brown Norway (BN) rats. The female F1 (F344 x BN) rats exhibited significantly increased pituitary growth after 10 weeks of diethylstilbestrol treatment, but the pituitary was not haemorrhagic. The haemorrhagic pituitaries in F2 rats were mostly massive, indicating that some genes regulate both phenotypes (Wendell *et al.*, 1996). Diethylstilbestrol increased pituitary mass to 10.6-fold in male ACI rats, and only to 4.4-fold in male Copenhagen (COP) rats. The pituitary growth response of the diethylstilbestrol-treated (5 mg at 63 ± 4 days until 12 weeks of age) in F1 (COPxACI) rats was intermediate (6.9-fold) to that exhibited by the parental ACI and COP strains (Strecker *et al.*, 2005).

See Table 3.3.

3.4 Perinatal exposure

3.4.1 Mouse

Methylcholanthrene treatment induced vaginal tumours (squamous cell carcinoma and mixed carcinoma (squamous cell carcinoma plus adenocarcinoma)) with significantly higher incidence in the CD-1 mice after prenatal exposure to diethylstilbestrol (Walker, 1988). Prenatal exposure to diethylstilbestrol with a high-fat diet increased the incidence of uterine glandular tumours but not of mammary tumours (Walker, 1990). Prenatal diethylstilbestrol induced

Table 3.3 Studies of cancer in experimental animals exposed to diethylstilbestrol (subcutaneous implantation)

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat Wistar-MS (F) 1 yr Suzuki *et al.* (1994)	Irradiated with 260 cGy of gamma rays on 21 d after parturition[a] + CHOL pellets containing 5 mg DES were implanted 1 mo after lactation. DES pellets[b] remained for 1 yr and were replaced every 8 wk 17–28 rats/group	Mammary tumours (no histological information) *Incidence, latency period (month)* 260 cGy + CHOL (6/17 (35%), 10.5 ± 0.2); O cGy + DES (3/11 (27%), 10.0 ± 1.2); 260 cGy + DES (27/28 (96%), 7.4 ± 0.5); Virgin rats: 0 cGy + DES (0/20)	$P < 0.001$ in the incidence and latency period, 260 cGy + DES vs 260 cGy + CHOL $P < 0.001$ in the incidence and latency period 260 cGy + DES vs DES alone	DES promoted radiation-induced mammary tumorigenesis

[a] Detailed location was not described
[b] The release of DES from the pellet was estimated to be 1 µg/day
CHOL, cholesterol; d, day or days; DES, diethylstilbestrol; F, female; mo, month or months; vs, versus; wk, week or weeks; yr, year or years

pituitary tumours in female CD-1 mice (Walker & Kurth, 1993).

In the CBA female descendants of mothers treated with prenatal diethylstilbestrol exposure, described as F2m, the incidence of uterine sarcomas, lymphomas, and ovarian tumours was significantly higher than in controls (Turusov *et al.*, 1992). The persistence of diethylstilbestrol effects was studied in one further generation (diethylstilbestrol-lineage-2 mice). Diethylstilbestrol-lineage-2 mice, exposed to low- or high-fat maternal diets, had significantly more tumours in their reproductive system and liver than control mice with the same dietary fat exposure (Walker & Haven, 1997). The incidence of uterine adenocarcinomas in F2 females with prenatal diethylstilbestrol exposure was significantly higher than in controls, whereas the incidence of tumours of the liver, lung or other organs examined in this study was not significantly different from that in control animals (Newbold *et al.*, 1998). In F2 males, a significant increase in the incidences of proliferative lesions of the rete testis (hyperplasia and tumours) was observed, suggesting that the rete testis is a target for the transgenerational effects of diethylstilbestrol in males (Newbold *et al.*, 2000).

Prenatal diethylstilbestrol treatment of female CBA mice increased the incidence of DMH-induced colon carcinoma (Turusov *et al.*, 1997). Effects of perinatal diethylstilbestrol exposure on mammary tumorigenesis were studied in female C3H/HeN/MTV+ mice. Neonatal treatment with a low dose of diethylstilbestrol increased the probability of mammary tumour formation (Lopez *et al.*, 1988). Effects of perinatal exposure to estrogens during the development stage of organs in the reproductive tract were studied in CD-1 mice. Uterine adenocarcinomas were induced in a time- and dose-related manner after diethylstilbestrol treatment (Newbold *et al.*, 1990). Male offspring of CD-1 mice with transplacental exposure to arsenite were treated with diethylstilbestrol neonatally. Total liver tumour incidence, the number of mice with multiple liver tumours, and urinary bladder proliferative lesions was higher in the arsenite plus diethylstilbestrol mice compared to the arsenite-alone

group (Waalkes *et al.*, 2006b). In female offspring CD-1 mice, the incidence of carcinoma of the cervix and of urinary bladder total proliferative lesions (hyperplasia plus papilloma plus carcinoma) in the arsenite plus diethylstilbestrol group was significantly higher than in the arsenite-alone group (Waalkes *et al.*, 2006a).

CD-1 and diethylstilbestrol induced-TGFα transgenic mice were neonatally treated with diethylstilbestrol. The presence of the TGFα transgene significantly increased the incidence of endometrial hyperplasia and benign ovarian cysts, whereas it did not promote uterine adenocarcinoma (Gray *et al.*, 1996). Transgenic MT-mER mice, which overexpress the estrogen receptor, driven by the mouse metallothionein I promoter were neonatally treated with diethylstilbestrol. The diethylstilbestrol-treated MT-mER mice demonstrated a significantly higher incidence of uterine adenocarcinomas (Couse *et al.*, 1997). Diethylstilbestrol-treated wild-type mice exhibited a relatively high frequency of uterus endometrial hyperplasia and granulosa cell tumours in the ovary, while αERKO mice (estrogen receptor α knockout mice) showed a complete lack of these lesions (Couse *et al.*, 2001). Lymphoma-prone Mlh1 or Msh2 knockout mice were treated with diethylstilbestrol. The combination of Mlh1 deficiency with diethylstilbestrol exposure was shown to accelerate lymphomagenesis (Kabbarah *et al.*, 2005). In murine PTEN (mPTEN) heterozygous mutant mice, demonstrated that neonatal diethylstilbestrol treatments exerted an inhibitory, rather than an enhancing, effect on PTEN-associated endometrial carcinogenesis via stromal alterations (Begum *et al.*, 2006).

3.4.2 Rat

Mammary tumours are induced in female ACI rats by either prenatal injections or by postnatal pellet implantation of diethylstilbestrol. The combination of both yielded significantly greater tumour multiplicity, and decreased tumour latency (Rothschild *et al.*, 1987). Vaginal epithelial tumours were induced in a dose-related manner in female Wistar rat following in-utero diethylstilbestrol exposure (Baggs *et al.*, 1991). Prenatal exposure to diethylstilbestrol produced uterine adenocarcinomas and pituitary adenomas in female Donryu rats, as reported in an earlier study in mice (Kitamura *et al.*, 1999). In Sprague Dawley rats, neonatal diethylstilbestrol exposure at a relatively low dose (1 μg/kg body weight) caused an increase in the incidence of mammary carcinomas induced by 1,2-dimethylbenz[*a*] anthracene (Ninomiya *et al.*, 2007). Female rats carrying the Eker mutation (*Tsc-2*$^{Ek/+}$) administered diethylstilbestrol neonatally had a significantly greater multiplicity of leiomyoma in the uterus (Cook *et al.*, 2005).

3.4.3 Hamster

The subcutaneous implantation of diethylstilbestrol pellets caused renal tumours in young Syrian hamsters (Liehr & Wheeler, 1983), and diethylstilbestrol pellets, implanted after orchiectomy, induced kidney tumours in the same species (Goldfarb & Pugh, 1990). Diethylstilbestrol-treated castrated hamsters exhibited interstitial lesions in the kidney as well as kidney tumours (Oberley *et al.*, 1991). In male and female Armenian hamsters, diethylstilbestrol pellets applied subcutaneously induced hepatocellular carcinomas (Coe *et al.*, 1990).

See Table 3.4.

3.5 Synthesis

The oral administration of diethylstilbestrol induced tumours of the ovary, endometrium and cervix, and mammary adenocarcinomas in female mice. Osteosarcomas and Leydig cell tumours were induced in rasH2 and Xpa/p53 male mice, respectively.

Table 3.4 Studies of cancer in experimental animals exposed to diethylstilbestrol (perinatal exposure)

Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse CD-1 (F), gestation Day 17 Walker (1988)	Prenatal: s.c. injection 1 µg/g bw DES in olive oil + 2% ethanol at Day 17 of pregnancy + Postnatal: sponges impregnated with MCA in beeswax were lodged against the cervix and vaginal fornices of the mice at 6 months of age Controls received olive oil Number/group at start NR	Vaginal tumours (SCC and mixed (SCC + adenocarcinoma) carcinomas): DES + MCA–(10/35, 29%) Vehicle + MCA–(2/28, 7%) DES + beeswax–(1/12, 8%) Vehicle + beeswax–(0/8, 0%)	$P < 0.05$ DES+MCA vs Vehicle+MCA	
Mouse CD-1 (F), gestation Day 16 Lifespan Walker & Kurth (1993)	i.p. injection 1 or 2 µg/g bw at 16–17 days postconception 132; 64 controls	Pituitary tumours: Control–(1/57, 2%) DES 1 µg–(19/34, 56%) DES 2 µg–(3/3, 100%)	 $P < 0.01$	
Mouse CBA (F), gestation Day 17 Lifespan Turusov *et al.* (1992)	Prenatal: i.p. injection 1 µg/g bw DES in olive oil + 2% ethanol at Day 17 of pregnancy (F2m,[a] the descendants of DES-treated grandmothers were used in this study) Number/group at start NR	Uterine (sarcomas): F2m female (17/84, 20%), control (6/108, 6%) Lymphomas:[b] F2m female (17/84, 20%), control (10/108, 9%) Benign ovarian tumours: F2m female (16/84, 19%), control (9/108, 8%)	 $P = 0.0022$ $P = 0.037$ $P = 0.004$	
Mouse CD-1 (F), gestation Day 17 Lifespan Walker & Haven (1997)	1 µg/g bw of DES in olive oil + 0.1% ethanol at Day 17 of pregnancy. DES-lineage2[c] mice were exposed to low- (LF, 2.6%) or high-fat (HF, 29%) diets Number/group at start NR	Reproductive system tumours: combination of ovarian, uterine, cervical (adenocarcinoma) and mammary tumours (adenocarcinoma + sarcoma) DES+LF (31/61, 51%), LF (11/66, 17%) DES+HF (25/54, 46%), HF (18/68, 26%) Liver tumours: DES+LF (17/61, 28%), LF (32/66, 48%) DES+HF (16/54, 30%), HF (22/68, 32%)	 $P < 0.001$ $P < 0.05$ $P = 0.03$ NS	The multi-generational effect of DES was observed in mice

Table 3.4 (continued)

Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse CD-1 (F), Day 1 for F2 Up to 24 mo Newbold *et al.* (1998)	s.c. injection G1: 2.5, 5, 10 µg/kg bw on Days 9–16 of gestation G2: 1000 µg/kg bw on Day 18 of gestation G3: 0.002 µg/pup/day on Days 1–5 after birth Female F2 mice[d] were examined in this study. Animals were held for 17–19 or 22–24 mo Number at start NR	Uterine adenocarcinomas in F2: Groups / at 17–19 mo / at 22–24 mo / total Control: 0/32 / 0/23 / 0/55 G1 2.5 g: 2/29 (7%) / 3/35 (9%) / 5/64 (8%) G1 5 g: 2/35 (6%) / 6/37 (16%) / 8/72 (11%) G1 10 g: 0/16 / 0/24 / 0/40 G2: 0/33 / 1/15 (7%) / 1/48 (2%) G3: 1/29 (3%) / 4/36 (11%) / 5/65 (8%)	vs. concurrent controls;[e] vs. historical controls[f] – ; – $P < 0.05$; $P < 0.001$ $P < 0.01$; $P < 0.001$ NS; NS NS; NS $P < 0.05$; $P < 0.001$	Pups, 1–3.5 g
Mouse CD-1 (M) Day 1 for F2 Up to 24 mo Newbold *et al.* (2000)	s.c. injection G1: 2.5, 5, 10 g/kg bw on Days 9–16 of gestation G2: 1000 µg/kg bw on Day 18 of gestation G3: 0.002 µg/pup/d on Days 1–5 after birth DES was dissolved in corn oil Male F2 mice[g] examined in this study were killed at 17–24 mo Number at start NR	Rete testis proliferative lesions (hyperplasia or tumours) in testis of DES-lineage (F2) male mice: Control (3/53, 6%) G1 2.5 µg (15/73, 21%) G1 5 µg (27/83, 32%) (1 tumour) G1 10 µg (17/49, 35%) G2 (5/52, 10%) (1 tumour) G3 (7/23, 30%)	– $P < 0.05$ $P < 0.01$ $P < 0.01$ NS $P < 0.01$	Pups, 1–3.5 g
Mouse, C3H (F) CBA (F) 2–3 months for F1 50 wk Turusov *et al.* (1997)	s.c. injection 0, 0.1, 0.3 mg/kg bw DES in olive oil + 0.1% ethanol at Day 17 of pregnancy and then descendants were treated with 1,2-dimethyl hydrazine (DMH)[h] Killed at Week 50 after the beginning of DMH 35–50; 84–101 controls/group	Colon (carcinomas): DMH / 0.1-DES+DMH / 0.3-DES+DMH C3H: 9/37 (24%) / 6/45 (13%) / 4/32 (12%) CBA: 2/38 (5%) / 10/54 (18%) / 10/27 (37%) Uterus (sarcomas): C3H: 0/37 / 4/45 (9%) / 0/32 CBA: 21/38 (55%) / 29/54 (53%) / 16/27 (59%)	NS P<0.01 (0.3–DES+DMH) NS NS	

Table 3.4 (continued)

Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse CD-1 (F) Day 1 of life Up to 18 mo Newbold *et al.* (1990)	s.c. injection of 0.002 or 2 µg/pup/d DES in corn oil for Days 1–5 of age Controls received corn oil Killed 1, 2, 4, 6, 8, 12, 18 mo of age Number at start NR	Uterus adenocarcinomas of 2 µg group at 1, 2, 4, 6, 8, 12, 18 mo: DES–0/18, 0/10, 0/10, 0/12, 0/5, 8/17 (47%), 9/10 (90%) Controls–0/15, 0/10, 0/10, 0/12, 0/5, 0/17, 0/10	$P = 0.0026$ at 12 mo, $P = 0.0002$ at 18 mo	
Mouse CD-1 (F) Day 1 of life (female offspring) 90 wk Waalkes *et al.* (2006a)	Transplacental exposure to arsenite (ARS)[i] 85 ppm from Days 8–18 of gestation + s.c. injection 2 µg/pup/d DES Days 1–5 of age Killed at 90 wk 35/group (offspring)	Cervix (carcinomas): Control (0/33), ARS (0/34), DES (6/33, 18%), ARS+DES (8/33, 24%) Urinary bladder (total proliferative lesions):[j] Control (1/33, 3%), ARS-alone (5/34, 15%), DES-alone (1/33, 3%), ARS+DES (13/33, 38%)	$P = 0.01$ (DES vs control) $P < 0.05$ (ARS+DES vs ARS) $P < 0.05$ (ARS+DES vs ARS)	Sodium arsenite in the drinking-water
Mouse CD-1 (F) TGF α[k] (F) Day 1 of life Gray *et al.* (1996)	s.c. injection 2 µg/pup/d DES in sesame oil on Days 1 – 5 of age Control remained untreated maintained 39, 52 wk or for life Number/group at start NR	Uterus (adenocarcinomas): Vehicle in CD-1, (0/26) Vehicle in TGF α (0/25) DES in CD-1 (7/16, 44%) DES in TGF α (7/15, 47%)	– – $P<0.01$ $P<0.01$	
Mouse, Wild type fvb/N (F) MT-mER[l] (F) Day 1 of life Up to 18 mo Couse *et al.* (1997)	s.c. injection 2 µg/pup/d DES in corn oil on Days 1 – 5 of age Control: untreated Killed at 4, 8, 12, and 18 mo of age Number/group at start NR	Uterus adenocarcinomas at 4, 8, 12, 18 mo: MT-mER +DES: 0/19, 19/26 (73%), 13/15 (87%), 12/13 (92%) Wild type+DES: 0/19, 11/24 (46%), 11/15 (73%), 13/14 (93%) MT-mER control: 0/14, 0/10, 0/15, 1/19 (5%) Wild type control: 0/15, 0/11, 0/15, 0/19	$P < 0.05$ at 8 months (MT-mER+DES vs Wild type+DES) $P < 0.05$ at 8, 12, 18 mo (Wild type+DES vs wild type)	

Table 3.4 (continued)

Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, ACI (F) gestation Day 15 Rothschild *et al.* (1987)	Prenatal: s.c. injection of 0.8 µg (Low) or 8.0 µg (High) DES in sesame oil on Days 15 and 18 of gestation Postnatal:[m] 2.5 mg DES pellet at 12 wk Control: sesame oil (vehicle/DES) Killed 7 mo post pellet 32–47, 32 controls	*Mean time to appearance of first mammary tumour* *mean No. of palpable mammary tumours/rat:* Vehicle/DES: 22.2 wk, 2.2 Low DES/DES: 19.4 wk,[1] 3.0 High DES/DES: 15.2 wk,[1] 4.3[2]	[1] $P < 0.05$ vs Vehicle/DES [2] $P < 0.05$ vs Vehicle/DES	
Rat, Wistar (F) gestation Day 18 > 120 d Baggs *et al.* (1991)	i.p. injection 0, 0.1, 0.5, 25, 50 mg/kg bw on Days 18, 19, and 20 of gestation 11–80, 147 controls	Vaginal epithelial tumours:[n] 2/147 (1%), 2/49 (4%), 4/80 (5%), 1/63 (2%), 3/11 (27%)	$P < 0.001$ (dose response)	
Rat, Donryu (F) gestation Day 17 18 mo Kitamura *et al.* (1999)	s.c. injection 0, 0.01, 0.1 mg/kg bw DES on Days 17 and 19 of gestation Control: olive oil 35–72, 23 controls	Uterus tumours (endometrial adenocarcinomas): 1/20 (5%), 4/39 (10%), 9/29 (31%) Pituitary (adenomas): 1/20 (5%), 0/39, 6/29 (20%)	$P < 0.05$, 0.1 mg vs control $P < 0.01$, 0.1 mg vs control	
Rat, Sprague Dawley (F) Day 1 of life Up to 300 d Ninomiya *et al.* (2007)	s.c injection 0, 0.1, 1, 10, 100 µg/kg bw DES in sesame oil at birth. 50 days after birth all rats received 1 mg DMBA in 1 mL sesame oil by gastric intubation Killed 50–300 d after birth 13–21, 22 controls	*Mammary tumour incidence and multiplicity* *(adenocarcinomas) at 300 d after birth:* 0.0 µg: 17/22 (77%), 1.50 ± 1.19 0.1 µg: 13/15 (86%), 2.80 ± 1.61[1] 1 µg: 21/21[1] (100%), 3.00 ± 1.22[2] 10 µg: 11/13 (85%), 2.54 ± 1.66[1] 100 µg: 12/16 (75%), 1.63 ± 1.45	[1] $P < 0.05$ vs control [2] $P < 0.01$ vs control	
Rat Eker Rats carrying a germ-line defect in the tuberous sclerosis complex 2 (Tsc-2) tumour suppressor gene (F) Day 3 of life Up to 16 mo Cook *et al.* (2005)	s.c. injections 10 µg/rat/d DES in sesame oil on Days 3, 4, and 5 after birth Controls: 50 µl sesame oil Killed up to 16 mo of age Number at start NR	Uterus (leiomyomas): *Incidence, multiplicity and size* In Ekar rats *Tsc-2*$^{Ek/+}$ Vehicle: 18/28 (64%), 0.82 ± 0.15, 2.30 ± 1.10 DES: 22/24 (92%), 1.33 ± 0.17[1], 10.50 ± 2.70[1] In wild type *Tsc-2*$^{+/+}$ Vehicle: 0/34, 0, 0 DES: 0/34, 0, 0	[1] $P < 0.02$ in multiplicity, and size in Eker rats (DES vs vehicle)	

Table 3.4 (continued)

Species, strain (sex), age Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Hamster Syrian (M), 4 wk 172 d Liehr & Wheeler (1983)	Two 40 mg pellets containing 90% DES + 10% cholesterol were implanted at Day 27 and one more (same composition) 3 mo later 15/group	Renal tumours: Control (0/9) DES (11/13, 85%)	$P < 0.05$	
Hamster, Syrian (M), 7 wk 2.5–6.2 mo Goldfarb & Pugh (1990)	Orchiectomy and 4 wk later 20 mg DES pellets every 3 mo, 9–13; 8–12 controls	Renal tumours: Controls at 2.5–3; 5–6.2 mo 0/8; 0/12 DES at 2.5; 4.6; 5.6; 6.2 mo 0/13; 2/12 (17%); 8/12 (67%); 7/9 (78%)	$P < 0.05$, DES vs control at 6.2 mo	
Hamster, Syrian (M) 2 mo Up to 9 mo Oberley *et al.* (1991)	Castrated and 20 mg of DES pellet every 2.5 mo 57, 4 controls	Interstitial foci in the kidney: DES at 1, 2, 3, 4, 5, 6, 7, 8, 9 mo 0/7; 0/7; 0/7; 0/7; 4/12 (33%); 5/8 (62%); NR; 2/3 (66%); 1/6 (17%) Kidney tumours:[m] DES at 1, 2, 3, 4, 5, 6, 7, 8, 9 mo 0/7; 0/7; 0/7; 0/7; 0/12; 3/8 (37%); NR; 2/3 (67%); 4/6 (67%) Controls: at 9 mo 0/4	NS	Age NR, Animal weight 90–100 g

[a] The descendants of DES-treated mothers, described as F1DES, were mated among each other or with untreated animals. F1DES males were successfully mated with untreated females (F2m).
[b] Both grossly visible tumours and microscopic cancers included in this category.
[c] The descendants of DES-treated mothers, described as DES-lineage were mated with control animals. DES-lineage2 mice were generated by mating DES-lineage female mice with control males.
[d] Female mice (F1) in each group were raised to sexual maturity and bred with control males. Female offspring (DES lineage or F2) from these matings were raised to maturity and housed with control males for 20 weeks.
[e] versus concurrent controls; relative to concurrent control rate of 0/55.
[f] versus historical controls; relative to historical control rate of 0.4% (2/482) in 21–24 month old female Charles River CD-1 mice.
[g] DES-exposed female mice (F(1)) were raised to maturity and bred with control males to generate DES-lineage (F(2)) descendants. The F(2) males obtained from these matings are the subjects of this report.
[h] The descendants, starting from the age of 2–3 months, received weekly s.c. injections of 1,2-dimethylhydrazine (DMH) (8 mg/kg bw), for a total of 20 injections.
[i] Pregnant CD-1 mice received 85 ppm arsenite in the drinking-water from gestation Days 8 to 18
[j] Total proliferative lesion (hyperplasia+ papilloma+ carcinoma).
[k] Homozygous TGF α transgenic female mice from the MT42 line.
[l] The transgenic construct consisted of a fragment of the mouse ER cDNA encoding the full-length ER protein driven by the mouse metallothionein I promoter.
[m] Pellets containing 2.5 mg DES+17.5 mg cholesterol (DES pellet) or 20 mg cholesterol were implanted s.c. into 12-week-old female offsprings.
[n] The types of epithelial tumours of the vagina were adenocarcinomas, squamous cell carcinomas, and mixed carcinomas.
bw, body weight; d, day or days; DES, diethylstilbestrol; DMBA, 7,12 dimethylbenz[a]anthracene; F, female; i.p., intraperitoneal; M, male; MCA, methylcholanthrene; mo, month or months; NR, not reported; NS, not significant; s.c., subcutaneous; vs, versus; wk, week or weeks

Subcutaneous implantation of diethylstilbestrol induced mammary tumours in female Wistar rats.

Perinatal exposure to diethylstilbestrol induced lymphomas, uterine sarcomas, adenocarcinomas and pituitary, vaginal, and ovarian tumours in female mice. Uterine adenocarcinomas and mammary and vaginal tumours were also induced in female rats. In hamsters, diethylstilbestrol perinatal exposure induced kidney tumours. In castrated hamsters, kidney tumours were also induced following implantation of diethylstilbestrol.

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

The toxicokinetics and metabolism of diethylstilbestrol (diethylstilbestrol) were reviewed by IARC, (1979b), and by Metzler & Fischer (1981).

Diethylstilbestrol is readily absorbed and distributed in the whole organism after oral administration (Marselos & Tomatis, 1992). In animal models used for the pharmacokinetics of diethylstilbestrol (with the exception of primates), it is apparent that the drug is almost exclusively eliminated through biliary excretion into the intestine, where it undergoes extensive enterohepatic circulation before being excreted in the faeces (Marselos & Tomatis, 1992). Only traces of diethylstilbestrol can be detected in urine (McMartin *et al.*, 1978).

Whole animal autoradiography experiments showed that radiolabelled diethylstilbestrol injected intravenously into rats is accumulated in the liver and small intestine within 4 hours, and radioactivity can still be detected in these organs after 4 days (Bengtsson, 1963). Peak plasma levels of radioactivity were found within 16 hours in sheep given radiolabelled diethylstilbestrol at single oral doses. Radioactivity disappeared almost completely after 120 hours (Aschbacher, 1972). Ten days after a single oral dose of radiolabelled diethylstilbestrol to steers, residues could be detected in the small intestine, the faeces, and the urine (Aschbacher & Thacker, 1974). In the rat, it was demonstrated that after intestinal intubation of diethylstilbestrol or diethylstilbestrol-glucuronide, free diethylstilbestrol is readily absorbed through the epithelium, whereas the conjugated form requires prior hydrolysis by the intestinal microflora (Fischer *et al.*, 1973).

Studies on diethylstilbestrol transfer across the placenta in mice have shown that it accumulates in the fetal genital tract, where it reaches levels that are three times higher than that found in the fetal plasma (Shah & McLachlan, 1976).

The kinetics of a single oral dose of radiolabelled diethylstilbestrol (10 mg) in cattle followed a biphasic depletion curve, attributed to hepatic clearance. An initial steeper slope represented a biological half-life of 17 hours, while the half-life for the later phase was 5.5 days (Rumsey *et al.*, 1975a). Furthermore, pellets of 24–36 mg diethylstilbestrol implanted subcutaneously in cattle or steers resulted in a systematic release of about 56–74 µg of diethylstilbestrol per day; the half-life was 80–90 days (Rumsey *et al.*, 1975b).

Subsequently, the oxidative quinone metabolite of diethylstilbestrol (4′,4″-diethystilbestrol quinone) was found to be reactive *in vitro*, binding to DNA (Liehr *et al.*, 1983, 1985a). The formation of the quinone is mediated by microsomal mono-oxygenase (Degen *et al.*, 1986; Roy *et al.*, 1991a), in particular cytochrome P450(CYP)1A1 (Roy *et al.*, 1992), by prostaglandin synthase (Ross *et al.*, 1985; Degen, 1993), and by peroxidases (Liehr *et al.*, 1983, 1985a; Metzler, 1984). The quinone metabolite is reduced by P450 reductase and xanthine oxidase, via the semiquinone and non-enzymatically, directly to diethylstilbestrol (Roy & Liehr, 1988; Roy *et al.*, 1991b). Diethylstilbestrol quinone is also formed *in vivo*, in the kidney of diethylstilbestrol-treated

male Syrian hamsters (Roy & Liehr, 1988), in the mammary gland tissue of diethylstilbestrol-treated ACI rats (Thomas *et al.*, 2004), and in the liver of diethylstilbestrol-treated rats (Green *et al.*, 2003). Diethylstilbestrol quinone is formed in the liver, kidney, uterus, and placenta of pregnant diethylstilbestrol-treated Syrian hamsters, and in the liver and kidney of their fetuses (Roy & Liehr, 1989). Diethylstilbestrol metabolites are also found in the female genital tract of adult mice and pregnant mice, and in tissues of their fetuses (Gottschlich & Metzler, 1984; Maydl *et al.*, 1985). The quinone metabolite was found to undergo a CYP-mediated process of redox cycling (Liehr *et al.*, 1985a), via a semiquinone intermediate (Kalyanaraman *et al.*, 1989).

During redox cycling of diethylstilbestrol, superoxide radicals are formed *in vitro* (Epe *et al.*, 1986; Roy & Liehr, 1988). In the kidney of diethylstilbestrol-treated hamsters, elevated levels of 8-hydroxy deoxyguanosine were found, indicating that diethylstilbestrol can induce oxidative DNA damage *in vivo* (Roy *et al.*, 1991c). Furthermore, increased levels of lipid hydroperoxides and of malondialdehyde-DNA adducts were also detected (Wang & Liehr, 1995a). Lipid hydroperoxides were also found to be increased in the mammary gland tissue of diethylstilbestrol-treated ACI rats (Gued *et al.*, 2003). These lipid hydroperoxides co-activate the CYP1A1-mediated oxidation of diethylstilbestrol to its quinone metabolite (Wang & Liehr, 1994). Diethylstilbestrol treatment reduced the activity of enzymes that protect against diethylstilbestrol-induced oxidative stress, such as glutathione peroxidase, quinone reductase, and superoxide dismutase (Segura-Aguilar *et al.*, 1990). In the mammary gland tissue of female rats, expression of the *Cyp1A1* gene was increased by diethylstilbestrol treatment, whereas the expression of the genes encoding glutathione-*S*-transferase and superoxide dismutase were depressed (Green *et al.*, 2007).

The oxidative metabolism of diethylstilbestrol almost certainly plays a central role in the induction of kidney tumours in Syrian hamsters, of genetic changes in various in-vitro assays, and probably also of other tumours in animals perinatally exposed to diethylstilbestrol *in utero*. Whether these events occur in target tissues of transplacental exposure to diethylstilbestrol in humans has not been determined.

4.2 Genetic and related effects

4.2.1 Direct genotoxicity

(a) Humans

No changes in DNA ploidy pattern and no mutations were found in specific cancer-related genes (H-*RAS* and K-*RAS* proto-oncogenes, *TP53* and the Wilms tumour (*WT-1*) tumour-suppressor genes) or in the coding region of the *estrogen receptor-α* (*ERα*) gene (Welch *et al.*, 1983; Boyd *et al.*, 1996; Waggoner *et al.*, 1996). The frequency of some known polymorphisms (exon 1, 3, and 8) in the *ERα* gene was not different from that expected in the general population (Boyd *et al.*, 1996).

In cervico-vaginal biopsies and smears from 19 women who had been exposed to diethylstilbestrol *in utero* and 19 controls, the frequencies of trisomy of chromosomes 1, 7, 11, and 17 were evaluated by the FISH technique. The trisomy frequencies were elevated in 4/19 (21%) diethylstilbestrol-exposed women. Trisomy of chromosomes 1, 7, and/or 11 was found, which frequently occurs in gynaecological tumours, but trisomy of chromosome 17 did not occur. No chromosomal trisomy was observed in samples from the control women (Hajek *et al.*, 2006).

In neoplastic and preneoplastic lesions of the breast, loss of heterozygosity and allelic imbalance at 20 microsatellite markers on nine chromosomal arms was comparable between women exposed *in utero* to diethylstilbestrol and control women (Larson *et al.*, 2006).

There are no data on the effects of diethylstilbestrol on cell proliferation or apoptosis in human target tissues of diethylstilbestrol-induced carcinogenicity.

Women with documented in-utero exposure to diethylstilbestrol had a higher mitogen-induced proliferation of peripheral blood lymphocytes compared to age- and menstrual-cycle phase-matched control women (Ways *et al.*, 1987; Burke *et al.*, 2001), suggestive of an increased cellular immune response. A hyperactive immune system may be related to the reported higher frequency of autoimmune disease, and immune-related inflammatory disorders such as arthritis following in-utero exposure to diethylstilbestrol, compared with control women (Wingard & Turiel, 1988; Noller *et al.*, 1988). However, natural killer-cell activity was not found to be altered in women exposed to diethylstilbestrol *in utero* (Ford *et al.*, 1983).

The developmental abnormalities and the disturbance of menstrual activity found in sons and daughters, respectively, of diethylstilbestrol daughters suggest that third-generation (F2) effects of human prenatal diethylstilbestrol exposure, including cancer development, are conceivable. However, there are no mechanistic data on this point in animal models, nor data about germ-line mutations or other heritable alterations.

Vaginal adenosis is an established, although non-obligatory, precursor of CCA. Although most women affected by vaginal adenosis do not develop CCA, adenosis is present in up to 100% of women with CCA (Herbst *et al.*, 1972, 1974; Robboy *et al.*, 1984a).

Other effects of in-utero exposure to diethylstilbestrol include infertility in female offspring, as reported in most but not all studies (Palmer *et al.*, 2001), and possibly in males (Perez *et al.*, 2005).

In most studies, changes in menstrual activity by decreasing the duration of menstrual bleeding were observed in comparison with control women (Hornsby *et al.*, 1994). Young women whose mothers had been exposed to diethylstilbestrol *in utero* had a 1.5- to 2-fold increased risk for self-reported menstrual irregularities and fertility problems (Titus-Ernstoff *et al.*, 2006b).

In a meta-analysis (Martin *et al.*, 2008) of three studies (Klip *et al.*, 2002; Palmer *et al.*, 2005; Pons *et al.*, 2005), in-utero exposure to diethylstilbestrol was associated with a 3.7-fold increased risk for hypospadias in men.

(b) Experimental systems

(i) In vivo

Diethylstilbestrol induced chromosomal aberrations in bone-marrow cells of mice treated *in vivo*, but data on in-vivo induction of sister chromatid exchange and micronuclei were equivocal (IARC, 1987b); it induced sister chromatid exchange in one study in rats (Gloser & Cerni, 1984). Diethylstilbestrol induced micronuclei in early haploid mouse spermatids 17 days after a single subcutaneous injection (Pylkkänen *et al.*, 1991a); chromosomal aberrations in cells of the renal cortex in male Syrian golden hamsters (the target tissue of diethylstilbestrol-induced carcinogenicity) (Banerjee *et al.*, 1994); sister chromatid exchange (but no changes in chromosome number) in uterine cervical epithelial cells, but not in the epithelium of the uterus or kidneys (Forsberg, 1991), and sister chromatid exchange, but no aneuploidy in mouse bone-marrow cells (Zijno *et al.*, 1989). Markedly increased aneuploidy was found in proximal tubular kidney cells of male Syrian hamsters with subcutaneously implanted diethylstilbestrol pellets (Li *et al.*, 1993, 1999).

In hamsters, diethylstilbestrol-induced kidney tumour point mutations were detected in the catalylic domain of the DNA polymerase β gene compared to control tissue (Yan & Roy, 1995), and at 44/365 random loci, seven of which were also present in non-tumorous kidney tissue (Singh & Roy, 2004). The expression of the DNA

polymerase β gene and a novel gene, *Etrg-1*, was reduced in tumorous and non-tumorous kidney tissues of diethylstilbestrol-treated hamsters compared to controls (Singh & Roy, 2008). Microsatellite instability was increased in early lesions induced by neonatal treatment of mice (Kabbarah *et al.*, 2003). In host-mediated assays using mice, no DNA-repair response was detected in *E. coli* strains (Kerklaan *et al.*, 1986).

Using [^{32}P]-postlabelling, adducted nucleotides were found in the kidney DNA of hamsters chronically treated with diethylstilbestrol but not in the kidneys of untreated animals (Liehr *et al.*, 1985b). Some adducts were chromatographically identical to those induced by estradiol and other estrogenic compounds, suggesting that some of these adducts may not be diethylstilbestrol-derived (Liehr *et al.*, 1986). The major diethylstilbestrol adduct formed *in vivo* in the hamster kidney and liver DNA was chromatographically identical to that observed after in-vitro reaction of DNA with 4′,4″-diethystilbestrol quinone in the presence of microsomes and hydroperoxide cofactors, suggesting that this metabolite is responsible for DNA damage by diethylstilbestrol *in vivo*, and that oxidative metabolism of diethylstilbestrol is required for its formation (Gladek & Liehr, 1989; Bhat *et al.*, 1994). The adduct was unstable with an in-vitro half-life of 4–5 days at 37°C, and an estimated in-vivo half-life of 14 hours, which is suggestive of in-vivo repair (Gladek & Liehr, 1989). Importantly, diethylstilbestrol adducts were also found in the mammary gland tissue of diethylstilbestrol-treated adult female rats (Green *et al.*, 2005), and in hamster fetal tissues after injection of their mothers with diethylstilbestrol, but the major adduct found was different from that identified in the kidneys of adult diethylstilbestrol-treated hamsters (Gladek & Liehr, 1991). The precise structures of the diethylstilbestrol-induced DNA adducts have not been elucidated, but it is probable that some are oxidative-stress-generated lipid–hydroperoxide– and malondialdehyde–DNA adducts (Wang & Liehr, 1995a, b). Although feeding of vitamin C reduced the incidence of kidney tumours, the generation of diethylstilbestrol quinone and the formation of adducts in the kidney of diethylstilbestrol-treated male Syrian hamsters (Liehr *et al.*, 1989), the biological significance of the diethylstilbestrol-generated adducts has not been determined, and specific mutations generated by exposure to diethylstilbestrol have not been identified thus far.

(ii) In vitro

Diethylstilbestrol induces aneuploidy and DNA strand breaks in human cells *in vitro* (IARC, 1987a, b; Rupa *et al.*, 1997; Schuler *et al.*, 1998; Quick *et al.*, 2008). Data on in-vitro induction of sister chromatid exchange, chromosomal aberrations, and mutations in human cells were inconclusive (IARC, 1987a, b). More recent studies found additional evidence of diethylstilbestrol-induced sister chromatid exchange in cultured human lymphocytes, but at cytotoxic diethylstilbestrol concentrations (Lundgren *et al.*, 1988; Konac *et al.*, 2005). Data on induction of micronuclei by diethylstilbestrol remain equivocal (Fauth *et al.*, 2000; Clare *et al.*, 2006), while studies on the induction of unscheduled DNA synthesis in human cells *in vitro* were mostly negative (IARC, 1987a, b). Diethylstilbestrol inhibited the polymerization of microtubules in human fibroblasts and prostate cancer cells, inducing metaphase arrest (Parry *et al.*, 1982; Hartley-Asp *et al.*, 1985), an effect that may underlie the induction of aneuploidy.

Diethylstilbestrol inhibited the in-vitro growth of human primary cervical cell strains, and inhibited colony formation at high concentrations (Johnstone *et al.*, 1984; Stanley *et al.*, 1985). Short-term exposure to diethylstilbestrol stimulated the growth of SV40-immortalized human endometrial stromal cells in soft agar, an effect that was inhibited by the anti-estrogen tamoxifen (Xu *et al.*, 1995). Chronic exposure of these cells to low concentrations of diethylstilbestrol

markedly increased growth in soft agar (Siegfried *et al.*, 1984; Rinehart *et al.*, 1996). Thus, diethylstilbestrol caused the transformation of human endometrial stromal cells.

Repeated treatment with low doses of diethylstilbestrol of MCF-10F immortalized, non-tumorigenic, human epithelial breast cells increased colony formation in a soft agar assay at diethylstilbestrol concentrations ranging from 0.007–70 nM (Russo *et al.*, 2001, 2003). Growth of these cells in collagen changed from differentiated ductular growth to solid spherical masses with the same dose–response relationship. Invasive growth in a Boyden chamber assay was increased more than 10-fold at a diethylstilbestrol concentration of 70 nM (Russo *et al.* 2001, 2003). Different effects are seen with high doses of diethylstilbestrol. Estrogen-receptor-positive MCF-7 human breast cancer cells growth in soft agar was inhibited by diethylstilbestrol at concentrations of 2 μM and higher (Brandes & Hermonat, 1983).

Block *et al.* (2000) found effects of exposure to diethylstilbestrol in Ishikawa (endometrial carcinoma) cells, HeLa (cervical carcinoma) cells, and SKOV-3 (ovarian carcinoma) cells on mRNA expression of homeobox (HOX) genes that are involved in the development of the reproductive tract and other tissues.

Tests for in-vitro transformation in rat and Syrian hamster embryo cells gave positive results, while results in mouse cells were negative (IARC, 1987b). No mutations were found in BALB/C 3T3 cells transformed by diethylstilbestrol (Fitzgerald *et al.*, 1989).

Aneuploidy and DNA strand breaks were induced in rodent cells *in vitro* (IARC, 1987b), as confirmed in additional studies (Hayashi *et al.*, 1996; Tsutsui & Barrett, 1997; Tsutsui *et al.*, 1997). Results for chromosomal aberrations, micronuclei, and sister chromatid exchange were equivocal (IARC, 1987b), but in more recent studies, chromosomal aberrations, micronuclei, and sister chromatid exchange, as well as aneuploidy were found in a variety of rodent cell lines (de Stoppelaar *et al.*, 2000; Aardema *et al.*, 2006; Wakata *et al.*, 2006; Tayama *et al.*, 2008).

In a comparison of diethylstilbestrol-induced aneuploidy in human foreskin fibroblasts and Syrian hamster embryo fibroblasts, the hamster cells appeared significantly more sensitive than the human cells (Tsutsui *et al.*, 1990).

The ability of diethylstilbestrol to bind covalently to tubulin in cell-free systems in the presence of an activating system (Sharp & Parry, 1985; Epe *et al.*, 1987), and to inhibit the polymerization of microtubules *in vitro* (Sharp & Parry, 1985; Sato *et al.*, 1987; Albertini *et al.*, 1993; Metzler & Pfeiffer, 1995), in Chinese hamster V79 cells and in Syrian hamster embryo cells (Tucker & Barrett, 1986; Sakakibara *et al.*, 1991; Ochi, 1999) may underlie the induction of aneuploidy. This microtubule-damaging property appears to be unique to diethylstilbestrol because it is not shared with estradiol or 17α-ethinyl estradiol, which are otherwise equally strong estrogens, and can be similarly genotoxic in some systems (Metzler & Pfeiffer, 1995).

Exposure to diethylstilbestrol did not induce mutations or unscheduled DNA synthesis (IARC, 1987b), except in a single study in Syrian hamster embryo cells, and in the presence of liver postmitochondrial supernatant from male rats pretreated with aroclor (Tsutsui *et al.*, 1984). Diethylstilbestrol did not inhibit intercellular communication and most studies did not find positive results for diethylstilbestrol in the mouse lymphoma assay using L5178 $tk^{+/+}$ cells (IARC, 1987b; Sofuni *et al.*, 1996). Exposure of phage and plasmid DNA to diethylstilbestrol quinone resulted in a variety of mutations and, under certain conditions, recombinations in LacZ(α) following transfection into *E. coli* (Korah & Humayun, 1993).

Diethylstilbestrol did not induce mutation in a variety of bacterial and insect systems, but it was mutagenic in plants (IARC, 1987b). In assays with *Saccharomyces cervisiae* and other yeasts,

diethylstilbestrol caused aneuploidy (IARC, 1987b), but it had mixed effects on induction of chromosomal losses (Albertini *et al.*, 1993), and, in most studies, it did not induce mutation, recombinations, or gene conversion (IARC, 1987b; Carls & Schiestl, 1994). DNA damage was not induced in fungi (yeasts) or bacteria, but diethylstilbestrol induced single-strand breaks in bacteriophage DNA in the presence of a horseradish peroxidase activation system (IARC, 1987b).

In vitro, rat liver and mammary gland mitochondria were able to oxidatively metabolize diethylstilbestrol to 4′,4″-diethylstilbestrol quinone and to reduce diethylstilbestrol quinone to diethylstilbestrol (Thomas & Roy, 1995; Thomas *et al.*, 2004). Treatment of Syrian hamsters with diethylstilbestrol resulted in the formation of adducts in kidney mitochondrial DNA by [^{32}P]-postlabelling detection (Thomas & Roy, 2001a), and diethylstilbestrol treatment of rats induced similar adducts in liver mitochondrial DNA at higher levels than in nuclear DNA (Thomas & Roy, 2001b). In addition, both functional ERα and ERβ have been identified in mitochondria (Yager & Chen, 2007). Thus, mitochondria may be a target of diethylstilbestrol, and its mitochondrial effects conceivably play a role in its carcinogenic activity.

4.2.2 Indirect effects related to genotoxicity

(a) Cell proliferation and apoptosis

Diethylstilbestrol increased the mitotic rate in Chinese hamster embryo cells, and in primary male hamster kidney tubular epithelial cells *in vitro* (Stopper *et al.*, 1994; Li *et al.*, 1995; Chen *et al.*, 1996). Chronic diethylstilbestrol treatment increased DNA synthesis in renal tubular cells isolated from male Syrian hamsters (Li *et al.*, 1993); this effect was blocked by co-treatment with a pure anti-estrogen (ICI 182780) (Chen *et al.*, 1996).

In-utero treatment of rats resulted in increased DNA synthesis in both the epithelium and stroma of the proximal portion of the Müllerian duct (which differentiate into the oviduct) on the last day of gestation, but not in the caudal portion (which differentiate into the upper vagina) where epithelial cell proliferation was actually depressed (Okada *et al.*, 2001). Neonatal exposure of mice to diethylstilbestrol resulted in markedly elevated DNA synthesis in epithelial, but not stromal cells of the vagina, whereas it increased the percentage of apoptotic stromal cells, but not epithelial cells at 90 days of age (Sato *et al.*, 2004). Following diethylstilbestrol treatment of pre-pubertal mice, DNA synthesis was markedly increased in the uterine and vaginal epithelium after 16–42 hours (Takahashi *et al.*, 1994). This effect was first apparent at 5 days of age and was still observed at 70 days (Suzuki *et al.*, 2006).

(b) Immune modulatory effects

There are several studies in mice that indicate some immune modulatory effects of diethylstilbestrol treatment. These appear to target the thymus, are highly dose-dependent, and differ in male and female animals (Calemine *et al.*, 2002; Utsuyama *et al.*, 2002; Brown *et al.*, 2006).

(c) Estrogen-receptor-mediated effects

(i) Female animals

Diethylstilbestrol exposure *in utero* reduced the response of the mouse uterus weight and morphology to estrogenic stimulation by diethylstilbestrol on Days 22–25 of life, but not on Day 21 (Maier *et al.*, 1985). Neonatal diethylstilbestrol treatment reduced the responsiveness of uterus weight to ovariectomy, with or without subsequent estrogen stimulation in young adult mice (Medlock *et al.*, 1992), and reduced vaginal weight (Suzuki *et al.*, 1996).

The morphological appearance of the mammary glands of 2- to 11-month-old mice neonatally treated with diethylstilbestrol

(0.1 μg daily for 5 days) was not different from that of untreated controls, but they developed hyperplasia more often in response to stimulation with estradiol. They showed the same response to stimulation with estradiol plus progesterone. The severity of the hyperplasia was increased in diethylstilbestrol-treated mice in response to both hormonal stimuli (Bern *et al.*, 1992).

Overexpression of ERα accelerated the onset of squamous metaplasia, atypical hyperplasia and adenocarcinoma of the uterus induced by neonatal diethylstilbestrol exposure by at least 4 months (Couse *et al.*, 1997). In αERKO mice, no uterine abnormalities, persistent vaginal cornification, or oviduct lesions were found following neonatal diethylstilbestrol treatment, and uterine weight was the same as in vehicle-treated αERKO mice (Couse *et al.*, 2001). This finding strongly suggests that the ERα is the mediator of the effects of neonatal diethylstilbestrol exposure in the female mouse genital tract (Couse & Korach, 2004). ERβ knockout mice (βERKO mice) had a normal morphological response to neonatal diethylstilbestrol treatment (Couse & Korach, 1999), related to the very low to absent expression of ERβ in the female mouse genital tract (Jefferson *et al.*, 2000).

In-utero diethylstilbestrol exposure caused persistent Müllerian duct structures resulting in a range of male and female genital tract abnormalities in mice, which are remarkably similar to those found in diethylstilbestrol-exposed humans (IARC, 1979a). Besides alterations in the uterus, cervix, and vagina, diethylstilbestrol also caused ovarian abnormalities in mice aged 3–14 months, exposed *in utero* (on Days 9–16 of gestation), and markedly increased ex-vivo ovarian production of progesterone, estradiol, and testosterone (Haney *et al.*, 1984).

(ii) Male animals

Neonatal diethylstilbestrol treatment of mice caused persistent decreases in weight of the male accessory sex glands at 12 months of age and the development of inflammation and dysplastic lesions in the posterior periurethral region of the accessory sex gland complex at 2, 12, and 18 months of age (Pylkkänen *et al.*, 1991b, 1993). After 12 and 18 months, there were also morphological changes in the testes (Pylkkänen *et al.*, 1991a, 1993). Treatment of these diethylstilbestrol-exposed mice at 2 months of age with estradiol caused squamous metaplasia in the periurethral prostatic ducts (Pylkkänen *et al.*, 1991b), and adult treatment with estradiol and 5α-dihydrotestosterone (via silastic implants) from 9–12 months of age exacerbated the inflammation and dysplasia at 12 months (Pylkkänen *et al.*, 1993). In contrast, prenatal diethylstilbestrol treatment did not have any lasting effects on the male accessory sex glands, except for occasional dysplasia in the ventral prostate lobe (Pylkkänen *et al.*, 1993). The prostatic weight decrease and lesion development were also found in mice exposed neonatally to diethylstilbestrol (Edery *et al.*, 1990). Neonatal exposure of rats to diethylstilbestrol enhanced the induction of prostatic dysplasia and cancer by subsequent chronic adult treatment with estradiol and testosterone (Yuen *et al.*, 2005). Diethylstilbestrol treatment of rats for 16 weeks with or without concomitant testosterone treatment resulted in increased levels of lipid peroxidation products, and altered antioxidant activity in the ventral and dorsolateral prostate (Tam *et al.*, 2003).

Neonatal diethylstilbestrol treatment of male mice also resulted in a decrease in size of male accessory sex glands, particularly the seminal vesicles. Inflammation and dysplastic lesions developed in the glands of the ventral and dorsolateral prostate between 6–18 months of age and increased in severity with time (Prins *et al.*, 2001). When the same treatment was given to αERKO mice, no morphological effects were found after 6–18 months, whereas the neonatal diethylstilbestrol effects in βERKO mice were indistinguishable from those in wild-type mice (Prins *et al.*, 2001).

(d) *Effects on gene expression (hormonal imprinting)*

(i) *Female animals*

In-utero treatment with diethylstilbestrol caused changes in the expression of several genes, including the estrogen-responsive lactoferrin gene and the developmental *Hox* and *Wnt* genes, in the Müllerian duct/uterus of the developing murine fetus and of mice on the first days of life (Newbold *et al.*, 1997; Ma *et al.*, 1998; Miller *et al.*, 1998; Okada *et al.*, 2001).

The expression of a range of genes in the mouse uterus and/or vagina was permanently altered by neonatal exposure to diethylstilbestrol on the first 4–5 days of life up to postnatal Days 60–90, and included alterations in developmental *Hox* and *Wnt* genes (Miller *et al.*, 1998; Block *et al.*, 2000; Couse *et al.*, 2001; Li *et al.*, 2003a; Miyagawa *et al.*, 2004a, b; Sato *et al.*, 2004; Huang *et al.*, 2005; Newbold *et al.*, 2007; Tang *et al.*, 2008).

A single injection of diethylstilbestrol in prepubertal mice acutely altered the expression of genes coding for 3 TGFß isoforms in the uterus (Takahashi *et al.*, 1994). Treatment of young adult mice also altered the expression of several genes in the vagina and uterus (Klotz *et al.*, 2000; Miyagawa *et al.*, 2004a; Suzuki *et al.*, 2006).

The persistently increased expression of lactoferrin, c-*fos*, and *Nsbp1* in mice that were treated neonatally with diethylstilbestrol was associated with the persistent hypomethylation of CpG sequences in the promoter regions of these genes (Li *et al.*, 1997, 2003a; Tang *et al.*, 2008). Other mechanisms may also be involved in gene expression (Miyagawa *et al.*, 2004a, Tang *et al.*, 2008). The persistently decreased expression of *Hox* genes found in the uterus after 5 days of neonatal treatment with diethylstilbestrol (Couse *et al.*, 2001) was not associated with changes in the methylation status of these genes (Li *et al.*, 2001). The decreased expression of most but not all developmental *Hox* and *Wnt* genes required the presence of ERα, because the expression of these genes is not affected when mice that lacked this estrogen receptor subtype are neonatally exposed to diethylstilbestrol (Couse *et al.*, 2001). The dose of diethylstilbestrol may be a major determinant of the size and direction of the effects on DNA methylation in the mouse uterus (Alworth *et al.*, 2002).

The mRNA expression of nucleosomal binding protein-1 (*Nsbp1*), which plays a role in chromatin remodelling, was permanently increased in mice treated neonatally with diethylstilbestrol for up to 18 months in a dose-related fashion (Tang *et al.*, 2008). A low-dose treatment resulted in a response in the expression and methylation pattern of the uterine *Nsbp*1 gene to the estrogen surge at puberty that was the opposite of that in control mice, but this phenomenon was dose-specific because a high diethylstilbestrol dose did not have this effect (Tang *et al.*, 2008). Ovarian hormones are important in the induction of uterine adenocarcinomas in mice treated neonatally with diethylstilbestrol, because prepubertally ovariectomized mice did not develop these tumours (Newbold *et al.*, 1990).

(ii) *Male animals*

Neonatal administration of diethylstilbestrol of mice caused a persistent upregulation of the c-*fos* and c-*myc* proto-oncogenes in all male accessory sex glands (Pylkkänen *et al.*, 1993; Salo *et al.*, 1997), and a marked increase in the response of c-*fos* expression to estradiol injection at 3–5 months (Salo *et al.*, 1997). In 30-day-old F344 rats treated neonatally with diethylstilbestrol, the expression of both ERα and ERβ was increased as well as circulating prolactin (Khurana *et al.*, 2000). Neonatal treatment of mice caused changes in the expression of several other genes and in DNA methylation patterns (Sato *et al.*, 2006).

Neonatal exposure of mice to diethylstilbestrol resulted in a persistent reduction of androgen-receptor-protein expression in the

ventral and dorsolateral prostate, ERβ expression was persistently decreased, and ERα expression (in stromal cells around prostatic ducts) was upregulated at postnatal Day 10 but not later in life (Prins *et al.*, 2001). This treatment also resulted in a persistent downregulation of a secretory protein, DLP_2, in the dorsolateral prostate. These effects of neonatal treatment with diethylstilbestrol were not seen in αERKO mice, whereas they were identical to those in wild-type mice in βERKO mice (Prins *et al.*, 2001).

4.3 Synthesis

Following exposure *in utero*, the oxidative metabolism of diethylstilbestrol can occur in fetal mouse tissues. There is some evidence that diethylstilbestrol binds covalently to DNA in fetal target tissue (uterus). In animal cells and tissues, diethylstilbestrol binds covalently to DNA and causes oxidative damage to DNA and lipids; some of these tissues are known targets of diethylstilbestrol-induced cancer in animals.

There is some evidence that diethylstilbestrol alters the expression of enzymes involved in diethylstilbestrol metabolism in rat.

Diethylstilbestrol causes aneuploidy in human and animal cells, most likely because of interference with microtubules, which requires oxidative metabolic activation. Diethylstilbestrol also induces chromosomal breaks and other chromosomal aberrations; this is likely to be a major mechanism of diethylstilbestrol-induced carcinogenicity.

Diethylstilbestrol can immortalize primary animal embryo cells *in vitro* and transform human breast cell lines. Diethylstilbestrol also increases the proliferation of human and animal cervical and uterine cells, and increases cell proliferation in diethylstilbestrol target tissues (uterus) in animals following neonatal and prepubertal exposure.

Neonatal exposure to diethylstilbestrol causes persistent changes in gene expression and DNA methylation patterns in diethylstilbestrol target tissues (prostate and uterus), and there is some evidence that hormone responsiveness is permanently altered in the mammary and prostate tissue of exposed mice.

Inflammatory and dysplastic prostate lesions are also observed in mice after neonatal exposure to diethylstilbestrol.

Several of the above effects of diethylstilbestrol, including mitogenic, gene expression, and prostatic effects, are mediated at least in large part by ERα.

There is some evidence of modulatory effects of perinatal exposure to diethylstilbestrol on the immune system in animals and humans.

It is likely that two or more of these factors in combination are responsible for the carcinogenic effects of diethylstilbestrol; estrogen-receptor-mediated effects and genotoxicity conceivably both being involved, while other factors may be contributory. The early developmental changes in the female and male genital tract caused by exposure to diethylstilbestrol *in utero* or – in rodents – neonatally, may result in epigenetic events that create a tissue and cellular environment conducive for the mechanisms responsible for the transplacental carcinogenic effects of diethylstilbestrol in humans and animals.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of diethylstilbestrol. Diethylstilbestrol causes cancer of the breast in women who were exposed while pregnant. Diethylstilbestrol also causes CCA in the vagina and cervix of women who were exposed *in utero*. Also, a positive association has been observed between exposure to diethylstilbestrol and cancer of the endometrium, and between in-utero exposure to diethylstilbestrol and squamous cell carcinoma of the cervix and cancer of the testis.

There is *sufficient evidence* in experimental animals for the carcinogenicity of diethylstilbestrol.

Diethylstilbestrol is *carcinogenic to humans (Group 1)*.

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ESTROGEN-ONLY MENOPAUSAL THERAPY

Estrogen therapy was considered by previous IARC Working Groups in 1987 and 1998 (IARC, 1987, 1999). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agents

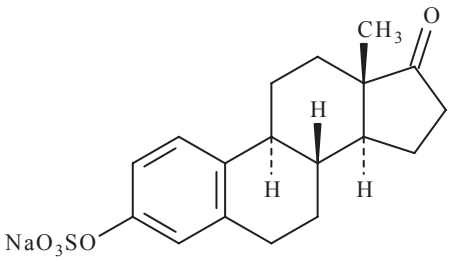
1.1.1 Conjugated estrogens

The term 'conjugated estrogens' refers to mixtures of at least eight compounds, including sodium estrone sulfate and sodium equilin sulfate, derived wholly or in part from equine urine or synthetically from estrone and equilin. Conjugated estrogens contain as concomitant components the sodium sulfate conjugates of 17α-dihydroequilin, 17β-dihydroequilin, and 17α-estradiol (United States Pharmacopeial Convention, 2007).

(a) Sodium estrone sulfate

Chem. Abstr. Serv. Reg. No.: 438-67-5
Chem. Abstr. Name: 3-(Sulfooxy)-estra-1,3,5(10)-trien-17-one, sodium salt
IUPAC Systematic Name: Sodium [(8*R*,9*S*,13*S*,14*S*)-13-methyl-17-oxo-7,8,9,11,12,14,15,16-octahydro-6*H*-cyclopenta[*a*]phenanthren-3-yl] sulfate
Synonyms: Estrone sodium sulfate; estrone sulfate sodium; estrone sulfate sodium salt; oestrone sodium sulfate; oestrone sulfate sodium; oestrone sulfate sodium salt; sodium estrone sulfate; sodium estrone-3-sulfate; sodium oestrone-3-sulfate

(i) Structural and molecular formulae, and molecular mass



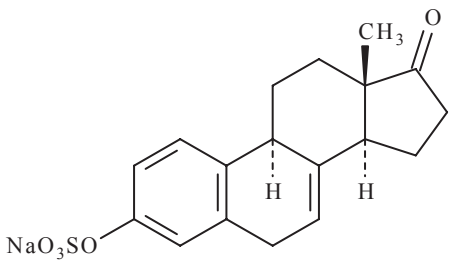
$C_{18}H_{21}O_5S.Na$
Relative molecular mass: 372.4

(b) Sodium equilin sulfate

Chem. Abstr. Serv. Reg. No.: 16680-47-0
Chem. Abstr. Name: 3-(Sulfooxy)-estra-1,3,5(10),7-tetraen-17-one, sodium salt
IUPAC Systematic Name: Sodium (13-methyl-17-oxo-9,11,12,14,15,16-hexahydro-6*H*-cyclopenta[*a*]phenanthren-3-yl) sulfate
Synonyms: Equilin, sulfate, sodium salt; equilin sodium sulfate; sodium equilin 3-monosulfate
Description: buff-coloured amorphous powder, odourless or with a slight characteristic odour [when obtained from natural sources]; white to light-buff-coloured crystalline or amorphous powder, odourless or with a slight odour [synthetic form] (Sweetman,

2008)

(i) Structural and molecular formulae, and molecular mass

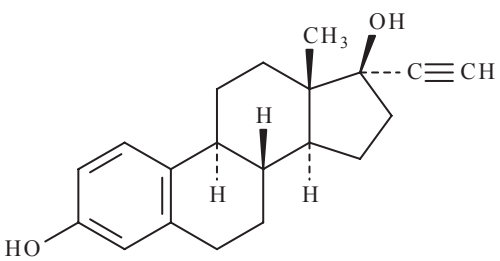


$C_{18}H_{19}O_5S.Na$
Relative molecular mass: 370.4

1.1.2 Ethinylestradiol

Chem. Abstr. Serv. Reg. No.: 57-63-6
Chem. Abstr. Name: (17α)-19-Norpregna-1,3,5(10)-trien-20-yne-3,17-diol
IUPAC Systematic Name: (8*R*,9*S*,13*S*,14*S*,17*R*)-17-Ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6*H*-cyclopenta[*a*]phenanthrene-3,17-diol
Synonyms: 17-Ethinyl-3,17-estradiol; 17-ethinylestradiol; 17α-ethinyl-17β-estradiol; ethinylestradiol; 17α-ethinylestradiol
Description: White to creamy- or slightly yellowish-white, odourless, crystalline powder (Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass

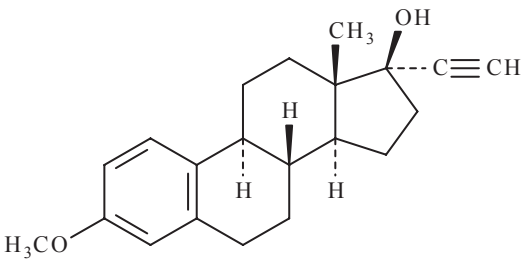


$C_{20}H_{24}O_2$
Relative molecular mass: 296.4

1.1.3 Mestranol

Chem. Abstr. Serv. Reg. No.: 72-33-3
Chem. Abstr. Name: (17α)-3-Methoxy-19-norpregna-1,3,5(10)-trien-20-yn-17-ol
IUPAC Systematic Name: (8*R*,9*S*,13*S*,14*S*,17*R*)-17-Ethynyl-3-methoxy-13-methyl-7,8,9,11,12,14,15,16-octahydro-6*H*-cyclopenta[*a*]phenanthren-17-ol
Synonyms: Ethinylestradiol 3-methyl ether; 17α-ethinylestradiol 3-methyl ether; ethinyloestradiol 3-methyl ether; 17α-ethinyloestradiol 3-methyl ether; ethynylestradiol methyl ether; ethynylestradiol 3-methyl ether; 17-ethynylestradiol 3-methyl ether; 17α-ethynylestradiol 3-methyl ether; 17α-ethynylestradiol methyl ether; ethynyloestradiol methyl ether; ethynyloestradiol 3-methyl ether; 17-ethynyloestradiol 3-methyl ether; 17α-ethynyloestradiol 3-methyl ether; 17α-ethynyloestradiol methyl ether; 3-methoxy-17α-ethinylestradiol; 3-methoxy-17α-ethinyloestradiol; 3-methoxy-17α-ethynylestradiol; 3-methoxyethynylestradiol; 3-methoxy-17α-ethynyloestradiol; 3-methoxyethynyloestradiol; 3-methylethynylestradiol; 3-*O*-methylethynylestradiol; 3-methylethynyloestradiol; 3-*O*-methylethynyloestradiol; Δ-MVE
Description: White or almost white to creamy-white, odourless, crystalline powder (Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass

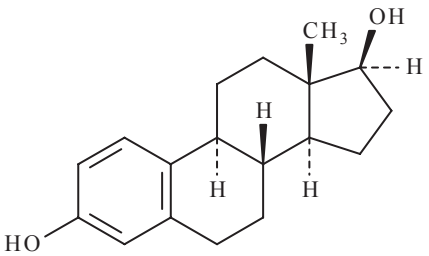


$C_{21}H_{26}O_2$
Relative molecular mass: 310.4

1.1.4 Estradiol

Chem. Abstr. Serv. Reg. No.: 50-28-2
Chem. Abstr. Name: (17*β*)-Estra-1,3,5(10)-triene-3,17-diol
IUPAC Systematic Name: (8*R*,9*S*,13*S*,14*S*,17*S*)-13-Methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthrene-3,17-diol
Synonyms: Dihydrofollicular hormone; dihydrofolliculin; dihydromenformon; dihydrotheelin; dihydroxyestrin; 3,17*β*-dihydroxyestra-1,3,5(10)-triene; 3,17-epidihydroxyestratriene; *β*-estradiol; 17*β*-estradiol; 3,17*β*-estradiol; (*d*)-3,17*β*-estradiol; oestradiol-17*β*; 17*β*-oestradiol
Description: White or creamy-white, odourless, crystalline powder (Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass

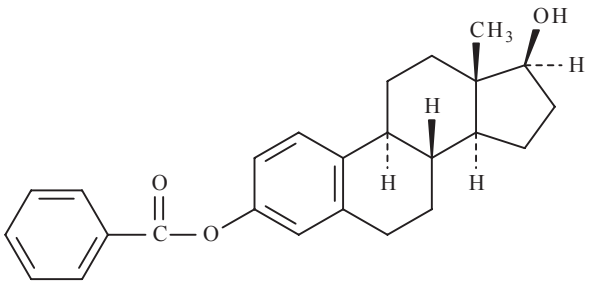


$C_{18}H_{24}O_2$
Relative molecular mass: 272.4

1.1.5 Estradiol benzoate

Chem. Abstr. Serv. Reg. No.: 50-50-0
Chem. Abstr. Name: Estra-1,3,5(10)-triene-3,17*β*-diol, 3-benzoate
IUPAC Systematic Name: [(8*R*,9*S*,13*S*,14*S*,17*S*)-17-Hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthren-3-yl] benzoate
Synonyms: Estradiol benzoate; *β*-estradiol benzoate; *β*-estradiol 3-benzoate; 17*β*-estradiol benzoate; 17*β*-estradiol 3-benzoate; estradiol monobenzoate; 1,3,5(10)-estratriene-3,17*β*-diol 3-benzoate; *β*-oestradiol benzoate; *β*-oestradiol 3 benzoate; 17*β*-oestradiol benzoate; 17*β*-oestradiol 3-benzoate; oestradiol monobenzoate; 1,3,5(10)-oestratriene-3,17*β*-diol 3-benzoate
Description: Almost white crystalline powder or colourless crystal (Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass



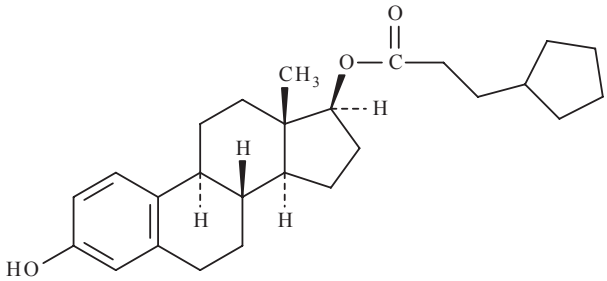
$C_{25}H_{28}O_3$
Relative molecular mass: 376.5

1.1.6 Estradiol cypionate

Chem. Abstr. Serv. Reg. No.: 313-06-4
Chem. Abstr. Name: (17*β*)-Estra-1,3,5(10)-triene-3,17-diol, 17-cyclopentanepropanoate
IUPAC Systematic Name: [(8*R*,9*S*,13*S*,14*S*,17*S*)-3-Hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthren-17-yl] 3- cyclopentylpropanoate
Synonyms: Cyclopentanepropionic acid, 17-ester with oestradiol; cyclopentanepropionic acid, 3-hydroxyestra-1,3,5(10)-trien-17*β*-yl ester; depo-estradiol cyclopentylpropionate; depoestradiol cypionate; estradiol 17*β*-cyclopentanepropionate; estradiol cyclopentylpropionate; estradiol 17-cyclopentylpropionate; estradiol

17β-cyclopentylpropionate; 17β-estradiol 17-cyclopentyl-propionate; estradiol cypionate; estradiol 17-cypionate; estradiol 17β-cypionate
Description: White to practically white crystalline powder, odourless or with a slight odour (Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass

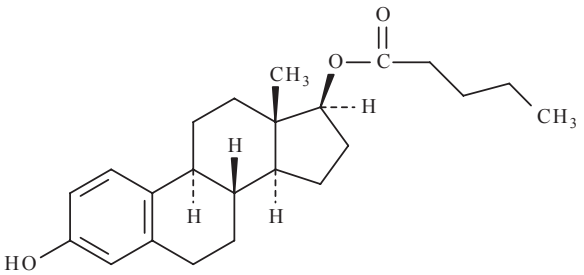


$C_{26}H_{36}O_3$
Relative molecular mass: 396.6

1.1.7 Estradiol valerate

Chem. Abstr. Serv. Reg. No.: 979-32-8
Chem. Abstr. Name: (17β)-Estra-1,3,5(10)-triene-3,17-diol, 17-pentanoate
IUPAC Systematic Name: [(8*R*,9*S*,13*S*,14*S*,17*S*)-3-Hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthren-17-yl] pentanoate
Synonyms: Oestradiol valerate; estradiol 17β-valerate; estradiol valerianate; estra-1,3,5(10)-triene-3,17β-diol 17-valerate; 3-hydroxy-17β-valeroyloxyestra-1,3,5(10)-triene
Description: White or almost white crystalline powder or colourless crystal, odourless or with a faint fatty odour (Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass

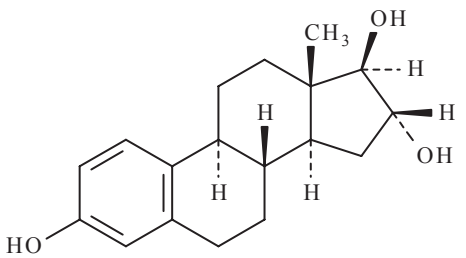


$C_{23}H_{32}O_3$
Relative molecular mass: 356.5

1.1.8 Estriol

Chem. Abstr. Serv. Reg. No.: 50-27-1
Chem. Abstr. Name: (16α,17β)-Estra-1,3,5(10)-triene-3,16,17-triol
IUPAC Systematic Name: (8*R*,9*S*,13*S*,14*S*,16*R*,17*R*)-13-Methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthrene-3,16,17-triol
Synonyms: Estra-1,3,5(10)-triene-3,16α,17β-triol; estratriol; 16α-estriol; 16α,17β-estriol; 3,16α,17β-estriol; follicular hormone hydrate; 16α-hydroxyestradiol; 3,16α,17β-trihydroxyestra-1,3,5(10)-triene; trihydroxyestrin
Description: White or practically white, odourless, crystalline powder (Sweetman, 2008)

(a) Structural and molecular formulae and relative molecular mass

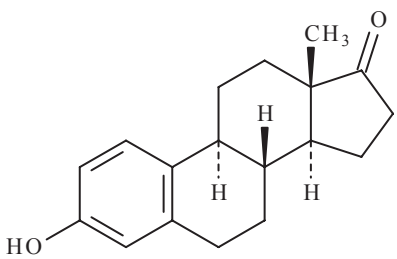


$C_{18}H_{24}O_3$
Relative molecular mass: 288.4

1.1.9 Estrone

Chem. Abstr. Serv. Reg. No.: 53-16-7
Chem. Abstr. Name: 3-Hydroxyestra-1,3,5(10)-trien-17-one
IUPAC Systematic Name: (8*R*,9*S*,13*S*,14*S*)-3-Hydroxy-13-methyl-7,8,9,11,12,14,15,16-octahydro-6*H*- cyclopenta[*a*]phenanthren-17-one
Synonyms: *d*-Estrone; *d*-oestrone
Description: Odourless, small white crystals or white to creamy-white crystalline powder (Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass

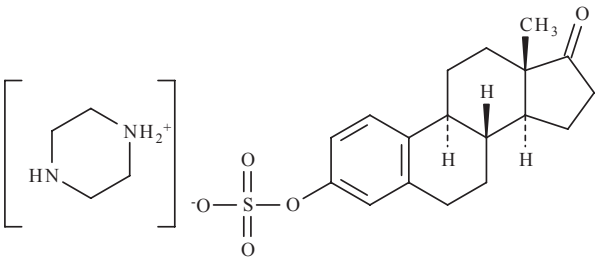


$C_{18}H_{22}O_2$
Relative molecular mass: 270.4

1.1.10 Estropipate

Chem. Abstr. Serv. Reg. No.: 7280-37-7
Chem. Abstr. Name: 3-(Sulfooxy)-estra-1,3,5(10)-trien-17-one, compd. with piperazine (1:1)
IUPAC Systematic Name: [(8*R*,9*S*,13*S*,14*S*)-13-Methyl-17-oxo-7,8,9,11,12,14,15,16-octahydro-6*H*-cyclopenta[*a*]phenanthren-3-yl] hydrogen sulfate: piperazine (1:1)
Synonyms: Piperazine estrone sulfate; piperazine oestrone sulfate; 3-sulfatoxyestra-1,3,5(10)-trien-17-one piperazine salt; 3-sulfatoxyoestra-1,3,5(10)-trien-17-one piperazine salt
Description: White or almost white to yellowish-white, fine crystalline powder, odourless or with a slight odour (Sweetman, 2008)

(a) Structural and molecular formulae, and relative molecular mass



$C_{22}H_{32}N_2O_5S$
Relative molecular mass: 436.6

1.2 Use of the agents

Information for Section 1.2 is taken from IARC (1999) and McEvoy (2007).

1.2.1 Indications

Menopausal estrogen therapy refers to the use of estrogen without a progestogen for women in the period around the menopause, primarily for the treatment of menopausal symptoms but also for the prevention of conditions that become more common in the postmenopausal period, such as osteoporosis and ischaemic heart disease. It is mainly given to women who have had a hysterectomy.

Conjugated estrogens, estradiol and its semi-synthetic esters (especially estradiol valerate), are the main estrogens used in the treatment of menopausal disorders. Their use has also been proposed in the prevention of cardiovascular diseases. Conjugated estrogens have been used extensively in the United Kingdom, Australia, Canada, and the United States of America for the treatment of climacteric [menopausal] symptoms. In Europe, micronised estradiol and estradiol valerate are used. Mestranol, estriol, and estropipate have also been used.

Estrogens may be used adjunctively with other therapeutic measures (e.g. diet, calcium, vitamin D, weight-bearing exercise, physical therapy) to

retard bone loss and the progression of osteoporosis in postmenopausal women, either orally (e.g. estradiol, estropipate, conjugated estrogens) or transdermally (e.g. estradiol).

Estrogens are also used in the treatment of a variety of other conditions associated with a deficiency of estrogenic hormones, including female hypogonadism, castration, and primary ovarian failure. In addition, estrogens may be used in the treatment of abnormal uterine bleeding caused by hormonal imbalance not associated with an organic pathology.

Oral conjugated estrogens and 'synthetic conjugated estrogens A' ['synthetic conjugated estrogens A' are a mixture of nine derivatives of estrone, equilin, estradiol, and equilenin] are used for the management of moderate-to-severe vasomotor symptoms associated with menopause, and for the management of vulvar and vaginal atrophy (atrophic vaginitis); for the latter, topical vaginal preparations are used.

'Synthetic conjugated estrogens B' [a mixture of ten derivatives of estrone, equilin, estradiol, and equilenin] are used for the management of moderate-to-severe vasomotor symptoms associated with menopause. Oral conjugated estrogens are also used for the management of female hypoestrogenism secondary to hypogonadism, castration, or primary ovarian failure.

Estradiol is the most active of the naturally occurring estrogens. Estradiol and its semisynthetic esters are used primarily as menopausal therapy. Estradiol may also be used for female hypogonadism or primary ovarian failure.

Although ethinylestradiol is used most extensively in oral contraceptives in combination with a progestogen, other indications include perimenopausal symptoms, hormonal therapy for hypogonadal women, treatment of postpartum breast engorgement, dysfunctional uterine bleeding, and therapy for carcinoma of the breast and prostate.

1.2.2 Dosages and preparations

Menopausal estrogen therapy is administered in a continuous daily dosage regimen or, alternatively, in a cyclic regimen. When estrogens are administered cyclically, the drugs are usually given once daily for 3 weeks followed by a 1 week washout period, or once daily for 25 days followed by 5 days washout, repeated as necessary.

Menopausal estrogen therapy is available as oral tablets, intranasal sprays, subcutaneous implants, topical applications for vulvovaginal use, intravaginal rings, and transdermal skin patches and gels.

(a) Conjugated estrogens

For the treatment of climacteric symptoms, conjugated estrogens are usually administered orally in a dose of 0.3–1.25 mg daily. Conjugated estrogens may also be administered intravaginally or by deep intramuscular or slow intravenous injection. When parenteral administration of conjugated estrogens is required, slow intravenous injection is preferred because of the more rapid response obtained following this route of administration compared to intramuscular injection. Topical vaginal therapy may be used specifically for menopausal atrophic vaginitis: 0.5–2 g of a 0.0625% cream may be used daily for 3 weeks of a 4-week cycle.

For the management of moderate-to-severe vasomotor symptoms associated with menopause, the usual oral dosage of 'synthetic conjugated estrogens A' is 0.45–1.25 mg daily, usually starting with 0.45 mg daily, and with any subsequent dosage adjustments dependent on the patient's response. For the management of vulvar and vaginal atrophy, the usual oral dosage of 'synthetic conjugated estrogens A' is 0.3 mg daily. The usual initial oral dosage of 'synthetic conjugated estrogens B' for the management of moderate-to-severe vasomotor symptoms associated with menopause is 0.3 mg daily, with any

subsequent dosage adjustment dependent on the patient's response.

For the prevention of osteoporosis, the usual initial oral dosage of conjugated estrogens is 0.3 mg once daily. Subsequent dosage should be adjusted based on the patient's clinical and bone mineral density responses. The drug may be administered in a continuous daily regimen or in a cyclic regimen (25 days on drug, followed by a 5-day washout period, repeated as necessary).

For therapy in female hypoestrogenism, the usual oral dosage of conjugated estrogens is 0.3–0.625 mg daily in a cyclic regimen (3 weeks on drug, followed by a 1-week washout period). For the management of female castration or primary ovarian failure, the usual initial oral dosage of conjugated estrogens is 1.25 mg daily in a cyclic regimen.

For the palliative treatment of prostatic carcinoma, an oral dose of 1.25–2.5 mg conjugated estrogens three times daily has been used. A dose of 10 mg three times daily for at least 3 months has been used for the palliative treatment of breast carcinoma in men and in postmenopausal women. Abnormal uterine bleeding has been treated acutely by giving 25 mg of conjugated estrogens by slow intravenous injection, repeated after 6–12 hours if required; the intramuscular route has also been used.

(b) Single estrogens

Ethinylestradiol has been used for menopausal therapy at doses of 10–20 µg daily.

Mestranol is rapidly metabolized to ethinylestradiol, therefore, acts in a similar fashion to that of estradiol. It has been used as the estrogen component of some preparations for menopausal therapy. It was usually given in a sequential regimen with doses ranging from 12.5–50 µg daily.

Estradiol may be used topically as transdermal skin patches that release between 14–100 µg of estradiol every 24 hours to provide a systemic effect. A low-dose patch supplying 14 µg daily is also available. Topical gel preparations can be applied to also provide a systemic effect; the usual dose is 0.5–1.5 mg of estradiol daily. A topical emulsion of estradiol is also available as the hemihydrate with a daily dose of 8.7 mg. This is also available as a nasal spray, delivering 150 µg of estradiol hemihydrate per spray. The usual initial dose is 150 µg daily. After two or three cycles the dose may be adjusted according to the response; the usual maintenance dose is 300 µg daily but may range from 150 µg once daily up to 450–600 µg daily in two divided doses.

Subcutaneous implants of estradiol may also be used in doses of 25–100 mg with a new implant being given after about 4–8 months, depending on whether therapeutic concentrations of estrogens are detected in the plasma.

Estradiol may be used locally either as 25 µg vaginal tablets, at an initial dose of one tablet daily for 2 weeks, followed by a maintenance dose of one tablet twice a week, or as a 0.01% vaginal cream in initial amounts of 2–4 g of cream daily for 1–2 weeks followed by half the initial dose for a similar period, then a maintenance dose of 1 g up to three times weekly. Also available for the relief of both local and systemic postmenopausal symptoms are local delivery systems using 3-month vaginal rings containing 2 mg of estradiol hemihydrate that release about 7.5 µg of estradiol daily or estradiol acetate that release either 50 or 100 µg of estradiol daily.

Intramuscular injections of estradiol benzoate or valerate esters have been used as oily depot solutions, given once every 3–4 weeks. The cypionate, dipropionate, enantate, hexahydrobenzoate, phenylpropionate, and undecylate esters have been used similarly. The enantate and cypionate esters are used as the estrogen component of combined injectable contraceptives (estradiol and other estrogens have sometimes been used at higher doses for the palliative treatment of prostate cancer and breast cancer in men, and breast cancer in postmenopausal women.)

Estriol is a naturally occurring estrogen with actions and uses that are similar to those described for estradiol valerate. For short-term treatment, oral doses of estriol are 0.5–3 mg daily given for 1 month, followed by 0.5–1 mg daily. Estriol has also been given with other natural estrogens, such as estradiol and estrone, with usual doses of estriol ranging from about 0.25–2 mg daily. Estriol is given intravaginally for the short-term treatment of menopausal atrophic vaginitis as a 0.01% or 0.1% cream or as pessaries containing 500 µg. It has also been given orally for infertility in doses of 0.25–1 mg daily on Days 6 to 15 of the menstrual cycle. Estriol succinate has also been given orally for menopausal disorders. The sodium succinate salt has been used parenterally in the treatment of haemorrhage and thrombocytopenia.

Estrone has been given in oral doses of 1.4–2.8 mg daily in a cyclic or continuous regimen for menopausal symptoms, and as a combination product with estradiol and estriol. Estrone has also been given by intramuscular injection in formulations with oily solutions or aqueous suspensions. When used for menopausal atrophic vaginitis, estrone has been given vaginally.

Estropipate is used for the short-term treatment of menopausal symptoms; suggested doses range from 0.75–3 mg daily, given cyclically or continuously orally; doses of up to 6 mg daily have also been given cyclically. When used long-term for the prevention of postmenopausal osteoporosis, a daily dose of 0.75–1.5 mg is given cyclically or continuously. Estropipate has also been used short-term for menopausal atrophic vaginitis as a 0.15% vaginal cream; 2–4 g of cream is applied daily. It can be given orally in the treatment of female hypogonadism, castration, and primary ovarian failure in doses of 1.5–3 mg daily, in a cyclic regimen; higher doses of up to 9 mg daily given cyclically have also been used.

1.2.3 Formulations

Conjugated estrogens are available as 0.3–1.25 mg tablets for oral administration, as a 25 mg solution for parenteral administration, and as a 0.0625% cream for topical administration. Synthetic conjugated estrogens A and B are available as 0.3–1.25 mg film-coated tablets for oral administration. Estradiol (alone) is available as a 0.06% gel and as a transdermal patch with doses ranging from 14–100 µg/24 hours for topical administration, as a 0.01% cream, and as a 2 mg/ring for vaginal administration. Estradiol (hemihydrate) is available as a 0.25% emulsion for topical administration and as 25 µg (of estradiol) film-coated tablets for vaginal administration. Estradiol (micronized) is available as 0.5–2 mg tablets for oral administration. Estradiol acetate is available as 0.45–1.8 mg tablets for oral administration, and as a 12.4–24.8 mg/ring (0.05–0.1 mg estradiol/24 hours) for vaginal administration. Estradiol cypionate is available as a 5 mg/mL injection solution in oil for parenteral administration. Estradiol valerate is available as a 10–40 mg/mL injection solution in oil for parenteral administration. Estropipate is available as 0.75–3 mg tablets for oral administration.

1.2.4 Trends in use

Early treatment regimens of menopausal symptoms included estrogen-only therapy. After a substantial increase in use in the 1960s and early 1970s, the use of these regimens declined after 1975 when a strong association with the development of endometrial cancer was found. Estrogen-only menopausal treatment is still prescribed for hysterectomized women.

2. Cancer in Humans

Studies were included in this review if they provided information regarding estrogen use (unopposed/alone).

2.1 Cancer of the breast

The previous *IARC Monograph* (IARC, 1999) on postmenopausal estrogen therapy and breast cancer considered the pooled analysis of original data from 51 studies, and also reviewed data from 15 cohort and 23 case–control studies. The majority of studies showed a small increased risk in current users who had been using estrogen for at least 5 years. Several of the studies reviewed included any hormone therapy and were not restricted to estrogen alone. Data were insufficient to determine whether the risk varied with type or dose of estrogen.

The present review of postmenopausal estrogen therapy taken without a progestogen (unopposed estrogen) and breast cancer includes papers published from 1996 to August 2008. It includes one systematic review, over 20 cohort and case–control studies combined, and one randomized trial. Studies were included if they reported estimated relative risks (RRs), hazard ratios (HRs) or odds ratios (ORs) and 95% confidence intervals (CI), and if they compared women who used unopposed estrogen for at least 1 year with women who used no estrogen.

2.1.1 Systematic review

Greiser *et al.* (2005) conducted a meta-analysis of unopposed estrogen therapy and breast cancer in postmenopausal women, which included 12 case–control studies, five cohort studies, and three clinical trials published in 1989–2004.

The summary relative risk for studies published before 1992 showed no increased breast cancer risk for case–control studies (OR, 1.02; 95%CI: 0.93–1.11). The summary estimate for case–control studies published later (OR, 1.18; 95%CI: 1.08–1.30) was similar to that of cohort studies published earlier (OR, 1.19; 95%CI: 1.10–1.28). The summary risks from cohort studies published in 1992 or later were largely driven by the Million Women Study results (OR, 1.30), which showed increased risks only in current hormone users.

2.1.2 Cohort studies

Of the ten cohort studies reported since 1999, four found increased risk of breast cancer from ever use of estrogen alone (Colditz & Rosner, 2000; Beral *et al.*, 2003; Bakken *et al.*, 2004; Ewertz *et al.*, 2005). The largest published cohort study is the United Kingdom Million Women Study (Beral *et al.*, 2003) that included 1084110 women aged 50–64, which found increased risk of breast cancer by duration of use, and type of preparation used (see Table 2.1 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-12-Table2.1.pdf). The increased risk was already evident for women with less than 5 years' estrogen therapy (OR, 1.21; 95%CI: 1.07–1.37). Several other cohort studies found increased risks of breast cancer with longer durations of use of estrogen-alone postmenopausal therapy: Schairer *et al.* (2000) from 1–2 years of use but not longer, Olsson *et al.* (2003) from 48 months or more of use of estriol, Lee *et al.* (2006) from 5 or more years of current use (with a dose–response relationship), and Rosenberg *et al.* (2008) from 10 or more years of use in those with a body mass index (BMI) of less than 25. In two of the cohort studies, there was no evidence of an increased risk of breast cancer from estrogen-alone postmenopausal therapy (Porch *et al.*, 2002; Fournier *et al.*, 2005).

2.1.3 Case–control studies

Of the 16 case–control studies, two studies found increased risk of breast cancer for ever use of estrogen-alone postmenopausal therapy (Newcomb *et al.*, 2002; Rosenberg *et al.*, 2008). In the study of Newcomb *et al.* (2002), the risk increase was restricted to those with 5 or more years of estrogen-alone menopausal therapy, and in current users with use within the previous 5 years. Some studies found increased risks only in subgroups: one for those diagnosed with invasive breast cancer (Henrich *et al.*, 1998), one for users of estrogen-alone postmenopausal therapy for 60 months or more (principally in those diagnosed with a lobular breast cancer) (Chen *et al.*, 2002), one in those who weighed 61.3 kg or more (Wu *et al.*, 2007), one in those with an in-situ breast cancer after 5 or more years of use of estrogen-alone postmenopausal therapy (Ross *et al.*, 2000), and one in those diagnosed with comedo carcinomas (Li *et al.*, 2006). Increased risks in those diagnosed with lobular cancer of the breast was not confirmed in the studies of Daling *et al.* (2002), Li *et al.* (2002), nor in that of Li *et al.* (2003). Kirsh & Kreiger (2002) found borderline increased risks for those with a duration of use of estrogen alone of 10 or more years, while Weiss *et al.* (2002) and Sprague *et al.* (2008) found no increases in risk.

See Table 2.2 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-12-Table2.2.pdf.

2.1.4 Clinical trials

The Women's Health Initiative estrogen only trial (WHI-ET) is the only large clinical trial of unopposed estrogen use (Anderson *et al.*, 2004). Women were required to have an annual mammogram to receive study medication (see Table 2.3 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-12-Table2.3.pdf). At baseline, almost half of the subjects reported prior postmenopausal hormone therapy before randomization. The trial was closed after an average 6.8 years follow-up, at which point 218 incident cases of invasive breast cancer were identified. There was no evidence that oral conjugated equine estrogen (0.625 mg daily) increased the risk of breast cancer. [The Working Group noted that the number of women who continued taking their assigned medication was low, which could have weakened any effects of estrogen.]

2.2 Cancer of the endometrium

The previous *IARC Monograph* summarized data from hree cohort studies and over 30 case–control studies. These consistently showed an increased risk of endometrial cancer in women who received menopausal estrogen therapy. Risk increased with duration of use, and decreased with time since last use, but the risk remained elevated for at least 10 years after cessation of treatment.

2.2.1 Cohort studies

Results from four cohort studies reported since the previous *IARC Monograph* are summarized in Table 2.4 (available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-12-Table2.4.pdf), and each found an increased risk of endometrial cancer from the use of unopposed estrogen therapy. In the Million Women Study (Beral *et al.*, 2005), the risk was somewhat lower in women with a BMI of < 25 kg/m^2 than in women with a BMI of 25 kg/m^2 or more. In two of the cohort studies (Lacey *et al.*, 2005, 2007), the risk of endometrial cancer increased with duration of use, and decreased with time since last use. The risk remained elevated over that of non-users after 5 or more years since cessation of use in one study (Lacey *et al.*, 2007), and 10 or more in the other (Lacey *et al.*, 2005).

2.2.2 Case–control studies

Results from five case–control studies reported since the previous *IARC Monograph* are summarized in Table 2.5 (available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-12-Table2.5.pdf), and each found an increased risk of endometrial cancer in users of unopposed estrogen of 6 months or more, with risks increasing with duration of use. In one study (Shields *et al.*, 1999) that reported additional analyses of data from a prior study, the trend in risk was observed in women with and without other risk factors for endometrial cancer (low parity, hypertension, and BMI), and in women with and without protective factors (oral contraceptive use and history of smoking). A total of 85% of the estrogen users reported using conjugated estrogens. In a population-based study in Sweden (Weiderpass *et al.*, 1999), the primary estrogen in use was estradiol. This study also included women with atypical endometrial hyperplasia. Risk of this condition was also increased in estrogen users, and the risk increased with duration of use. In a third study (Beard *et al.*, 2000), the risk of endometrial cancer was increased for users of both conjugated estrogens and non-conjugated steroidal estrogens. [The Working Group noted that steroidal estrogens were not further defined in the published report.] In one study (Weiss *et al.*, 2006), the aggressiveness of endometrial cancers that had been the subject of a series of population-based cased–control studies was categorized, based on both tumour grade and extent of disease. The risk of tumours of all three levels of aggressiveness increased with duration of use of estrogen for menopausal therapy. The increase in risk was greatest for the cancers with low tumour aggressiveness.

2.3 Cancer of the colorectum

In the previous *IARC Monograph* (IARC, 1999), 12 case–control and seven cohort studies provided information on the use of estrogen therapy and the risk of colorectal cancer. The risk was not increased and appeared to be reduced in half of the studies, though the reduced risk was observed among recent users, and was not related to duration of use.

2.3.1 Case–control studies

Results from the two case–control studies reported since the previous *IARC Monograph* are summarized in Table 2.6 (available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-12-Table2.6.pdf). In one study, no association with colon cancer was found following recent use of estrogen therapy for a 5- or 10-year period (Jacobs *et al.*, 1999). In the second study, the odds ratio of colon cancer for women who had estrogen therapy alone was 0.5 (95%CI: 0.2–0.9) both for those who had used estrogen within the last year, and for those who had used estrogen for 5 or more years (Prihartono *et al.*, 2000).

2.4 Cancer of the ovary

In the previous *IARC Monograph* (IARC, 1999), 12 case–control and four cohort studies that addressed the risk for ovarian cancer were considered. No clear association between estrogen therapy and the risk of ovarian cancer was found. Since then, there have been seven case–control and seven cohort studies investigating estrogen-alone exposure and the risk of ovarian cancer (see Table 2.7 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-12-Table2.7.pdf and Table 2.8 at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-12-Table2.8.pdf).

In five of the case–control studies, an increased risk of ovarian cancer was found with estrogen alone, though in one, this was only significant for 5 or more years of use (Rossing *et al.*, 2007), and in two after 10 or more years of use of estrogen for all types of ovarian cancer combined (Riman *et al.*, 2002; Moorman *et al.*, 2005). Of the remaining two case–control studies, one study (Pike *et al.*, 2004) found a relative risk for 5 years of use of 1.16 (95%CI: 0.92–1.48), and the other did not find an overall elevation in risk for unopposed estrogen use for 5 or more years (Sit *et al.*, 2002). In the three case–control studies that evaluated risks for different histological types of ovarian cancer, the findings were not consistent. Thus, there was a suggestion that risk was elevated for the endometroid type of ovarian cancer and for all types of ovarian cancer in women who had not had a hysterectomy or tubal ligation in one study (Purdie *et al.*, 1999); in the study in Sweden, risk was increased for serous type borderline ovarian cancer (Riman *et al.*, 2001) and for invasive mucinous epithelial ovarian cancer (Riman *et al.*, 2002), while in the study in the USA, risk was increased only for serous epithelial cancer (Moorman *et al.*, 2005), but the numbers were often very small for the different types. In one of the case–control studies (Rossing *et al.*, 2007), the risk among those women who had ceased estrogen use 3 of more years ago was also evaluated. No increases in risk of ovarian cancer were found, even among those who had taken estrogen for 5 or more years (see Table 2.7 online).

In one of the cohort studies, there were only two cases of ovarian cancer, and the relative risk was not elevated (Bakken *et al.*, 2004). In the remaining six cohort studies, the effect of duration of exposure was evaluated (see Table 2.8 online). In one study (Rodriguez *et al.*, 2001), an elevated risk of death from ovarian cancer was seen after 10 years of use of estrogen, in another for incident cases after 10 years of use (Lacey *et al.*, 2006), in the other four (of incident cases), after 5 years of use (Lacey *et al.*, 2002; Folsom *et al.*, 2004; Beral *et al.*, 2007; Danforth *et al.*, 2007). In the study of Lacey *et al.* (2002), risk was documented as continuing to increase with increasing duration of use, the maximum after 20 or more years (RR, 3.4; 95%CI: 1.6–7.5, based on 14 cases). In another (Rodriguez *et al.*, 2001), risk of death from ovarian cancer did not seem to further increase after 5 years of use of estrogen therapy. In the one cohort study that reported risk by histological type, risk was significantly increased for the serous type and the mixed/other/not-otherwise-specified grouping (Beral *et al.*, 2007).

In a meta-analysis that included data from 13 case–control studies, three cohort studies and the WHI-ET trial, the relative risks for ever use were 1.28 (95%CI: 1.18–1.40), and per year of estrogen therapy, 1.07 (95%CI: 1.06–1.08) (Greiser *et al.*, 2007). In another meta-analysis of 13 population-based case–control and cohort studies, but not the WHI-ET trial data, [The Working Group noted that these studies include three recent studies not included in the Greiser *et al.* (2007) meta-analysis] the relative risk per 5 years of use of estrogen use was 1.22 (95%CI: 1.18–1.27) (Pearce *et al.*, 2009). In an additional meta-analysis, reviewing essentially the same data, Zhou *et al.* (2008) computed odds ratios from the case–control studies of 1.19 (95%CI: 1.01–1.40), and 1.51 (95%CI: 1.21–1.88) from the cohort studies for ovarian cancer from ever use of estrogen therapy alone.

2.5 Cancer of the urinary bladder

Since the previous *IARC Monograph* (IARC, 1999), three cohort studies from the USA provide data on cancer of the urinary bladder in users of estrogen therapy (Cantwell. *et al.*, 2006; McGrath *et al.*, 2006; Prizment *et al.* 2007; see Table 2.9 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-12-Table2.9.pdf). No associations were found between the use of estrogen therapy and the risk of bladder cancer.

Risk by trend of duration of use was only reported in one study, and there was no trend in risk with increasing duration of use.

2.6 Cancer of the pancreas

A cohort of 387981 postmenopausal women in the USA, the Cancer Prevention Study (CPS)-II (Teras *et al.*, 2005), found no significant positive trends in pancreatic cancer mortality rates with years of estrogen therapy use, both for current and former users.

2.7 Exogenous estrogen use and melanoma risk

A review of the published literature, including a pooled analysis, concluded that neither oral contraceptives nor hormone therapy are associated with melanoma risk (Lens & Bataille, 2008). Two previous pooled analyses, based on 18 and 10 case–control studies, showed summary odds ratios of 2.0 (95%CI: 1.2–3.4) and 0.86 (95%CI: 0.74–1.01), respectively (Feskanich *et al.*, 1999; Karagas *et al.*, 2002).

2.8 Cancer of the cervix

Only two cohort studies and one case–control study investigated the relationship between the use of post-menopausal estrogen therapy and the risk for invasive cervical cancer. On balance, the limited evidence available suggests that postmenopausal estrogen therapy is not associated with an increased risk for invasive cervical carcinoma. The results provide some suggestion that postmenopausal estrogen therapy is associated with a reduced risk for cervical cancer, but the finding could be due to more active screening for pre-invasive disease among women who have received postmenopausal estrogen therapy (IARC, 1999).

2.9 Cancer of the thyroid

Seven case–control studies reporting on thyroid cancer and use of postmenopausal estrogen therapy did not show an effect on risk (IARC, 1999).

2.10 Synthesis

Estrogen-only menopausal therapy causes cancer of the endometrium, and of the ovary. Also, a positive association was observed between exposure to estrogen-only menopausal therapy and cancer of the breast. The Working Group also noted that an inverse relationship is established between exposure to estrogen-only menopausal therapy and cancer of the colorectum.

In addition, for cancer of the endometrium, the risk is increased in users of exogenous estrogen, and increases with duration of use. The excess in risk declines with time after use, but persists for over 10 years after exposure. The risk is also increased for atypical endometrial hyperplasia, a presumed precursor of endometrial cancer.

In addition, for cancer of the breast, a minority of case–control studies show an association between ever use or current use of estrogen therapy and breast cancer risk. Although the evidence is less consistent with regard to duration of estrogen therapy use, a few studies point to an increased risk of breast cancer with longer term use. The evidence is also inconsistent for the possibility that estrogen therapy increases the risk of lobular breast carcinoma. Although at the time of writing the evidence remains scant, estrogen therapy does not appear to be related differently to in-situ versus invasive breast cancer, to tumour stage, or to hormone receptor status.

The Working Group also concluded that the use of estrogen-only menopausal therapy is unlikely to alter the risk of cancer of the thyroid, bladder, pancreas, cervix, and of melanoma.

3. Cancer in Experimental Animals

3.1 Summary of the previous *IARC Monograph*

3.1.1 Conjugated estrogens

(a) Subcutaneous implantation

(i) Hamster

Hydrolysed conjugated equine estrogens, equilin and *d*-equilenin were tested in male hamsters by subcutaneous implantation. The hydrolysed estrogens and equilin induced microscopic renal carcinomas, whereas *d*-equilenin was inactive (Li *et al.*, 1983).

3.1.2 Estradiol

Estradiol and its esters were tested in neonatal mice, mice, rats, hamsters, guinea-pigs, and monkeys by subcutaneous injection or implantation, and in mice by oral administration.

(a) Subcutaneous injection or implantation

(i) Mouse

Subcutaneous injections of estradiol to neonatal mice resulted in precancerous and cancerous cervical and vaginal lesions in later life, and an increased incidence of mammary tumours. Its subcutaneous administration to mice resulted in increased incidences of mammary, pituitary, uterine, cervical, vaginal and lymphoid tumours, and interstitial cell tumours of the testis (IARC, 1999).

(ii) Rat

Invasive pituitary tumours were induced in rats treated subcutaneously with estradiol dipropionate (Satoh *et al.*, 1997).

(iii) Hamster

In hamsters, a high incidence of malignant kidney tumours occurred in intact and castrated males, and in ovariectomized females treated with estradiol, but not in intact females (Li *et al.*, 1983; Goldfarb & Pugh, 1990).

The 4-hydroxy metabolite of estradiol induced renal cell carcinomas in castrated male hamsters (Liehr *et al.* 1986; Li & Li, 1987).

(iv) Guinea-pig

In guinea-pigs, diffuse fibromyomatous uterine and abdominal lesions were observed (Lipschutz & Vargas, 1941).

(b) Oral administration

(i) Mouse

Oral administration of estradiol to mice bearing the murine mammary tumour virus increased the incidences of uterine (endometrial and cervical) adenocarcinomas and mammary tumours (Highman *et al.*, 1980).

(c) Administration with known carcinogens

Estradiol was tested in two-stage carcinogenesis models in mice with the known carcinogens *N*-methyl-*N*-nitrosourea (MNU), *N*-ethyl-*N*-nitrosourea or 3-methylcholanthrene, and in two-stage carcinogenesis models in rats with MNU, 2-acetylaminofluorene, *N*-nitrosodiethylamine, 7,12-dimethylbenz[*a*]anthracene (DMBA) or *N*-butyl-*N*-nitrosourea.

(i) Mouse

In mice, estradiol enhanced the incidences of endometrial adenomatous hyperplasia, atypical hyperplasia and adenocarcinomas induced by MNU and *N*-ethyl-*N*-nitrosourea (Niwa *et al.* 1991, 1996). A continuously high serum concentration of estradiol and a low concentration of progesterone appeared to be important for the development of endometrial adenocarcinomas in mice (Takahashi *et al.*, 1996). Estradiol decreased the development of uterine cervical carcinomas induced by 3-methylcholanthrene (Das *et al.*, 1988).

(ii) Rat

In rats, large doses of estradiol alone or estradiol with progesterone suppressed the development of mammary carcinomas induced by MNU (Grubbs *et al.*, 1983). Combined treatment of ovariectomized rats with estradiol and MNU induced vaginal polyps (Sheehan *et al.*, 1982). In a two-stage model of liver carcinogenesis in rats, estradiol showed no initiating activity (Sumi *et al.*, 1984). It did not show promoting effects in the liver of rats initiated with *N*-nitrosodiethylamine (Yager *et al.*, 1984). In one study, pretreatment with estradiol increased the number of liver foci positive for γ-glutamyl transferase induced by *N*-nitrosodiethylamine (Wotiz *et al.*, 1984). Estradiol did not affect mammary tumour development in intact or ovariectomized female rats treated with DMBA. Estradiol benzoate enhanced the incidence of mammary tumours in rats treated with γ-rays (Inano *et al.*, 1995).

3.1.3 Estriol

(a) Subcutaneous implantation

(i) Mouse

In castrated mice, estriol increased the incidence and accelerated the appearance of mammary tumours in both male and female mice (Rudali *et al.*, 1975).

(ii) Hamster

In hamsters, estriol produced kidney tumours (Kirkman, 1959a).

(b) Administration with known carcinogens

(i) Mouse

In female mice, estriol slightly increased the incidence of MNU-induced endometrial adenocarcinomas (Niwa *et al.*, 1993).

(ii) Rat

In several studies in female rats, estriol inhibited the induction of mammary tumours by DMBA when administered before the carcinogen; continuous treatment with estriol resulted in a decreased incidence of mammary tumours (Wotiz *et al.*, 1984). In one study in female rats, estriol inhibited the induction of mammary carcinomas when administered 13–15 days after irradiation with γ-rays (Lemon *et al.*, 1989).

3.1.4 Estrone

(a) Oral administration

(i) Mouse

Estrone was tested for carcinogenicity by oral administration in one study in castrated male mice. The incidence of mammary tumours was increased (Rudali *et al.*, 1978).

(b) Subcutaneous and/or intramuscular administration

(i) Mouse

Mammary tumours were induced in male mice, and the average age at the time of appearance of mammary tumours in female mice was reduced (Shimkin & Grady, 1940). In two studies of subcutaneous or intramuscular administration, estrone benzoate induced mammary tumours in male mice (Bonser, 1936; Shimkin & Grady, 1940).

(ii) Rat

In castrated male and female rats, subcutaneous injection of estrone resulted in mammary tumours (Geschickter & Byrnes, 1942). In one study in rats, subcutaneous injection of estrone benzoate induced mammary and pituitary tumours in animals of each sex (Chamorro, 1943).

(c) Subcutaneous implantation

(i) Mouse

In several studies involving subcutaneous implantation of estrone, the incidences of mammary and lymphoid tumours were increased in mice (Bittner, 1941; Gardner & Dougherty, 1944).

(ii) Hamster

In intact and castrated male hamsters, implantation of estrone resulted in malignant kidney tumours (Kirkman, 1959b). The estrone metabolite, 4-hydroxyestrone, induced renal tumours at a low incidence in castrated male hamsters (Li & Li, 1987).

(d) Administration with known carcinogens

(i) Mouse

The incidence of endometrial adenocarcinomas induced by MNU in the uterine corpus of mice was significantly increased in those receiving an estrone-containing diet; furthermore, the incidences of pre-neoplastic endometrial lesions in the MNU-treated and untreated uterine corpora were significantly increased in mice receiving the estrone-containing diet (Niwa *et al.*, 1993).

(ii) Toad

In one study in female toads, subcutaneous administration of estrone enhanced the incidence of hepatocellular carcinomas induced by subcutaneous injection of *N*-nitrosodimethylamine (Sakr *et al.*, 1989).

3.2 Studies published since the previous *IARC Monograph*

See Table 3.1

3.2.1 Estradiol

(a) Rat

In a study the objective of which was to characterize some of the genetic bases of estrogen-induced tumorigenesis in the rat, Schaffer *et al.* (2006) used young adult female ACI, BN, (BN x ACI)F1 (F1), and (BN x ACI)F2 (F2) rats that were treated with estradiol. Whereas nearly 100% of the ACI rats developed mammary cancer when treated continuously with estradiol, BN rats did not develop palpable mammary cancer during the 196-day course of estradiol treatment. Susceptibility to estradiol-induced mammary cancer segregated as a dominant or incompletely dominant trait in a cross between BN females and ACI males.

3.2.2 Administration with known carcinogens or other modifying agents

(a) Rat

Ting *et al.* (2007) used three mammary cancer carcinogen models. DMBA, MNU and estradiol were combined with local ovarian DMBA administration to induce progression to mammary and ovarian cancer concurrently in rats. Mammary hyperplasia was observed in DMBA/DMBA- (mammary carcinogen/ovarian carcinogen) and MNU/DMBA-treated rats; however, ovarian pre-neoplastic changes were seldom observed after these treatments. All estradiol/DMBA-treated rats had mammary hyperplasia, atypia, ductal carcinoma *in situ* and/or invasive adenocarcinoma, and half also developed pre-neoplastic changes in the ovary (ovarian epithelial and stromal hyperplasia and inclusion cyst formation).

Aiyer *et al.* (2008) fed female ACI rats with either AIN-93M standard diet or diets supplemented with either powdered blueberry or black raspberry or ellagic acid. They received estradiol implants and were killed after 24 weeks. A high incidence of mammary carcinomas was observed in the AIN-93M group. No differences were found in tumour incidence at 24 weeks; however, tumour volume and multiplicity were reduced significantly in the ellagic acid and black raspberry groups. The blueberry group showed a reduction (40%) only in tumour volume.

Callejo *et al.* (2005) evaluated the influence of different hormonal environments on the induction of breast cancer in the DMBA-induced mammary cancer model in rats. Breast cancer was induced by using a single intragastric dose

Table 3.1 Studies of cancer in experimental animals exposed to estradiol with known carcinogens or other modifying agents

Species, strain (sex) Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Female F344 (F) Ting *et al.* (2007)	Group 1: Controls Group 2: DMBA (10 mg/kg bw, p.o.)/ DMBA[a] Group 3: MNU (50 mg/kg bw, i.p.)/ DMBA[a] Group 4: E_2, 3.0 mg, pellet implant/ DMBA[a] 8; 6 controls/group 3 and 6 mo groups	Mammary carcinoma multiplicity at 3 and 6 mo, respectively: 0; 0 0; 1.00 ± 0.78 0.67 ± 0.21; 0.80 ± 0.20 2.40 ± 0.68; 3.50 ± 0.50 Ovarian carcinoma multiplicity at 3 and 6 mo, respectively: 0.83 ± 0.48; 0.75 ± 0.11 3.58 ± 0.69; 4.40 ± 1.20 3.42 ± 0.49; 4.00 ± 0.82 7.40 ± 0.25; 9.50 ± 0.88	 NS; NS NS; $P < 0.05$ $P < 0.05$; $P < 0.05$ $P < 0.05$[a]; $P < 0.05$[a] –; – $P < 0.05$; $P < 0.05$ $P < 0.05$; $P < 0.05$ $P < 0.05$[b]; $P < 0.05$[b]	Age at start NR; weight, 50–55 g
Rat, ACI (F) Aiyer *et al.* (2008)	AIN-93M diet for 2 wk; then silastic implant of E_2 (0 or 27 mg over 24 wk) Group 1: diet Group 2: diet + 2.5% powdered blueberry Group 3: diet + 2.5% black raspberry Group 4: diet + 400 ppm ellagic acid; 24 wk 19–25; 6 controls/group	Mammary carcinomas 100% in all E_2-treated animals at 24 wk	Reduction of tumour volume; reduction of tumour multiplicity –; – $P < 0.0001$; NS $P < 0.003$; NS $P < 0.001$; $P < 0.027$	Control incidence not specified but assumed to be 0%
Rat, ACI, BN, (BN x ACI)F1 (F1), and (BN x ACI)F2 (F2) (F) Schaffer *et al.* (2006)	E_2: 27.5 mg, implanted ACI: 23 rats BN: 13 rats F1: 22 rats F2: 257 rats	Mammary carcinomas (animals at risk): 94% 0% 86% 58%	Significance relative to ACI strain – – $P < 0.05$ $P < 0.05$	

Table 3.1 (continued)

Species, strain (sex) Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague Dawley (F) Callejo *et al.* (2005)	Single dose of DMBA 20 mg, dissolved in 0.5 to 1 mL corn oil; estradiol valerate, one daily dose, 5 mg/kg bw i.m. starting at 11 wk	Mammary carcinomas (mean tumours/group):	Differences with controls	
	Group 1: control lpt + DMBA	4	–	
	Group 2: lpt + ovariectomy + DMBA	0.1	$P < 0.001$	
	Group 3: lpt + ovariectomy + DMBA + tibolone	0.3	$P < 0.001$	
	Group 4: lpt + ovariectomy + DMBA + raloxifene	0.1	$P < 0.001$	
	Group 5: lpt + ovariectomy + DMBA + E_2	0.1	$P < 0.001$	
	10 animals/group			
Rat, Sprague Dawley (F) Kang *et al.* (2004)	DMBA, 10 mg in sesame oil; MNU, 50 mg/kg bw in saline; E_3B 30 or 300 µg, implanted	Mammary carcinomas (incidence; multiplicity):		E_3B tends to decrease the multiplicity of DMBA or MNU-induced mammary gland tumours
	DMBA alone	11/12 (92%); 4.17 ± 0.96	–; –	
	DMBA + E_3B 30	6/12 (50.0%); 3.08 ± 1.17	$P < 0.05$; NS	
	DMBA + E_3B 300	8/12 (67%); 2.5 ± 1.52	NS; NS	
	MNU alone	7/12 (58%); 1.67 ± 0.57	–; –	
	MNU + E_3B 30	4/12 (33%); 0.58 ± 0.30	NS; NS	
	MNU + E_3B 300	7/12 (58%); 1.17 ± 0.46	NS; NS	
	16 animals/group 4 animals/group killed at 5 wk 12 animals/group killed at 21 wk			

[a] in the ovary

[b] differs significantly from Groups 1 and 3

bw, bodyweight; d, day or days; F, female; DMBA, 7,12-dimethylbenz[*a*]anthracene; E_2, 17β-estradiol; E_3B, estradiol-3-benzoate; i.m., intramuscular; lpt, laparotomy; MNU: *N*-methyl-*N*-nitrosourea; mo, month or months; NR, not reported; NS, not significant; wk, week or weeks

of 20 mg of DMBA in pre-pubertal Sprague-Dawley rats randomized into five groups: Group 1 (control); Group 2 (castrated pre-pubertal animals); and Groups 3, 4, and 5 (castration of pre-pubertal animals followed by hormonal treatment starting at puberty [11 weeks] with tibolone, raloxifene, and estradiol, respectively). For Group 5 (estradiol valerate), a single daily dose of 5 mg/kg from a suspension of 1.5 mg/mL was administered orally. Absence of ovarian activity was observed in Groups 2, 3, 4, and 5, as well as the expected variations in hormone levels in all groups. Breast cancers were induced in 100% of the animals in the control group, with an average of four (2–7) tumours per animal in this group. Only one cancer appeared in Groups 2, 3, and 4, and none appeared in Group 5.

Kang *et al.* (2004) used the DMBA and MNU mammary carcinogenesis models to evaluate the effects of estradiol-3-benzoate. The hormone decreased the multiplicity of DMBA- or MNU-induced mammary gland tumours. There was also increased branching of the mammary gland, and a decrease of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ). The inhibitory effect on mammary carcinogenesis may be associated with the differentiation of mammary gland and modulation of ERα and ERβ.

3.2.3 Metabolites

Possible cancer suppressor effects of the physiological metabolite of estradiol, 2-methoxyestradiol were evaluated by Cicek *et al.* (2007) using a murine metastatic breast cancer cell line injected to BALB/C mice. They found that 2-methoxyestradiol inhibited tumour growth in soft tissue, metastasis to bone, osteolysis, and tumour growth in bone. Tumour-induced osteolysis was also significantly reduced in mice receiving 2-methoxyestradiol.

3.3 Synthesis

Estradiol causes malignant mammary tumours in mice and malignant kidney tumours in hamsters. Estrone causes malignant mammary tumours in mice.

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

The absorption, distribution, metabolism and excretion of estrogens have been extensively reviewed previously in the *IARC Monograph* on combined estrogen-progestogen menopausal therapy (IARC 1999, 2007). In summary, cytochrome P450 1A1 and 1B1 catalyse the production of catechol estrogens that are further oxidized to estrogen *o*-quinones that can induce the formation of DNA damage. This is counteracted by the detoxification enzymes, catechol-*O*-methyltransferase, sulfotransferase, and uridine 5′-diphosphate(UDP)-glucuronosyl transferase which reduce the levels of catechols by forming methoxyestrogens, sulfates, and glucuronide conjugates, respectively (IARC 1999, 2007). As far as detoxification of the *o*-quinones are concerned, there have been some reports that the quinones can be detoxified through reduction by quinone reductase and/or conjugation with glutathione (GSH) catalysed by glutathione *S*-transferases (GSTs) (Hachey *et al.*, 2003; Zahid *et al.*, 2008), although the non-enzymatic reduction by nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and Michael addition with GSH are very facile reactions, enhanced enzymatic catalysis may be of questionable importance. A large body of epidemiological data has failed to identify a consistent association between exposure to estrogenic hormones and risk for cancer with any single enzyme variant of these phase I and phase II enzymes (Saintot *et al.*, 2003; Boyapati *et al.*,

2005; Cheng *et al.*, 2005; Rebbeck *et al.*, 2006; Hirata *et al.*, 2008; Justenhoven *et al.*, 2008; Van Emburgh *et al.*, 2008), but possible interactions between these genes remain to be examined in more detail.

4.2 Genetic and related effects

The genetic effects of endogenous estrogens, estradiol, and equine estrogens have been reviewed previously (IARC, 1999, 2007). New data that have appeared since are summarized below.

4.2.1 Direct genotoxicity

(a) DNA adducts

Estrogen quinoids can directly damage cellular DNA (see diethylstilbestrol, this volume; Liehr, 2000; Bolton *et al.*, 2004; Russo & Russo, 2004; Prokai-Tatrai & Prokai, 2005; Cavalieri *et al.*, 2006; IARC, 2007; Bolton & Thatcher, 2008; Gaikwad *et al.*, 2008). The major DNA adducts produced from 4-hydroxyoestradiol-*o*-quinone are depurinating N^7-guanine and N^3-adenine adducts resulting from 1,4-Michael addition both *in vitro* and *in vivo* (Stack *et al.*, 1996; Cavalieri *et al.*, 2000, 2006; Li *et al.*, 2004; Zahid *et al.*, 2006; Saeed *et al.*, 2007; Gaikwad *et al.*, 2008). Interestingly, only the N^3-adenine adduct may induce mutations because this adduct depurinates extremely rapidly, whereas the half-life of the N^7-guanine adduct is 6–7 hours (Saeed *et al.*, 2005; Zahid *et al.*, 2006). In contrast, the considerably more rapid isomerization of the 2-hydroxyestradiol-*o*-quinone to the corresponding quinone methides results in 1,6-Michael addition products with the exocyclic amino groups of adenine and guanine (Stack *et al.*, 1996; Debrauwer *et al.*, 2003). In contrast to the N^3- and N^7-purine DNA adducts, these adducts are stable which may affect their repair and mutagenicity *in vivo*. A depurinating N^3-adenine adduct of 2-hydroxyestradiol quinone methide has recently been reported in reactions with adenine and DNA (Zahid *et al.*, 2006). The levels of this adduct in DNA were considerably lower than corresponding depurinating adducts observed in similar experiments with 4-hydroxyestradiol-*o*-quinone. This is consistent with the suggestion that 2-hydroxylation does not lead to cancer, whereas 4-hydroxylation results in carcinogenesis. This same study (Zahid *et al.*, 2006) provided evidence to suggest that depurinating DNA adducts of estrogen quinoids were formed in much greater abundance than stable adducts. [The Working Group noted that this implies a causal role for the depurinating adducts in estrogen carcinogenesis; but these adducts were analysed by methods (high-perfomance liquid chromatography with electrochemical detection) that differed from those used to detect the stable adducts (^{32}P-postlabelling/thin-layer chromatography), making direct quantitative comparisons somewhat problematic.]

It is important to mention that stable DNA adducts at extracyclic aminogroups have been detected by ^{32}P-postlabelling in DNA from Syrian hamster embryo cells treated with estradiol and its catechol metabolites. The rank order of DNA adduct formation was 4-hydroxyestradiol > 2-hydroxyestradiol > estradiol. The adduct formation correlated with cellular transformation (Hayashi *et al.*, 1996). [The Working Group noted that these data do not clarify the relative importance of depurinating adducts versus stable DNA adducts in catechol estrogen carcinogenesis.]

For the major equine estrogens (equilin, equilenin, and 17β-ol derivatives) the data strongly suggests that the majority of DNA damage also results from reactions of 4-hydroxyequilenin-*o*-quinone through a combination of oxidative damage (ie. single-strand cleavage and oxidation of DNA bases) and through generation of apurinic sites as well as stable bulky cyclic adducts (Bolton & Thatcher, 2008). For example, a depurinating guanine adduct was

detected in in-vivo experiments with rats treated with 4-hydroxyequilenin, following liquid chromatography-tandem mass spectrometry (LC/MS-MS) analysis of extracted mammary tissue (Zhang *et al.*, 2001). However, analysis of this rat mammary tissue DNA by LC/MS-MS also showed the formation of stable cyclic deoxyguanosine and deoxyadenosine adducts as well as the aforementioned oxidized bases. Single-strand breaks were also detected (Ding *et al.*, 2003; Kolbanovskiy *et al.*, 2005; Yasui *et al.*, 2006; Ding *et al.*, 2007). [The Working Group noted that, interestingly, the ratio of the diasteriomeric adducts detected *in vivo* differs from in-vitro experiments. This suggests differential repair of these stereoisomeric lesions.] Using highly sensitive nano LC/MS-MS techniques to analyse DNA in five human breast tumours and five adjacent tissue samples, including samples from donors with a known history of Premarin-based hormone replacement therapy, cyclic 4-hydroxyequilenin-dC, -dG, and -dA stable adducts were detected for the first time in 4/10 samples (Embrechts *et al.*, 2003). [The Working Group noted that although the sample size in this study was small, and the history of the patients is not fully known, these results suggest that the equilin metabolite 4-hydroxyequilenin has the potential to form a variety of DNA lesions in humans.]

(b) Oxidative damage to DNA

As indicated earlier, these estrogens are oxidized to *o*-quinones which are electrophiles as well as potent redox active compounds (Bolton *et al.*, 2000). They can undergo redox cycling with the semiquinone radical generating superoxide radicals mediated through cytochrome P450/P450 reductase (Bolton *et al.*, 2000; IARC, 2007; see diethylstilbestrol, this volume) which gives rise to hydroxyl radicals. In support of this mechanism, various free radical effects have been reported in animals treated with estradiol (IARC, 2007), including DNA single-strand breaks (Nutter *et al.*, 1991; Roy & Liehr, 1999), 8-hydroxydeoxyguanosine formation (Cavalieri *et al.*, 2000; Lavigne *et al.*, 2001; Rajapakse *et al.*, 2005), and chromosomal abnormalities (Li *et al.*, 1993; Banerjee *et al.*, 1994; Russo & Russo, 2006). The estradiol catechol metabolite 4-hydroxyestradiol also induces oxidative stress and apoptosis in human mammary epithelial cells (MCF-10A) (Chen *et al.*, 2005). [The high concentrations used in this study (> 10 μM) have questionable physiological relevance.] It has been further shown that the equilenin catechol, 4-hydroxyequilenin, is also capable of causing DNA single-strand breaks and oxidative damage to DNA bases both *in vitro* and *in vivo* (IARC, 2007; Okamoto *et al.*, 2008). These and older data provide evidence for the generation of reactive oxygen species by redox cycling of estrogen metabolites – reactive oxygen species that are known to damage DNA. This is a proposed mechanism of estrogen-induced tumour initiation/promotion (Cavalieri *et al.*, 2000; Bolton & Thatcher, 2008).

(c) Genetic effects in women

Two studies have identified catechol estrogen adducts in breast tissue of women with breast cancer (see IARC, 2007), and the excessive production of reactive oxygen species in breast cancer tissue has been linked to metastasis in women with breast cancer (Malins *et al.*, 1996, 2006; Karihtala & Soini, 2007). [However, the Working Group was not aware of studies on markers of oxidative stress in women on estrogen therapy.]

(d) Genetic effects in animals

There is some in-vivo evidence for estradiol inducing DNA strand breaks, sister chromatid exchange, chromosomal aberrations, and aneuploidy, but not micronuclei or covalent binding of estrogen metabolites to DNA (IARC, 1999, 2007).

Injection of 4-hydroxyequilenin into the mammary fat pads of Sprague-Dawley rats

resulted in a dose-dependent increase in single-strand breaks and oxidized bases as analysed by the comet assay, and the formation of 8-hydroxydeoxyguanosine and 8-hydroxydeoxyadenine (Zhang *et al.*, 2001). In mice treated with equilenin, the levels of 8-hydroxydeoxyguanosine were increased 1.5-fold in the uterus (Okamoto *et al.*, 2008).

(e) Genetic effects in human and animal cells

There is some evidence for estradiol inducing DNA strand breaks, sister chromatid exchange, and chromosomal aberrations in various human cells, including MCF-7 breast cancer cells, although the evidence for induction of aneuploidy in human cells is equivocal (IARC, 1999, 2007).

Repeated treatment at physiological (70 nM) and at much lower (0.007 nM) doses of estradiol of MCF-10F immortalized human breast epithelial cells, which are ERα-negative, increased colony formation in a soft agar assay (Russo *et al.*, 2001, 2003). When grown in collagen, these cells changed from differentiated ductular growth to solid-spherical masses with the same dose–response relationship, and invasive growth in a Matrigel assay was increased (Russo *et al.*, 2002, 2006). Following estradiol exposure at a concentration of 70 nM of MCF-10F cells for four alternating 24-hour periods, several passages of the cells and subsequent selection, four clones grew in Matrigel, two of which formed tumours in nude mice (Russo *et al.*, 2006). Both clones had loss of chromosomal region 9p11–3 and all other subclones had variable other chromosomal losses, deletions, and gains. [The Working Group noted that this study established that estradiol is capable of inducing malignant transformation in cultured immortalized human breast cells that are ER-negative.]

The same types of genetic damage induced *in vivo* are also induced in cells in culture by estradiol, estrone, and their catechol metabolites as well as cell transformation, but mutations have not consistently been observed (IARC, 1999, 2007). The mutagenic properties of the aforementioned stable DNA adducts derived from 2-hydroxyestrogen-quinone-methide have been evaluated using oligonucleotides containing site-specific adducts transfected into simian kidney (COS-7) cells where G→T and A→T mutations were observed (Terashima *et al.*, 2001).

4.2.2 Indirect effects related to genotoxicity

(a) Estrogen-receptor-mediated effects

Receptor-mediated mechanisms by which estrogens may cause or contribute to carcinogenesis have previously been reviewed extensively (IARC, 1999, 2007). Estrogen therapy in the menopause increases the rate of cell proliferation in the postmenopausal human breast (IARC, 1999, 2007). This effect is mediated through nuclear ER-mediated signalling pathways, and this results in an increased risk of genomic mutations during DNA replication (Nandi *et al.*, 1995; Feigelson & Henderson, 1996; Henderson & Feigelson, 2000; Flötotto *et al.*, 2001; Yager & Davidson, 2006). [The Working Group noted that direct evidence for this mechanism in relevant experimental models of target tissues is, however, not available at present.] Similarly acting non-genomic pathways, potentially involving newly discovered membrane-associated estrogen receptors, appear to regulate extranuclear estrogen signalling pathways that can affect cell proliferation (Björnström & Sjöberg, 2005; Revankar *et al.*, 2005; Song *et al.*, 2006; Yager & Davidson, 2006; Hammes & Levin, 2007; Pietras & Márquez-Garbán, 2007; Levin & Pietras, 2008). Recent studies have shown the presence of ERα and ERβ in the mitochondria of various cells and tissues, which may be involved in deregulation of mitochondrial bioenergetics, and conceivably contribute to estrogen-exposure-related cancers (Yager & Davidson, 2006; Chen *et al.*, 2008). Cross-talk between these genomic and non-genomic second-messenger pathways may have

an important role in estrogenic control of cell proliferation, inhibition of apoptosis, and induction of DNA damage (Yager & Davidson, 2006).

4.3 Synthesis

Receptor-mediated responses to hormones are a plausible and probably necessary mechanism for estrogen carcinogenesis. In addition, there is support for a genotoxic effect of estrogenic hormones or their associated by-products such as reactive oxygen species. The types of DNA damage found in cells and tissues exposed to estrogens are consistent with such genotoxic effects. Current knowledge does not allow a conclusion as to whether either of these mechanisms is the major determinant of estrogen-induced cancer. It is entirely possible that both mechanisms contribute to and are necessary for estrogen carcinogenesis. Although there appears to be a direct link between exposure to estrogens, metabolism of estrogens, and increased risk of breast cancer, the factors that affect the formation, reactivity, and cellular targets of estrogen quinoids remain to be fully explored.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of estrogen-only menopausal therapy. Estrogen-only menopausal therapy causes cancer of the endometrium and of the ovary. Also, a positive association has been observed between exposure to estrogen-only menopausal therapy and cancer of the breast.

For cancer of the colorectum, there is *evidence suggesting lack of carcinogenicity*. An inverse relationship has been established between exposure to estrogen-only menopausal therapy and cancer of the colorectum.

There is *sufficient evidence* in experimental animals for the carcinogenicity of some estrogens used in estrogen-only menopausal therapy.

Estrogen-only menopausal therapy is *carcinogenic to humans (Group 1*).

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COMBINED ESTROGEN–PROGESTOGEN MENOPAUSAL THERAPY

Combined estrogen–progestogen menopausal therapy was considered by previous IARC Working Groups in 1998 and 2005 (IARC, 1999, 2007). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

Combined estrogen–progestogen menopausal therapy involves the co-administration of an estrogen and a progestogen to peri- or menopausal women. The use of estrogens with progestogens has been recommended to prevent the estrogen-associated risk of endometrial cancer. Evidence from the Women's Health Initiative (WHI) of adverse effects from the use of a continuous combined estrogen–progestogen has affected prescribing. Patterns of exposure are also changing rapidly as the use of hormonal therapy declines, the indications are restricted, and the duration of the therapy is reduced (IARC, 2007).

1.1 Identification of the agents

1.1.1 Estrogens

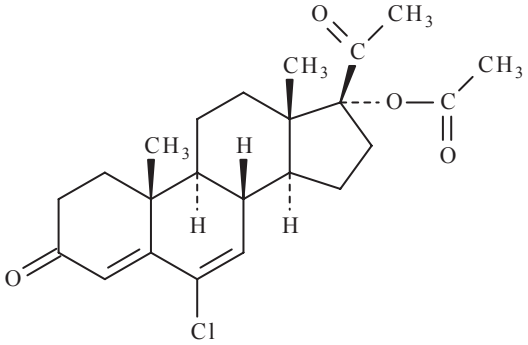
For Estrogens, see the *Monograph* on Estrogen-only Menopausal Therapy in this volume.

1.1.2 Progestogens

(a) Chlormadinone acetate

Chem. Abstr. Serv. Reg. No.: 302-22-7
Chem. Abstr. Name: 17-(Acetyloxy)-6-chloropregna-4,6-diene-3,20-dione
IUPAC Systematic Name: 6-Chloro-17-hydroxypregna-4,6-diene-3,20-dione, acetate
Synonyms: 17α-Acetoxy-6-chloro-4,6-pregnadiene-3,20-dione; 6-chloro-Δ^6-17-acetoxyprogesterone; 6-chloro-Δ^6-[17α] acetoxyprogesterone

Structural and molecular formulae, and relative molecular mass

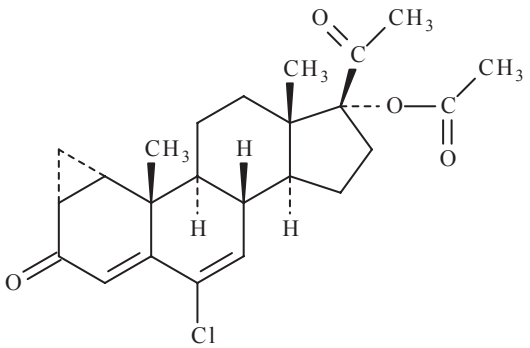


$C_{23}H_{29}ClO_4$
Relative molecular mass: 404.9

(b) Cyproterone acetate

Chem. Abstr. Serv. Reg. No.: 427-51-0
Chem. Abstr. Name: (1*β*,2*β*)-17-(Acetyloxy)-6-chloro-1,2-dihydro-3′*H*-cyclopropa[1,2]pregna-1,4,6-triene-3,20-dione
IUPAC Systematic Name: 6-Chloro-1*β*,2*β*-dihydro-17-hydroxy-3′H-cyclopropa[1,2]pregna-1,4,6-triene-3,20-dione acetate
Synonyms: Cyproterone 17-*O*-acetate; cyproterone 17*α*-acetate; 1,2*α*-methylene-6-chloro-17*α*-acetoxy-4,6-pregnadiene-3,20-dione; 1,2*α*-methylene-6-chloro-$\Delta^{4,6}$-pregnadien-17*α*-ol-3,20-dione acetate; 1,2*α*-methylene-6-chloro-pregna-4,6-diene-3,20-dione 17*α*-acetate; methylene-6-chloro-17-hydroxy-1*α*,2*α*-pregna-4,6-diene-3,20-dione acetate

Structural and molecular formulae, and relative molecular mass

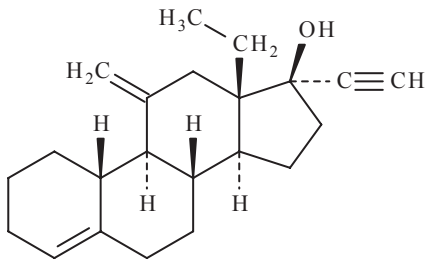


$C_{24}H_{29}ClO_4$
Relative molecular mass: 416.9

(c) Desogestrel

Chem. Abstr. Serv. Reg. No.: 54024-22-5
Chem. Abstr. Name: (17*α*)-13-Ethyl-11-methylene-18,19-dinorpregn-4-en-20-yn-17-ol
IUPAC Systematic Name: 13-Ethyl-11-methylene-18,19-dinor-17*α*-pregn-4-en-20-yn-17-ol
Synonyms: 13-Ethyl-11-methylene-18,19-dinor-17*α*-4-pregnen-20-yn-17-ol; 17*α*-ethynyl-18-methyl-11-methylene-Δ^4-oestren-17*β*-ol

Structural and molecular formulae, and relative molecular mass

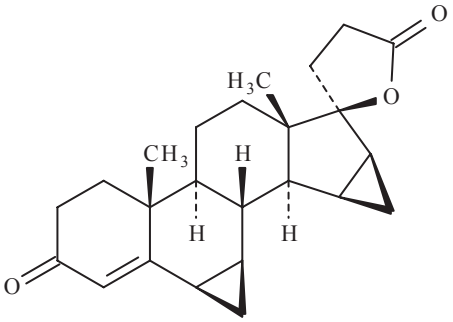


$C_{22}H_{30}O$
Relative molecular mass: 310.5

(d) Drospirenone

Chem. Abst. Services Reg. No.: 67392-87-4
Chem. Abstr. Name: (2′*S*,6*R*,7*R*,8*R*,9*S*,10*R*,-13*S*,14*S*,15*S*,16*S*)-1,3′,4′,6,7,8,9,10,11,-12,13,14,15,16,20,21-Hexadecahydro-10,13-dimethyl-spiro[17*H*-dicyclopropa[6,7:15,16]cyclopenta[*a*]phenanthrene-17,2′ (5′*H*)-furan]-3,5′ (2*H*)-dione
Synonyms: Dihydrospirorenone; 1,2-dihydrospirorenone; drospirenona; spiro[17*H*-dicyclopropa[6,7:15,16]cyclopenta[*a*]phenanthrene-17,2′(5′H)-furan]-3,5′(2H)-dione, 1,3′,4′,6,7,8,9,10,11,12,13,14,15,16,20,21-hexadecahydro-10,13-dimethyl-, [6*R*-(6*α*,7*α*,8*β*,9*α*,10*β*,13*β*,14*α*,15*α*,16*α*,17*β*)]-

Structural and molecular formulae, and relative molecular mass

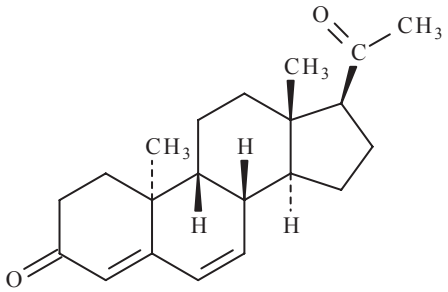


$C_{24}H_{30}O_3$
Relative molecular mass: 366.5

(e) Dydrogesterone

Chem. Abstr. Serv. Reg. No.: 152-62-5
Chem. Abstr. Name: (9*β*,10*α*)-Pregna-4,6-diene-3,20-dione
IUPAC Systematic Name: 10*α*-Pregna-4,6-diene-3,20-dione
Synonyms: 10*α*-Isopregnenone; dehydroretroprogesterone; dehydroprogesterone

Structural and molecular formulae, and relative molecular mass

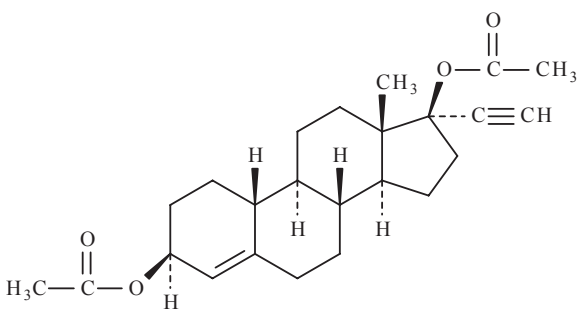


$C_{21}H_{28}O_2$
Relative molecular mass: 312.5

(f) Ethynodiol diacetate

Chem. Abstr. Serv. Reg. No.: 297-76-7
Chem. Abstr. Name: (3*β*,17*α*)-19-Norpregn-4-en-20-yne-3,17-diol, diacetate
IUPAC Systematic Name: 19-Nor-17*α*-pregn-4-en-20-yne-3*β*,17*β*-diol, diacetate
Synonyms: Ethinodiol diacetate; ethynodiol acetate; *β*-ethynodiol diacetate

Structural and molecular formulae, and relative molecular mass

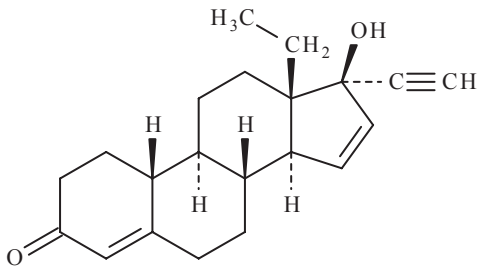


$C_{24}H_{32}O_4$
Relative molecular mass: 384.5

(g) Gestodene

Chem. Abstr. Serv. Reg. No.: 60282-87-3
Deleted CAS Reg. No.: 110541-55-4
Chem. Abstr. Name: (17*α*)-13-Ethyl-17-hydroxy-18,19-dinorpregna-4,15-dien-20-yn-3-one
IUPAC Systematic Name: 13-Ethyl-17-hydroxy-18,19-dinor-17*α*-pregna-4,15-dien-20-yn-3-one

Structural and molecular formulae, and relative molecular mass

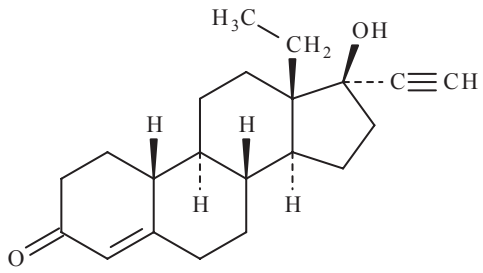


$C_{21}H_{26}O_2$
Relative molecular mass: 310.4

(h) Levonorgestrel

Chem. Abstr. Serv. Reg. No.: 797-63-7
Deleted CAS Reg. No.: 797-62-6; 4222-79-1; 121714-72-5
Chem. Abstr. Name: (17*α*)-13-Ethyl-17-hydroxy-18,19-dinorpregn-4-en-20-yn-3-one
IUPAC Systematic Name: 13-Ethyl-17-hydroxy-18,19-dinor-17*α*-pregn-4-en-20-yn-3-one
Synonyms: 13-Ethyl-17-ethynyl-17*β*-hydroxy-4-gonen-3-one; 13-ethyl-17*α*-ethynyl-17-hydroxygon-4-en-3-one; 13-ethyl-17*α*-ethynylgon-4-en-17*β*-ol-3-one; 13*β*-ethyl-17*α*-ethynyl-17*β*-hydroxygon-4-en-3-one; 13-ethyl-17-hydroxy-18,19-dinor-17*α*-pregn-4-en-20-yn-3-one; 17-ethynyl-18-methyl-19-nortestosterone; 18-methylnorethindrone; L-norgestrel; DL-norgestrel; D-norgestrel

Structural and molecular formulae, and relative molecular mass

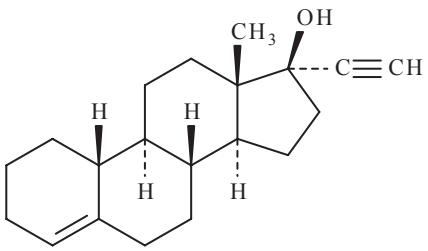


$C_{21}H_{28}O_2$
Relative molecular mass: 312.5

(i) Lynestrenol

Chem. Abstr. Serv. Reg. No.: 52-76-6
Deleted CAS Reg. No.: 60416-16-2
Chem. Abstr. Name: (17α)-19-Norpregn-4-en-20-yn-17-ol
IUPAC Systematic Name: 19-Nor-17α-pregn-4-en-20-yn-17-ol
Synonyms: 3-Desoxynorlutin; Δ^4-17α-ethinylestren-17β-ol; Δ^4-17α-ethinyloestren-17β-ol; ethynylestrenol; ethynyloestrenol; 17α-ethynylestrenol; 17α-ethynyloestrenol; 17α-ethynyl-17β-hydroxy-Δ^4-estrene; 17α-ethynyl-17β-hydroxy-Δ^4-oestrene

Structural and molecular formulae, and relative molecular mass

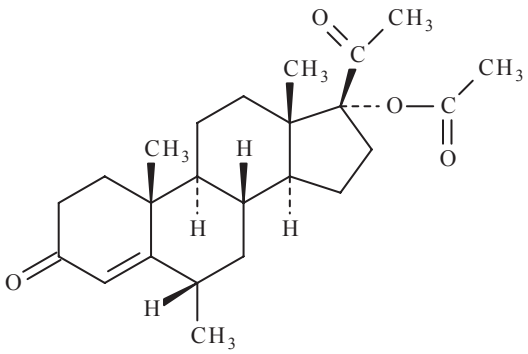


$C_{20}H_{28}O$
Relative molecular mass: 284.4

(j) Medroxyprogesterone acetate

Chem. Abstr. Serv. Reg. No.: 71-58-9
Chem. Abstr. Name: (6α)-17-(Acetyloxy)-6-methylpregn-4-ene-3,20-dione
IUPAC Systematic Name: 17-Hydroxy-6α-methylpregn-4-ene-3,20-dione, acetate
Synonyms: 17α-Acetoxy-6α-methylprogesterone; depomedroxyprogesterone acetate; depo-progestin; depot-medroxyprogesterone acetate; DMPA; 17-hydroxy-6α-methylprogesterone, acetate; 17α-hydroxy-6α-methylprogesterone acetate; MAP; MPA; medroxyprogesterone 17-acetate; 6α-methyl-17-acetoxyprogesterone; 6α-methyl-17α-hydroxyprogesterone acetate

Structural and molecular formulae, and relative molecular mass

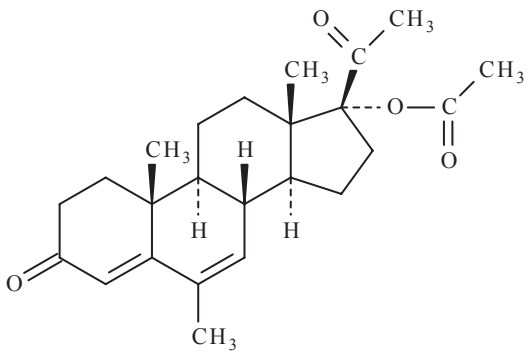


$C_{24}H_{34}O_4$
Relative molecular mass: 386.5

(k) Megestrol acetate

Chem. Abstr. Serv. Reg. No.: 595-33-5
Chem. Abstr. Name: 17-(Acetyloxy)-6-methylpregna-4,6-diene-3,20-dione
IUPAC Systematic Name: 17-Hydroxy-6-methylpregna-4,6-diene-3,20-dione, acetate
Synonyms: DMAP; megestryl acetate; MGA

Structural and molecular formulae, and relative molecular mass



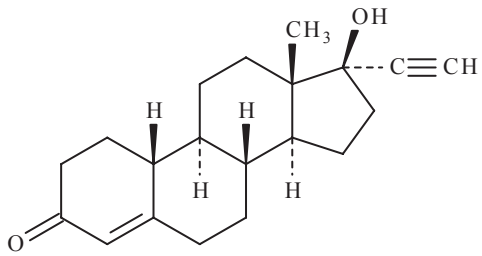
$C_{24}H_{32}O_4$

Relative molecular mass: 384.5

(l) Norethisterone

Chem. Abstr. Serv. Reg. No.: 68-22-4
Chem. Abstr. Name: (17α)-17-Hydroxy-19-norpregn-4-en-20-yn-3-one
IUPAC Systematic Name: 17-Hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one
Synonyms: Ethinylnortestosterone; 17α-ethinyl-19-nortestosterone; ethynylnortestosterone; 17-ethynyl-19-nortestosterone; 17α-ethynyl-19-nortestosterone; norethindrone; norethisteron; norethynodrone; 19-nor-17α-ethynyltestosterone; norpregneninolone

Structural and molecular formulae, and relative molecular mass



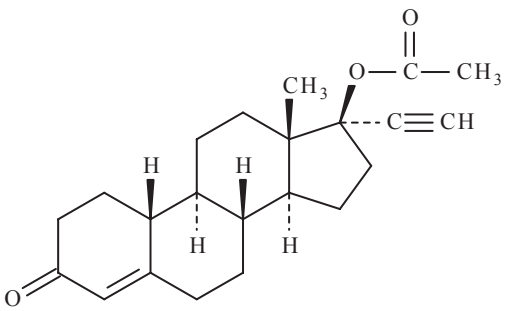
$C_{20}H_{26}O_2$

Relative molecular mass: 298.4

(m) Norethisterone acetate

Chem. Abstr. Serv. Reg. No.: 51-98-9
Chem. Abstr. Name: (17α)-17-(Acetyloxy)-19-norpregn-4-en-20-yn-3-one
IUPAC Systematic Name: 17-Hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one, acetate
Synonyms: 17α-Ethinyl-19-nortestosterone 17β-acetate; 17α-ethinyl-19-nortestosterone acetate; 17α-ethynyl-19-nortestosterone acetate; norethindrone acetate; norethindrone 17-acetate; norethisteron acetate; norethisterone 17-acetate; 19-norethisterone acetate; norethynyltestosterone acetate; 19-norethynyltestosterone acetate; norethysterone acetate

Structural and molecular formulae, and relative molecular mass



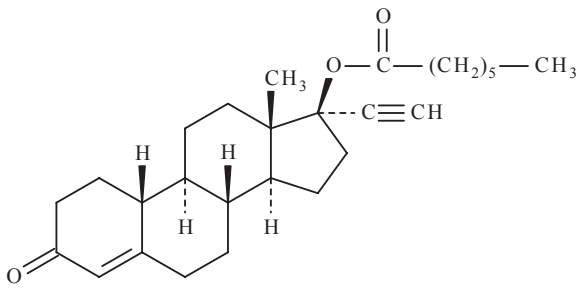
$C_{22}H_{28}O_3$

Relative molecular mass: 340.5

(n) Norethisterone enanthate

Chem. Abstr. Serv. Reg. No.: 3836-23-5
Chem. Abstr. Name: (17α)-17-(Heptanoyl)-19-norpregn-4-en-20-yn-3-one
IUPAC Systematic Name: 17-Hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one, heptanoate
Synonyms: Norethindrone enanthate; norethindrone oenanthate; norethisterone enanthate; norethisterone heptanoate; 17β-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one heptanoate

Structural and molecular formulae, and relative molecular mass

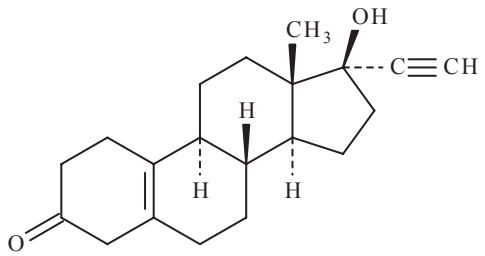


$C_{27}H_{38}O_3$
Relative molecular mass: 410.6

(o) Norethynodrel

Chem. Abstr. Serv. Reg. No.: 68-23-5
Chem. Abstr. Name: (17α)-17-Hydroxy-19-norpregn-5(10)-en-20-yn-3-one
IUPAC Systematic Name: 17-Hydroxy-19-nor-17α-pregn-5(10)-en-20-yn-3-one
Synonyms: Enidrel; noretynodrel

Structural and molecular formulae, and relative molecular mass

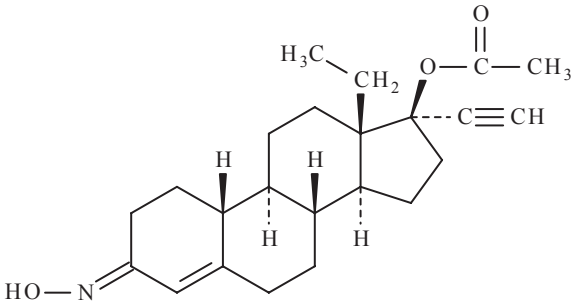


$C_{20}H_{26}O_2$
Relative molecular mass: 298.4

(p) Norgestimate

Chem. Abstr. Serv. Reg. No.: 35189-28-7
Chem. Abstr. Name: (17α)-17-(Acetyloxy)-13-ethyl-18,19-dinorpregn-4-en-20-yn-3-one, 3-oxime
IUPAC Systematic Name: 13-Ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one oxime acetate (ester)
Synonyms: 17α-Acetoxy-13-ethyl-17-ethynylgon-4-en-3-one oxime; dexnorgestrel acetime

Structural and molecular formulae, and relative molecular mass

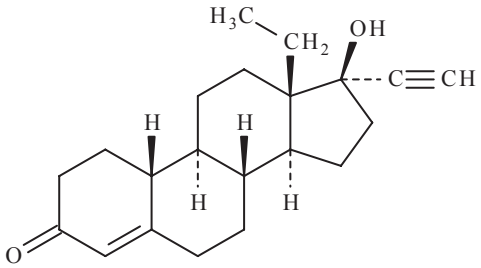


$C_{23}H_{31}NO_3$
Relative molecular mass: 369.5

(q) Norgestrel

Chem. Abstr. Serv. Reg. No.: 6533-00-2
Chem. Abstr. Name: (17α)-dl-13-Ethyl-17-hydroxy-18,19-dinorpregn-4-en-20-yn-3-one
IUPAC Systematic Name: dl-13-Ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one
Synonyms: (17α)-13-Ethyl-17-hydroxy-18,19-dinorpregn-4-en-20-yn-3-one; methylnorethindrone; α-norgestrel; dl-norgestrel; DL-norgestrel

Structural and molecular formulae, and relative molecular mass

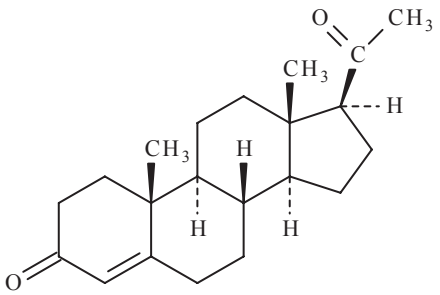


$C_{21}H_{28}O_2$
Relative molecular mass: 312.5

(r) Progesterone

Chem. Abst. Services Reg. No.: 57-83-0
Chem. Abstr. Name: Pregn-4-ene-3,20-dione
Synonyms: Corpus luteum hormone; luteal hormone; luteine; luteohormone; Δ^4-pregnene-3,20-dione

Structural and molecular formulae, and relative molecular mass



$C_{21}H_{30}O_2$

Relative molecular mass: 314.5

1.2 Use of the agents

Information for Section 1.2 is taken from IARC (2007), McEvoy (2007), and Sweetman (2008).

1.2.1 Indications

Estrogen–progestogen combinations are used for the treatment of moderate-to-severe vasomotor symptoms, vulvar and vaginal atrophy associated with menopause, and for the prevention and treatment of osteoporosis. Women with an intact uterus are prescribed a progestogen in addition to estrogen to reduce the increased risk of endometrial carcinoma.

1.2.2 Dosages and formulations

A variety of products are available for use in combined estrogen–progestogen menopausal therapy, either as individual estrogen and progestogen components that can be co-administered, or as a combined tablet.

Available products can be defined by their estrogen form, dose, and mode of delivery. The most common estrogens available for menopausal therapy are conjugated equine estrogen, conjugated plant-based estrogens (A and B; see the *Monograph* on Estrogen-only Menopausal Therapy in this volume), estradiol and ethinylestradiol. A range of 3–5 different doses are often available for each product, varying from low doses (0.3–0.5 mg orally) to higher doses (2.5–5 mg). The doses of estrogens used are generally lower than those used in combined oral contraceptives, and do not therefore provide contraception. They are available as oral tablets, intranasal sprays, transdermal skin patches and gels, subcutaneous implants, topical applications for vulvovaginal use, and intravaginal rings.

Generally, if prolonged therapy (for more than 2–4 weeks) with an estrogen by any route is envisaged in a woman with an intact uterus, a progestogen is given to prevent endometrial proliferation. A range of progestogens are available for use in combined therapy. Those most commonly used are medroxyprogesterone acetate, norethisterone, levonorgestrel, and micronized progestogens. Several doses of each progestogen are usually available. While oral forms predominate, progestogens are also available as a vaginal pessary, a systemically absorbed vaginal gel, a transdermal patch and an intrauterine device. The administration of progestogens may follow one of three types of schedule. In continuous combined therapy, the same dose of both estrogen and progestogen is administered each day. Cyclic or sequential therapy consists of estrogen-alone daily, followed by progestogen with estrogen for 7–20 days and then 5–7 days with no hormones. The duration of each phase can vary.

Combined oral products contain both estrogen and progestogen. The various preparations available differ in their estrogen component, their progestogen component, the dose of these components, and the schedule and mode of drug administration. Continuous exposure to both hormones (both estrogen and progestogen at fixed daily doses) is common, particularly in the United States of America, whereas cyclic dosing, in which progestogen is added periodically to daily estrogen, is prevalent in other

countries. Other scheduling strategies are also used occasionally.

When conjugated estrogens (A or B) are used with medroxyprogesterone acetate for the management of moderate-to-severe vasomotor symptoms associated with menopause or for the management of vulvar and vaginal atrophy, conjugated estrogens are administered in a continuous daily dosage regimen while medroxyprogesterone acetate is administered in a continuous daily dosage regimen or cyclically. When both drugs are administered in a continuous daily dosage regimen, conjugated estrogens are administered in a daily dosage of 0.3 mg in conjunction with oral medroxyprogesterone acetate in a daily dosage of 1.5 mg. Alternatively, conjugated estrogens are administered in a daily dosage of 0.45 mg in conjunction with medroxyprogesterone acetate in a daily dosage of 1.5 mg, or conjugated estrogens are administered in a daily dosage of 0.625 mg in conjunction with medroxyprogesterone acetate in a daily dosage of 2.5 or 5 mg. When conjugated estrogens are administered in a continuous daily dosage regimen and medroxyprogesterone acetate is administered cyclically, conjugated estrogens are administered in a daily dosage of 0.625 mg, while oral medroxyprogesterone acetate is administered in a daily dosage of 5 mg on Days 15–28 of the cycle.

1.2.3 Trends in use

When the addition of a progestogen to estrogens was introduced after 1975 as a strategy to reduce the risk of endometrial cancer, the use of the combination for menopausal therapy increased steadily in the 1980s, particularly in developed countries. Combined estrogen–progestogen menopausal therapy is now administered to women who have not undergone a hysterectomy.

Combined hormonal therapy is much more commonly used in developed countries than in developing countries. At the peak of use in 1999, approximately 20 million women in developed countries used combined hormonal therapy. Use has fallen by more than 50% since 2002, particularly for continuous combined hormonal therapy. Use in some developing countries has also declined modestly, although the data are more limited. Among peri- and postmenopausal women in developed countries, current users of combined hormonal therapy tend to be younger and more highly educated, to have a lower body mass, and to use health care more regularly than non-users (IARC, 2007).

2. Cancer in Humans

2.1 Cancer of the breast

At the time of the first *IARC Monograph* of hormones and breast cancer (IARC, 1999), almost all of the epidemiological evidence came from studies that evaluated estrogen prescribed without a progestogen. Data on breast cancer risk related to estrogen plus a progestogen were deemed insufficient to reach any firm conclusions about the carcinogenicity of combined hormone therapy.

The next *IARC Monograph* on this topic (IARC, 2007) reviewed two randomized trials, ten cohort studies, and seven case–control studies on combined postmenopausal hormone therapy and breast cancer published up to and including 2004. These studies consistently reported an increased risk for breast cancer in users of estrogen plus progestogen therapy compared with non-users. The risk increased with increasing duration of use, was largely confined to current or recent users, and decreased soon after hormone treatment was stopped. Although the previous IARC evaluation concluded that there is *sufficient* evidence in humans for the carcinogenicity of combined estrogen–progestogen menopausal therapy in the breast, it was not possible to evaluate whether

breast cancer risk varied according to the type of progestogen or its dose, or according to the number of days each month that the progestogen was taken.

The present review of studies published through August 2008 includes four new systematic reviews, additional analyses from two clinical trials, five cohort studies, and four case–control studies, as well as many studies of time trends, and two trials of hormone therapy in breast cancer survivors. Studies were included if the authors provided risk estimates (odds ratios [OR], hazard ratios [HR] or relative risks [RR]) and 95% confidence intervals (CI) comparing breast cancer risk in women who used combined (estrogen plus progestogen) hormone therapy with non-hormone users for at least 1 year, or if the authors specified that at least 80% of estrogen-taking women were likely to be using combined therapy. Evidence from many studies of varying breast cancer incidence during recent years of increasing and then decreasing prescription of combined hormone therapy is also reviewed.

2.1.1 *Systematic reviews*

Four systematic reviews published after 2004 comprise studies of combined postmenopausal hormone use and breast cancer risk (Campagnoli *et al.*, 2005; Collins *et al.*, 2005; Greiser *et al.*, 2005; Shah *et al.*, 2005). All support the conclusion that the use of estrogen plus progestogen increases the risk of breast cancer in women, although each review included a somewhat different set of studies, offered some distinct conclusions based on the different studies chosen, had different definitions of hormone exposure, and different subset analyses related to different hormone regimens, duration, and recency of use.

Campagnoli *et al.* (2005) reviewed several publications, mainly those reviewed in the previous *IARC Monograph*, and confirmed a significantly increased risk of breast cancer risk with combined hormone therapy. In a subset analysis of ten studies comparing continuous combined progestogens with sequential progestogens, half of the studies suggested a higher risk with continuous combined therapy, but most differences were small and had overlapping 95% confidence intervals (see Table 2.1 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-13-Table2.1.pdf).

Another meta-analysis of papers published between 1989–2004 included 21 case–control studies, 15 cohort studies, and six controlled clinical trials. Trials and cohort studies had more cancer cases than case–control studies, but provided separate stratified analyses of estrogen plus progestogen for only three ever-never use comparisons, and four duration-of-use analyses. Most of the relevant results are from the subset of ten case–control studies that reported combined hormone treatment separately. The summary statistics showed an increased cancer risk for estrogen plus progestogen, especially for data after 1992, when estrogen plus progestogen in a single tablet became more widely available (Greiser *et al.*, 2005).

Shah *et al.*, (2005) published a meta-analysis of eight studies of current hormone therapy and breast cancer excluding women with a history of oral contraceptive use. The summary analysis showed that estrogen plus progestogen use for less than 5 years significantly increased breast cancer risk (OR, 1.35; 95%CI: 1.16–1.57), and use for more than 5 years showed a somewhat greater risk (OR, 1.63; 95%CI: 1.22–2.18).

A systematic review by Collins *et al.* (2005) included published data on estrogen plus progestogen and breast cancer from four randomized trials, two of which – WHI and Heart and Estrogen/Progestin Replacement Study (HERS) – were included in the previous *IARC Monograph*, and two other small earlier trials that together added only five cancer cases (all in the placebo group), which did not change the overall risk estimates. This review includes

very useful pre-planned subset analyses with the following results:

- the relative risk for breast cancer was lower in the intent-to-treat analysis (OR, 1.24; 95%CI: 1.01–1.54) than in the adherent women analysis (OR, 1.49; 95%CI: 1.13–1.96), compatible with the high drop-out and crossover rate in the WHI-Estrogen-Progestogen Trial (EPT);
- the dominance of the Million Women Study (Beral *et al.*, 2003) (1934 estrogen user cases) did not explain the excess breast cancer risk associated with combined hormone therapy in published studies, in that the published summary risk in the seven epidemiological studies that included the Million Women Study (OR, 1.70; 95%CI: 1.36–2.13) was very similar to the summary risk for the other six studies that did not include the Million Women Study (OR, 1.67; 95%CI: 1.29–2.17);
- the analysis confirms the WHI-EPT data showing a significantly increased risk of breast cancer begins within 5 years of initiating combined therapy and increases with increasing years of use; the analysis highlights an important result from the Million Women Study, showing similar relative risks for equine estrogen (OR, 1.29; 95%CI: 1.16–1.43) and estradiol (OR, 1.24; 95%CI: 1.12–1.37); and,
- an analysis (based on pooled data from the Million Women Study and the Danish Nurses Cohort) showed essentially identical risk for C21 progestogens (medroxyprogesterone acetate) and C19 progestogens (norethisterone, levonorgestrel) (OR, 2.14 for each).

[The Working Group noted that more than 400 user cases for the estradiol versus equine estrogen comparison and the more than 1900 user cases for the progestogen comparisons make it less likely that lack of power concealed clinically meaningful differences.]

2.1.2 Studies of changing breast cancer incidence in the context of changing patterns of menopausal hormone therapy use

The widely publicized increased risk of breast cancer in the WHI-EPT trial published in 2002 (Rossouw *et al.*, 2002) was followed by rapid and substantial drops in the incidence of invasive breast cancer in the USA (Clarke *et al.*, 2006; Glass *et al.*, 2007; Jemal *et al.*, 2007; Kerlikowske *et al.*, 2007; Ravdin *et al.*, 2007; Robbins & Clarke, 2007), Germany (Katalinic & Rawal, 2008), France (Allemand *et al.*, 2008), and Switzerland (Bouchardy *et al.*, 2006). The most striking changes were observed in countries with the highest rates of postmenopausal estrogen use before the WHI-EPT trial results were known. Declines were not reported in Norway or Sweden (Zahl & Maehlen, 2007) or in African-Americans in the USA (Hausauer *et al.*, 2007). Most of the studies were based on representative regional or national cancer registries, with validated diagnoses, but had only self-reported data on individual hormone treatment or mammography.

A recent review of 21 papers published from 1987–2007 on the incidence of breast cancer in the USA in the 1980s (Krieger, 2008) documented the rise in breast cancer in women aged 50 years and older (9.9% per year in the 1980s) and the decrease (by 50%) since 2002 in the context of changing frequency of hormone therapy. In a French study (Allemand *et al.*, 2008), the age-adjusted breast cancer incidence increased 2.1% per year during 2000–04; decreased 4.3% during 2004–05, and 5.3% during 2005–06, a decrease observed only in women aged 50 years and older. This decrease was not likely to be explained by decreased mammography after cessation of hormones because the use of mammography increased by 335% during 2000–06.

The only study of time trends in breast cancer incidence that provides data on cancer incidence by cancer stage and estrogen receptor positive (ER+) status, mammography use, and hormone prescription rates in the same cohort comes from a large pre-paid health care plan in the USA, which included screening mammography, pharmacy dispensing of hormone therapy, and a tumour registry (Glass *et al.*, 2007). The age-adjusted incidence rates of breast cancer increased by 25% from the early 1980s to 1992–93, then increased an additional 15% through 2000–01, and dropped by 18% in 2003–04. Increases were mainly for ER+ breast cancers in women aged 45 years and older. During 1993–2006, 75–79% of women older than 45 years of age were screened within the previous 2 years. Postmenopausal hormone prescriptions, primarily estrogen plus progestogen, increased during 1988–2002, and dropped by approximately 75% after 2002, coincident with the publication of the WHI-EPT (estrogen plus progestogen) clinical trial results that showed that combined hormone therapy increased the risk of heart disease and breast cancer (Rossouw *et al.*, 2002).

Overall, the studies of time trends in ER+ breast cancers in women aged 45 or older are compatible with a substantial increase in breast cancer risk associated with increasing menopausal hormone use, with a remarkable decrease concurrent with the release of the WHI-EPT results. Although the increase in breast cancer could be partly explained by the increasing use of mammography in women concordant with increasing hormone therapy, the decrease in breast cancer concurrent with reduced hormone was not explained by a decrease in mammography.

2.1.3 Cohort studies

All five cohort studies (Stahlberg *et al.*, 2004; Fournier *et al.*, 2005; Lee *et al.*, 2006; Rosenberg *et al.*, 2006a; Corrao *et al.*, 2008) reported an increased risk of breast cancer in postmenopausal women using estrogen plus progestogen (see Table 2.2 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-13-Table2.2.pdf).

In a cohort study using data from a previously published Danish Nurses Study, Stahlberg *et al.* (2004) reported that current estrogen plus cyclical progesterone-like progestogen, cyclical testosterone-like progestogen, or continuous testosterone-like progestogen were each significantly associated with an increased risk of ductal carcinoma (OR, 3.10; 95%CI: 1.69–5.67; OR, 2.15; 95%CI: 1.31–3.54; and OR, 4.10; 95%CI: 2.29–7.30, respectively).

Fournier *et al.* (2005) reported the risk of breast cancer in 54548 postmenopausal French women who were followed an average of 2.8 years postmenopause, during which time 948 new invasive breast cancers were diagnosed. There was a similar increased risk of breast cancer with transdermal/percutaneous (RR, 1.4; 95%CI: 1.2–1.7) and oral estrogens (RR, 1.5; 95%CI: 1.1–1.9) when combined with a synthetic progestogen, compared to the risk in non-hormone users. There was no increased risk when estrogen was combined with micronized progesterone (RR, 0.9; 95%CI: 0.7–1.2) (*P* test for heterogeneity < 0.001).

Lee *et al.* (2006) reported a prospective study of combined hormone therapy and breast cancer in 55371 African-American, Native Hawaiian, Japanese-American, Latina, and Caucasian postmenopausal women from the US Multiethnic Study. The authors provide no information about the type of estrogen or progestogen used, but combined equine estrogen and medroxyprogesterone acetate accounted for more than 80% of combined hormone prescriptions in the USA during the mid-and late 1990s. Current use of estrogen plus progestogen was associated with a significantly increased risk of breast cancer within the first 5 years of use (adjusted RR, 1.43; 95%CI: 1.06–1.93), and this risk increased with duration of use. The increased risk was

associated with both ductal and lobular cancer, more advanced cancer (based on standard pathological criteria), and ER+/Progesterone Receptor positive (PR+), ER+/PR-, and ER-/PR- tumours. It was statistically significant in all ethnic groups, and persisted after adjusting for the lower frequency of mammograms in women not taking hormones.

Rosenberg *et al.* (2006a) reported an 8-year follow-up study of 32559 African-American women. Compared to non-hormone users, the incidence rate ratio associated with 10 or more years of estrogen plus progestogen was 1.45 (95%CI: 0.94–2.23). Shorter durations of use were not associated with estrogen plus progestogen therapy in these African-American women.

Corrao *et al.* (2008) followed 73505 Italian women in Lombardia who had received at least one prescription for postmenopausal hormone therapy in 1998–2000, and were followed until 2005. More than 88% began treatment using transdermal estradiol; and combined hormone use was assumed because few Italian women have had a hysterectomy. Breast cancer risk increased with duration of therapy and was greater with oral than with transdermal estradiol. The odds ratio for at least 25 months of transdermal estradiol was 1.27 (95%CI: 1.07–1.51), compared to 2.14 (95%CI: 1.43–3.21) for oral estradiol (P for heterogeneity < 0.01). This difference is consistent with and somewhat larger than that reported in the initial report from the Million Women Study (Beral *et al.*, 2003), with a similar mean follow-up of 2.6 years.

A new analysis from Prentice *et al.* (2008) combined data from the WHI-EPT trial (n = 16608) and the subset of women from the WHI observational study who were either not taking hormones at baseline (n = 32084) or were users at enrollment of the same hormone regimen used in the WHI-EPT trial (n = 25328). Women included from the observational study were also required to have had a mammogram in the past two years, to parallel the protocol in the trial. Hazard ratios for estrogen plus progestogen users compared to non-users were close to 2 (95%CI: 1.86–2.20) in the trial and the observational study groups, except for the clinical trial participants without prior hormone use who had a much smaller risk ratio (1.13). Women who initiated hormone therapy within 5 years of the menopause had a significantly higher risk of breast cancer than those who initiated hormone therapy later; and the risk increased with duration of use (HR, 1.85; 95%CI: 1.03–3.34) for 2–5 years of use and (HR, 2.75; 95%CI: 1.73–4.39) for more than 5 years of use.

A new analysis of the Million Women Study by Reeves *et al.* (2006) assessed the relative risk of current hormone therapy for different histological types of breast cancer. In analyses of current combined hormone use versus never use, the risk was significantly increased for all types of cancers for which there were more than 50 cases, including lobular cancer (n = 503, RR, 2.80; 95%CI: 2.46–3.18), tubular cancer (n = 186, RR, 3.51; 95%CI: 2.80–4.41), and ductal cancer (n = 2241, RR, 2.0; 95%CI: 1.89–2.12). The risk for these three most common types of breast cancer increased significantly with increasing duration of therapy.

2.1.4 Case–control studies

Four case–control studies also showed an increased risk of breast cancer in women using estrogen plus progestogen (Li *et al.*, 2006; Rosenberg *et al.*, 2006b, c; Wu *et al.*, 2007; see Table 2.3 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-13-Table2.3.pdf).

In a Swedish study (Rosenberg *et al.*, 2006c), women who had used a medium-potency estrogen (i.e. not estriol) plus a progestogen for 5 or more years had a significantly increased risk of lobular cancer (OR, 5.6; 95%CI: 3.2–9.7), tubular cancer (OR, 6.5, 95%CI: 2.8–14.9), and ductal cancer (OR, 2.3; 95%CI: 1.6–3.3). In another report

from the same study (Rosenberg *et al.*, 2006b), estrogen (mainly estradiol) plus a progestogen (mainly levonorgestrel or norethisterone) for at least 5 years significantly increased the risk of ER+/PR+ cancers (OR, 3.0; 95%CI: 2.1–4.1) but not ER-/PR- tumours (OR, 1.3; 95%CI: 0.7–2.5).

A study of Asians (Chinese, Japanese or Filipino) living in the USA (Wu *et al.*, 2007) found a 26% increased risk of breast cancer among current users of estrogen plus progestogen for each 5 years of use (OR, 1.26; 95%CI: 1.04–1.52).

Li *et al.* (2006) reported breast cancer histology in a multicentre case–control study in the USA. Compared to never use, current use of estrogen plus progestogen was associated with an increased risk of all types of breast cancer; the excess risk was statistically significant for ductal-lobular (OR, 2.9; 95%CI: 1.7–4.9) and tubular (OR, 3.2; 95%CI: 1.3–7.5) cancers.

2.1.5 Postmenopausal breast cancer risk with prior oral contraceptive use, hormone therapy use, or both

Dumeaux *et al.* (2005) reported breast cancer risk in current hormone users according to prior use of hormones in a French cohort of 68670 postmenopausal women. The most widely used postmenopausal hormone regimen was transdermal estrogen in combination with either micronized progesterone or a progesterone derivative. In women currently using hormone therapy, a history of prior oral contraceptive use did not further increase the risk (OR, 0.91; 95%CI: 0.81–1.03), but women with a history of postmenopausal hormone use did have an increased risk (OR, 1.41; 95%CI: 1.26–1.59). Results were similar for women who had used both oral contraceptives and postmenopausal hormones (OR, 1.43; 95%CI: 1.25–1.64).

In contrast, in a Norwegian cohort study of 30118 postmenopausal women, reported by Lund *et al.* (2007), current users of hormone therapy sustained a significantly greater risk of breast cancer if they were former oral contraceptives users (RR, 2.45; 95%CI: 1.92–3.12) than if they had never used oral contraceptives (RR, 1.67; 95%CI: 1.32–2.12) (P = 0.002). The odds ratios in current hormone users who were past oral contraceptives users were very similar in women currently using estrogen (primarily estradiol) alone (OR, 2.63; 95%CI: 1.65–4.20) or estrogen plus a progestogen (primarily a testosterone derivative) (OR, 2.55; 95%CI: 1.94–3.35).

2.1.6 New WHI-EPT clinical trial analyses

Anderson *et al.* (2006) compared breast cancer rates in women in the WHI-EPT trial according to their history of menopausal hormone use before beginning the trial. Despite controlling for an extensive list of potential confounders, there was a significantly greater risk of invasive breast cancer in women who reported pre-WHI postmenopausal hormone use (HR, 1.96; 95%CI: 1.17–3.27) compared to the women without such a history (HR, 1.02; 95%CI: 0.77–1.36). These women also had a significantly higher risk of a larger tumour, higher number of positive nodes, and more regional/metastatic disease, but the number of women in these subsets was small and the 95% confidence intervals were large. The authors note that they did not adjust for multiple comparisons and that there were significant differences between the women who did or did not use hormones before entering the WHI-EPT trial, not all of which could be characterized well enough to exclude residual confounding.

Heiss *et al.* (2008) examined the effect of stopping combined hormone treatment 2–3 years after the WHI-EPT trial was stopped. More invasive breast cancers occurred in women assigned to continuous estrogen and progestogen than women assigned to placebo (HR, 1.27; 95%CI: 0.91–1.78), with a risk similar to that observed for combined therapy during the trial (HR, 1.26; 95%CI: 1.02–1.55). These data are the strongest evidence for a continued excess risk 2–3 years

after abrupt cessation of hormone therapy. [The Working Group noted that one limitation of this study is that women who abruptly stopped hormone therapy at the end of the trial may have restarted within a year when menopause symptoms recurred.]

In conclusion, many new studies confirm the earlier studies reporting that estrogen plus progestogen increases the risk of breast cancer in postmenopausal women. The more recent studies provide additional evidence that the increased risk begins within 5 years of initiating combined therapy and increases with increasing duration of therapy. The data are insufficient to determine whether the risk differs by estrogen type, dose, or route of administration or the progestogen type (progesterone or progesterone-derived versus testosterone derived) or regimen (continuous versus cyclic) progestogen. The data provide no consistent evidence that any histological type of cancer is more often associated with combined hormone therapy in postmenopausal women.

2.1.7 *Postmenopausal hormone therapy after breast cancer*

Col *et al.* (2005) published a meta-analysis of two uncontrolled and unblinded randomized trials and eight observational studies of breast cancer survivors, of whom 1316 reported unspecified hormone therapy and 2839 did not. The trials showed that hormone therapy increased breast cancer recurrence (RR, 3.41; 95%CI: 1.59–7.33), while the observational studies suggested it reduced risk (RR, 0.64; 95%CI: 0.50–0.82). The apparently protective associations in observational studies were attributed by the authors to probable selection for hormone treatment of younger, more often node-negative women.

Holmberg & Anderson (2004) reported preliminary results from HABITS, a randomized clinical trial of hormone therapy for menopause symptoms in Scandinavian patients aged 40–70 years old who had a history of breast cancer (up to Stage II), were free of recurrence an average of 3 years after cancer treatment, had no other serious disease, and had severe menopause symptoms. Women were randomly assigned to hormones or to the best available symptom treatment without hormones. Hormone treatment in women without a hysterectomy included either cyclic or continuous estrogen and progestogen. At the time the trial was stopped early for harm, after an average of 2 years with at least one follow-up visit, the hazard ratio for women assigned to any estrogen regimen with or without a progestogen was 3.5 (95%CI: 1.5–8.1). The risk was not changed after adjusting for prior use of hormone replacement therapy, tamoxifen, and ER positivity.

In a follow-up study, Holmberg *et al.* (2008) showed persistence of harm after an extended 4-year follow-up of 442 of the 447 women in the HABITS trial; 39 of the 221 women in the hormone-treated group and 17 of the 221 women in the untreated group had experienced recurrent breast cancer (HR, 2.4; 95%CI: 1.3–4.2), i.e. 19 additional cases since the trial had been stopped. Compared to the 100 women taking estrogen plus continuous progestogen, the hazard ratio for recurrence was increased in women taking different types of hormone therapy (HR, 1.4 for the 150 women taking estrogen plus a continuous progestogen; 1.4 for the 100 taking sequential progestogen, and 1.4 for the 53 women taking progestogen, primarily norethisterone acetate), but numbers in each group were small and confidence intervals were wide.

Concurrent with the HABITS trial, the Stockholm randomized clinical trial also enrolled Swedish women with a history of breast cancer (von Schoultz & Rutqvist, 2005). The authors reported that the 77% of women who had had a hysterectomy were treated with estradiol valerate, and 23% with estradiol plus cyclic or spaced low-dose medroxyprogesterone acetate. After a median follow up of 4.1 years, there were 11 breast cancer recurrences in

the hormone-treatment group and 13 in the control group (HR, 0.82; 95%CI: 0.35–1.9). The authors speculated that the absence of harm in the Stockholm trial could reflect the low dose of medroxyprogesterone instead of the norethisterone acetate progestogen used in HABITS, but they also report that the women in this trial had less node-positive cancer, and were more likely to have had prior treatment with tamoxifen than women in the HABITS trial.

In summary, there is consistent evidence that combined estrogen–progestogen menopausal therapy increases the risk of breast cancer. There is evidence for an increasing risk with increasing duration of use among current users. However, determining whether all current formulations and treatment regimens are equally carcinogenic is not possible on the available data.

2.2 Cancer of the endometrium

The previous *IARC Monograph* (IARC, 2007) concluded that there is *sufficient evidence* that estrogen plus progestogen for at least 14 days can prevent estrogen-induced endometrial cancer. The most compelling evidence was from the Million Women Study (Beral *et al.*, 2005), and from the WHI-EPT trial (Anderson *et al.*, 2003), where endometrial cancer rates were low and were not increased by 5 years of continuous combined estrogen plus progestogen in a single tablet. The Million Women Study (1320 endometrial cancer cases) showed that the protective effect of a progestogen added to daily estrogen increased with increasing number of days/month that progestogen was used, while the WHI-EPT results (58 endometrial cancer cases) related only to daily (continuous) combined hormone therapy and only to one progestogen, medroxyprogesterone acetate.

Since the last *IARC Monograph*, two new case–control studies and two cohort studies have been published on the association of estrogen plus progestogen with endometrial cancer. All are from the USA, where more than 80% of combined estrogen regimens were conjugated equine estrogen plus medroxyprogesterone acetate. The newer studies provide contradictory evidence about the minimum progestogen required to reduce the estrogen-induced risk of endometrial cancer.

In a population-based case–control study in Pennsylvania, Strom *et al.* (2006) compared 511 endometrial cancer cases detected by active surveillance of regional hospitals with 1412 controls of similar age identified mainly from random-digit dialling. The history of hormone use was determined mainly by telephone, using a structured questionnaire and memory aids mailed in advance. Use of combined hormones of any duration was not associated with an increased risk of endometrial cancer (OR, 0.8; 95%CI: 0.6–1.1) (see Table 2.4 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-13-Table2.4.pdf). Numbers were too small for useful comparisons of continuous and sequential progestogen therapy.

Using data from three population-based case–control studies in different counties of Washington state, Weiss *et al.* (2006) examined the aggressiveness of endometrial cancer (based on pathology review) in 1304 cases from the state cancer registry, and 1779 controls of similar age who were recruited by random-digit dialling. Combined hormone therapy was not significantly associated with aggressive endometrial cancer (OR, 1.6; 95%CI: 0.4–7.2). The risk for the least aggressive endometrial cancer was increased with combined therapy when the progestogen was used for less than 10 days a month for at least 4 years (OR, 6.2; 95%CI: 3.2–12.0) and for 10–24 days/month for at least 4 years (OR, 2.9; 95%CI: 1.6–5.0).

In a cohort study of 30379 women from the US National Cancer Institute Breast Cancer Detection Demonstration Project, started in 1979 with a 13-year follow-up, combined hormone use was first queried in 1987–89, and

periodically thereafter by mail and telephone (Lacey *et al.*, 2005). Cancer was identified from self-reports, medical records, cancer registries, and death certificates. Endometrial cancer risk was increased with exclusive use of estrogen plus progestogen for less than 15 days/month (RR, 3.0; 95%CI: 2.0–4.6, 32 cases) and for more than 15 days/month (RR, 2.3; 95%CI: 1.3–4.0, 15 cases) (see Table 2.5 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-13-Table2.5.pdf). Risk increased with increasing duration of use for both regimens.

A cohort study of 73211 women from the National Institutes of Health-AARP Diet and Health Study included 51312 women who either never used estrogen or only used estrogen plus progestogen for at least 10 days/cycle (Lacey *et al.*, 2007). Hormone use was self-reported by mail, and cases were ascertained from state cancer registries and death indices. Compared to non-hormone users, neither estrogen plus progestogen for 10–14 days/cycle (RR, 0.74; 95%CI: 0.39–1.40, based on 11 cases) or for at least 20 days/cycle (RR, 0.80; 95%CI: 0.55–1.15, based on 35 cases) was associated with an increased risk of endometrial cancer. Similar results were seen in analyses restricted to women who had used combined therapy with either regimen for at least 3 years.

In conclusion, three of the four new US studies do not show consistent prevention of estrogen-associated endometrial cancer risk in women taking estrogen plus sequential progestogen (mainly medroxyprogesterone acetate) for at least 15 days a month, and contradict findings reported in many earlier studies (Anderson *et al.*, 2003; Beral *et al.*, 2005). [The Working Group noted that the reason for these differences is not clear, but poor hormone regimen recall or adherence cannot be excluded.]

There is consistent evidence that the risk of endometrial cancer is increased in women taking unopposed estrogen, and the increased risk remains evident when the opposing progestogen is taken for less than 15 days per month. The risk of endometrial cancer is inversely associated with the number of days per month that progestogens are allied to the regimen. It is not known whether continuous use (or daily use) reduces the risk of endometrial cancer compared to baseline.

2.3 Cancer of the colorectum

At the time of the previous *IARC Monograph* (IARC, 2007), data from two randomized trials, four cohort studies, and three case–control studies showed no elevated risks of colorectal cancer in women taking combined postmenopausal hormones. In fact, all but one study showed a relative risk estimate less than one (with a statistically significant reduction in two of these) suggesting protection. The reduced risk was mainly in recent users and unrelated to duration of use. Since the last review, there have been two new case–control studies and one cohort study that included analyses largely restricted to (or almost entirely restricted to) estrogen plus progestogen regimens.

A US population-based case–control study conducted in Washington state of 578 cancer cases and 590 controls, in which a history of hormone use and covariates was based on a 60-minute telephone interview, showed a 40% reduced risk of colorectal cancer (OR, 0.60; 95%CI: 0.05–0.09) in women who used combined hormone therapy exclusively for at least 5 years compared to non-users (Newcomb *et al.*, 2007) (see Table 2.6 available at http://monographs.iarc.fr/ ENG/Monographs/vol100A/100A-13-Table2.6.pdf). The reduced risk was restricted to current users. There was no association with cancer stage at diagnosis.

A German case–control study of 354 cases and 1422 age-matched controls (Dinger *et al.*, 2007) showed no significant associations with colorectal cancer by progestogen type (medroxyprogesterone use was rare), duration of progestogen use, or sequential versus continuous progestogen

use. The number of cancer cases in each subset analysis was small.

A cohort study from Lund, Sweden (Nazeri *et al.*, 2006) included 2452 women who reported estrogen plus progestin therapy, primarily a fixed-dose preparation containing 2 mg of estradiol and 2 mg of norethisterone, and 3600 women not taking postmenopausal hormones. There was a reduced risk of colorectal cancer in women taking combined hormones (OR, 0.18; 95%CI: 0.04–0.84) based on only 16 cases in the no-hormone group and two cases in the women reporting mostly combined hormone therapy (see Table 2.7 available at http://monographs.iarc.fr/ ENG/Monographs/vol100A/100A-13-Table2.7.pdf). Information about the type of estrogen or progestogen was not provided.

[The Working Group noted that one of two case–control studies and a single cohort study found that combined hormone therapy reduced the risk of colorectal cancer, which is compatible with suggestive evidence in the previous *IARC Monograph*. One of these three studies found that reduced risk was seen only with current hormone use of combined hormone therapy. These results are suggestive but insufficient to conclude that estrogen plus progestogen therapy protects against colorectal cancer, or to conclude that this putative effect varies by type of estrogen or progestogen used.]

2.4 Cancer of the ovary

In the previous *IARC Monograph* (IARC, 2007), results from two randomized trials, four cohort studies, and three case–control studies were inadequate to establish an association between ovarian cancer and combined estrogen–progestogen therapy. The largest clinical trial (WHI), in which 16608 women were assigned to conjugated equine estrogen and continuous medroxyprogesterone acetate or placebo, did not show a significantly increased risk of ovarian cancer (RR, 1.58; 95%CI: 0.77–3.24) (Anderson *et al.*, 2003).

Since the previous *IARC Monograph*, there have been two new case–control studies, three new cohort studies, and one new meta-analysis on estrogen plus progestogen therapy and risk of ovarian cancer.

Moorman *et al.* (2005) reported a North Carolina (USA) population-based case–control study of ovarian cancer with 364 cases and 370 controls in postmenopausal women (see Table 2.8 available at http://monographs.iarc.fr/ ENG/Monographs/vol100A/100A-13-Table2.8.pdf). Exposure to hormone therapy and covariates was obtained by a 90-minute interview. Sources of cancer cases included a rapid case ascertainment system and a state-wide cancer registry (only 70 cases and 87 controls used combined estrogen and progestogen exclusively). The only significant association was in women who had used combined hormones, but not exclusively, for > 119 months (22 cases, 14 controls); these women had an odds ratio of 2.4 (95%CI: 1.1–5.3). Types of cancer did not differ by hormone use history.

Rossing *et al.* (2007) reported on a US case–control study of 1054 women (440 cases, 614 controls) in western Washington state. Hormone exposure was determined by interview, aided by photographs to identify pills. Cancer data were from the regional cancer registry. No increased risk was reported among current users who used only estrogen plus progestogen, regardless of duration of use (OR, 1.1; 95%CI: 0.8–1.5).

The Million Women Study of postmenopausal women is the most compelling new cohort study based on its size, prospective design, large number of ovarian cancer cases, data on histological subtypes, high proportion of women taking hormone therapy (30%), and data for the most important covariates including hysterectomy (Beral *et al.*, 2007). In analyses limited to estrogen plus progestogen use (different stratified analyses included 69263 cancer cases), the

risk of ovarian cancer was significantly increased only in women who had taken combined hormones for at least 5 years (RR, 1.53; 95%CI: 1.27–1.84), and was similar for continuous or combined regimens. The risk was significantly increased for estrogen plus norethisterone, non-significantly increased for norgestrel, and not increased for medroxyprogesterone acetate. The overall increased risk of hormone therapy was primarily for epithelial serous tumours; histological data were not shown stratified by combined hormone therapy (see Table 2.9 available at http://monographs.iarc.fr/ ENG/Monographs/vol100A/100A-13-Table2.9.pdf).

A second cohort study from the NIH-AARP Diet and Health Study (Lacey *et al.*, 2006), which included 97638 postmenopausal women (of whom 73483 did not undergo a hysterectomy, and 51698 had used no hormone therapy or only estrogen plus progestogen), reported a significantly increased risk associated with 5 or more years of estrogen plus progestogen use either sequentially (RR, 3.09; 95%CI: 1.68–5.68) or continuously (RR, 1.82; 95%CI: 1.03–3.23).

The US Nurses Health Study followed 7394 postmenopausal women who had at some point reported current use of combined estrogen plus progestogen and 20853 never users (Danforth *et al.*, 2007) (the cohort was followed from 1976–2002, but few women were using estrogen plus progestin until the 1980s, and the duration of relevant follow-up and the number of cases for different analyses are not clear). No significant association between estrogen plus progestogen use and epithelial ovarian cancer was observed (RR, 1.04; 95%CI: 0.82–1.32, based on 82 cases), or by duration or recency of use, but there were only ten ovarian cancer cases among women using estrogen plus progestogen exclusively. There was also no association with serous (RR, 1.12; 95%CI: 0.84–1.51, based on 49 cases) or endometrioid tumours (RR, 1.04; 95%CI: 0.53–2.03, based on 15 cases).

A meta-analysis of 42 studies of hormone therapy and ovarian cancer published from 1966–2006 included 30 case–control studies, seven cohort studies, one randomized clinical trial and four cancer registry studies, with 12238 ovarian cancer cases. The summary risk for ever versus never estrogen plus progestin use, based on 31 data sets but no explicitly stated number of cancer cases, was 1.11 (95%CI: 1.02–1.21). There was no evidence for a significantly increased risk per year of use (22 data sets). Risks were slightly greater for European women (OR, 1.06; 95%CI: 1.03–1.09, based on 14 data sets) than for North American women (OR, 1.00; 95%CI: 0.96–1.04, based on seven data sets). Within the estrogen plus progestogen data sets, there were no differences by histological subtypes of ovarian cancer. Funnel plots suggest publication bias (not publishing small studies showing increased cancer risk) (Greiser *et al.*, 2007).

The overall risk estimate in a recent meta-analysis was 1.1 for ever versus never use. The two new case–control studies and three new cohort studies of ovarian cancer and combined hormone therapy suggest only very small increased ovarian cancer risk after use for 5 or more years; however, the evidence is not consistent across studies.

2.5 Cancer of the skin

Since the previous *IARC Monograph* (IARC, 2007), an updated analysis of data from a hospital-based case–control study in San Francisco, USA (Lea *et al.*, 2007), and a report on a hospital-based case-control study in Italy (Naldi *et al.*, 2005) have been published. These were based on 318 cases and 395 frequency-matched controls, and 316 cases and 308 controls, respectively. Neither studies showed any association between menopausal therapy and risk of cutaneous melanoma.

2.6 Cancer of the thyroid

Since the previous *IARC Monograph* (IARC, 2007), results from a population-based case–control study in New Caledonia, France, an area with an unusually high incidence of thyroid cancer, have been published (Truong *et al.*, 2005). Answers to in-person interviews of 293 cases and 354 controls selected from electoral rolls were compared. The odds ratio in women who ever took menopausal therapy at age 45 years or above was 0.9 (95%CI: 0.4–2.2), and no trend in risk with duration of use to 5 years or more was observed.

2.7 Lymphomas and leukaemias

Since the previous *IARC Monograph* IARC (2007), a Danish population-based cohort study of menopausal therapy and risk of non-Hodgkin lymphoma was published and was based on 157024 women aged 40 years or more of whom 23708 were users of menopausal therapy, followed for 13 years with linkage to the health service to determine menopausal therapy prescriptions and to the Danish Cancer Registry to identify cases (40 among users and 310 among non-users) (Nørgaard *et al.*, 2006). The odds ratio for ever use of menopausal therapy was 0.99 (95%CI: 0.71–1.39), no trend in risk with duration of use to 20 or more years was observed.

Ross *et al.* (2005) evaluated the effect of menopausal therapy on risk of leukaemia in a cohort of 37172 postmenopausal women (aged 55–69 years at entry) in Iowa, USA. A total of 201 cases of leukaemia were identified over 16 years of follow-up of which 71 had ever used menopausal therapy. The relative risk for ever use of menopausal therapy was 0.87 (95%CI: 0.65–1.16), with little difference according to type of leukaemia, and no trend in risk with duration of use of 5 or more years was observed.

2.8 Cancers of the central nervous system

In the cohort study based on the Canadian National Breast Screening study (Silvera *et al.*, 2006), 59 incident glioma cases occurred during an average 16.4 years of follow-up in postmenopausal women. Based on answers to a self-administered questionnaire at recruitment into the cohort, the hazard ratio for gliomas was 0.92 (95%CI: 0.54–1.55) in women who ever used menopausal therapy, and no trend in risk with duration of over 3 years of use was observed.

In a population-based case–control study of 45 postmenopausal women with gliomas and 182 controls in Sweden (Wigertz *et al.*, 2006), the odds ratio in women who ever used menopausal therapy was 0.9 (95%CI: 0.4–1.7), and risk did not vary appreciably with duration of use. Among 108 cases of meningioma compared to 185 controls, the odds ratio for ever use of menopausal therapy was 1.7 (95%CI: 1.0–2.8) but no consistent duration–response relationship was observed, the risk being highest among those who had used menopausal therapy for less than a year.

In a cohort study based upon records from the Mayo clinic of 335318 women aged 26–86 years, 18037 were ever users of menopausal therapy (Blitshteyn *et al.*, 2008). Among 1390 women with meningioma, 156 were ever users of menopausal therapy. The odds ratio for ever use of menopausal therapy was 2.2 (95%CI: 1.9–2.6, adjusted for age), with little or no variation by age over 55 years. No analysis of risk by duration of use was reported.

2.9 Cancer of the urinary tract

In a Canadian cohort study of women enrolled in a breast cancer screening trial (Kabat *et al.*, 2007), the hazard ratio for renal cell cancers in women who ever used menopausal therapy was

0.98 (95%CI: 0.69–1.41), and no trend in risk with duration of use was observed.

Two cohort studies in the USA showed no increased risks of cancers of the urinary bladder in users of menopausal therapy. During approximately 26 years of follow-up of 116598 women enrolled in the Nurse's Health Study (McGrath *et al.*, 2006), 22540 were postmenopausal, among which 307 cases of bladder cancer were diagnosed. The use of hormones was ascertained periodically during the follow-up period by mailed questionnaire. The relative risk in postmenopausal women with current use of menopausal therapy (defined in the study as estrogen plus progestogen) was 0.75 (95%CI: 0.44–1.26, based on 18 cases). No analysis of risk by duration of use was reported.

During an average follow-up of 15.3 years, 167 cases of bladder cancer developed in a cohort of 54308 women who were enrolled in the Breast Cancer Detection Demonstration Project (Cantwell. *et al.*, 2006). Menopausal therapy use was based on answers to telephone interviews at the time of recruitment. The relative risk of bladder cancer was 0.98 (95%CI: 0.71–1.37) in women who ever used menopausal therapy. No analysis of risk by duration of use was reported.

2.10 Cancer of the lung

La Vecchia (2006) reviewed the studies relating to menopausal therapy and lung cancer, largely comprising the data considered in the previous *IARC Monograph* (IARC, 2007), and concluded that there was no consistent association between menopausal therapy and risk for lung cancer.

A case–control study nested in the Royal College oral contraceptive study (Elliott & Hannaford, 2006) found no increased risk of lung cancer in ever users of menopausal therapy. No analysis of risk by duration of use was reported. The odds ratio for current users (at the time of diagnosis) was 1.2 (95%CI: 0.2–6.4).

In the Canadian cohort study of women enrolled in a breast cancer screening trial (Kabat *et al.*, 2007), the hazard ratio for lung cancer in women who ever used menopausal therapy was 1.05 (95%CI: 0.85–1.32). No analysis of risk by duration of use was reported.

Liu *et al.* (2005) reported on a population-based cohort study of 44677 middle-aged never-smoking Japanese women, followed for 8–12 years, with 153 lung cancers diagnosed. For women with a natural menopause, the relative risk from hormone use [not further characterized] was 1.19 (95%CI: 0.61–2.30), but for women with an induced menopause, it was 2.40 (95%CI: 1.07–5.40). No analysis of risk by duration of use was reported. [The Working Group considered that the majority of women with an induced menopause were likely to have received estrogen alone.]

Chen *et al.* (2007) conducted a case–control study of 826 women with lung cancer and 531 healthy controls in Taiwan, China. The odds ratio for ever use of menopausal therapy was 0.70 (95%CI: 0.53–0.94). Although no findings by duration of use were presented, many stratified analyses were conducted (e.g. by smoking status, age, exposure to cooking fumes, and family history of lung cancer), all showed similar inverse associations though several of the upper confidence intervals were > 1.0.

2.11 Cancer of the pancreas

In a Canadian cohort study of women enrolled in a breast cancer screening trial (Navarro Silvera *et al.*, 2005), the hazard ratio for pancreatic cancer in women who formerly used menopausal therapy was 0.86 (95%CI: 0.55–1.35), and 0.76 (95%CI: 0.47–1.24) among current users. However, no trend in risk with duration of use was observed.

2.12 Cancer of the stomach

A population-based case–control study in ten Canadian provinces (Frise *et al.*, 2006) compared answers to a self-administered questionnaire by 326 women with gastric adenocarcinoma to answers from an equal number of age-matched controls. The odds ratio in women who ever used menopausal therapy was 0.72 (95%CI: 0.37–1.40). A slight trend in reduction in risk with increasing duration of use was observed, with an odds ratio for 15 or more years of use of 0.42 (95%CI: 0.15–1.16).

2.13 Cancer of the cervix

No further evidence has been published that alters the conclusions reached since the previous *IARC Monograph* (IARC, 2007). There is little evidence to suggest that combined estrogen–progestogen therapy alters the risk for cervical cancer.

2.14 Cancer of the liver

No further evidence has been published that alters the conclusions reached since the previous *IARC Monograph* (IARC, 2007). The data for liver cancer remains too sparse for evaluation.

2.15 Synthesis

A large body of evidence was evaluated for several organ sites, among which the Working Group concluded combined estrogen–progestogen menopausal therapy causes cancer of the breast, and of the endometrium. The increased risk for estrogen-induced endometrial cancer decreases with the number of days per month that progestogens are added to the regimen.

For cancer of the colorectum, the Working Group concluded that it is unlikely that the use of combined estrogen–progestogen menopausal therapy increases the risk of cancers of the colon or rectum. The Working Group concluded that the use of combined estrogen–progestogen menopausal therapy is unlikely to alter the risk of cancer of the thyroid, lung, stomach, liver, urinary tract, pancreas, ovary, cervix, or the risk of lymphoma and leukaemia, cutaneous melanoma, and tumours of the central nervous system.

3. Cancer in Experimental Animals

3.1 Summary of the previous *IARC Monograph*

Oral administration of combined hormonal therapy in mice that are prone to develop mammary tumours resulted in similar incidences of mammary tumours in controls, and in animals treated with conjugated equine estrogens alone or with conjugated equine estrogens plus medroxyprogesterone acetate. However, tumour latency was reduced in animals treated with conjugated equine estrogens plus medroxyprogesterone acetate. Conjugated equine estrogens plus medroxyprogesterone acetate suppressed the development of uterine adenomyosis (Sakamoto *et al.*, 1997a; IARC, 2007).

Oral administration of conjugated equine estrogens alone or with medroxyprogesterone acetate to ovariectomized rats pretreated with the carcinogen 7,12-dimethylbenz[*a*]anthracene increased the incidence of mammary tumours with equal frequency, and to a level equal to that in non-ovariectomized controls (Sakamoto *et al.*, 1997b; IARC, 2007).

3.2 Studies published since the previous *IARC Monograph*

3.2.1 Estradiol and progesterone

(a) Mouse

Medina *et al.* (2007) and Rajkumar *et al.* (2007) transplanted mammary ducts from the glands of *p53* null BALB/c female mice into the cleared mammary fat pads of *p53* wild-type female mice, and also used the activated Her-2/neu transgenic mouse (FVB) model to show in transplanted mice and FVB mice that short-term exposure (2 and 3 weeks) to estradiol and progesterone significantly decreases mammary carcinogenesis in pre-pubertal and mature mice.

(b) Rat

Blank *et al.* (2008) used intact and ovariectomized ACI rats to study the role of progesterone in mammary carcinogenesis. The animals were subcutaneously implanted with low- or high-dose estradiol, progesterone alone, low-dose estradiol plus progesterone, and ovariectomized ACI rats with high-dose estradiol plus progesterone. Also, ovariectomized ACI rats were treated with high-dose estradiol plus progesterone plus testosterone propionate to determine the role of the androgen in hormonal mammary carcinogenesis. In intact but not in ovariectomized rats, continuous exposure to high concentrations of estradiol alone induced mammary carcinogenesis. In ovariectomized ACI rats, mammary carcinogenesis require continuous exposure to high concentrations of estradiol and progesterone. Testosterone had no effect on tumour incidence.

3.2.2 Administration with a known carcinogen

(a) Rat

Yuri *et al.* (2006) examined the effects of different durations of exposure to estradiol and progesterone pregnancy levels on mammary carcinogenesis risk in Lewis rats. Mammary carcinomas were induced with *N*-methyl-*N*-nitrosourea at 28 days of age. One group was left untreated (control group), and one was subcutaneously implanted with estradiol and progesterone pellets. Rats that received long- or short-term estradiol plus progesterone treatment had a decreased incidence of any mammary carcinomas or of mammary carcinomas with a diameter greater or equal to 1 cm, compared to control rats. Long-term (but not short-term) estradiol plus progesterone treatment increased the incidence of fibroadenomas.

Tsukamoto *et al.* (2007) used short-term treatment with estradiol and progesterone to mimic pregnancy in aged female Lewis rats treated with *N*-methyl-*N*-nitrosourea to show promotion of mammary carcinogenesis. Development of *N*-methyl-*N*-nitrosourea-induced mammary carcinomas was accelerated after short-term estradiol plus progesterone treatment, compared with estradiol plus progesterone-untreated rats: the incidence of ≥ 1-cm mammary carcinomas increased (60 versus 44%), latency was shorter (28.7 versus 34.6 weeks), and cancer multiplicity increased significantly (number of all-sized carcinomas per rat; 1.8 versus 0.8).

The effects of hormones on mammary tumorigenesis were studied by Thordarson *et al.* (2004) in growth-hormone-deficient spontaneous dwarf rats. The rats were divided into several groups treated with: bovine growth hormone; estradiol plus progesterone; bovine growth hormone plus estradiol plus progesterone; and a control group. After 1 week, all animals were injected intraperitoneally with the carcinogen *N*-methyl-*N*-nitrosourea. Growth hormone treatment alone increased mammary tumour incidence from 4.8% in controls to 100%. Estradiol plus progesterone treatment did not significantly alter tumorigenesis (0% tumour incidence); estradiol plus progesterone and growth-hormone obliterated the growth hormone-stimulated increase in tumour development (16.7% tumour incidence).

See Table 3.1.

Table 3.1 Studies of cancer in experimental animals exposed to estrogen–progestogen combinations

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, BALB/c (F) 45–58 wk Rajkumar *et al.* (2007) Medina *et al.* (2007)	s.c. implant 50 µg of E_2 and 20 mg of P Initial number: NR	Mammary carcinomas (tumours/transplants):		BALB/c mice used in this experiment were transplanted with mammary ducts from *p53* null BALB/c mice
	Untreated	16/66 (24%)		
	E_2/P (5–7)[a]	2/66 (3%)	$P < 0.05$	
	Untreated	10/20 (50%)		
	E_2/P (5–7)[a]	3/20 (15%)	$P < 0.05$	
	E_2/P (23–25)[a]	4/20 (20%)	$P < 0.05$	
	Untreated, trans. (3)[a]	0/20 (0%)		
	E_2/P (5–7)[a], trans. (3)[a]	3/15 (20%)	$P < 0.05$	
	Untreated, trans. (11)[a]	9/20 (45%)		
	E_2/P (5–7)[a], trans. (11)[a]	4/20 (20%)	$P < 0.05$	
Mouse, FVB (F) 24–32 wk	s.c. implant 100 µg of E_2 and 15 mg of P Initial number: NR			
	Untreated	15/15 (100%)		
	E_2/P (for 3 wk)	6/15 (37%)	$P < 0.05$	
	E_2 (for 3 wk)	5/15 (33%)	$P < 0.05$	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, ACI (F) 6 wk 39 wk Blank *et al.* (2008)	s.c. implant – intact rats Initial number: NR	Mammary carcinomas:		
	Untreated control	0/10		
	Low E_2 (10 µg)	0/15	NS	
	High E_2 (30→3 mg)	5/7	$P < 0.007$	
	30 mg P	0/15	NS	
	Low E_2 plus 30 mg of P	0/15	NS	
	s.c. implant – ovariectomized rats			
	Untreated control	0/10		
	Low E_2	0/15	NS	
	High E_2	0/8	NS	
	30 mg P	0/15	NS	
	Low E_2 plus 30 mg of P	0/14	NS	
	High E_2 plus 30 mg of P	11/11	$P < 0.0001$	
	High E_2 plus 30 mg of P plus 30 mg of TP	14/14	$P < 0.0001$	
Rat, Lewis (F) Dependent on tumour volume (≥ 1 cm) or 29 wk Yuri *et al.* (2006)	MNU: 50 mg/kg bw i.p. E_2/P s.c. implant: 0.5 mg/32.5 mg Initial number: NR	Mammary carcinomas ≥ 1 cm in diameter, any carcinoma:		The E_2/P pellet was replaced every 3–4 wk (long-term) or implanted only once (short-term); 5/14 rats of The MNU + long-term E_2/P group developed fibroadenomas vs 0/20 MNU-treated rats ($P < 0.01$)
	MNU	19/20 (95%), 20/20 (100%)		
	MNU + Long-term E_2/P	5/14 (36%), 9/14 (64%)	$P < 0.01$, $P < 0.01$	
	MNU + Short-term E_2/P	4/19 (21%), 11/19 (58%)	$P < 0.01$, $P < 0.01$ MNU + E_2/P vs MNU	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Lewis (F) 48 wk Tsukamoto *et al.* (2007)	MNU, 20 mg/kg bw (i.p.) at 7 wk of age MNU, 20 mg/kg bw (i.p.) at 7 wk of age + E_2 (0.5 mg)/P (32.5 mg), one pellet s.c. implanted at 24 wk 29 animals/group	Mammary carcinomas: 11/25 (44%) vs 9/15 (60%) Tumour multiplicity: 0.8 ± 0.2 vs 1.8 ± 0.5 Latency was shorter (28.7 vs 34.6 wk, $P < 0.05$)	$P < 0.05$	
Rat, SDR (F) 20 wk Thordarson *et al.* (2004)	bGH, 40–50 mg/kg bw in 50 µl weekly s.c. injections; MNU, 50 mg/kg bw (i.p.), 1 wk after hormone treatment	Mammary carcinomas:		Spontaneous dwarf rats are growth-hormone-deficient
	Control (MNU only)	1/21 (4.8%)		
	bGH	8/8 (100%)		
	E_2 (30 µg) + P (30 mg/2 mo) (s.c.)	0/11 (0%)		
	bGH and E_2 + P Initial number NR	1/6 (16.7%)	[$P < 0.003$] (bGH and E_2 + P vs bGH)	

[a] from week x to week y or at week x

bGH, bovine growth hormone; bw, body weight; E_2, estradiol; F, female; MNU, *N*-methyl-*N*-nitrosourea; mo, month or months; NR, not reported; NS, not significant; P, progesterone; TP, testosterone propionate; trans., transplanted; i.p., intraperitoneal; s.c., subcutaneously; vs, versus; wk, week or weeks

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

Various combinations of estrogens and progestogens are used for hormonal menopausal therapy. Because steroids penetrate normal skin easily, a variety of systems have been developed that deliver estrogens and progestogens parenterally (e.g. transdermal patches), thus bypassing the liver.

While the mechanisms of absorption and distribution of estrogens and progestogens have been known for several years, only recently has an understanding of the genes that encode the enzymes which control the enzymatic steps involved in steroid metabolism been acquired. This applies especially to the oxidative metabolism of estrogens. The phase I enzymes cytochrome P450 1A1 and 1B1 catalyse the production of catechol estrogens further oxidized to estrogen quinones that can induce the formation of DNA adducts. This is counteracted by the phase II enzymes, catechol-*O*-methyltransferase and glutathione *S*-transferase P1, which reduce the levels of catechol and quinones by forming methoxyestrogens and glutathione conjugates. Polymorphic variants of these and other enzymes occur frequently in the population and several are associated with altered enzyme function (IARC, 2007).

One of the particular areas of research on genetic variations concerns the capacity to metabolize hormones. Two common polymorphisms in the cytochrome P450 1A1 (CYP1A1) gene were examined in Chinese women with or without breast cancer. Homozygosity for both alleles was associated with a reduction of risk of borderline significance and the reduction was greater in slender women or those with a long history of menstrual cycles. Use of hormone replacement therapy (HRT) was not considered in this study (Boyapati *et al.*, 2005). In a case–control study of American women, sequence variations in the genotypes of the progesterone receptor and eight enzymes involved in the metabolism of estrogen were assessed in relation to endometrial cancer risk. Women with a particular polymorphism in the *SULT1A1* gene were found to have a significantly elevated risk of endometrial cancer if they received HRT (Rebbeck *et al.*, 2006). In another study of American women with breast cancer and their case–controls, polymorphisms in the genes for progesterone receptor and CYP3A4 were assessed in relation to breast cancer. It was reported that there was an elevated risk of ductal breast cancer or PR+ breast cancers among women with PGR331A alleles and greater than 3 years of combined HRT use. Women with at least one CYP3A4*1B allele who did not have a history of HRT use had a higher risk of ER- breast cancers (Rebbeck *et al.*, 2007).

4.2 Genetic and related effects

4.2.1 Direct genotoxicity

Data on the genetic effects of estrogens and their derivatives indicate that these compounds give rise to reactive metabolites and reactive oxygen species that can induce DNA damage. In recent years, it has been reported that metabolites of estrogen can form adducts on DNA and, based on this, it has been suggested that these lesions could induce the genetic alterations found in cancers (IARC, 2007; see also *Monograph* on Estrogen-only Menopausal Therapy in this volume). The evidence reported since the previous evaluation further substantiates the premise that these mechanisms could contribute to the induction of cancer by estrogens. Since the previous *IARC Monograph* (IARC, 2007), it has been shown that DNA adducts derived from equine estrogens can interfere with DNA synthesis. Using an in-vitro DNA primer extension assay past adducts with bypass polymerases

kappa and eta, it has been shown that 4-hydroxyequilenin adducts are highly mutagenic giving rise to both A→T transversions and A→G transitions. This finding offers a plausible mechanism for the contribution of estrogen adducts to the induction of cancer (Yasui *et al.*, 2006). [The Working Group noted that although these new findings increase the plausibility of these pathways as mechanisms of estrogen-related carcinogenesis, they do not prove that these are the major pathways to estrogen-related cancers. The way in which progestogens might influence the genotoxicity of estrogens is not known.]

Targeted studies have explored how sequence variations in specific genes influence cancer risk, including their effects on hormone receptor function or metabolism of hormones. One of these recently published studies showed that certain genetic variants in the *ERα* gene or progesterone receptor genes were linked to increased mammographic density following HRT (van Duijnhoven *et al.*, 2006). In another study, a specific mutation in the ERα was shown to be linked to a higher risk of breast cancer (Conway *et al.*, 2007). In contrast, in a study of Hispanic and non-Hispanic women in the South western USA, particular genetic variants in the *ERα* or androgen receptor genes were not found to be related to breast cancer risk (Slattery *et al.*, 2007a). In another study by the same group and the same study population, genetic variants in the genes of the insulin-related pathways were assessed with regard to breast cancer risk. In this study, some of the variants examined were related to breast cancer and to an involvement with HRT, with positive or negative relationships observed for particular variants (Slattery *et al.*, 2007b). [The Working Group noted that the study of single-nucleotide polymorphisms (SNPs) and other sequence variations as factors affecting the risk of breast cancer and increased breast density in patients treated with estrogen plus progestogen menopausal therapy are at an early stage of development. Some intriguing findings have been made in individual studies but their interpretation awaits repetition of findings particularly in other laboratories and other study populations.]

Other studies have considered the relationships between various genetic variants and the risk of colon cancer. In a study considering polymorphisms in the genes for IGF-1 and the IGF-binding protein 3 (IGF-BP3), women with the GG genotype of IGF-BP3 who received HRT had a reduced incidence of colon cancer compared with women who did not receive HRT (Morimoto *et al.*, 2005). Genetic variants of ERα, ERβ, and the androgen receptor were studied in relationship to colorectal cancer. It was found that women having an R allele (absence of an Rsa1 restriction site) at 1,082G > A in ERβ had reduced risk of colorectal cancer if they received HRT (Slattery *et al.*, 2005). In another study, SNPs were evaluated in ten estrogen-metabolism-related genes. None of the studied SNPs were associated with an elevated risk of colon cancer. There were no significant differences between women receiving HRT and those who did not (Huber *et al.*, 2005). In a study reflecting the expression levels of DNA mismatch repair genes and measuring the frequencies of mismatch repair defects detected in single- and di-nucleotide repeats, use of estrogen or estrogen–progestogen combinations were evaluated for their effect on colorectal cancer incidences. For all women taking combinations of estrogen and progestogen, there was a reduced risk of colorectal cancer. For those women with little or no evidence of mismatch repair defects, the colon cancer risk reduction was greater (40%). Neither of these effects was observed in women treated with estrogen alone (Newcomb *et al.*, 2007).

4.2.2 Receptor-mediated effects

The literature on the receptor-mediated effects of estrogen–progestogen menopausal therapy was reviewed in the previous *IARC Monograph* (IARC, 2007).

In more recently reported studies, colon cancer cells were transfected to transiently overexpress ERα and cultured with or without estrogen. Gene expression for hTNF-α and DNA fragmentation were evaluated as measures of apoptosis and β-catenin signalling was evaluated as a measure of cell proliferation. ERα overexpression, with or without estrogen treatment, activated DNA fragmentation. ERα overexpression combined with estrogen treatment increased both expression of hTNF-α and DNA fragmentation, and treatment of cells with antibodies to hTNF-α reduced the DNA fragmentation. ERα overexpression combined with estrogen treatment increased expression of proliferation inhibitory molecules p21 and p27 and decreased expression of β-catenin and its downstream pro-proliferation target genes, cyclin D1 and Rb (Hsu *et al.*, 2006). [The Working Group noted that this would be consistent with an estrogen-mediated inhibition of cell proliferation in the colon.]

The expression of steroid receptor coactivator AIB1 was examined in endometrial specimens from patients with endometrial cancer, comparing areas with cancer to normal areas or to those with complex atypical hyperplasia. Expression of AIB1 was assessed by immunohistochemistry in comparison to ERα and progesterone receptors. AIB1 was most highly expressed in endometrial cancers. There were no differences detected between the morphological groups for the expression of other co-regulators tested. It was suggested that when AIB1 and ER are expressed together, ER activity is enhanced, contributing to hyperplasia and malignancy (Balmer, *et al.*, 2006).

Transcriptional activation in vivo by ERα, ERβ and the androgen receptor were compared for estradiol and various equine estrogens with unsaturated B-rings found in conjugated equine estrogens used for HRT. Differences in binding of these estrogens to the ligand-binding domain of ERα were determined by crystallography. In comparison to binding by estradiol, decreased ligand flexibility and hydrophobicity was found for the equine estrogens with unsaturated B-rings (Hsieh *et al.*, 2008).

The effects of synthetic progestogens (progestins) on androgen receptors were considered in a review article. Evidence was reviewed indicating that synthetic hormones that act like progestins may exert their effect in part through their ability to bind to androgen receptors and, through linked pathways, act to suppress estrogen-induced functions. The activity of these progestins occurs independently of their effects through the progesterone receptors. The authors propose that some of the reported excess of breast cancer associated with synthetic progestins, such as medroxyprogesterone acetate, may occur because of their endocrine receptor effects on the androgen receptor (Birrell *et al.*, 2007).

The relationship between radiological breast density and breast histology was evaluated in a study of American breast cancer patients treated with HRT as compared to an equal number of patients not being treated with HRT. Studies focused on areas of the breast not involved with cancer and evaluated radiological breast density and histological fibrous stroma, ducts and lobules, and evidence of cell proliferation rates. The higher breast density in patients receiving HRT was correlated significantly with the presence of fibrous stroma and type I lobules, as well as increased proliferation in ducts and lobules. Estrogen receptor and progesterone receptor levels in the breast tissue did not correlate with HRT therapy or breast density. Because the increase in breast stroma and type I lobules was not related to hormone receptor levels, it is speculated that this effect may be mediated by paracrine factors (Harvey *et al.*, 2008).

In an in-vitro study, medium conditioned by progesterone-treated human breast cancer cells was shown to produce paracrine factors that induced the proliferation of endothelial cells and epithelial breast tumour cells through VEGF receptors. Inhibition of VEGF receptor 1 by antibody and VEGF receptor 2 using SU-1498 blocked the induced proliferation of the epithelial breast tumour cells and endothelial cells, as did anti-progestin mifepristone (RU486) treatment of the tumour cells that conditioned the medium. These results were interpreted as implicating VEGF and possibly other paracrine factors in the progestin-induced proliferation of endothelial cells surrounding breast tumours (Liang & Hyder, 2005).

In a study of ER+ and ER- breast cell lines, both malignant and non-malignant, it was shown that both estradiol and iron (as ferrous sulfate) increased cell proliferation as measured by Ki-67 and proliferating cell nuclear antigen levels and that the combination of estradiol and iron caused even greater increases (Dai *et al.*, 2008).

Norwegian women on systemic HRT had elevated plasma estrogen levels that were comparable to those in premenopausal women. Among women not on HRT, plasma levels of estradiol and serum hormone-binding globulin were influenced by the women's basal metabolic index (Waaseth *et al.*, 2008). In a study of Swedish ER+ breast cancer patients, HRT caused altered expression of 276 genes as compared to their expression in a larger control group of ER+ tumours from patients with unknown HRT usage. It was concluded that patients with postmenopausal HRT use had lower ER protein levels, a distinct gene expression profile, and better disease-free survival (Hall *et al.*, 2006).

4.3 Synthesis

Current knowledge indicates that hormone-receptor-mediated responses are a plausible and probably necessary mechanism for hormonal carcinogenesis by combined estrogen progestogen menopausal therapy . There is also support for the potential involvement of genotoxic effects of combined estrogen–progestogen menopausal therapy estrogenic hormones or their associated metabolic by-products including the formation of DNA adducts, and reactive oxygen species that damage DNA. Recent data suggests that these adducts slow down or block DNA replication and invoke bypass replication, which is prone to mutagenesis.

The predominant effects of combined estrogen–progestogen menopausal therapy associated with hormonal carcinogenesis are likely to be the result of one or more receptor-mediated processes. Progestogens including those used for combined estrogen–progestogen menopausal therapy appear to have the capacity to stimulate cell proliferation in the breast while they inhibit proliferation in the uterus. The magnitude of these effects vary for different synthetic progestogens, with a suggestion that medroxyprogesterone acetate is very active.

Cessation of hormonal treatment may reduce some receptor-mediated effects. The hormone-induced genotoxic effects may be persistent.

Use of combined estrogen–progestogen menopausal therapy was linked to increases in breast density, which is an established risk factor for breast cancer.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of combined estrogen–progestogen menopausal therapy. Combined estrogen–progestogen menopausal therapy causes cancer of the breast, and of the endometrium. The increased risk for estrogen-induced endometrial cancer decreases with the number of days per month that progestogens are added to the regimen.

There is *limited evidence* in experimental animals for the carcinogenicity of conjugated equine estrogens plus medroxyprogesterone acetate.

There is *limited evidence* in experimental animals for the carcinogenicity of estradiol plus progesterone.

Combined estrogen–progestogen menopausal therapy is *carcinogenic to humans (Group 1).*

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COMBINED ESTROGEN–PROGESTOGEN CONTRACEPTIVES

Combined estrogen–progestogen contraceptives were considered by previous IARC Working Groups in 1998 and 2005 (IARC, 1999, 2007). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

Combined hormonal contraceptives consist of an estrogen and a progestogen, and act primarily by preventing ovulation through the inhibition of the follicle-stimulating hormone and luteinizing hormone. The progestogen component also renders the cervical mucus relatively impenetrable to sperm, and reduces the receptivity of the endometrium to implantation (IARC, 2007).

A variety of innovations have been developed since combined hormonal contraceptives were first available in the late 1950s, including changes in drug components, doses used, and the temporal sequencing of exposure to drugs. The dominant trends have been towards less androgenic progestogens, lower doses of estrogen and progestogen, the near abandonment of hormonal contraceptives with an estrogen-only phase, a proliferation of different product formulations, and continuing development of novel delivery systems (IARC, 2007).

1.1 Identification of the agents

See the *Monographs* on Estrogen-only Menopausal Therapy and Combined Estrogen–Progestogen Menopausal Therapy.

1.2 Use of the agents

Information for Section 1.2 is taken from IARC (2007), McEvoy (2007), and Sweetman (2008).

1.2.1 Indications

Oral, intravaginal, injectable and transdermal estrogen–progestogen combinations are used for the prevention of conception in women.

A short-course, high-dose regimen of an oral estrogen–progestogen combination is used in women for the prevention of conception after unprotected intercourse (postcoital contraception, "morning-after" pills) as an emergency contraceptive.

Certain oral estrogen–progestogen combinations have been used for the treatment of moderate acne vulgaris in females 15 years of

age or older who are unresponsive to topical anti-acne medication.

An estrogen–progestogen combination of ethinylestradiol with drospirenone can be used for the treatment of pre-menstrual disorders.

1.2.2 Dosages

The large number of products that are currently available differ in several respects, including the estrogen compound used and its dose, the progestogen used, the schedule of exposure to the drugs, and the route of administration. Identical formulations may carry different brand names in different countries or even within the same country. [These products and their ingredients are presented in Annexes 1–3 of Vol.91 of the IARC *Monographs* (IARC, 2007).]

The most common estrogen in combined hormonal contraceptives is ethinylestradiol. Other estrogens have been used, including mestranol (a prodrug of ethinylestradiol) and, more recently, estradiol. In the early combined hormonal contraceptives, doses of estrogen in the range of 100–150 μg were commonly used. Contemporary combined hormonal contraceptives may be classified by estrogen dose into 'high-dose' (50 μg or more), 'moderate-dose' (30–35 μg), and 'low-dose' (15–20 μg).

A variety of progestogens are used in combined hormonal contraceptives. Currently, progestogens are often distinguished as 'first-generation' estranes (such as norethynodrel or norethisterone), 'second-generation' gonanes (such as levonorgestrel or norgestimate), 'third-generation' gonanes (gestodene and desogestrel), and 'fourth-generation' drospirenone. An additional class of progestogens, the pregnanes (eg. cyproterone and chlormadinone), may also be used. Estranes are highly androgenic, while pregnanes and drospirenone have anti-androgenic activity. The later gonanes are less androgenic than the earlier compounds in that series. The affinity of individual progestogens for progesterone receptors varies considerably, and determines the daily doses required to produce endometrial differentiation. Drospirenone has the lowest affinity (typical daily dose, 3 mg), while the later gonanes have the greatest affinity (0.05–0.15 mg daily dose).

The schedule by which exposure to the drugs occurs may also vary. Most commonly, a constant combination of estrogen and progestogen is used for 3 weeks of a 4-week cycle. The doses of progestogen and (less often) estrogen may vary in two or three phases followed by a drug-free phase. Sequential exposure regimens that used prolonged exposure to estrogen-alone are no longer used (IARC, 1999), but a short, 5-day, estrogen-only sequence has been re-introduced. Cycle lengths shorter and longer than 4 weeks may be used with the aim of limiting the duration of menses or eliminating menses altogether.

Injection of an estrogen and progestogen was used early on in the development of hormonal contraception, and remains available. Innovations in drug delivery have generated transdermal patches and a vaginal device. Hormonal intrauterine contraceptive devices are also available.

(a) Contraception

(i) Oral dosage

Combined estrogen–progestogen oral contraceptives are usually classified according to their formulation: preparations containing 50 μg of estrogen; preparations containing less than 50 μg of estrogen (usually 20–35 μg); those containing less than 50 μg of estrogen with two sequences of progestogen doses; those containing less than 50 μg of estrogen with three sequences of progestogen doses; and those containing three sequences of estrogen (eg. 20, 30, 35 μg) with a fixed dose of progestogen.

Although the progestogen content of the formulations also varies, oral contraceptives are usually described in terms of their estrogen

content. The estrogenic or progestogenic dominance of an oral contraceptive may contribute to hormone-related adverse effects, and may be useful in selecting an alternative formulation when unacceptable adverse effects occur with a given formulation.

Most fixed combinations are available as 21- or 28-day dosage preparations (conventional-cycle oral contraceptives). Some 28-day preparations contain 21 hormonally active tablets and seven inert or ferrous-fumarate-containing tablets; other 28-day preparations contain 24 hormonally active tablets and four inert or ferrous-fumarate-containing tablets.

One fixed-combination extended-cycle oral contraceptive is available as a 91-day dosage preparation containing 84 hormonally active tablets and seven inert tablets. Another extended-cycle oral contraceptive is available as a 91-day preparation with 84 hormonally active tablets containing estrogen–progestogen and seven tablets containing low-dose estrogen.

(ii) Intravaginal dosage

Each vaginal contraceptive ring containing ethinylestradiol and etonogestrel is intended to be used for one cycle which consists of a 3-week period of continuous use of the ring followed by a 1-week ring-free period. After a 1-week ring-free period, a new ring is inserted on the same day of the week as in the previous cycle. Withdrawal bleeding usually occurs within 2–3 days after removal of the ring.

(iii) Transdermal dosage

When used for contraception, the transdermal system (containing ethinylestradiol 0.75 mg and norelgestromin 6 mg) is applied once weekly for 3 weeks, followed by a 1-week drug-free interval, then the regimen is repeated. Systemic exposure to estrogen is greater with the transdermal system than with oral contraceptive preparations.

(b) Postcoital contraception

When an emergency contraceptive kit is used for postcoital contraception, two tablets of an estrogen–progestogen contraceptive (each tablet containing ethinylestradiol 50 µg and levonorgestrel 0.25 mg, for a total dose of ethinylestradiol 100 µg and levonorgestrel 0.5 mg) are administered orally within 72 hours after unprotected intercourse, repeating the dose 12 hours later.

Several other regimens employing short-course, high-dose oral combinations of ethinylestradiol and norgestrel or levonorgestrel have been used for postcoital contraception. One of the most widely used regimens consists of an oral dose of 100 µg of ethinylestradiol and 1 mg of norgestrel (administered as two tablets, each containing 50 µg and 0.5 mg of the drugs, respectively) within 72 hours after unprotected intercourse, with a repeat dose 12 hours later.

Alternative combination regimens that have been used consist of a dose of 120 µg of ethinylestradiol and 1.2 mg of norgestrel or 0.5–0.6 mg of levonorgestrel within 72 hours after intercourse, repeating the dose 12 hours later.

1.2.3 Trends in use

At the time of writing, more than 100 million women worldwide, an estimated 10% of all women of reproductive age, use combined hormonal contraceptives, most as oral preparations. A higher proportion of women receive these drugs in developed countries (16%) than in developing countries (6%). Proportions of 'ever use' higher than 80% have been reported for some developed countries. In developing countries, 32% of women are estimated to have ever used hormonal contraception, but there is extreme variability between countries. In many countries, these preparations are mainly used by women of a younger age and a higher level of education, and who have greater access to health care (UN, 2004).

The UN (2004) has compiled data from multiple sources on worldwide patterns of combined hormonal contraceptive use. It was estimated that, among women in marriage or sexual unions, 7.3% currently use combined hormonal contraception orally, and 2.9% currently use hormonal injections or implants. The use of injectable preparations is greater in developing countries than in developed countries.

Data on sales of combined hormonal contraceptives indicate increasing use worldwide; a 19% increase was noted from 1994–99, and a subsequent 21% increase from 1999–2004. The largest increases occurred in eastern Europe, the Eastern Mediterranean, South-East Asia and the Western Pacific, and modest increases in Africa and South America. It should be noted that these data may not include a large amount of hormonal contraceptives that are provided by national and international family planning programmes. Several other trends are indicated from the sales data: (i) the use of higher estrogen doses (≥ 50 μg) has continued to decline; (ii) growth in the use of later progestogen-containing products (gestodene, desogestrel) has slowed down, and in some countries, there has been a shift back to earlier progestogens (norethynodrel, norethisterone); and (iii) monophasic hormonal formulations have continued to predominate with some shift away from multiphasic forms (IARC, 2007).

1.2.4 Sequential oral contraceptives

The sequential oral contraceptive regimen consisted of estrogen treatment in the follicular phase of the cycle when estrogens are normally present with no progesterone, and a combination of a progestational agent and the estrogen in the luteal treatment phase, a period when estrogens plus progesterone are normally present (Dorfman, 1974).

Sequential oral contraceptives were taken off the market in 1976 (NCI, 2003). No information was available on the prevalence or duration of use of these products prior their discontinuation.

2. Cancer in Humans

The epidemiological evidence that combined oral contraceptives may alter risks of specific cancers in women was most recently reviewed in a previous *IARC Monograph* (IARC, 2007). The results of studies published since then, through May 2008, are summarized in this section, and a new assessment of the overall evidence is provided. All tables except Table 2.2 and Table 2.5 are from the prior *IARC Monograph*; they have been updated where appropriate to provide new information from previously cited and new studies with results published in the interim.

2.1 Cancer of the breast

The results of a meta-analysis of most of the epidemiological data on oral contraceptives and breast cancer were published in 1996 (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). The previous evaluation (IARC, 2007) relied heavily on this effort, which included data from more than ten cohort studies and 60 case–control studies that included over 60 000 women with breast cancer. There was little, and inconsistently observed, increase in risk of breast cancer overall in women who had ever used oral contraceptives. However, the sum of the evidence suggested an increase in the relative risk of breast cancer among current and recent users. This effect was noted particularly among women under 35 years of age at diagnosis who had begun using contraceptives when young (< 20 years), whereas the increased risk declined sharply with older age at diagnosis. Ten years after cessation of use, the risk in women who had used combined hormonal contraceptives appeared to be similar to that in women who

had never used them. Confounding by important known risk factors did not appear to account for the association. The possibility that the association seen for current and recent users is due to detection bias was not ruled out, but it was considered to be unlikely in explaining the association observed in young women. Other results from individual studies that were not considered conclusive but that warranted additional investigation included: a stronger association with invasive lobular than with ductal carcinoma, absence of an association between oral contraceptives and ductal carcinoma *in situ*, an association particularly in women with a family history of breast cancer or a mutation in the *BRCA1* (but not *BRCA2*) gene, and a stronger association in women aged under 35 years using higher rather than lower dose preparations.

Updated results of two long-term cohort studies in the United Kingdom indicate that risk of breast cancer does not increase even a long time after initial exposure. The Oxford Family Planning Association Contraceptive Study (hereafter referred to as the Oxford study) included 17032 women who were 25 to 39 years old when they were recruited into the study between 1968–74 (Vessey & Painter, 2006). No associations with breast cancer risk were observed in women who ever had used oral contraceptives (relative risk [RR], 1.0; 95%CI: 0.8–1.1), with duration of use, or with time since use, including women whose last exposure was over 20 years previously (RR, 0.9; 95%CI: 0.7–1.1).

The Royal College of General Practitioners' Oral Contraceptive study (hereafter referred to as the Royal College study) recruited approximately 23000 oral contraceptive users, and an equal number of non-users in 1968–69 (Hannaford *et al.*, 2007). No associations with risk of breast cancer were observed in women who had ever taken oral contraceptives (RR, 1.0; 95%CI: 0.9–1.1), and no significant trends were observed with duration of use or time since last use (RR in women who last used oral contraceptives over 20 years previously, 0.54; 95%CI: 0.35–0.82).

From 1989–91, approximately 267000 women were enrolled in a randomized trial of breast self-examination in Shanghai, People's Republic of China (hereafter referred to as the Shanghai BSE trial). Information on duration of oral contraceptive use was ascertained at enrollment by in-person interviews. The women in the cohort were followed through to July 2000. The relative risk in women who ever used oral contraceptives was 0.90 (95%CI: 0.78–1.03), and there was no trend in risk with duration of use up to over 10 years of exposure (RR, 0.94; 95%CI: 0.66–1.32) (Rosenblatt *et al.*, 2008).

Hospital based case–control studies in the Islamic Republic of Iran (Yavari *et al.*, 2005), Kuala Lumpur, Malaysia (Kamarudin *et al.*, 2006), Kelantan, Malaysia (Norsa'adah *et al.*, 2005), and Turkey (Beji & Reis, 2007), reported relative risks in women who ever used oral contraceptives of 1.95 (95%CI: 1.32–2.87), 0.71 (95%CI: 0.46–1.08), 2.5 (95%CI: 1.3–4.8), and 1.98 (95%CI: 1.38–2.85), respectively. No results by duration of use, time since use, or age at use or diagnosis were given. [No attempts to validate use of oral contraceptives were made in any of these studies, and the possibility of more complete recall of oral contraceptive use by cases than controls cannot be ruled out. In addition some controls may have had conditions that precluded use of oral contraceptives. For these reasons, the Working Group did not believe that these results were sufficiently compelling to conclude that oral contraceptives, as they have been used in the countries in which these studies were conducted, had altered the overall risk of breast cancer.]

Two population-based case–control studies have yielded results that corroborate the increase in risk of breast cancer in young and recent users (see Table 2.1 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.1.pdf). In one study (Jernström *et al.*, 2005), 245 cases were recruited from the South

Swedish Health Care region, and three controls were randomly selected for each case from a population-based cohort study being conducted in the same region. The odds ratio of breast cancer in women who ever used oral contraceptives was 1.65 (95%CI: 0.95–2.87). The odds ratios were significantly elevated in women who used oral contraceptives before the birth of their first child (OR, 1.63; 95%CI: 1.02–2.62), and before the age of 20 years (OR, 2.10; 95%CI: 1.32–3.33), and the risk increased with duration of use at these times in life. The risk was not significantly increased in women who used oral contraceptives after the age of 20 years. The odds ratios per year of use before the age of 20 years were 1.31 (95%CI: 1.07–1.62) in women born in 1955 or later, when most use was of low dose preparations, but only 0.95 (95%CI: 0.74–1.20) in women born in 1954 or earlier, when there was more use of higher dose products. Each year of low-dose oral contraceptives before the age of 20 years was associated with an odds ratio of 1.80 (95%CI: 1.24–2.61). No comparable estimate was given for high-dose oral contraceptives.

In a population-based case–control study conducted in four states in the United States of America, in-person interviews were conducted with 796 Hispanic cases and 919 Hispanic controls, and with 1522 non-Hispanic white cases and 1596 non-Hispanic white controls (Sweeney *et al.*, 2007). Odd ratios were not significantly elevated in women who ever used oral contraceptives in either Hispanics (OR, 1.10; 95%CI: 0.88–1.37) or non-Hispanics (OR, 1.08; 95%CI: 0.90–1.29). When both groups were combined, the odds ratio in women who used oral contraceptives in the past 5 years was 1.27 (95%CI: 0.99–1.63), with no difference between the two ethnic groups. Risk was also increased in users of over 20 years' duration (OR, 1.50; 95%CI: 1.04–2.17), with similar estimates for both groups; however, there was no apparent trend in the magnitude of risk associated with duration of use.

The odds ratios for ever use were slightly higher for users prior 1980 compared with after 1980, suggesting that the more recently marketed low-dose products may be less strongly associated with risk of breast cancer than older, higher dose preparations, but the differences were small, and risk in relation to time since last use was not presented by decade of use.

A population-based case–control study was conducted in Los Angeles County, USA, in which 567 cases of breast carcinoma *in situ* were compared to 614 controls (Gill *et al.*, 2006). No association was observed with any of the following features of oral contraceptive use: any use, years of use, use before first live birth, time since last use, age at first use, use of high-dose products, and use of low-dose products. [The Working Group concluded that there was insufficient evidence to determine whether newer, lower dose oral contraceptives altered the risk of breast cancer differently than older, higher dose products.]

Five studies have provided estimates of risk in relation to oral contraceptive use separately for ductal and lobular carcinomas of the breast. In a population-based case–control study of women under the age of 75 years in four states in the USA (Newcomer *et al.*, 2003), the relative risks of ductal and lobular carcinomas were, respectively, 1.0 (95%CI: 0.7–1.3) and 1.7 (95%CI: 0.9–3.5) in women who used oral contraceptives for 15 years or more. Similarly, in a population-based case–control study in Washington State, USA (Li *et al.*, 2003), the relative risks for lobular and ductal carcinomas in women who used oral contraceptives for 15 or more years were 2.6 (95%CI: 1.3–5.3) and 1.6 (95%CI: 1.0–2.6), respectively. In the study in four states described previously (Sweeney *et al.*, 2007), odds ratios for ductal and lobular carcinomas were, respectively, 1.05 (95%CI: 0.90–1.22) and 1.20 (95%CI: 0.82–1.75) in ever users; 1.23 (95%CI: 0.94–1.62) and 1.21 (95%CI: 0.59–2.45) in recent users (within the past 5 years); and, 1.36 (95%CI: 0.91–2.04) and

2.08 (95%CI: 0.93–4.62) in users of over 20 years' duration.

These findings of a stronger association with lobular compared with ductal carcinoma were not confirmed in two other studies. In a case–control study in Sweden (Rosenberg *et al.*, 2006), the use of oral contraceptives was not associated with any of three histological types of breast cancer, and the odds ratio estimates for users of over 5 years' duration were similar: 0.9 (95%CI: 0.7–1.1) for ductal, 0.9 (95%CI: 0.6–1.4) for lobular, and 1.0 (95%CI: 0.5–1.9) for tubular carcinomas. In the portion of the population-based Woman's Interview Study of Health (WISH) conducted in Atlanta, Seattle-Puget Sound, and New Jersey, USA (Nyante *et al.*, 2008), odds ratios for ductal and lobular carcinomas were, respectively, 1.21 (95%CI: 1.01–1.45) and 1.10 (95%CI: 0.68–1.74) in ever users; 1.45 (95%CI: 1.08–1.96) and 0.33 (95%CI: 0.08–1.40) in recent users (within the past 2 years); and, 1.30 (95%CI: 1.06–1.59) and 0.92 (95%CI: 0.53–1.59) in users of over 4 years' duration. [The Working Group concluded that there is no convincing evidence that oral contraceptives use is more strongly associated with lobular carcinoma than with ductal carcinoma of the breast.]

Studies have also been conducted to assess risk in relation to oral contraceptive use separately for breast cancers with and without estrogen and progesterone receptors (ER and PR). Cotterchio *et al.* (2003) combined data from two case–control studies in Ontario, Canada. In both studies, cases were identified from the Ontario Cancer Registry, and controls were selected from roles of the Ministry of Finance. ER/PR status of the tumours was ascertained from laboratory records. Oral contraceptive use was obtained from a mailed questionnaire. The odds ratios for ER+/PR+ tumours, and for ER-/PR- tumours in women who used oral contraceptives for 10 or more years were, respectively, 0.92 (95%CI: 0.61–1.37) and 1.33 (95%CI: 0.79–2.25) in premenopausal women, and 0.95 (95%CI: 0.71–1.27) and 1.41 (95%CI: 0.96–2.08) in postmenopausal women.

In analyses of data from the study by Sweeney *et al.* (2007), based on 1214 ER+ cases, 339 ER- cases, and 2513 controls, odds ratios for ER+ and ER- breast cancers, respectively, were estimated to be 1.02 (95%CI: 0.87–1.21) and 1.38 (95%CI: 1.04–1.84) for ever users; 1.25 (95%CI: 0.93–1.70) and 1.53 (95%CI: 0.98–2.40) for users within the past 5 years; and, 1.39 (95%CI: 0.90–2.14) and 2.23 (95%CI: 1.17–4.25) in users of over 20 years' duration.

In a population-based case–control study in Los Angeles County, USA, 1794 cases from 20–49 years of age were identified from a population-based cancer registry, and compared to 444 age- and race-matched control women who were selected by a neighbourhood walk algorithm (Ma *et al.*, 2006). ER and PR status of the tumours were ascertained from medical records. Risk of neither ER+/PR+ nor ER-/PR- tumours was significantly associated with duration of oral contraceptive use, but the odds ratios in women who used oral contraceptives for over 10 years were 0.76 (95%CI: 0.49–1.18) for ER+ PR+ tumours, and 1.27 (95%CI: 0.75–2.14) for ER- PR- tumours. [The Working Group concluded that the evidence was insufficient to determine whether oral contraceptives use is more strongly associated with ER– tumours than with ER+ tumours.]

In a population-based case–control study in North Carolina (Conway *et al.*, 2007), with cases recruited in 1993–96, paraffin-embedded tumour blocks from 684 cases were successfully screened for mutations in the ERα gene (*ESR1*), which may render tissue hypersensitive to estrogen, and which has been observed in hyperplastic breast tissue. Results of in-person interviews with the 37 cases with an *ESR1* mutation, and with the 616 cases without the mutation, were compared with those from 790 control women. Although many of the odds ratio estimates for mutation-positive tumours were based on small

numbers of exposed cases, the odds ratios in relation to multiple features of oral contraceptive use were consistently greater for mutation-positive than mutation-negative tumours. Odds ratios for mutation-positive and mutation-negative tumours were, respectively, 1.72 (95%CI: 0.66–4.44) and 1.15 (95%CI: 0.87–1.52) in ever users; 3.73 (95%CI: l.l6–12.03) and 1.18 (95%CI: 0.77–1.81) in users of over 10 years duration; 3.63 (95%CI: 0.80–16.45) and 1.06 (95%CI: 0.65–1.72) in recent users (in the past 10 years); and, 6.49 (95%CI: 1.32–31.89) and 1.32 (95%CI: 0.73–2.38) in women who used oral contraceptives for over 10 years and had stopped using oral contraceptives within the past 10 years. [The Working Group noted that these results are at variance with those for ER+ and ER- tumours, and require independent confirmation before firm conclusions can be made.]

There is concern that oral contraceptives may preferentially alter the risk of breast cancer in women at high risk of this disease because of the occurrence of breast cancer in one or more family members, or because they carry a specific genetic mutation. In an investigation based on the Canadian National Breast Screening studies (Silvera *et al.*, 2005), 89835 women between the ages of 40–59 years were recruited during 1980–85, and completed a self-administered questionnaire, which included items on oral contraceptive use. The cohort was followed through 1998, 1999, or 2000, depending on the area, and cases were identified by linkage to provincial and national cancer registries. Hazard ratios of breast cancer in ever users, current users (at baseline), and users of over 7 years' duration were 0.88 (95%CI: 0.73–1.07), 1.01 (95%CI: 0.56–1.81), and 0.74 (95%CI: 0.55–0.99), respectively, in women with any family history of breast cancer. A significant decreasing trend in risk (P = 0.03) with increasing duration of use was observed. Results were broadly similar for women with first- and second-degree relatives with breast cancer. Comparable results for women with no family history were not given.

Four studies have assessed the risk of breast cancer in oral contraceptive users with mutations in the *BRCA1* or *BRCA2* genes. This can only be done in studies in which both cases and comparable non-cases are tested for the mutations, for instance, studies in which only cases were tested are not considered in this review. In a multicentre study conducted in 52 centres in 11 countries (Narod *et al.*, 2002), 1311 women with breast cancer and a mutation in *BRCA1* or *BRCA2* were compared to an equal number of unaffected controls with the same mutations, matched to the cases on year of birth, country, and mutation (*BRCA1* or *BRCA2*). In a study in Poland (Gronwald *et al.*, 2006), 348 cases of breast cancer were compared to 348 age-matched controls, who had not developed either breast or ovarian cancer; all were carriers of one of three Polish founder *BRCA1* mutations. A collaborative international case–control study (Haile *et al.*, 2006) included 195 cases and 302 controls with *BRCA1* mutations, and 128 cases and 179 controls with *BRCA2* mutations. In another international collaborative study (Brohet *et al.*, 2007), a cohort of 1593 women who had *BRCA1* (n = 1181) or *BRCA2* (n = 412) mutations was followed up and the hazard ratio of developing breast cancer in relation to several features of oral contraceptive use was estimated, based on 597 cases with a *BRCA1* mutation and 249 cases with a *BRCA2* mutation. The results of all four studies are summarized in Table 2.2 (available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.2.pdf). The risk of breast cancer in women who ever used oral contraceptives was increased in *BRCA1* mutation carriers (Narod *et al.*, 2002; Haile *et al.*, 2006; Brohet *et al.*, 2007), and there were increasing trends in risk with total duration of use. However, the risk was not consistently increased in women who used oral contraceptives before their first full-term pregnancy, or at an early age. Among

BRCA2 mutation carriers, the risk was increased in two of the studies in women who ever used oral contraceptives (Haile *et al.*, 2006; Brohet *et al.*, 2007), and the risk was particularly increased in long-term users in these two studies. An increase in risk was consistently observed in relation to use before a first full-term pregnancy, and at an early age. [The Working Group noted that the preponderance of the evidence suggests that use of oral contraceptives is associated with an increased risk of breast cancer in carriers of *BRCA1* or *BRCA2* mutations. The Working Group further noted that if this association reflects a causal relationship, then it could, at least in part, explain the observation summarized in the 2005 *IARC Monograph* (IARC, 2007) that risk of breast cancer was increased in women under the age of 35 years who had begun using oral contraceptives at a young age and who were current or recent users.]

2.2 Cancer of the endometrium

The previous *IARC Monograph* (IARC, 2007) on oral contraceptives and endometrial cancer was based on several cohort and case–control studies (see Table 2.3 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.3.pdf and Table 2.4 at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.4.pdf). The results of these studies consistently showed that the risk of endometrial cancer in women who had ever taken oral contraceptives was approximately halved. The reduction in risk was generally greater with longer duration of use, and persisted for at least 15 years after cessation of use, although the extent of the protective effect could lessen over time. Few data were available on the more recent, low-dose formulations of oral contraceptives.

The cohort study in the BSE trial in Shanghai (Rosenblatt, *et al.*, 2008) reported hazard ratios of 0.68 (95%CI: 0.45–1.04) in women who had ever used oral contraceptives, and 0.48 (95%CI: 0.27–0.85) in women who had used those for 1 or more years. The updated results from the two British cohort studies provide additional information on the effect of long-term use on the risk of endometrial cancer, and on the duration of the apparent protective effect. Estimates of the relative risks from the Royal College study (Hannaford *et al.*, 2007) and the Oxford study (Vessey & Painter, 2006) for users of over 8 years' duration were, respectively, 0.57 (95%CI: 0.27–1.19) and 0.1 (95%CI: 0.0–0.4), and for last use more than 20 years ago were 0.63 (95%CI: 0.23–1.78) and 0.5 (95%CI: 0.3–0.9).

These results were confirmed in a population-based case–control study in Shanghai (Tao *et al.*, 2006), in which 1204 women with endometrial cancer, who were identified through the Shanghai cancer registry, were compared to 1629 controls that were selected from the Shanghai resident registry. Women who reported ever using oral contraceptives had an odds ratio of 0.75 (95%CI: 0.60–0.93). The risk decreased with increasing duration of use (*P*-trend = 0.14), and the risk in women who had last used oral contraceptives 25 or more years in the past was 0.57 (95%CI: 0.42–0.78).

A case–control study was conducted in three hospitals in Japan (Okamura *et al.*, 2006; Table 2.4 online). Cases were identified from hospital admissions, and controls were selected from cervical cancer screening clinics. Based on 155 cases and 96 controls, only three and ten of which, respectively, had ever used oral contraceptives, the odds ratio in ever users was 0.16 (95%CI: 0.04–0.66). [The Working Group noted that the controls were probably not representative of the population from which the cases came.]

Data from the population-based Cancer and Steroid Hormone (CASH, 1987a, b) case–control study in the USA were re-analysed to assess the risk of endometrial cancer in relation to the potency of the estrogens and progestogens in the oral contraceptives women had taken (Maxwell *et al.*, 2006). Based on data from in-person

interviews of 434 cases identified through the Surveillance, Epidemiology, and End Results (SEER) programme in the USA, and 2557 controls selected by random-digit dialling, the odds ratios in women who took low- and high-progestogen potency products were, respectively, 0.39 (95%CI: 0.27–0.57) and 0.20 (95%CI: 0.10–0.41). The comparable estimates for women with a body mass index (BMI) less than 22.1 kg/m^2 who took low- and high-progestogen potency products were, respectively, 0.30 (95%CI: 0.11–0.83) and 0.26 (95%CI: 0.13–0.52), and for heavier women were 0.16 (95%CI: 0.06–0.45) and 0.51 (95%CI: 0.33–0.80). The risk was not significantly different in users of high- and low-estrogen potency products. However, eight controls, but no cases, had used products classified as high-progestogen/low-estrogen potency, suggesting that preparations with a high ratio of progestogen to estrogen may offer particularly strong protection against endometrial cancer.

2.3 Cancer of the cervix

In an initial review of five cohort and 16 case–control studies of oral contraceptives and invasive cervical cancer (IARC, 1999), the Working Group could not rule out biases related to sexual behaviour, screening, and other factors as possible explanations for an observed trend in risk of cervical cancer with increasing duration of use. The previous *IARC Monograph* (IARC, 2007) considered results from three additional cohort studies and seven more case–control studies that provided information on invasive or in-situ cervical carcinoma and use of oral contraceptives. All but three of the most recent studies were summarized in a meta-analysis of published data (Smith *et al.*, 2003) that was used in the previous *IARC Monograph*. The sum of the evidence indicated that, overall, the risk of both in-situ and invasive cervical cancer increased with increasing duration of use of oral contraceptives. The increase in risk with duration of use was observed in studies that were restricted to women with high-risk human papilloma virus (HPV) infections, and in studies that controlled for the presence of this infection. The increase was observed for both in-situ and invasive disease, and for both squamous cell carcinoma and adenocarcinoma. The relative risk declined after cessation of use. The results were broadly similar regardless of adjustment for number of sexual partners, cervical screening, tobacco smoking, and the use of barrier contraceptives. Although the possibility that the observed associations were due to residual confounding or detection bias could not be completely ruled out, they were considered unlikely to explain fully the observed relationships.

The updated results from the two British cohort studies also show increasing risks of invasive cervical cancer with duration of use, and declining trends in risk with time since last use. Estimates of relative risks from the Royal College (Hannaford *et al.*, 2007) and Oxford (Vessey & Painter, 2006) studies for users of over 8 years' duration were 2.73 (95%CI: 1.61–4.61) and 6.1 (95%CI: 2.5–17.9), respectively. The relative risks were not elevated between 15–20 years since last use (RR, 0.65; 95%CI: 0.23–1.83) and 20 or more years since last use (RR, 0.78; 95%CI: 0.11–5.71) in the Royal College study; and not elevated after 20 years since cessation of use (OR, 1.3; 95%CI: 0.1–7.2) in the Oxford study.

Results from a second meta-analysis have been published since the 2005 Working Group review (Appleby *et al.*, 2007). Data on 16573 women with cervical cancer and 35509 women without this disease, from 24 epidemiological studies, were included in the analysis. The percentage of control women who had used oral contraceptives was higher in women who had had at least one Pap smear, multiple sexual partners, early age at first sexual intercourse, borne children, smoked, and who were more educated, than in women without these attributes. All analyses were controlled for these potentially confounding factors, although

the results differed little from those that were controlled only for age and study, suggesting that residual confounding was unlikely to account for the observed associations. The relative risks (floated standard error, FSE) of invasive cervical cancer in women who used oral contraceptives for less than 5 years, 5–9 years, and 10 or more years were 0.96 (FSE, 0.04), 1.20 (FSE, 0.05), and 1.56 (FSE, 0.08), respectively; and the trend was statistically significant ($P < 0.0001$). There was also a significant ($P < 0.0001$) decreasing trend in relative risks with time since last use: 1.65 (FSE, 0.08), 1.28 (FSE, 0.08), 1.12 (FSE, 0.06), 1.05 (FSE, 0.06), and 0.83 (FSE, 0.05) in current users and in women who last used oral contraceptives 2–4, 5–9, 10–14, and 15 or more years in the past, respectively.

As shown in Table 2.5 (available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.5.pdf), the risk of in-situ disease was increased in users of less than 5 years' duration, but the risk of invasive disease was increased only after 5 years of use. The risk of both conditions declined with time since last use, and there was no elevation in risk of invasive disease 10 years since exposure. The relative risks of invasive cancer in women with evidence of oncogenic HPV DNA in exfoliated cervical cells was 0.80 (95%CI: 0.38–1.22) in users of less than 5 years' duration, and 1.45 (95%CI: 0.86–20.4) for 5 or more years' duration. Data from 12 studies could not be included in the meta-analysis, but evidence was presented that indicated their exclusion was unlikely to have altered the overall results. Subsequent studies of invasive cervical carcinoma (Matos *et al.*, 2005), and in-situ disease (Castle *et al.*, 2005; Massad *et al.*, 2005; Syrjänen *et al.*, 2006) did not consider risk in relation to duration of oral contraceptive use or time since last use.

2.4 Cancer of the ovary

The relationship between oral contraceptive use and beteen risk of ovarian cancer was extensively reviewed in the previous *IARC Monograph* (IARC, 2007). Based on results from six cohort studies and more than 30 case–control studies, plus six pooled analyses of data from multiple studies (see Table 2.6 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.6.pdf and Table 2.7 at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.7.pdf), it was clearly shown that women who had used oral contraceptives were at reduced risk of ovarian cancer. Risk declined with duration of use, and the apparent protective effect persisted for at least 20 years after last use. In most studies of specific histological types of ovarian cancer, reductions in risk of all types were observed in oral contraceptive users, although the association tended to be weaker and less consistently observed for mucinous than for other tumour types. Results for all histological types combined were confirmed in updated analyses of the two British cohort studies, although not in the Shanghai cohort, in which there were few long-term users.

Extension and clarification of all of these observations are provided by results of a meta-analysis of nearly all of the known epidemiological data on oral contraceptives and ovarian cancer available at the time of writing (Beral *et al.*, 2008). This analysis was of data from 13 prospective studies, 19 case–control studies with population controls, and 13 case–control studies with hospital controls, and included information on 23257 women with ovarian cancer and 87303 controls. Relative risks of women who used oral contraceptives for less than 1, 1–4, 5–9, 10–14, and 15 or more years were estimated to be 1.00 (95%CI: 0.91–1.10), 0.78 (95%CI: 0.73–0.83), 0.64 (95%CI: 0.59–0.69), 0.56 (95%CI: 0.50–0.62), and 0.42 (95%CI: 0.36–0.49), respectively. The apparent protective effect declined with time

since last use, but persisted for over three decades. For any time since last use, the reduction in risk was greater the longer the time oral contraceptives had been taken. The relative risk in women who had ever used oral contraceptives, and who had last used them over 30 years previously was 0.86 (95%CI: 0.76–0.97). The reduction in risk was seen for all histological types of malignant tumours, although it was weaker for mucinous when compared with other epithelial types (clear cell, endometrioid, and serous) and non-epithelial types. The reduction in risk was also less for borderline than for malignant serous tumours, and there was no reduction in risk of borderline mucinous tumours.

Independent analyses of data from a population-based study in Denmark (Huusom *et al.*, 2006) confirmed the absence of a relationship between oral contraceptive use and borderline mucinous ovarian tumours, and the presence of a relationship with borderline serous tumours. Another population-based study in Denmark (Soegaard *et al.*, 2007) showed decreasing trends in risk with duration of oral contraceptive use for invasive serous and endometrioid ovarian cancers, but not for invasive mucinous types. [The Working Group concluded that oral contraceptives are protective against epithelial ovarian cancers, and that the protective effect may be less for mucinous than for other histological types.]

The previous *IARC Monograph* (IARC, 2007) found no evidence that newer oral contraceptives with generally lower levels of estrogen and progestogen offered less protection than older products with generally higher levels. A recent meta-analysis also showed that the reduction in risk was similar in women who used oral contraceptives in the 1960s, 1970s, and 1980s, when most products contained relatively high, intermediate, and low doses of estrogen, respectively. In a population-based case–control study of 20–74-year-old women in North Carolina (Moorman *et al.*, 2008), cases diagnosed in 1999–2006 were identified from a local cancer registry, and controls were selected by random-digit dialling. The odds ratios did not significantly vary by time since first or last use after controlling for duration of use; and the odds ratios by duration of use were consistently lower for ovarian cancer in premenopausal women (who would be more likely to have used lower dose products) than in postmenopausal women (who would be more likely to have used higher dose products). The odds ratios for users of over 10 years' duration were 0.3 (95%CI: 0.2–0.6) for premenopausal women and 0.9 (95%CI: 0.6–1.5) for postmenopausal women.

In the previous *IARC Monograph* (IARC, 2007), the three studies that reported risk in relation to specific dosages all found lower odd ratios in users of relatively low- compared to high-estrogen potency products. This was also observed in an analysis of data from a population-based case–control study in Hawaii and Los Angeles (Lurie *et al.*, 2007) that included 745 cases and 943 controls. Products with 0.035 mg or more of ethinyl estradiol were considered as having high estrogen potency, and products containing progestogens with 0.3 mg norgestrel equivalent or more were considered as having high progestogen potency. The odds ratios in women who ever used products of high- and low-estrogen potency (regardless of progestogen potency) were 0.61 (95%CI: 0.42–0.89) and 0.33 (95%CI: 0.21–0.52), respectively; and the odds ratios in relation to ever use of high- and low-progestogen potency products (regardless of estrogen potency) were 0.54 (95%CI: 0.38–0.75) and 0.41 (95%CI: 0.18–0.94), respectively. The comparable odds ratios in users of products with high doses of both hormones, high estrogen–low progestogen, low estrogen–high progestogen, and low doses of both, were 0.62 (95%CI: 0.43–0.92), 0.55 (95%CI: 0.19–1.59), 0.45 (95%CI: 0.28–0.72), and 0.19 (95%CI: 0.05–0.75), respectively. [The Working Group concluded that, although some of the differences in the odds ratio estimates for high- and low-dose products could have occurred

by chance, in the aggregate, the consistency of the results across studies suggests that the newer, lower dose products may actually offer more protection than the older preparations.]

The meta-analysis (Beral *et al.*, 2008) found no significant differences in odds ratio estimates in women with and without a family history of breast cancer (presumably used as a rough surrogate for the possible presence of a *BRCA* gene mutation). The previous *IARC Monograph* (IARC, 2007) included four studies in which cases and controls with mutations in the *BRCA1* or *BRCA2* genes were compared. All four showed reductions in risk of ovarian cancer in oral contraceptive users who were carriers of a mutation in one of these genes. These observations were confirmed in two subsequent investigations.

In the case–control study of breast and ovarian cancers in women with *BRCA1* mutations in Poland (described previously in the section on breast cancer), 150 cases of ovarian cancer and 150 controls with one of three Polish founder mutations in *BRCA1* were compared. Relative risks in ever users of oral contraceptives and in users of 2 or fewer years and more than 2 years' duration were 0.4 (95%CI: 0.2–1.0), 0.8 (95%CI: 0.2–2.5), and 0.2 (95%CI: 0.1–0.7), respectively (Gronwald *et al.*, 2006). In an expansion of one of the studies included in the previous *IARC Monograph*, 670 cases with a *BRCA1* mutation and 128 with a *BRCA2* mutation were compared to 2043 controls with a *BRCA1* mutation and 380 controls with a *BRCA2* mutation (McLaughlin *et al.*, 2007). Subjects came from 11 different countries and were primarily identified through high breast cancer risk genetic testing and counselling clinics. The odds ratios in women who ever used oral contraceptives were 0.56 (95%CI: 0.45–0.71) in *BRCA1* mutation carriers, and 0.39 (95%CI: 0.23–0.66) in *BRCA2* mutation carriers. The odds ratios in carriers of either gene declined with duration of use. The odds ratio estimates for users of up to 1, 1–3, 3–5, and over 5 years in *BRCA1* mutation carriers were 0.69 (95%CI: 0.50–0.95), 0.67 (95%CI: 0.47–0.96), 0.41 (95%CI: 0.27–0.63), and 0.48 (95%CI: 0.35–0.66), respectively. The corresponding estimates for *BRCA2* mutation carriers were 0.56 (95%CI: 0.28–1.10), 0.42 (95%CI: 0.20–0.88), 0.14 (95%CI: 0.05–0.46), and 0.37 (95%CI: 0.19–0.72), respectively.

2.5 Cancer of the liver

In the previous *IARC Monograph* (IARC, 2007), it was noted that long-term use of combined oral contraceptives was associated with an increase in the risk of hepatocellular carcinoma in populations that had low prevalence of hepatitis B virus (HBV) infection and chronic liver disease, each of which are major causes of liver cancer. This association was also seen in analyses in which women with such infections were excluded. Three cohort studies showed no significant association between the use of oral contraceptives and the incidence of, or mortality from, liver cancer, but the expected number of cases was very small, which resulted in low statistical power. Few data were available on the more recent, low-dose formulations. In the three case–control studies conducted in populations that had a high prevalence of infection with hepatitis viruses, no statistically significant increase in the risk of hepatocellular carcinoma was associated with oral contraceptive use, but little information was available on long-term use.

Results for liver cancer were not reported in the updated results from the Oxford study, and the updated results from the Royal College study (Hannaford *et al.*, 2007) only included results for cancers of the liver and gallbladder combined (OR, 0.55; 95%CI: 0.26–1.17, in ever users, based on 14 cases that were users and 13 that were non-users); and there was no significant trends in risk with duration of use or time since last use. Consistent with the case–control studies in HBV-endemic areas, the cohort study in the BSE trial cohort in Shanghai (Rosenblatt *et al.*, 2008) did not find an increase in risk in women who

ever used oral contraceptives (RR, 0.82; 95%CI: 0.60–1.13), and no trend in risk with duration of use. The odds ratio in users of 10 or more years' duration was 0.67 (95%CI: 0.32–1.44).

A meta-analysis (Maheshwari *et al.*, 2007) of published data from 12 case–control studies of oral contraceptive use and hepatocellular carcinoma (see Table 2.8 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.8.pdf) provided an estimated risk of 1.57 (95%CI: 0.96–2.45) for ever users. However, there was significant heterogeneity of results among the studies. As expected, the large study conducted in eight HBV-endemic areas (WHO Collaborative Study of Neoplasia and Steroid Contraceptives, 1989a, b) found no association with ever use of oral contraceptives (OR, 0.71; 95%CI: 0.40–1.21), and no trend in risk with duration of use. A second study in South Africa, based on seven cases and eight controls, reported an odds ratio of 1.9 (95%CI: 0.5–5.6) in ever users (P = 0.19) (Kew *et al.*, 1990), with no trend in risk with duration of use. All of the other reviewed studies had been conducted in areas not endemic for HBV, and all but one showed an increased risk of hepatocellular carcinoma in relation to oral contraceptive use. The exception was a collaborative study in six European countries (Heinemann *et al.*, 1997) which reported an odds ratio of 0.75 (95%CI: 0.54–1.03) in ever users. However, in women with no serological evidence of HBV or hepatitis C virus (HCV) infection, and no history of hepatic cirrhosis, the odds ratio in users of over 6 years' duration was 2.29 (95%CI: 1.05–5.02). In the other studies in non-HBV-endemic areas, with sufficient numbers of study subjects to assess risk in relation to duration of use, risk was observed to increase with years of exposure.

2.6 Cancer of the skin

At the time of the previous *IARC Monograph* (IARC, 2007) four cohort and 19 case–control studies provided information on the use of combined oral contraceptives and risk of cutaneous malignant melanoma (see Table 2.9 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.9.pdf and Table 2.10 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.10.pdf). No consistent evidence for an association was found with respect to current use, duration of use, time since last use or age at first use.

The relative risks of cutaneous melanoma from the Oxford and Royal College studies did not increase with duration of use, and were, respectively, 1.0 (95%CI: 0.6–1.7) and 1.71 (95%CI: 0.96–3.06) for users of over 8 years' duration; and, they were 0.8 (95%CI: 0.4–1.5) and 0.62 (95%CI: 0.24–1.59) 20 or more years after last use. An updated analysis of data from a hospital-based case–control study in San Francisco (Lea *et al.*, 2007) did not show an association between oral contraceptive use and risk of cutaneous melanoma (see Table 2.10 online).

2.7 Cancer of the colorectum

At the time of the previous *IARC Monograph* (IARC, 2007), nine cohort and 14 case–control studies provided information on oral contraceptives and risk of colorectal cancer (see Table 2.11 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.11.pdf and Table 2.12 at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.12.pdf). Most studies did not show an increase in risk in women who had ever used oral contraceptives, or in relation to duration of use. The results were generally similar for colon and rectal cancer when examined separately, and two case–control studies showed a significant reduction in risk of colorectal cancer in users of oral contraceptives.

The two updated British cohort studies show no significant associations between oral contraceptive use and cancers of the colon and rectum combined. Odds ratio estimates from the Oxford and Royal College studies were, respectively, 0.8 (95%CI: 0.5–1.2) and 0.95 (95%CI: 0.59–1.54) in users of over 8 years' duration, and 0.9 (95%CI: 0.6–1.4) and 1.09 (95%CI: 0.60–1.99) 20 or more years after cessation of use. In the Shanghai BSE trial cohort study (Rosenblatt *et al.*, 2008), 655 women developed cancer of the colon and 368 developed cancer of the rectum. Relative risks of colon and rectal cancer were 1.09 (95%CI: 0.86–1.37) and 1.31 (95%CI: 0.98–1.75), respectively, in women who had ever used oral contraceptives. Weak increasing trends in risk with duration of use were observed for both cancers (*P*-values for trend: 0.16 and 0.017, respectively), and the relative risks in users for 10 or more years were 1.56 (95%CI: 1.01–2.40) and 1.34 (95%CI: 0.71–2.52), respectively. However, two additional cohort studies showed inverse associations between oral contraceptive use and colorectal cancer. Of 39680 American women aged 45 years or older who were enrolled in a randomized trial of aspirin and vitamin E (Lin *et al.*, 2007), women who had ever used oral contraceptives at baseline were at significantly reduced risk of colon and rectal cancers, but among users, there was no significant trend in risk with duration of exposure up to 60 or more months of use. The hazard ratios for both colon and rectal cancers were also reduced in oral contraceptive users in a cohort of 89835 women between 40–59 years of age who were enrolled in a randomized trial of breast screening in Canada (Kabat *et al.*, 2007a). The hazard ratios were similar for cancers of the proximal and distal colon. However, there were no significant trends in risk with duration of use.

One additional population-based case–control study provided results similar to those of the cohort studies in the USA and Canada. A total of 1404 colon and rectal cancer cases that were identified from cancer registries in Ontario, Newfoundland and Labrador were compared to 1203 population controls (Campbell *et al.*, 2007). A self-administered questionnaire was used to collect information on the use of hormonal contraceptives [presumably largely combined oral contraceptives]. The odds ratio for colorectal cancer in women who had ever used any type of hormonal contraceptive was 0.77 (95%CI: 0.65–0.91). Among users, no trend in risk with duration of use was observed.

2.8 Cancer of the thyroid

In the previous *IARC Monograph* (IARC, 2007), results from a pooled analysis of data from 13 studies, and reports from six additional investigations (see Table 2.13 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.13.pdf), revealed weak or no associations between the use of oral contraceptives and cancer of the thyroid. In the cohort study in the Shanghai BSE trial (Rosenblatt *et al.*, 2008), no increase in risk of thyroid cancer in women who ever used oral contraceptives was observed (RR, 0.75; 95%CI: 0.46–1.23, based on 161 cases, 20 of whom were users). Results of one additional population-based case–control study, in New Caledonia, France, an area with an unusually high incidence of thyroid cancer, were published (Truong *et al.*, 2005) where answers to in-person interviews of 293 cases and 354 controls selected from electoral rolls were compared. The odds ratio was 1.1 (95%CI: 0.8–1.7) for ever users of oral contraceptives, and no trend in risk with duration of use up to over 5 years was observed.

2.9 Lymphomas

The previous *IARC Monograph* (IARC, 2007) included two studies that did not find associations between the use of oral contraceptives and the risk of lymphomas (see Table 2.14 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-14-Table2.14.pdf). In the most

recent results from the Oxford study (Vessey & Painter, 2006), no increased risk of lymphomas and leukaemias combined in women who ever used oral contraceptives and no trend in risk with duration of use were observed.

2.10 Cancers of the central nervous system

One study was cited in the previous *IARC Monograph* (IARC, 2007) that showed no association between risk of tumours of the central nervous system and the use of oral contraceptives. However, the most recent results from the Royal College study (Hannaford *et al.*, 2007) showed an increased risk of cancers of the central nervous system or pituitary gland with 8 or more years of use.

In the cohort study based on the Canadian National Breast Screening study (Silvera *et al.*, 2006), 120 incident glioma cases occurred during an average 16.4 years follow-up. Based on answers to a self-administered questionnaire at recruitment into the cohort, the hazard ratio for gliomas was 1.01 (95%CI: 0.68–1.52) in women who ever used oral contraceptives, and there was no trend in risk with duration of use up to over 6 years of use. In a population-based case–control study of 115 women with gliomas and 323 controls in Sweden (Wigertz *et al.*, 2006), the odds ratio in women who ever used oral contraceptives was 0.8 (95%CI: 0.5–1.4), and the risk did not vary appreciably with duration of use. In a hospital-based case–control study conducted at multiple sites in the USA (Hatch *et al.*, 2005), 212 women with gliomas were compared to 436 controls. Based on responses to in-person interviews, the odds ratio for glioma was 0.66 (95%CI: 0.44–1.00) in women who ever used oral contraceptives, and there trend in risk with duration of use was observed.

Four case–control studies and one cohort study of meningiomas that provided information on possible associations with oral contraceptive use were reviewed by Claus *et al.* (2007). None of the studies showed statistically significant associations with ever user of oral contraceptives, and two studies showed no increase in risk with over 10 years of use.

2.11 Cancer of the urinary tract

The most recent results from the Oxford study showed no association between kidney or bladder cancer combined with the use of oral contraceptives. The relative risk in women who ever used oral contraceptives was 0.8 (95%CI: 0.6–1.2), and there was no trend in risk with duration of use up to over 8 years, and no increase in risk up to over 20 years since last use (Vessey & Painter, 2006).

In the Canadian cohort study of women enrolled in a breast cancer screening trial (Kabat *et al.*, 2007a), the hazard ratio for renal cell cancers in women who ever used oral contraceptives was 0.80 (95%CI: 0.58–1.09), and no trend in risk with duration of use was observed. In the previous *IARC Monograph* (IARC, 2007), one case–control study was cited that also showed no association between risk of renal cell cancer and ever use of oral contraceptives.

Two prospective studies in the USA have shown no increases in risk of cancers of the urinary bladder in users of oral contraceptives. During approximately 26 years of follow-up of 116598 women enrolled in the Nurse's Health Study (McGrath *et al.*, 2006), 336 cases of bladder cancer were detected. The use of oral contraceptives was ascertained periodically during the follow-up period by mailed questionnaire. The relative risk in women who ever used oral contraceptives was 0.84 (95%CI: 0.65–1.08), and there was no trend in risk with duration of use, up to over 6 years of use. During an average follow-up of 15.3 years, 167 cases of bladder cancer developed in a cohort of 54308 women who were enrolled in the Breast Cancer Detection Demonstration Project (Cantwell *et al.*, 2006). Oral contraceptive use was based on answers to telephone interviews

at the time of recruitment. The relative risk of bladder cancer was 1.14 (95%CI: 0.77–1.70) in women who ever used oral contraceptives, and no trend in risk with duration of use up to over 5 years of exposure was observed.

2.12 Cancer of the lung

The most recent results from the Oxford (Vessey & Painter, 2006) and Royal College (Hannaford *et al.*, 2007) studies, from the Shanghai BSE trial cohort (Rosenblatt *et al.*, 2008), and from the Canadian National Breast Screening Study (Kabat *et al.*, 2007b) showed no increased risk of lung cancer in oral contraceptive users, and no trends in risk with duration of use, and the two British studies also showed no increase up to 20 years or more since last use. One case–control study, summarized in the previous *IARC Monograph* (IARC, 2007) found a reduced risk in smokers who ever used oral contraceptives (OR, 0.50; 95%CI: 0.34–0.74), but not in non-smokers. Another case–control study nested in the Royal College study (Elliott & Hannaford, 2006) found no increases in the risk of lung cancer in women who ever used oral contraceptives or in relation to duration of use, or time since first or last use. Current users (at the time of diagnosis) had an odds ratio of 0.5 (95%CI: 0.1–3.3).

2.13 Cancer of the pancreas

In the previous *IARC Monograph* (IARC, 2007), no association was observed between ever users of oral contraceptives and risk of pancreatic cancer, and there was no trend in risk with duration of use. A cohort of 387981 postmenopausal women in the USA, the CPS-II (Teras *et al.*, 2005), also found no significant trend ($P = 0.19$) in pancreatic cancer mortality rates with years of oral contraceptive use.

2.14 Cancer of the gallbladder

Four of five studies reviewed in the previous *IARC Monograph* (IARC, 2007) showed no association between risk of cancer of the gallbladder and ever users of oral contraceptives. In the Shanghai BSE trial cohort (Rosenblatt, *et al.*, 2008), no significant increase in risk in users of oral contraceptives for cancer of the gallbladder was observed.

2.15 Cancer of the stomach

The Oxford study found no association between risk of oesophageal and stomach cancers combined and use of oral contraceptives. The relative risks in ever users, and in users for over 8 years were 0.6 (95%CI: 0.3–1.3) and 0.5 (95%CI: 0.2–1.2), respectively (Vessey & Painter, 2006). The Shanghai BSE trial cohort (Rosenblatt *et al.*, 2008) found a relative risk for stomach cancer of 1.02 (95%CI: 0.82–1.27), and no trend with duration of use. A hazard ratio for stomach cancer of 1.05 (95%CI: 0.70–1.58) was found in women who had ever used oral contraceptives at entry into another cohort study in Shanghai (Freedman *et al.*, 2007), based on 154 cases of stomach cancer that occurred in 73442 women followed from 1997–2004. A population-based case–control study in ten Canadian provinces (Frise *et al.*, 2006) compared answers to a self-administered questionnaire by 326 women with gastric adenocarcinoma to answers from an equal number of age-matched controls. The odds ratio in women who ever used oral contraceptives was 0.79 (95%CI: 0.43–1.45).

2.16 Other cancers

A pooled analysis of data from three hospital-based case–control studies was cited in the previous *IARC Monograph* (IARC, 2007) that estimated the odds ratio of squamous cell oesophageal cancer in women who ever used

oral contraceptives to be 0.24 (95%CI: 0.06–0.96) (Gallus *et al.*, 2001). The prior review also included two case–control studies that showed the risk of gestational trophoblastic disease to be increased in women who ever used oral contraceptives, with increasing trends with duration of use (Palmer *et al.*, 1999; Parazzini *et al.*, 2002). Also, the risk of neuroblastoma in children whose mothers took oral contraceptives during their pregnancy was observed to be increased in one study (Schüz *et al.*, 2001), but not in another (Olshan *et al.*, 1999).

2.17 Synthesis

There are increased risks for cancer of the breast in young women among current and recent users only, for in-situ and invasive cancer of the uterine cervix, and for cancer of the liver in populations that are at low risk for HBV infection (this risk is presumably masked by the large risk associated with HBV infection in HBV-endemic populations).

In addition, for cancer of the uterine cervix, the magnitude of the associations is similar for in-situ and invasive disease, and the risks increase with duration of use, and decline after cessation of use.

For cancer of the endometrium, the Working Group concluded that oral contraceptives are protective against endometrial cancer, that the magnitude of the protective effect increases with duration of use, and that it lasts for at least two decades after cessation of use. There is also evidence that the level of the protective effect is proportional to the progestogen potency of the preparation, and inversely proportional to its estrogen potency.

For cancer of the ovary, the Working Group concluded that oral contraceptives are protective against ovarian cancer. The reduction in risk increases with duration of use and persists for at least 30 years after cessation of use. The level of protection is at least as great for newer, lower dose preparations or for older, higher dose oral contraceptives. The reduced risk is seen in women with and without a genetic predisposition to ovarian cancer.

For cancer of the colorectum, the Working Group concluded that it is unlikely that the use of oral contraceptives increases the risk of cancers of the colon or rectum. The aggregate information suggests that oral contraceptives may reduce the risk of colorectal cancer.

The Working Group concluded that the use of oral contraceptives is unlikely to alter the risk of cancer of the thyroid, lung, stomach, urinary tract, gallbladder, pancreas, or the risk of lymphoma, cutaneous melanoma, and tumours of the central nervous system.

3. Cancer in Experimental Animals

The carcinogenicity of combined estrogen–progestogen contraceptives was extensively reviewed in the previous *IARC Monograph* (IARC, 2007). Since then, no new relevant studies have been published.

The data evaluated showed a consistent carcinogenic effect of several estrogen–progestogen combinations across different animal models in several organs.

3.1 Estrogen–progestogen combinations

The incidence of malignant mammary tumours was increased in female and male mice by ethinylestradiol plus megestrol acetate, in female and male rats by ethinylestradiol plus ethynodiol diacetate, and in female rats by mestranol plus norethisterone and mestranol plus norethynodrel. The incidence of benign mammary tumours was increased in male rats by ethinylestradiol plus norethisterone acetate, in intact and castrated male mice by

Table 3.1 Effects of combinations of various estrogens and progestogens on tumour incidence in mice

Combination	Mammary tumours			Pituitary adenomas		Uterine tumours	Cervical/vaginal tumours
	Benign	*Malignant*					
	Male	Male	Female	Male	Female		
Ethinylestradiol + chlormadinone acetate	+/c						
Ethinylestradiol + ethynodiol diacetate				+	+	+[b]	
Ethinylestradiol + megestrol acetate		+[a]	+[a]				
Ethinylestradiol + norethisterone					+		
Ethinylestradiol + norethisterone acetate				+/?	+/?		
Mestranol + chlormadinone acetate				+	+		
Mestranol + ethynodiol diacetate				+	+		
Mestranol + lynestranol			+/–				
Mestranol + norethisterone				+	+		
Mestranol + norethynodrel	c		+/?	+	+		+[b]

[a] same study

[b] only one study

+, increased tumour incidence; +/–, slighly increased tumour incidence; +/c, increased tumour incidence in intact and castrated animals; c, increased tumour incidence in castrated animals; +/?, increased tumour incidence, but not greater than that with the estrogen or progestogen alone

From IARC (1979, 1999, 2007)

ethinylestradiol plus chlormadinone acetate, and in castrated male mice by mestranol plus norethynodrel. Ethinylestradiol plus norethisterone acetate did not cause tumour formation in any tissue in one study in female monkeys (IARC, 1999, 2007; Table 3.1; Table 3.2).

In female and male mice, the incidence of pituitary adenoma was increased by administration of mestranol plus chlormadinone acetate, mestranol plus ethynodiol diacetate, ethinylestradiol plus ethynodiol diacetate, mestranol plus norethisterone, ethinylestradiol plus norethisterone (females only), and mestranol plus norethynodrel. The latter combination also increased the incidence of pituitary adenomas in female rats (IARC, 1999, 2007).

In female mice, the incidence of malignant non-epithelial uterine tumours was increased by ethinylestradiol plus ethynodiol diacetate, and the incidence of vaginal or cervical tumours was increased by norethynodrel plus mestranol. In female mice treated with 3-methylcholanthrene to induce genital tumours, ethinylestradiol plus lynestrenol, ethinylestradiol plus norgestrel, and mestranol plus norethynodrel increased the incidence of uterine tumours; however, this occurred only at the highest doses of ethinylestradiol plus lynestrenol and ethinylestradiol plus norgestrel that were tested. Lower doses inhibited tumorigenesis induced by 3-methylcholanthrene alone (IARC, 1999, 2007).

Ethinylestradiol plus norethisterone acetate and mestranol plus norethisterone increased the incidence of liver adenomas in male rats. Liver foci, which are putative preneoplastic lesions, were induced in female rats by mestranol plus norethynodrel. In female rats initiated for hepatocarcinogenesis with *N*-nitrosodiethylamine, mestranol plus norethynodrel increased the formation of altered hepatic foci (IARC, 1999, 2007).

Table 3.2 Effects of combinations of various estrogens and progestogens on tumour incidence in rats

Combination	Mammary tumours			Liver				Pituitary adenomas	
	Benign	*Malignant*		*Adenomas*		*Carcinomas*			
	Male	Male	Female	Male	Female	Male	Female	Male	Female
Ethinylestradiol + ethynodiol diacetate		+	+						
Ethinylestradiol + megestrol acetate	+/–	+/–	+/–	+/?	+/?				
Ethinylestradiol + norethisterone acetate	+			+		–	+/–		
Ethinylestradiol + norgestrel	+/–								
Mestranol + ethynodiol diacetate		?	?						
Mestranol + norethisterone			+[a]	+	–				
Mestranol + norethynodrel	+/?	+/?	+	+/?	–	–	–	+/?	+

[a] one study only

+, increased tumour incidence; +/–, slighly increased tumour incidence; +/?, increased tumour incidence, but not greater than that with the estrogen or progestogen alone; ? conflicting result; –, no effect

From IARC (1979, 1999, 2007)

In one study, subcutaneous administration of levonorgestrel with ethinylestradiol or estradiol to female rabbits clearly induced deciduosarcomas in several organs (uterus, spleen, ovary, liver, and lung) (Jänne *et al.*, 2001; IARC, 2007).

3.2 Estrogens

The incidence of malignant mammary tumours in female and male mice and female rats was increased by ethinylestradiol and mestranol; however, mestranol did not increase the incidence of mammary tumours in female dogs in a single study.

In female mice, ethinylestradiol alone was associated with the development of uterine cancer. Ethinylestradiol also increased the incidence of cervical tumours in female mice.

The incidence of pituitary adenomas was increased by ethinylestradiol and mestranol in female and male mice, and by ethinylestradiol in female rats.

In female and male mice, ethinylestradiol increased the incidence of hepatocellular adenomas. In female rats, ethinylestradiol and mestranol increased the numbers of altered hepatic foci. In rats, ethinylestradiol increased the incidence of adenomas in females and males, and that of hepatocellular carcinomas in females, whereas mestranol increased the incidence of hepatic nodules and carcinomas combined in females.

The incidence of microscopic malignant kidney tumours was increased in male hamsters exposed to ethinylestradiol. In male hamsters, subcutaneous implantation of estradiol was associated with the development of renal tumours of unspecified histology.

In female mice initiated for liver carcinogenesis and exposed to unleaded gasoline, ethinylestradiol increased the number of altered hepatic foci; however, when given alone after the liver carcinogen, it reduced the number of such foci.

In female rats initiated for liver carcinogenesis, ethinylestradiol and mestranol increased the number of altered hepatic foci and the incidence of adenomas and carcinomas. Ethinylestradiol also increased the incidence of kidney adenomas, renal cell carcinomas and liver carcinomas in male rats initiated with *N*-nitrosoethyl-*N*-hydroxyethylamine. In female hamsters initiated

Table 3.3 Effects of ethinylestradiol and mestranol on tumour incidence in mice

Estrogen	Malignant mammary tumours		Vaginal/cervical tumours	Malignant uterine tumours	Pituitary adenomas		Liver adenomas	
	Male	Female			Male	Female	Male	Female
Ethinylestradiol	+	+	+[a]	+	+	+	+	+
Mestranol					+	+	–	–

[a] one study only
+, increased tumour incidence; –, no effect
From IARC (1979, 1999, 2007)

with *N*-nitrosobis(2-oxopropyl)amine, ethinylestradiol increased the incidence of renal tumours and the multiplicity of dysplasias.

In female rabbits, subcutaneous administration of ethinylestradiol alone was associated with the proliferation of hepatic bile duct cells.

Subcutaneous injection of 2-hydroxy- and 4-hydroxyestradiol induced uterine adenocarcinomas in female mice.

Oral administration of ethinylestradiol to *p53*-deficient female mice in combination with an intraperitoneal injection of the known carcinogen *N*-ethyl-*N*-nitrosourea increased the incidence of uterine atypical hyperplasias and stromal sarcomas.

In female mice initiated with *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine, subcutaneous implantation of estradiol, estrone, estriol, 16β-hydroxyestrone diacetate, 16α-hydroxyestrone, and 17-epiestrol increased the incidence of endometrial adenocarcinomas (IARC, 1999, 2007; Table 3.3; Table 3.4).

3.3 Progestogens

The incidence of malignant mammary tumours was increased in female mice by lynestrenol, megestrol acetate, and norethynodrel. In female rats, lynestrenol and norethisterone slightly increased the incidence of malignant mammary tumours. Norethisterone also slightly increased the incidence of malignant mammary tumours in male rats, while norethynodrel increased the incidence of both benign and malignant mammary tumours in male rats. In female dogs, chlormadinone acetate, lynestrenol and megestrol acetate increased the incidence of benign and malignant mammary tumours; however, lynestrenol had a protective effect at a low dose but enhanced tumour incidence at two higher doses. Levonorgestrel did not increase the incidence of mammary tumours in one study in dogs (IARC, 1999, 2007).

Megestrol acetate increased the incidence of liver adenomas in female mice. Cyproterone

Table 3.4 Effects of ethinylestradiol and mestranol on tumour incidence in rats

Estrogen	Malignant mammary tumours	Liver				Pituitary adenomas
		Adenomas		*Carcinomas*		
	Female	Male	Female	Male	Female	Female
Ethinylestradiol	+	+	+		+	+
Mestranol	+[a]				+/–	

[a] one study only
+, increased tumour incidence; –, no effect; +/–, slightly increased tumour incidence
From IARC (1979, 1999, 2007)

Table 3.5 Effects of various progestogens on tumour incidence in mice

Progestogen	Mammary tumours		Liver				Pituitary adenomas	
	Benign	*Malignant*	*Adenomas*		*Carcinomas*			
	Male	Female	Male	Female	Male	Female	Male	Female
Chlormadinone acetate			+/–					
Cyproterone acetate			+[a]	+/–[a]	+[a]	+[a]		
Ethynodiol diacetate	c		+/–					
Lynestrenol		+	+					
Megestrol acetate		+[b]		+				
Norethisterone acetate			+/–					
Norethisterone			+/–					+
Norethynodrel	c	+		+/–			+	+

[a] dose exceeded the maximum tolerated dose
[b] one study only
+, increased tumour incidence; +/–, slightly increased tumour incidence; –, no effect; c, increased incidence in castrated males
From IARC (1979, 1999, 2007)

acetate increased the incidence of liver adenomas and hepatocellular carcinomas in female and male mice, but at levels that exceeded the maximum tolerated dose. In rats, the incidence of liver adenomas was increased by norethisterone acetate (females and males), norethisterone (males), norethynodrel and cyproterone acetate (females and males). The numbers of altered hepatic foci in female rats were also increased by norethisterone acetate and cyproterone acetate. In male mice treated with chlormadinone acetate, ethynodiol diacetate, lynestrenol, norethisterone or norethisterone acetate, the incidence of liver adenomas was increased. In female rats treated with *N*-nitrosodiethylamine to initiate hepatocarcinogenesis, norethynodrel increased the number of altered hepatic foci. Norethynodrel alone was shown to increase the incidence of hepatocarcinomas in male rats (IARC, 1999, 2007).

The incidence of pituitary adenomas was increased by norethisterone in female mice and by norethynodrel in female and male mice, and male rats (IARC, 1999, 2007).

In female mice treated with 3-methylcholanthrene to induce uterine tumours, norethynodrel further increased the tumour incidence. Levonorgestrel in combination with *N*-nitrosobis(2-oxopropyl)amine did not increase the incidence of renal dysplastic lesions or tumours in female hamsters (IARC, 1999, 2007).

Oral administration of dienogest induced mammary gland proliferation in female dogs but not in female rats or monkeys (Ishikawa *et al.*, 2000; IARC, 2007).

See Table 3.5, Table 3.6.

3.4 Synthesis

Ethinylestradiol plus ethynodiol diacetate caused malignant mammary tumours in rats. Mestranol plus norethynodrel caused malignant mammary tumours in rats. Ethinylestradiol plus levonorgestrel caused deciduosarcomas of the uterus, spleen and liver in rabbits. Estradiol plus levonorgestrel caused deciduosarcomas of the uterus, spleen, ovary, liver and lung in rabbits.

Ethinylestradiol caused malignant mammary tumours in mice and rats and liver cancer in rats. Mestranol caused malignant mammary tumours in mice.

Table 3.6 Effects of various progestogens on tumour incidence in rats

Progestogen	Mammary tumours			Liver			Pituitary adenomas
	Benign	*Malignant*		*Adenomas*		*Carcinomas*	
	Male	Male	Female	Male	Female	Male	Male
Cyproterone acetate				+[a]	+[a]		
Ethynodiol diacetate	+						
Lynestrenol			+/–				
Norethisterone acetate				+	+		
Norethisterone	+/–	+/–	+/–	+			
Norethynodrel	+	+[b]		+	+	+[b]	+

[a] liver adenomas detected only at high doses
[b] one study only
+, increased tumour incidence; +/–, slightly increased tumour incidence; –, no effect
From IARC (1979, 1999, 2007)

Norethynodrel caused malignant mammary tumours in mice. Lynestrenol caused malignant mammary tumours in mice.

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

The formulations of combined hormonal contraceptives continue to evolve, especially with the introduction of new progestogens (Practice Committee of the American Society for Reproductive Medicine, 2006; Sitruk-Ware, 2006; Spitz, 2006; Madauss *et al.*, 2007). In general, the chemical structure of a progestogen determines its relative binding affinities for the progesterone receptor and other steroid receptors, as well as sex hormone-binding globulin, which both determine its biological effects.

Estrogenic and progestogenic compounds in oral contraceptives are readily absorbed and are metabolized to varying extents by bacterial enzymes, enzymes in the intestinal mucosa, and especially enzymes in the liver. The metabolism typically involves reduction, hydroxylation, and conjugation. First-pass metabolism through the liver reduces the overall bioavailability of oral contraceptives. Peak concentration levels in the systemic circulation are observed between 0.5–4 hours after intake. Hydroxylated metabolites are usually conjugated as glucuronides or sulfates, and are eliminated rapidly with half-lives of 8–24 hours (IARC, 2007).

Estrogens are discussed in the Monograph on Combined Estrogen–Progestogen Menopausal Therapy (IARC, 2007, and this volume).

4.2 Genetic and related effects

4.2.1 Direct genotoxicity

Since the previous *IARC Monograph* (IARC, 2007), there is additional evidence to support the hypothesis that certain estrogens are carcinogenic through genotoxic effects in addition to their presumed action via a receptor-mediated mechanism (see also Estrogen-only Menopausal Therapy in this volume). Some of the more recent data suggest that some progestogens used in combined hormonal contraceptives may also be genotoxic. In the presence but not in the absence of liver microsomes (S9), norethynodrel induced significant increases in sister chromatid exchange and chromosomal abberrations, and reduced replication index in cultured human lymphocytes, suggesting a genotoxic effect that requires a metabolic process (Siddique & Afzal,

2005). [The Working Group noted that supratherapeutic concentrations seem to have been used in this study.] In a similarly structured study using medroxyprogesterone acetate (MPA) as the progestogen, MPA treatment of cells with S9 and NADPH were found to have significant increases in sister chromatid exchange and chromosomal aberrations. Addition of superoxide dismutase increased genotoxicity, and addition of catalase reduced genotoxicity. The results suggest that reactive oxygen species generated during drug metabolism were responsible for the genotoxicity (Siddique *et al.*, 2006a). In studies that focused on agents protecting against genotoxicity in cultured human lymphocytes, the progestogen norgestrel together with cyproterone were shown to increase sister chromatid exchange and chromosomal aberrations (Siddique *et al.*, 2006b, 2008). In an assay that detects DNA double-strand breaks by the presence of phosphorylated histone H2AX as marker for genotoxicity, norethindrone was weakly positive but only at supratherapeutic concentrations (Gallmeier *et al.*, 2005). No data were available on the genetic effects of combined exposures to estrogens and progestogens.

Triplet repeat length polymorphisms in the androgen receptor were evaluated with regard to serum testosterone levels in women, oral contraceptive use, and familial breast cancer risk. Larger numbers of GGC repeats were associated with lower testosterone levels, whereas shorter repeats, particularly if bi-allelic, were more common in women with familial excess of breast cancer. No association was found with oral contraceptive use (Hietala *et al.*, 2007).

Polymorphisms in genes for enzymes that metabolize estrogen were examined in non-Hodgkin lymphoma patients. Although there were some relationships between polymorphism and haplotypes between cases and controls, the most pronounced finding was the significant reduction of risk among female patients who had taken oral contraceptives (Skibola *et al.*, 2005).

4.2.2 Receptor-mediated effects

(a) Cell proliferation

Exposure to combined hormonal contraceptives increases the proliferation of human breast epithelial cells, as observed in biopsies and fine-needle aspirate samples collected during small randomized studies (IARC, 2007). Several recent studies have evaluated the effects of progestogens alone or combined with estrogens on proliferation or proliferation-related end-points in human breast cells. MPA-alone induced proliferation and the expression of pro-proliferative gene procyclin D1 in PR+ human breast cancer cells (Saitoh *et al.*, 2005). In normal explants of premenopausal and postmenopausal human breast tissue from reduction mammoplasty, estrogen and MPA increased the expression of pro-proliferative gene products cyclin D1 and Ki-67, and decreased the expression of anti-proliferative gene products p21 and p27 (Eigėlienė *et al.*, 2008). MPA was also shown to induce expression of caveolin-1 in a murine breast cancer cell line, and this in turn was shown to activate the MAPK and PI-3K signalling pathways that induce cell growth (Salatino *et al.*, 2006). In studies comparing normal (MCF-10A) and malignant human breast cells (MCF-7), effects of MPA or norethisterone were compared on cells treated with estrogens and growth factors. It was found that estrogen and growth factors reduced the ratio of apoptosis to proliferation; and MPA, and to a lesser extent norethisterone, reduced this effect in both cell types (Seeger *et al.*, 2005). In MCF-10A cells and breast cancer cell line HCC1500, MPA decreased the ratio of apoptosis to proliferation, norethisterone produced a lesser decrease, and progesterone had no significant effect (Krämer *et al.*, 2005). MPA and chlormadinone acetate both induced proliferation in MCF-10A cells (Krämer *et al.*, 2006). The results of these studies indicate that progestogens increase the proliferation of breast tissue cells, and the extent of the proliferative stimulus depends on the specific

progestogen (Seeger *et al.*, 2005; Krämer *et al.*, 2005, 2006).

In organ cultures of breast tissue, estradiol, MPA or estradiol plus MPA increased proliferation and decreased apoptosis (Eigėlienė *et al.*, 2006). MPA also stimulated proliferation in xenografts of human breast cancer cell lines grown in nude mice (Liang *et al.*, 2007). When MCF-7 and HCC1500 cells were incubated with ethinylestradiol, a common constituent of oral contraceptives, using different durations of exposure, there was a significant increase in cell proliferation with no difference observed between two treatments (Merki-Feld *et al.*, 2008). In another study which included breast cancer cell lines and organoid cultures of normal, benign and malignant breast, estradiol and progesterone stimulated cell proliferation while tamoxifen and mifepristone (RU486), an anti-progestogen, inhibited cell proliferation (Calaf, 2006). In a study that considered the effects of MPA, megesterol acetate, norethynodrel, and norethindrone on the expression of fatty acid synthase in ER- and PR+ MCF-7 breast cancer cells, only norethynodrel and norethindrone induced the expression significantly. This is important because fatty acid synthase is required for progestogen-induced anchorage-independent growth and survival of these cells (Menendez *et al.*, 2005). In contrast, testosterone prevented the cell proliferation increase, determined by immunohistochemistry for Ki-67/MIB-1, in breast cells collected by fine-needle aspiration from women treated with testosterone in addition to estradiol and norethisterone (Hofling *et al.*, 2007). In a review, the issue of the binding and activation of estrogen receptor and androgen receptor by MPA was considered in relation to breast cancer risk. It was argued that the disruptive effect of MPA may affect androgen action, and thereby reduce the cancer-protective benefits of androgen action in the breast (Birrell *et al.*, 2007). [In a commentary on the results of the WHI study, it was suggested that chossing MPA may have contributed to the results observed due to its side-effect profile (Lauritzen, 2005).]

These hormones have also been evaluated for their effects on cell proliferation in other tissues. Combined hormonal contraceptives have atrophic and anti-proliferative effects on the endometrium that are apparently independent of the regimen or the progestogen used (IARC, 2007). In more recent studies, endometrial biopsies from women receiving depot MPA for contraception and then treated with mifepristone were examined for ERα, progesterone receptors A and B, Ki-67, capsase-3, and apoptosis by TUNEL assay or immunohistochemistry. The treatment with mifepristone initially produced increased cell proliferation and decreased apoptosis, but this effect was lost on prolonged (10 weeks) treatment (Jain *et al.*, 2006). Transient transfection of progesterone receptor into endometrial carcinoma cells followed by treatment with MPA induced the expression of anti-proliferative proteins p21 and p27 (Kawaguchi *et al.*, 2006). Proliferation of human ovarian cancer cells (OVCAR-3) was stimulated by both low and high concentrations of mifepristone and progesterone (Fauvet *et al.*, 2006). In another study with OVCAR-3 cells, treatments with progesterone, MPA, and norethisterone were evaluated for their effects on proliferation and on growth-factor-induced cell proliferation. MPA and norethisterone but not progesterone induced cell proliferation without growth-factor induction; with growth factors, MPA but not norethisterone or progesterone inhibited proliferation (Seeger *et al.*, 2006). Progesterone receptors A and B were evaluated in the fallopian tube and uterus of mice treated with progesterone. Progesterone reduced progesterone receptors A and B expression in both tissues and decreased p27, cyclin D2, and proliferating cell nuclear antigen only in the uterus. Treatment with anti-progestogens increased progesterone receptors A and B, and induced apoptosis (Shao *et al.*, 2006).

The effects of progesterone and mifepristone on cell proliferation were examined in two astrocytoma cell lines. Progesterone increased cell proliferation, mifepristone reduced cell proliferation when used alone and decreased progesterone-induced proliferation. The effects of mifepristone were not the result of apoptosis (González-Agüero *et al.*, 2007). In rat liver, ethinylestradiol reduced the number of cycling cells together with a reduction of pro-proliferative markers and an increase in anti-proliferative gene expression consistent with a cell-cycle block before S-phase (Koroxenidou *et al.*, 2005). In male rats treated with estradiol, in addition to a reduction of the differentiation of sperm, there was a rise in lipid peroxidation, a fall in catalase and superoxide dismutase, and a rise in cells showing signs of apoptosis in the testicular tissue (Chaki *et al.*, 2006).

Estrogens or progestogens may enhance HPV gene expression in the human cervix via progesterone-receptor mechanisms, and hormone-response elements in the viral genome. In-vitro studies support this concept, and mechanisms other than those that are receptor-mediated may be involved. Experiments in transgenic mouse models that express HPV-16 genes in the cervix showed that estrogens can cause cervical cancer, probably via a receptor-mediated process. This effect was diminished after cessation of treatment with estrogens (IARC, 2007). There are a few new reports on the increase in incidence of squamous cell carcinoma of the cervix among users of oral contraceptives. In a report comparing COX-2 protein concentrations in biopsies of cervical lesions and normal tissue from a small group of patients, there was a much greater quantity of COX-2 protein in CIN1 and CIN2 lesions than in controls, but there were no significant associations with oral contraceptive use (Saldivar *et al.*, 2007). In an uncontrolled study of 80 cervical cancer patients, oral contraceptive use was not associated with any of the several tumour markers investigated. In contrast, strong c-myc staining was associated with high concentrations of progesterone; low epithelial growth factor receptor staining was associated with high concentrations of estradiol in serum, and current smoking was strongly associated with an absence of p53 staining (Lindström *et al.*, 2007).

It was proposed that the thick and viscous cervical mucus of oral contraceptive users might prolong contact of the cervix with carcinogenic agents (Guven *et al.*, 2007).

Ethinylestradiol plus levonorgestrel induces ovarian epithelial cell apoptosis in intact monkeys (Rodriguez *et al.*, 2002).

Colon carcinogenesis in animal models is inhibited by estrogens, and there is adequate evidence to suggest that estrogens have inhibitory effects on colon cancer cells via ERβ (IARC, 2007).

(b) Cell differentiation

Recent reports have considered the effects of hormones in oral contraceptives on the regulation of expression of several gene products. Progesterone-induced blocking factor was shown to be induced by progesterone and this induction was counteracted by mifepristone in several cell populations (Srivastava *et al.*, 2007). Estradiol and progesterone inhibited the expression of gonadotropin-releasing hormone I receptor in a gonadotroph-derived cell line α-T3–1 (Weiss *et al.*, 2006). Expression of breast cancer resistance protein was downregulated by estradiol in an ER-related manner whereas progesterone or progesterone plus estradiol upregulated breast cancer resistance protein, but these effects of progesterone appeared to be independent of progesterone receptors (Wang *et al.*, 2006). In mouse mammary tumour cell lines, MPA treatment strongly induced the expression of signal transducer and activator of transcription (Stat) and its binding to DNA; mifepristone inhibited induction (Proietti, *et al.*, 2005). In a gene expression microarray study of several synthetic progestogens used in oral contraceptives, the

altered pattern of genes expression induced by progestogen were quite comparable among the progestogens tested; drospirenone, a spironolactone analogue, had the most divergent profile (Bray *et al.*, 2005). In ER- and PR+ MCF-7 cells, the effects of 4-hydroxytamoxifen alone or in combination with mifepristone were evaluated in relation to the expression of retinoblastoma protein. The combined treatment had cytostatic and cytotoxic effects; the apoptotic cytotoxic effect was mediated by a drug-induced decrease of retinoblastoma protein (Schoenlein *et al.*, 2007). In human T47D breast cancer cells, treatment with progesterone or MPA, with or without estradiol, increased the expression of myoepithelial cytokeratins, which is indicative of a luminal epithelial to myoepithelial transition (Sartorius *et al.*, 2005). Human breast cancer cells were treated with estradiol plus progestogens either continuously or in a sequential combined regimen, and the expression of estrogen-activating and -inactivating enzymes were evaluated. MPA, and to a lesser extent progesterone, induced the mRNA and protein expression of estrogen-activating genes but did not influence the expression of estrogen-inactivating enzymes. Levonorgestrel, norethindrone and dienogest had no detectable effect on either type of enzymes. This raises the question of whether MPA might be associated with a higher risk of breast cancer than other progestogens (Xu *et al.*, 2007). Matrix metalloproteinases (MMP) 2 and 9 were evaluated in T47D breast cancer cells treated with combinations of estradiol–progesterone or estradiol–MPA, and in MCF-7 breast cancer cells treated with estradiol–progesterone, estradiol–MPA or equilin–MPA. All treatments increased MMP-2 in both cell types and MMP-9 in MCF-7 cells. Only one combined treatment was found to increase MMP-9 in T47D cells (Abdallah *et al.*, 2007).

Endometrial specimens from women treated with progestogens for hyperplasia, with either MPA administered systemically or with a levonorgestrel intrauterine device, were evaluated for expression of Bcl-2 (anti-apoptotic) and BAX (pro-apoptotic) by immunohistochemistry and apoptosis using the TUNEL method. The levonorgestrel intrauterine device reversed all hyperplasias whereas with MPA only 50% hyperplasias were reversed. Both treatments reduced glandular Bcl-2 expression with increased apoptosis upon prolonged therapy; in each case, the effects of the levonorgestrel intrauterine device therapy exceeded those of the systemic MPA (Vereide *et al.*, 2005). In a similar study, progestogen therapy reduced the expression of progesterone receptor A, progesterone receptor B, ERα and ERβ in the endometrium with the effects of the levonorgestrel intrauterine device exceeding those of MPA (Vereide *et al.*, 2006). In a review considering the effects of levonorgestrel intrauterine devices on the endometrium, treatment was associated with decidualization of the stromal cells, atrophy of glandular and surface epithelial cells, downregulation of sex steroid receptors with perturbation of locally acting progesterone-regulated mediators (Guttinger & Critchley, 2007). MPA enhanced expression of Forkhead Transcription Factor FOXO1 in differentiating human endometrial stromal cells with induction of cytoplasmic retention and inactivation. Upon withdrawal of MPA, FOXO1 accumulated in the nucleus, the expression of *BIM*, a pro-apoptotic target gene, was induced, and cell death occurred (Labied *et al.*, 2006). Endometrial epithelial and stromal cells were treated with estradiol, MPA and the combination of estradiol and MPA, and the expression of interleukins (IL) 13 and 15 were evaluated. All hormone treatments induced expression of IL-13 and IL-15 but with some cell- and hormone-related variations, affecting proliferation- and inflammation-related functions (Roberts *et al.*, 2005).

In a gene-expression microarray study of a human endometrial epithelial cell line, treatments with estradiol, MPA, estradiol plus MPA, or tibolone were compared. Tibolone-induced

gene-expression profiles resembled those found after MPA treatment (Hanifi-Moghaddam *et al.*, 2006). Endometrial carcinoma cells made resistant to the growth-suppressive effect of progesterone by prolonged culture with progesterone were evaluated for expression of ERα, ERβ, and PR-B as well as transforming growth factor α, and epidermal growth factor receptor. Chronic exposure to progesterone reduced the expression of ERα and PR-B, and increased the expression of ERβ, transforming growth factor α, and epidermal growth factor receptor (Zhao *et al.*, 2007).

(c) Other effects

Exposure to exogenous hormones affects the proliferative activity and quantity of stromal and epithelial tissue in the breast, thereby increasing breast density assessed by mammography, a factor highly correlated with breast cancer risk (Boyd *et al.*, 2006). In a study in Norwegian women, there was a significant dose-dependent increase in mammographic density in current users of estradiol plus norethisterone (Bremnes *et al.*, 2006). In a related Norwegian study, in this case only considering women not currently using exogenous hormone therapy, there was an association between breast density and plasma steroid hormone-binding globulin concentration, and a weaker association with plasma estrone concentrations (Bremnes *et al.*, 2007). In another Norwegian study, lower prevalence of mammary ductal hyperplasia in women was associated with current long-term (8 years or more) oral contraceptive use, while the onset of oral contraceptive use after the age of 35 years was associated with an increased prevalence of ductal carcinoma *in situ* (Vamre *et al.*, 2006). The effect of hormonal contraceptives on steroid hormone receptors in biopsies of the vaginal epithelium was investigated in a Swedish study. The progesterone receptor level was significantly reduced in women receiving depot MPA as compared to controls or women receiving levonorgestrel implants or oral contraceptives. In addition, the estrogen receptor level was significantly elevated in women treated with depot MPA, and all treatments reduced serum estradiol levels (Ildgruben *et al.*, 2005). In review articles on human hepatic adenoma, the relationship between this lesion and oral contraceptive use has been considered. The frequency of this rare benign tumour is greater in young women and women with a history of oral contraceptive use. There is a relationship between duration of use and risk (Giannitrapani *et al.*, 2006; Lizardi-Cervera *et al.*, 2006). Use of MPA did not have a significant effect on risk of high-grade cervical intra-epithelial neoplasia in young women (Massad *et al.*, 2005).

4.3 Synthesis

Hormone-receptor-mediated responses are probably a necessary mechanism for hormonal carcinogenesis by combined estrogen–progestogen oral contraceptives. Progestogens including those used for combined estrogen–progestogen oral contraceptives appear to have the capacity to stimulate cell proliferation in human breast cells and to inhibit proliferation in human endometrial cells. The magnitude of these effects vary for different synthetic progestogens. Because estrogen mediates the expression of progesterone-receptor expression, the presence of estrogen in these combined estrogen–progestogen oral contraceptives may be essential for progestogen-mediated cell proliferation. Combined estrogen–progestogen oral contraceptives were also shown to produce increased radiological breast density, and increased proliferation of cells removed from the breast by needle biopsy. In animal models, estrogen potentiated cervical cancer, and inhibited colon cancer development.

There is also support for the involvement of genotoxic effects of the metabolic by-products of estrogenic hormones in combined estrogen–progestogen oral contraceptives or of the reactive oxygen species generated in response to them.

The estrogenic metabolites can bind to DNA, and the reactive oxygen species can also damage DNA. There are also some data consistent with some genotoxic effects of progestogens.

Current evidence suggests that the predominant effects of combined estrogen–progestogen oral contraceptives associated with hormonal carcinogenesis occur via one or more receptor-mediated process.

Cessation of hormonal treatment may reduce some receptor-mediated effects. It is plausible that hormone-induced genotoxic effects may be persistent.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of combined estrogen–progestogen oral contraceptives. Combined estrogen–progestogen oral contraceptives cause cancer of the breast, in-situ and invasive cancer of the uterine cervix, and cancer of the liver.

For cancer of the endometrium, ovary, and colorectum, there is *evidence suggesting lack of carcinogenicity*. An inverse relationship has been established between exposure to combined estrogen–progestogen oral contraceptives and cancer of the endometrium, ovary, and colorectum.

There is *sufficient evidence* in experimental animals for the carcinogenicity of several combinations of estrogen–progestogen used in oral contraceptives.

Combined estrogen–progestogen oral contraceptives are *carcinogenic to humans (Group 1)*.

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AZATHIOPRINE

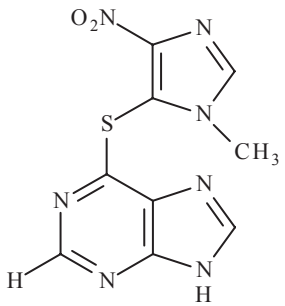
Azathioprine was considered by previous IARC Working Groups in 1980 and 1987 (IARC, 1981, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 446-86-6
Chem. Abstr. Name: 9*H*-Purine, 6-[(1-methyl-4-nitro-1*H*-imidazol-5-yl)thio]-
IUPAC Systematic Name: 6-(3-Methyl-5-nitroimidazol-4-yl)sulfanyl-7*H*-purine
Synonyms: 6-(1-Methyl-4-nitroimidazol-5-yl)thiopurine; 6-(1-methyl-4-nitromidazol-5-ylthio)purine; 1*H*-purine, 6-[(1-methyl-4-nitro-1*H*-imidazol-5-yl) thio]-
Description: Pale yellow, odourless powder (Sweetman, 2008)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_9H_7N_7O_2S$
Relative molecular mass: 277.3

1.2 Use of the agent

Information for Section 1.2 is taken from McEvoy (2007), Royal Pharmaceutical Society of Great Britain (2007), and Sweetman (2008).

1.2.1 Indications

Azathioprine is used as an adjunct for prevention of the rejection of kidney allografts. The drug is usually used in conjunction with other immunosuppressive therapy including local radiation therapy, corticosteroids, and other cytotoxic agents. Azathioprine is also used for the management of the signs and symptoms of rheumatoid arthritis in adults. It is considered to be a prodrug which converts into 6-mercaptopurine after absorption. 6-Mercaptopurine is an important component of treatment programmes for acute lymphocytic leukaemia in children and adults.

1.2.2 Dosage

Azathioprine is usually administered orally. Following renal transplantation, azathioprine may initially be given intravenously to patients unable to tolerate oral medication. Oral therapy should replace parenteral therapy as soon as possible.

Azathioprine sodium aqueous solution (10 mg/mL) may be given by direct intravenous injection or further diluted in 0.9% sodium chloride or 5% dextrose injection for intravenous infusion. Intravenous infusions of the drug are usually administered over 30–60 minutes; however, infusions have been given over periods ranging from 5 minutes to 8 hours.

(a) Renal allotransplantation

The usual oral dosage of azathioprine in children and adults undergoing renal transplantation is 3–5 mg/kg daily. Dosage reduction may be necessary due to leukopenia.

(b) Autoimmune disorders

For the treatment of severe active rheumatoid arthritis and autoimmune disorders the usual initial oral adult dosage of azathioprine is 1 mg/kg (approximately 50–100 mg) daily; daily dosage is increased as necessary at 4-week intervals by 0.5 mg/kg up to a maximum of 2.5 mg/kg. Daily maintenance dosage may be reduced to the lowest possible effective level in increments of 0.5 mg/kg (or approximately 25 mg) every 4 weeks, while keeping other therapy constant. The optimum duration of maintenance therapy has not been determined. Azathioprine may be administered in a single dose or twice-daily doses.

Azathioprine is available as 25, 50, 75, and 100 mg tablets for oral administration. Azathioprine sodium is also available as 50 and 100 mg (of azathioprine) powders for reconstitution for injection and intravenous use for parenteral administration.

1.2.3 Trends in use

No information was available to the Working Group.

2. Cancer in Humans

2.1 Transplant recipients

Assessment of the carcinogenicity of single agents used in solid organ transplant recipients can be complex owing to the multiple drugs used in modern transplantation, and to the drugs' respective mechanisms of action.

One large prospective cohort study (Kinlen *et al.*, 1979; also reported in IARC, 1981) on renal transplant recipients that received azathioprine examined the incidence and mortality from different types of cancer compared with expected numbers, based on the incidence and mortality rates for the relevant country (Australia, New Zealand, the United Kingdom). An almost 60-fold increase in risk for non-Hodgkin lymphoma was observed, as well as a 30-fold excess of squamous cell skin cancer in patients from the UK (3 observed, 0.13 expected).

A smaller cohort study in Japan did not find an association between azthioprine use as immunosuppressant in renal transplant patients and the development of all cancers combined (Imao *et al.*, 2007).

Recent reviews on transplant immunosuppression in solid organ recipients reported increased risks for cancers at several sites following azathioprine use (Kauffman *et al.*, 2006; Dantal & Pohanka, 2007).

2.2 Autoimmune disorders

Azathioprine is used in a greater number of autoimmune patients than of transplant recipients. Exposure to immunosuppressive drugs is often sporadical or prolonged, depending on the clinical course (see Table 2.1 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-15-Table2.1.pdf).

Excesses in non-Hodgkin lymphoma (relative risk (RR), 10.9) and squamous cell skin

cancer (RR, 5.0) were also found in non-transplant patients receiving azathioprine, though to a smaller extent (Kinlen, 1985).

Kandiel *et al.* (2005) performed a meta-analysis using six published cohort studies on patients with inflammatory bowel disease, and found a meta-relative risk for non-Hodgkin lymphoma associated with the use of azathioprine of 4.18 (2.07–7.51). [The Working Group noted that this study maintained its power when any one of the comprised studies was excluded.]

Bernatsky *et al.* (2008) examined a nested case-control study of 246 cancer cases and 538 controls selected from an original cohort of approximately 5500 patients with systemic *lupus erythaematosus*. The adjusted hazard ratio for developing all types of cancer after treatment with azathioprine only was 0.75 (0.43–1.31), and for haematological malignancies, 1.19 (0.48–2.92). Taking into account a 5-year lag exposure did not change the results.

Fraser *et al.* (2002) performed an analysis on 626 patients who received azathioprine, selected from a retrospective cohort of 2204 patients with inflammatory bowel disease. Treatment with azathioprine did not significantly increase the risk for cancer at all sites. For patients with ulcerative colitis, risk for colorectal cancer was not increased.

Matula *et al.* (2005) examined 96 patients who received 6-mercaptopurine and azathioprine from a larger cohort of 315 patients with inflammatory bowel disease. The hazard ratio for progression to high-grade dysplasia or colorectal cancer was 1.30 (0.45–3.75).

3. Cancer in Experimental Animals

Azathioprine was tested for carcinogenicity by oral administration in one study in mice and one study in rats, by intraperitoneal administration in two studies in mice and one study in rats, and also by subcutaneous or intramuscular injection in five studies in mice (see Table 3.1).

Oral administration of azathioprine caused increased incidences of systemic lymphomas and uterine haemangioendotheliomas in female mice (Ito *et al.*, 1989). Lymphohaematopoietic malignancies were also observed in mice after intraperitoneal, subcutaneous, or intramuscular injections of azathioprine. However, these studies did not report full experimental details, the age-adjusted comparisons, and/or the early death from autoimmune disease of the hybrid mice used, precluding direct comparison with treated mice. Studies in rats were negative though squamous cell ear-duct carcinomas were observed in male and female rats treated orally (IARC, 1981, 1987a).

Several studies were also conducted in which azathioprine was administered in conjunction with other chemical or biological agents. Mice treated with azathioprine in drinking-water, alone or in combination with antigenic stimulation in the form of a single intraperitoneal injection of lactic-dehydrogenase-elevating virus, subcutaneous injections of complete Freund's adjuvant, intramuscular injections of HeLa cells, or vaccination with smallpox vaccine, developed an increase in malignant lymphomas (IARC, 1981, 1987a). Azathioprine also enhanced the photocarcinogenesis of ultraviolet radiation in hairless mice (Kelly *et al.*, 1987). Oral administration of azathioprine did not enhance the hepatocarcinogenesis of *N*-hydroxy-*N*-2-fluorenyl-acetamide- or of 7,12-dimethylbenz[*a*]anthracene-induced tumours when tested in rats (IARC, 1981, 1987a).

Table 3.1 Studies of cancer in experimental animals exposed to azathioprine

Species, strain (sex) Duration Reference	Route Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, B6C3F1 (M, F) 94 wk Ito et al. (1989)	Feed 0, 5 or 20 ppm azathioprine 50 sex/treatment group	Lymphomas: F–1/50, 5/49, 6/48	$P < 0.05$ for high dose	
		Uterine haemangioendotheliomas: F–0/50, 0/49, 7/48	$P < 0.01$ for high dose	
Mouse, C57BL (M, F) 65 wk Imamura et al. (1973)	s.c. 100 mg/kg bw azathioprine in basic saline twice/wk for 2 wk and then once/wk for 7 mo. Surviving animals kept for an additional 33 wk 25 M, 21 F	Haematopoietic tumours: 11/38 treated animals (sex NR)– Thymic lymphoma ($n = 8$) Reticulum cell neoplasm ($n = 1$) Non-thymic lymphoma ($n = 2$) No leukaemia in the 16 untreated controls (10 M, 6 F) evaluated	NR	No data on survival provided
Mouse, NZB/NZW (F) 15–17 mo Mitrou et al. (1979a, b)	s.c. 0.2 mg azathioprine in basic saline 5 ×/wk 25 mice (120-d-old), 23 mice (180-d-old) 0.4 mg azathioprine 5 ×/wk 24 mice (120-d-old), 24 mice (180-d-old) 2.0 mg azathioprine once/wk 15 mice (120-d-old), 12 mice (180-d-old). 96 untreated or saline- or solvent-treated mice served as controls. Study terminated at the beginning of the 21st month of age for each group	Incidence of neoplasms: Low dose–10/48 High dose–29/48 2.0 mg/wk dose–4/27 Controls–3/96	$P < 0.05$ for high and low dose compared to controls	Tumours were observed in 43 treated animals in all groups and consisted of 33 lymphomas, 3 undifferentiated sarcomas, 4 squamous cell carcinomas of the anal region and 2 adenocarcinomas of the lung. However, they were not tabulated according to individual groups and there were no age-adjusted comparisons provided. The early deaths of NZB hybrid mice from autoimmune disease precluded direct comparison with treated mice

Table 3.1 (continued)

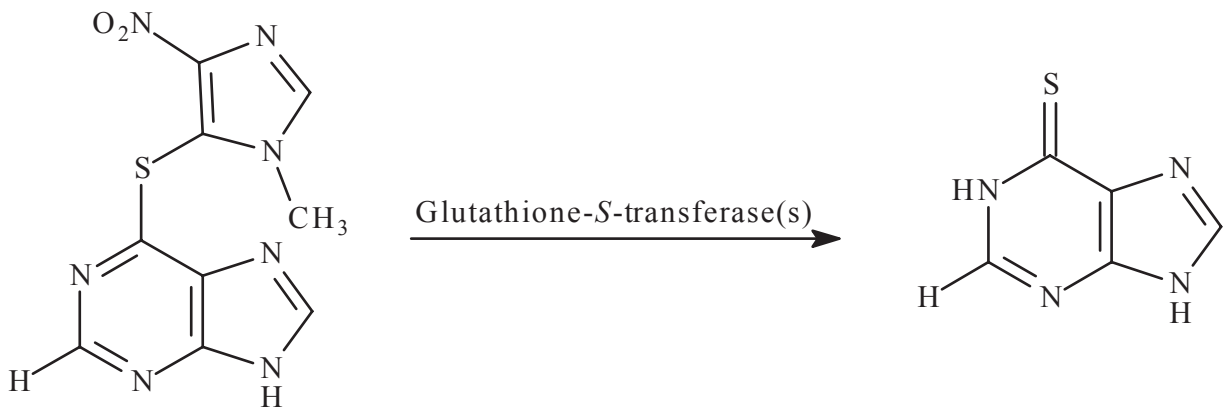
Species, strain (sex) Duration Reference	Route Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, NZB/NZW (F) 7–8 mo Mitrou et al. (1979b)	s.c. 0.2 mg azathioprine in basic saline 5 ×/wk Four groups of 9–10 mice. The same control group as that described in a previous study (Mitrou et al., 1979a) was used	Incidence of neoplasms: Treated animals–10/39 Controls–3/96		10 treated animals developed 11 tumours, which were, histologically, 9 lymphomas, 1 mammary adenocarcinoma and 1 adenocarcinoma of the lung. However, they were not tabulated according to individual groups and there were no age-adjusted comparisons provided. The early deaths of NZB hybrid mice from autoimmune disease precluded direct comparison with treated mice
Mouse, NZB (M) 6 mo Casey (1968a, b)	i.m. 0 or 100 mg/kg bw azathioprine in basic saline 3 ×/wk for 4 wk followed by twice/wk for 1 wk and then once/wk, total treatment period 6 mo 8, 8	Thymic lymphoma: Treated–6/8 Controls–0/6	NR	No age-adjusted comparisons were provided and the early deaths of NZB hybrid mice from autoimmune disease precluded direct comparison with treated mice
Mouse, NZB/NZW (F) 6 mo Casey (1968b)	i.m. 0 or 100 mg/kg bw azathioprine in basic saline 3 ×/wk for 3 wk followed by twice/wk for 1 wk and then once/wk, total treatment period 10 mo 12, 12	Thymic lymphoma: Treated–7/12 Controls–0/6	NR	Mean survival times and latent periods were not specified. No age-adjusted comparisons were provided and early death of NZB hybrid mice from autoimmune disease precluded direct comparison with treated mice

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, Swiss-Webster (M, F) 15 mo Weisburger (1977)	i.p. 7.5 (M or F), 15 (M) or 30 (F) mg/kg bw azathioprine in basic saline 3 ×/wk for 6 mo Two groups of 25 males and females 254 untreated mice as controls. Animals that survived over 100 d observed for up to 12 mo	Incidence of neoplasms: M–9/21 (43%) F–25/40 (63%) [reported as 1.5–2 times higher than that in controls (26%)] Histology: M– Lymphosarcomas (n = 4) Lung tumours (n = 5) F– Lymphosarcomas (n = 11) Lung tumours (n = 6) Uterine tumours (n = 2)	NR	Inadequate reporting of survival times, the amalgamation of various experimental groups and tumour types, as well as the lack of age-adjustment in the analyses precluded a complete evaluation of this study
Rat, Sprague-Dawley CD (M, F) 15 mo Weisburger (1977)	i.p. 18 or 37 mg/kg bw azathioprine in basic saline 3 ×/wk for 6 mo Two groups of 25 males and females Approximately 180 untreated rats of each sex served as controls. Animals that survived over 100 d observed for up to 12 mo	Incidence of neoplasms: M–15/34 F–22/44 (reported as similar to that seen in controls)	NR	Inadequate reporting of survival times, the amalgamation of various experimental groups and tumour types, as well as the lack of age-adjustment in the analyses precluded a complete evaluation of this study
Rat, F344 (M, F) 52 wk Frankel et al. (1970)	Feed 100 mg/kg feed azathioprine/d for 52 wk 50 sex/group 10 males and females (6–8-wk-old) as controls	Squamous cell carcinomas of the ear-duct: Controls–0/20 M–3/17 F–3/25	[NS]	Toxicity observed in females, 40% died within first 12 wk of study. All surviving animals killed after 52 wk. No difference in incidence of other tumour types

bw, body weight; d, day or days; F, female; i.m., intramuscular; i.p., intraperitoneal; M, male; mo, months; NR, not reported; NS, not significant; s.c., subcutaneous; wk, week or weeks; yr, year or years

Fig. 4.1 Conversion of azathioprine to 6-mercaptopurine



Azathioprine

6-Mercaptopurine

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

Azothioprine is essentially a prodrug for 6-mercaptopurine (see Fig. 4.1; for review, see Aarbakke *et al.*, 1997).

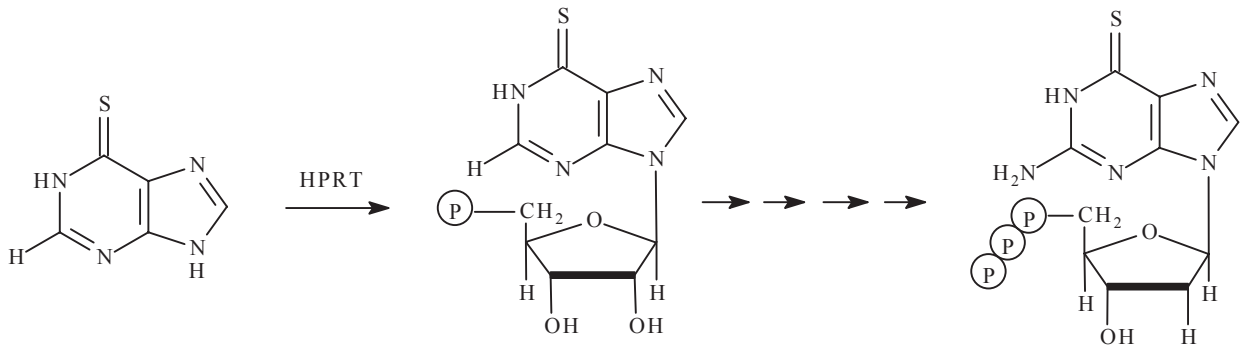
In humans, azathioprine is readily absorbed from the gut. Following oral administration of ^{35}S-azathioprine, 12% of the radioactivity is found in faeces as unabsorbed material, and 50% in the urine over 24 hours. After oral administration of ^{14}C-azathioprine, about 30% is bound to serum proteins in the blood, but it appears to be dialysable (Elion, 1972).

In vivo, azathioprine is split primarily by chemical conversion (Elion & Hitchings, 1975) but possibly also by enzymatic conversion (Watanabe *et al.*, 1978), with the release of 6-mercaptopurine. In addition, splitting occurs such that the sulfur is part of the methylnitroimidazole ring.

In humans, azathioprine is catabolized to a variety of oxidized and methylated derivatives, which are excreted by the kidneys; very little azathioprine or 6-mercaptopurine are excreted intact. At least 11 different metabolites have been identified, with the major one, 6-thiouric acid, found in urine. Other metabolites resulting from the biotransformation of the methylnitroimidazole moiety include 5-mercapto-1-methyl-4-nitroimidazole, 1-methyl-4-nitro-5-thioimidazole, 8-hydroxyazathioprine, and inorganic sulfate (Chalmers *et al.*, 1967; Elion, 1972; Elion & Hitchings, 1975). Some of these metabolites are also found in the urine of rodents and dogs. The profile of the methylnitroimidazole urinary metabolites in the dog is similar to that in humans but different from that in the rat (de Miranda *et al.*, 1973, 1975; IARC, 1981).

Most of the biological and biochemical effects of azathioprine depend on its in-vivo conversion to 6-mercaptopurine. Following removal of the protective methylnitroimidazole group in reactions involving glutathione (mediated most likely by glutathione-*S*-transferase) (Watanabe *et al.*, 1978), 6-mercaptopurine enters the purine salvage pathway. It is converted to thioinosine monophosphate (TIMP) by hypoxanthine(guanine)phosphoribosyl transferase (HPRT), and then by a series of enzymatic

Fig. 4.2 Salvage pathway of 6-mercaptopurine, metabolite of azathioprine

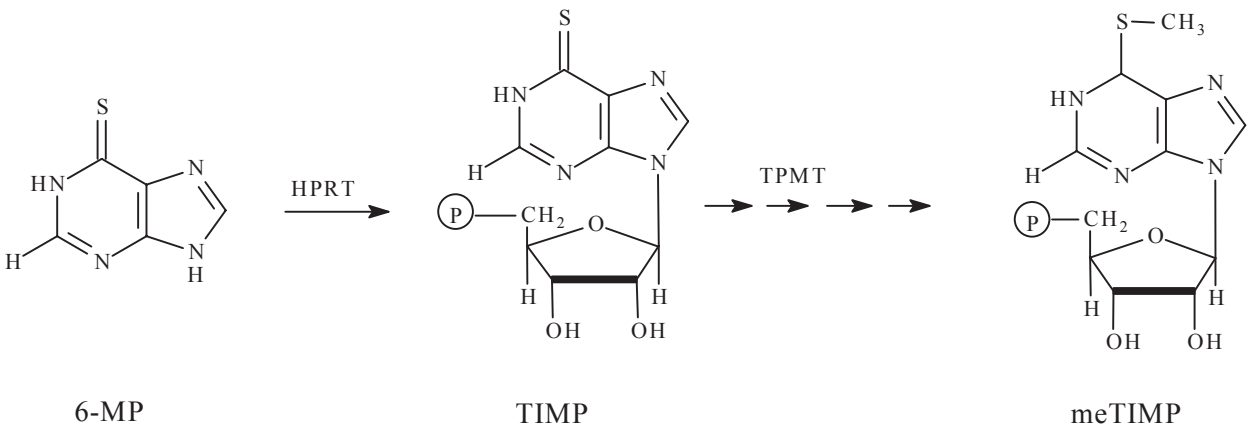


6-MP, 6-mercaptopurine; dthioGTP, 6-thioguanine deoxynucleoside triphosphate; HPRT, hypoxanthine(guanine)phosphoribosyl transferase; TGN, thioguanine nucleotide; TIMP, thioinosine monophosphate

steps to the thioguanine nucleotide (TGN), 6-thioguanine deoxynucleoside triphosphate (dthioGTP) (see Fig. 4.2). Thioguanine nucleotide is a precursor of DNA synthesis, and a good substrate for replicative DNA polymerases, which also copy template 6-thioguanine with reasonable efficiency and accuracy (Spratt & Levy, 1997). In an important catabolic step (see Fig. 4.3), thiopurine methyltransferase (TPMT) methylates thioinosine monophosphate to methylthioinosine monophosphate (meTIMP), a potent inhibitor of purine biosynthesis (Stet *et al.*, 1993; Dervieux *et al.*, 2002). The balance between thioguanine nucleotide and methylthioinosine monophosphate formation is the key to the pharmacological action of the thiopurine drugs. There are several polymorphic variants of thiopurine methyltransferase (Weinshilboum & Wang, 2006). These vary in their efficiency and when patients who are homozygous (or compound heterozygous) for alleles encoding inefficient thiopurine methyltransferase are treated with azathioprine, they experience severe and often life-threatening myelotoxicity – essentially an azathioprine overdose. The past 20 years or so has seen a significant body of research into the occurrence and effects of polymorphisms of the thiopurine methyltransferase gene. A screening test has been introduced (reviewed in Evans & Relling, 1999; Relling & Dervieux, 2001; Evans, 2004). In general, patients with high thiopurine methyltransferase levels have clinically poor responses to azathioprine therapy. Despite this, the formation of methylthioinosine monophosphate and potential inhibition of *de novo* purine biosynthesis are considered by some to be the basis of the pharmacological effects of azathioprine. This paradoxical position may have arisen from the choice of mismatch repair (MMR)-deficient cell lines in which the effects of azathioprine were examined *in vitro*; in the absence of MMR, purine depletion may be a significant factor in toxicity (Karran, 2006). Nevertheless, since many bona fide inhibitors of *de novo* purine biosynthesis have similar effects on purine biosynthesis (Shipkova *et al.*, 2005), a contribution of deoxynucleotide pool imbalance to the immunosuppressive and anti-inflammatory activities of azathioprine has not been formally excluded.

Thioguanine nucleotide levels are an alternative determinant of the effects of azathioprine (Lennard *et al.*, 1987). There is a correlation between thioguanine nucleotide levels and

Fig. 4.3 Catabolism of 6-mercaptopurine



6-MP, 6-mercaptopurine; meTIMP, methyl-TIMP; TIMP, thioinosine monophosphate; TPMT, thiopurine methyltransferase; HPRT, hypoxanthine (guanine) phosphoribosyl transferase

clinical effectiveness in inflammatory bowel disease (Osterman *et al.*, 2006). Thioguanine nucleotides enter the deoxynucleotide pool and are used as substrates for replicative DNA polymerases. This results in the incorporation of 6-thioguanine in place of DNA guanine, and levels of around 0.5% (around 10^5 to 10^6 per cell) replacement have been reported (Warren *et al.*, 1995; Cuffari *et al.*, 2004). DNA-6-thioguanine forms reasonable base pairs (Bohon & de los Santos, 2003) and is not, in itself, cytotoxic. The biological effects of this substitution require an active DNA MMR system and depend on the particular chemical reactivity of DNA thioguanine. The thiol group of DNA-6-thioguanine is more reactive than the corresponding oxygen of guanine. DNA-6-thioguanine is susceptible to chemical methylation – most likely by *S*-adenosylmethionine. This reaction converts a small fraction of DNA-6-thioguanine, approximately one in 10^4 or 10^5 incorporated 6-thioguanine bases, into *S*-methylthioguanine (me6-TG) which, unlike unmodified DNA-6-thioguanine, is highly miscoding during replication; DNA-6-methylthioguanine preferentially base pairs with thymine (T) during replication (Swann *et al.*, 1996). The formation of me6-TG:T base pairs triggers recognition by MMR which leads to cell death. Therefore, cells in which MMR is inactive are highly resistant to 6-mercaptopurine and 6-thioguanine. Importantly, loss of MMR confers a significant selective advantage; and in the laboratory, thiopurine treatment of human cells *in vitro* can be used to isolate rare variants with defective MMR from populations of cells with proficient MMR (Offman *et al.*, 2004).

4.2 Genotoxic effects

There are conflicting reports of effects on the incidence of chromosomal aberrations in lymphocytes and bone-marrow cells of patients treated with azathioprine. In one study, the incidence of sister chromatid exchange in lymphocytes of treated patients was not increased. In animals treated *in vivo*, azathioprine induced dominant lethal mutations in mice, chromosomal aberrations in rabbit lymphocytes and Chinese hamster bone-marrow cells, and micronuclei in mice, rats and hamsters; it did not induce sister chromatid exchange in Chinese hamster bone-marrow cells.

Azathioprine induced chromosomal aberrations but not sister chromatid exchange in human

lymphocytes *in vitro*. It induced chromosomal aberrations in *Drosophila*, was weakly mutagenic to fungi, and was mutagenic to bacteria (IARC, 1987b).

4.3 Mechanisms of carcinogenesis in organ-transplant patients

Until recently, azathioprine was the most commonly prescribed immunosuppressant in solid organ transplant patients. It was usually given combined with steroids and/or ciclosporin. Organ transplant patients get two types of cancer: post-transplant lymphoproliferative disorders and skin cancer, predominantly squamous cell carcinoma.

4.3.1 Post-transplant lymphoproliferative disorders

After skin cancer, these are the most common malignancies associated with transplantation. They tend to affect around 5% of transplant recipients (LaCasce, 2006). Most are associated with the Epstein-Barr virus (EBV). Most arise in the first year after transplant during which immunosuppression is intense, and are of recipient origin.

The development of post-transplant lymphoproliferative disorders almost certainly reflects immunosuppression. EBV is incorporated into B-lymphocytes during primary infection, and immunocompetent hosts mount both antibody and cellular immune responses. Post-transplant lymphoproliferative disorders develops as a consequence of compromised immunosurveillance. EBV-naïve transplant recipients typically acquire the infection from donor B cells, and post-transplant lymphoproliferative disorders is more than 20 times more common in this group (LaCasce, 2006). Because azathioprine is an immunosuppressant, it may contribute to the development of post-transplant lymphoproliferative disorders.

4.3.2 Squamous cell skin carcinoma

The frequency of squamous cell skin carcinoma in transplant patients is up to 250-fold higher that in the normal population (Euvrard *et al.*, 2003). There is no association with defective MMR in transplant-related squamous cell skin carcinoma. Although immunosuppression *per se* undoubtedly contributes to this increased incidence, the identification of sunlight as a cofactor (Bavinck *et al.*, 1993) suggests a possible alternative contributory mechanism.

The skin of patients taking azathioprine contains DNA-6-thioguanine (O'Donovan *et al.*, 2005). Unlike the canonical DNA bases, 6-thioguanine acts as an endogenous ultraviolet A (UVA) chromophore. UVA comprises more than 95% of the UV radiation that reaches the Earth's surface. 6-Thioguanine has been shown to be a UVA photosensitizer, and to generate reactive oxygen species, including singlet oxygen $^1O_2$, when exposed to UVA. $^1O_2$ is highly damaging for DNA and oxidizes DNA guanine to the promutagenic 8-oxoguanosine, which has been implicated in the development of cancer (Cadet *et al.*, 2003, 2006). DNA-6-thioguanine itself is also susceptible to oxidation leading to DNA lesions that block replication and transcription, and are likely to be promutagenic (O'Donovan *et al.*, 2005). Patients taking azathioprine are selectively sensitive to erythema induction by UVA (Perrett *et al.*, 2008). This suggests that these potentially hazardous photochemical reactions of DNA-6-thioguanine, which may generate promutagenic DNA changes, occur in the skin of patients.

4.4 Synthesis

Azathioprine is carcinogenic via two mechanisms:

- as an immunosuppressant, it is associated with post-transplant lymphoproliferative disorders that generally have a viral etiology;

- because it causes 6-thioguanine to accumulate in patients' DNA, it also contributes to cancer development by DNA damage.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of azathioprine. Azathioprine causes cancer of the skin (squamous cell carcinoma), and non-Hodgkin lymphoma.

There is *sufficient evidence* in experimental animals for the carcinogenicity of azathioprine.

Azathioprine is *carcinogenic to humans (Group 1).*

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CHLORNAPHAZINE

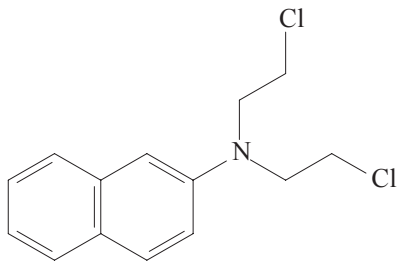
Chlornaphazine was considered by previous IARC Working Groups in 1973 and 1987 (IARC, 1974, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 494-03-1
Chem. Abstr. Name: 2-Naphthalenamine, *N*,*N*-bis(2-chloroethyl)-
IUPAC Systematic Name: *N*,*N*-Bis(2-chloroethyl)naphthalen-2-amine
Synonyms: Bis(2-chloroethyl)-2-naphthylamine; chloronaphthine; di(2-chloroethyl)-β-naphthylamine; *N*,*N*-naphthylamine mustard; β-naphthylbis(β-chloroethyl) amine; β-naphthyldi(2-chloroethyl)amine
Description: platelets (O'Neil, 2006)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_{14}H_{15}Cl_2N$
Relative molecular mass: 268.2

1.2 Use of the agent

1.2.1 Indications

Chlornaphazine was used clinically in several countries as a chemotherapeutic agent for the treatment of Hodgkin lymphoma, as well as for the control of *polycythaemia vera* (Thiede *et al.*, 1964; Videbaek, 1964a).

The US National Toxicology Program (NTP, 1980) found no evidence that chlornaphazine had ever been produced or used commercially in the United States of America.

1.2.2 Dosage

No information (other than that given above) was available to the Working Group.

1.2.3 Trends in use

Chlornaphazine is not known to be used currently.

Table 3.1 Studies of cancer in experimental animals exposed to chlornaphazine

Species, strain (sex) Duration Reference	Route Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Rat (strain & sex NR) Duration NR Koller (1953)	s.c. Injections of 40 mg over "several" months Initial number NR	Local sarcomas in 7 animals	NR	Purity NR; no control animals; very few details provided
Mouse, A/J (M, F) 39 wk Shimkin *et al.* (1966)	i.p. Injection in 200 µl 0.5% acacia of 0, 280, 1119, 4477 and 17910 µmol/kg bw (total dose) 3 injections/wk for 4 wk 360, 30, 30, 30, 30	Lung (adenomas and adenocarcinomas): 124/330, 12/30, 18/29, 25/29 and 25/25 Tumours per mouse: 0.48, 0.5, 0.9, 2.0 and 3.6	[p<0.01] (three highest doses; incidence)	Purity NR

bw, body weight; F, female; i.p., intraperitoneal; M, male; NR, not reported; s.c., subcutaneous; wk, week or weeks

2. Cancer in Humans

The previous evaluation of chlornaphazine was based on one report of three cases (Videbaek, 1964b), and one analytical study (Thiede & Christensen, 1975), described below.

Among 61 patients (34 men and 27 women) with *polycythaemia vera* treated with chlornaphazine in one Danish hospital department during 1954–62 and followed up until 1974, eight developed invasive carcinoma of the bladder, five developed papillary carcinoma grade II of the bladder, and eight had abnormal urinary cytology. [The Working Group noted that approximately 0.7 cases of bladder cancer and papillomas would have been expected using appropriate Danish incidence rates, giving a relative risk of about 20.] The invasive carcinomas were seen in four of five patients treated with a cumulative dose of 200 g or more, in two of 15 patients given 100–199 g, in one of ten patients given 50–99 g, and in one of 31 patients given less than 50 g (Thiede & Christensen, 1975). [The Working Group noted that no relative risk were reported.]

No additional relevant data were available to the Working Group.

3. Cancer in Experimental Animals

In one study, subcutaneous injection of rats with chlornaphazine induced local sarcomas; and in another, intraperitoneal injection of mice with chlornaphazine increased the incidence of combined benign and malignant lung tumours. (IARC, 1974, 1987a; Table 3.1).

No additional studies were available to the Working Group.

4. Other Relevant Data

The only data available on the metabolism of chlornaphazine was a study in rats administered chlornaphazine. Sulfate esters of 2-naphthylamine were excreted in the urine (Boyland & Manson, 1963).

In the previous *IARC Monograph* (IARC, 1987b), it was reported that chlornaphazine exhibits mutagenic/genotoxic activity in various experimental systems. Chlornaphazine induced chromosomal aberrations in Chinese hamster cells, mutations in mouse lymphoma cells, and unscheduled DNA synthesis in rat hepatocytes *in vitro*. A single study of cell transformation

in virus-infected Syrian hamster embryo cells was inconclusive. Chlornaphazine induced sex-linked recessive lethal mutations and chromosomal aberrations in *Drosophila*.

In a subsequent study, chlornaphazine was shown to induce chromosomal aberrations in Chinese hamster lung cells, micronuclei in the bone-marrow cells of mice and rats, and to be mutagenic to *Salmonella typhimurium*, with and without, a metabolic activating system (Ashby *et al.*, 1988). After intraperitoneal administration, chlornaphazine induced dominant lethal mutations in mouse germ cells (Barnett & Lewis, 2003).

No data were available on the genetic and related effects of chlornaphazine in humans.

Chlornaphazine is a bifunctional alkylating agent with mutagenic/genotoxic activity. In addition, the presence of sulfate esters of 2-naphthylamine as intermediates in the metabolism of chlornaphazine in rats is consistent with the production of 2-naphthylamine, and the increased incidence of bladder tumours in humans (IARC, 2010, and Volume 100F, IARC, 2012).

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of chlornaphazine. Chlornaphazine causes cancer of the urinary bladder.

There is *limited evidence* in experimental animals for the carcinogenicity of chlornaphazine.

Chlornaphazine is *carcinogenic to humans (Group 1)*.

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CICLOSPORIN

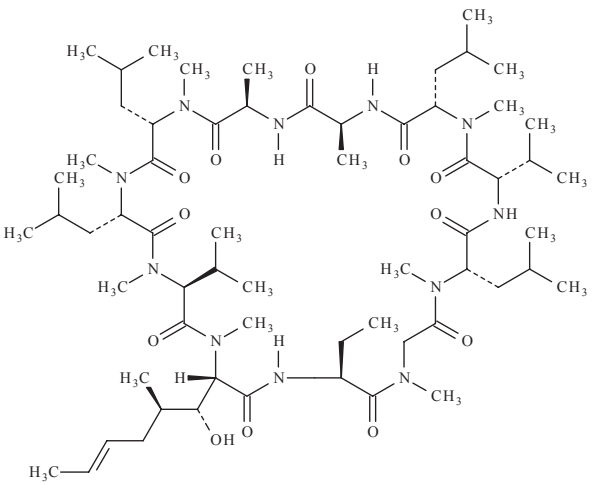
Ciclosporin was considered by a previous IARC Working Group in 1989 (IARC, 1990). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 59865-13-3
Chem. Abstr. Name: Cyclosporin A
IUPAC Systematic Name:
30-Ethyl-33-[(*E*)-1-hydroxy-2-methylhex-4-enyl]-1,4,7,10,12,15,19,25,28-nonamethyl-6,9,18,24-tetrakis(2-methylpropyl)-3,21-di(propan-2-yl)-1,4,7,10,13,16,19,22,25,28,31-undecazacyclotritriacontane-2,5,8,11,14,17,20,23,26,29,32-undecone
Synonyms: Cyclo{-[4-(*E*)-but-2-enyl-*N*,4-dimethyl-L-threonyl]-L-homoalanyl-(*N*-methylglycyl)-(*N*-methyl-L-leucyl)-L-valyl-(*N*-methyl-L-leucyl)-L-alanyl-D-alanyl-(*N*-methyl-L-leucyl)-(*N*-methyl-L-leucyl)-(*N*-methyl-L-valyl)-}; cyclosporin; cyclosporine; cyclosporin A
Description: White prismatic needles (O'Neil, 2006); white or essentially white, fine crystalline powder (McEvoy, 2007; Sweetman, 2008)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_{62}H_{111}N_{11}O_{12}$
Relative molecular mass: 1202.6

1.2 Use of the agent

Information for Section 1.2 is taken from Royal Pharmaceutical Society of Great Britain (2007), McEvoy (2007), Thomson Healthcare (2007), and Sweetman (2008).

1.2.1 Indications

Ciclosporin, a calcineurin inhibitor, is a potent immunosuppressant that is virtually non-myelotoxic but markedly nephrotoxic. It is used in organ and tissue transplantation, for prevention of graft rejection following bone-marrow, kidney, liver, pancreas, heart, lung, and heart-lung transplantation, and for prophylaxis and treatment of graft-versus-host disease. Ciclosporin is also used for the treatment of chronic allograft rejection in patients previously treated with other immunosuppressive agents (e.g. azathioprine).

Oral ciclosporin is used in the management of the active stage of severe rheumatoid arthritis in selected adults who have an inadequate therapeutic response to methotrexate. The drug may be used in combination with methotrexate in those who do not respond adequately to methotrexate monotherapy.

Oral ciclosporin is used in immunocompetent adults with severe (i.e. extensive and/or disabling) recalcitrant plaque psoriasis that is not adequately responsive to at least one systemic therapy (e.g. retinoids, methotrexate, psoralen and ultraviolet A (UVA) light [PUVA] therapy) or in patients for whom other systemic therapy is contraindicated or cannot be tolerated. It is also used to treat atopic dermatitis.

Ciclosporin ophthalmic emulsion is used to increase tear production in adults whose tear production is suppressed secondary to ocular inflammation related to keratoconjunctivitis sicca.

1.2.2 Dosage

Ciclosporin is administered orally as liquid-filled capsules or oral solution. Alternatively, the drug may be administered orally as modified liquid formulations (with increased bioavailability) that form emulsions in aqueous fluids; the modified formulations are available as oral solutions for emulsion, and as oral liquid-filled capsules.

For the prevention of allograft rejection in adults and children, ciclosporin is administered at 5–10 mg/kg/day. In the postoperative period, dosage is given twice a day. Initial levels are maintained at 250 ng/mL during the first three months followed by a subsequent weaning period as tolerated. For solid organ transplantation, ciclosporin is rarely administered as a single agent. Often, an induction antibody is administered at the time of transplantation with ciclosporin and an antimetabolite (mycophenolic acid or azathioprine). To prevent a cytokine response from the antibody induction agent, an initial dose of steroids is also administered. These steroids are then often eliminated from the treatment. For bone-marrow transplantation, prevention and treatment of graft-versus-host disease, ciclosporin is administered to adults and children over 3 months of age, at a dose of 3–5 mg/kg daily intravenously then converted to 12.5 mg/kg daily orally for 3–6 months then tailed off (may take up to a year after transplantation).

For the treatment of nephrotic syndrome, ciclosporin is administered orally, at a dose of 5–6 mg/kg daily in divided doses. Maintenance treatment is reduced to the lowest effective dose according to proteinuria and serum creatinine measurements, and discontinued after 3 months if no improvement is observed.

For the management of rheumatoid arthritis, the usual initial dosage is 1.25 mg/kg twice daily. Lack of benefit by Week 16 usually leads to the discontinuation of the therapy.

For the management of psoriasis in adults, the usual initial dosage is 1.25 mg/kg twice daily continued for at least 4 weeks unless adverse effects occur. Dosage may be increased in these increments to a maximum of 4 mg/kg daily based on the patient's tolerance and response.

It is also used for the short-term treatment of severe atopic dermatitis (usually less than

8 weeks) in adults and adolescents over 16 years of age.

Ciclosporin is applied topically to the eye as an ophthalmic emulsion in the management of keratoconjunctivitis sicca in adults as one drop of a 0.05% emulsion in each eye twice daily.

1.2.3 Trends in use

The current trend is for minimization of use of calcineurin inhibitors in general.

2. Cancer in Humans

At the time of the previous *IARC Monograph* (IARC, 1990), both lymphoma and Kaposi sarcoma had been associated frequently with exposure to ciclosporin in case reports of transplant recipients. In two of the five previously reported cohort studies of people receiving ciclosporin for transplant, a higher incidence of lymphoma was identified (IARC, 1990). In several cases, there was a well-documented regression of lymphoma following withdrawal of the drug (IARC, 1990). In these studies, the effect of ciclosporin alone is difficult to delineate due to the multiple immunosuppressive drugs administered, the cumulative dose, and the overall global immunosuppression. Since then, new studies have been published, and are summarized below (see Table 2.1 available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-17-Table2.1.pdf).

Grulich *et al.* (2007) performed a random-effect meta-analysis of the log of standardized incidence ratios (SIRs) in immunosuppressed patients. In the transplant recipient cohort (n = 31977), comparison was made to the general population. [The Working Group noted that these patients were transplanted during the era of three-drug immunosuppression. The majority of the patients would have received ciclosporin, mycophenolic acid mofetil (MMF), and steroids. Steroids are not known to be carcinogenic. The antimetabolite MMF has known antineoplastic, antireplicative and antiviral properties, and has been shown in several studies to be protective against malignancy development (O'Neill *et al.*, 2006; Lake *et al.*, 2005; Robson *et al.*, 2005). This leaves ciclosporin as the only possible carcinogenic agent in these mixtures.] For 20 of 28 types of cancers examined, there was a significantly increased risk. Included in these cancers are non-Hodgkin lymphoma, Kaposi sarcoma, squamous cell cancers (skin, oral cavity, vagina, cervix, colon, rectum), and liver cancer. [The Working Group noted the majority of these malignancies are known to have specific viral causes (Epstein-Barr virus, cytomegalovirus, Kaposi sarcoma herpes virus, hepatitis C virus, and several serotypes of human papilloma virus).]

Väkevä *et al.* (2008) reported on short-term ciclosporin therapy for inflammatory skin disorders, and did not identify any increase in SIRs. [The Working Group noted the short-term and limited drug exposure in this study, which may be the reason for this result.]

Bustami *et al.* (2004) examined a large cohort of 41000 first-time cadaveric transplant recipients from the Scientific Registry of Transplant Recipients. The use of antibody induction therapy significantly increased the risk for lymphoma and for *de novo* cancers in this study. No effect of either ciclosporin or tacrolimus was noted in patients receiving induction therapy. Ciclosporin patients had a higher relative risk of lymphoma compared to tacrolimus patients when antibody induction was not used.

Kasiske *et al.* (2004) examined a large cohort of 35765 first-time kidney transplant recipients, and neither ciclosporin nor microemulsion ciclosporin patients had increased relative risks for non-skin cancer (1.01 and 0.98, respectively) or non-melanoma skin cancer (1.02 and 1.01, respectively). [The Working Group noted the large proportion of live donors in this study, which would result in lower requirement for immunosuppression.]

Table 3.1 Studies of cancer in experimental animals exposed to ciclosporin

Species, strain (sex) Duration Reference	Route Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, AKR (M) Up to 34 wk Hattori et al. (1986)	Feed 0, 150 mg/kg in diet daily 30 animals/group	Thymic lymphoma: 0/1, 1/3 at 19 wk 2/12, 13/18 between 20–29 wk 3/9, 9/9 between 30–34 wk	 [NS] [$P < 0.004$] [$P < 0.005$]	Screening assay in a strain (AKR) highly susceptible to the development of leukaemia
Rats, Wistar (M) Duration NR Reddi et al. (1991)	Gavage 0, 10 mg/kg bw for 20 wk 13–16/group	Kidney: 2/16, 7/13	 [$P < 0.05$]	Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (60 mg/kg bw). No tumours were observed in a group of 10 non-diabetic control rats
Macaque monkeys (sex NR) Duration NR Bieber et al. (1982)	i.m. 25 mg/kg bw/d for 14 d and then every other day or 17 mg/kg bw/d continuously 16 animals	B-cell lymphoma: 2/16		Intracytoplasmic viral particles found in animals was a concern No untreated control values provided

bw, body weight; d, day or days; i.m., intramuscular; M, male; NR, not reported; NS, not significant; wk, week or weeks

Opelz & Döhler (2004) examined a large cohort of 200000 renal transplant recipients, and reported an 11.8-fold increase in lymphoma in those recipients compared to a matched non-transplant population. In this study, ciclosporin did not confer an increased risk over patients treated with azathioprine/prednisone.

Kessler *et al.* (2006) examined SIRs in 488 ciclosporin-treated renal transplant recipients. Over 4638 patient–years of exposure, 51 (10.4%) transplant recipients developed a first non-melanoma skin cancer, which was associated with older age at transplant and period of transplant (1991–95). The SIRs for all cancers was 2.2 for men, and 3.0 for women. The SIRs for native renal cell carcinoma was 13.0, for post-transplant lymphoproliferative disorder 9.5, and for cervical cancer 25.3. [The Working Group noted that native renal cell carcinoma has been linked to prolonged end-stage renal disease and haemodialysis, and may be confounding in this study.]

3. Cancer in Experimental Animals

Ciclosporin has been tested in mice and rats by oral administration, alone and in combination with other treatments, and by intramuscular injection in monkeys (macaques) that had received heart or heart-lung transplants (allografts). See Table 3.1

Mice and rats fed diets containing ciclosporin did not develop an increased incidence of tumours, except in one study where an increased incidence of thymic lymphoma was observed in male mice given ciclosporin alone (Hattori *et al.*, 1986; IARC, 1990). Two B-cell lymphomas were also reported in 16 macaques receiving ciclosporin via intramuscular injection (Bieber *et al.*, 1982; Ryffel, 1992).

Renal tumour incidence was increased in streptozotocin-induced diabetic rats administered ciclosporin by gavage (Reddi *et al.*, 1991).

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

Ciclosporin is rapidly absorbed and widely distributed in humans and in experimental animals (IARC, 1990). It is extensively metabolized by the cytochrome P450 3A4 (CYP3A4) (Delaforge *et al.*, 2001). The major route of ciclosporin metabolite excretion is via the biliary system, and renal elimination plays a minor role.

4.2 Cytogenetic effects

In a single study, ciclosporin was reported to increase the incidence of chromosomal aberrations in the lymphocytes of kidney transplant patients. Ciclosporin did not induce dominant lethal mutations in mice, chromosomal aberrations in the bone marrow of Chinese hamsters or micronuclei in the bone marrow of Chinese hamsters or mice *in vivo*. It induced sister chromatid exchange in human peripheral lymphocytes *in vitro* but did not induce gene mutations in Chinese hamster cells. Cyclosporin did not induce mutations in *Salmonella typhimurium* (IARC, 1990).

4.3 Mechanisms of carcinogenesis

4.3.1 Immunosuppressive activity

Ciclosporin, a cyclic lipophilic undecapeptide, inhibits calcineurin (also known as protein phosphatase 2B). The major effect of this is inhibition of cytokine (and some cell surface receptors) production by activated T cells (Matsuda & Koyasu, 2000; Rovira *et al.*, 2000; Hamawy, 2003; Mascarell & Truffa-Bachi, 2003; Grinyó & Cruzado, 2004).

The effects of ciclosporin are mediated via inhibition of the nuclear factor of activated T cells (NFAT) family of transcription factors that regulate inducible cytokine expression. The key interaction is between ciclosporin – bound to its cytoplasmic receptor protein, cyclophilin – and the A subunit of the heterodimeric calcineurin (CnA). Under normal conditions, activation of T cells by engagement of the T-cell receptor with its cognate ligand causes an increase in intracellular Ca^{2+} concentration that activates the calmodulin protein. This activated calmodulin interacts with calcineurin to release an autoinhibitory domain and activates its latent protein phosphatase activity. In non-stimulated T cells, the three relevant isoforms of NFAT are maintained in a highly phosphorylated form within the cytoplasm. Activation of calcineurin allows dephosphorylation of NFAT and their translocation to the nucleus where these DNA-binding proteins interact with *cis* regulatory elements of activation-induced factors. By binding directly to calcineurin at the interface between the CnA and CnB subunits, the cyclosporin–cyclophilin complex blocks access to the active site of calcineurin, and inhibits its phosphatase activity (Matsuda & Koyasu, 2000).

The immunosuppressive activity of ciclosporin is consistent with an increased risk for cancer due to impaired immune surveillance, particularly for virus-related cancers such as Epstein-Barr virus-related lymphoma, and cervical cancer which is caused by human papillomaviruses in most cases. However, there are almost certainly other mechanisms involved in the carcinogenic action of ciclosporin. For example, inactivation of interleukin-2 (IL-2) or of NFAT in transgenic mice had an effect on immune function, which is not the same as treatment with ciclosporin, and does not completely explain the carcinogenic effects of ciclosporin observed in humans (Ryffel *et al.*, 1992; Nabel, 1999).

Furthermore, immunosuppression *per se* cannot explain the peculiar pathological features of the skin cancers found in humans treated with

ciclosporin and other immunosuppressive drugs (Hojo *et al.*, 1999; Yarosh *et al.*, 2005).

4.3.2 Signalling pathways: relevant effects

(a) Effect on tumour-suppressor growth factor-β (TGF-β)

Ciclosporin also induces increased synthesis of TGF-β and a consequent activation of its dependant transcriptional activators, the (small mothers against decapentaplegic proteins) SMADs (Hojo *et al.*, 1999; Akool *et al.*, 2008). TGF-β is produced by many tumours and is associated among other things with increased invasiveness (Teicher, 2001; Bachman & Park, 2005; Leivonen & Kähäri, 2007). Ciclosporin treatment of cultured human pulmonary adenocarcinoma cells causes increased expression of TGF-β and induces changes in properties consistent with acquisition of a more invasive cellular phenotype. In immunodeficient SCID beige mice, ciclosporin treatment was associated with an increased number of pulmonary metastases of a transplanted renal cell adenocarcinoma. Both in-vitro and in-vivo effects were blocked by anti-TGF-β antibodies (Hojo *et al.*, 1999). These effects on signalling are independent of any immunosuppressive properties of ciclosporin, but they may ultimately contribute to cancer. It seems likely that they are secondary to some other effects of ciclosporin, however.

(b) Oxidative stress and DNA damage

One likely effect of ciclosporin relevant to carcinogenesis is its ability to generate reactive oxygen species (ROS). The literature suggests that antioxidants protect against some of the side-effects (nephrotoxicity, hepatotoxicity, and cardiotoxicity) of ciclosporin (Rezzani, 2006). In addition, the upregulation of TGF-β (and consequent activation of the SMAD downstream targets) is prevented by antioxidants (specifically *N*-acetylcysteine or superoxide dismutase) (Akool *et al.*, 2008).

These findings are consistent with the effects of ciclosporin on signalling being secondary to the ability of the drug to induce oxidative stress. Excess ROS generated by ciclosporin indicate the possibility of oxygen radical damage to DNA. This is known to be linked to cancer development. A recent publication (O'Driscoll & Jeggo, 2008) provides direct evidence that implicates ciclosporin treatment in the induction of DNA double-strand breaks. Importantly, this acute effect was seen in replicating cells that were defective in the repair of DNA double-strand breaks. Ciclosporin, by acting as a source of ROS, causes DNA single-strand breaks that are converted in DNA double-strand breaks during replication. On acute treatment, DNA double-strand breaks only accumulate to detectable levels in repair-defective cells. The implication is that chronic treatment will be associated with increases in DNA double-strand breaks – although in repair-proficient cells, the steady-state levels may fall below the level of detection. Nevertheless, because DNA double-strand breaks are precursors of deletions and/or translocations and chromosomal rearrangements, a chronic, low-level increase in steady-state DNA double-strand break levels is potentially mutagenic and carcinogenic.

In view of the structure and proposed mechanism of ciclosporin, it seems unlikely that it is directly mutagenic by interacting with or damaging DNA. There are several claims that ciclosporin inhibits the repair of ultraviolet-induced DNA damage (Herman *et al.*, 2001; Ori *et al.*, 2005, Yarosh *et al.*, 2005). The first two studies compared spontaneous or UVC-induced unscheduled DNA synthesis (UDS) in lymphocytes. UDS in lymphocytes from patients treated with triple therapy (ciclosporin/azathioprine/prednisolone) was lower than those from double therapy (azathioprine/prednisolone) or untreated patients. In this same study, UDS induced by UVC in lymphocytes was reduced by ciclosporin treatment *in vitro* (Herman *et al.*, 2001). In the second

study, ciclosporin was shown to reduce UDS in untreated lymphocytes (Ori *et al.*, 2005). The third examined DNA cyclobutane–pyrimidine dimer removal which appeared to be reduced, but not abolished by ciclosporin treatment (Yarosh *et al.*, 2005). Although none of these studies makes an overwhelmingly convincing case for inhibition of DNA repair, UDS measurements are a particularly indirect measure of repair, and the possibility that ciclosporin causes the persistence of promutagenic DNA lesions cannot be discounted until ruled out by a more rigorous experimental approach. These effects would be obviously directly relevant only to ciclosporin-related skin cancers.

(c) Effect on P-glycoprotein

Ciclosporin is also well known as an inhibitor of the function of P-glycoprotein, a membrane-associated transporter that facilitates efflux from the cell of a variety of toxins and anticancer drugs (Vaalburg *et al.*, 2005). Inhibition of P-glycoprotein function may allow retention in the cell of toxins including potential mutagens, but potentiation of carcinogenic activity has not been demonstrated. However, the doses of ciclosporin used to produce immunosuppression, and the serum levels achieved, are much lower than the concentrations needed to modulate the multidrug resistant (MDR) phenotype *in vitro*, and are also lower than the ciclosporin doses used in clinical trials attempting to decrease resistance to chemotherapy (List *et al.*, 2001; Ross *et al.*, 1994).

4.4 Synthesis

Ciclosporin is an immunosuppressant and long-term immunosuppression is linked to an increased risk of cancer. There are at least two facets to this. First, immunosuppression *per se* is associated with cancer, for example in individuals positive for the human immunodeficiency virus (HIV). Pharmacological immunosuppression is associated with an increased incidence of a similar spectrum of malignancies. These generally have a viral etiology (Grulich *et al.*, 2007). Examples include the EBV-related post-transplant lymphoproliferative disorders (LaCasce, 2006), and HPV-related cervical carcinoma. In addition to these malignancies that usually arise early after immunosuppression is initiated, there are late effects – such as the development of skin cancer – that may have a different etiology that could reflect direct or indirect effects of ciclosporin on DNA.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of ciclosporin. Ciclosporin causes cancer of the skin (squamous cell carcinoma), cancer at multiple other sites, and non-Hodgkin lymphoma.

There is *limited evidence* in experimental animals for the carcinogenicity of ciclosporin.

Ciclosporin is *carcinogenic to humans (Group 1).*

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PLANTS CONTAINING ARISTOLOCHIC ACID

Plants containing aristolochic acid were considered by a previous IARC Working Group in 2002 (IARC, 2002). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

For the purpose of this *Monograph*, unless otherwise specified, the term 'aristolochic acids' refers to an extract of *Aristolochia* species comprising a mixture of aristolochic acid I and its demethoxylated derivative, aristolochic acid II. *Aristolochia* species also contain the related aristolactams, which are phenanthrene cyclic amides (EMEA, 2000). In some of the older literature, it is unclear whether individual compounds or mixtures are being discussed when referring to 'aristolochic acid'.

1.1 Identification of the agent

Aristolochia species refers to several members of the genus (family *Aristolochiaceae*) (WHO, 1997) that are often found in traditional Chinese medicines, e.g. *Aristolochia debilis*, *A. contorta*, *A. manshuriensis*, and *A. fangchi*. The medicinal parts of each plant (i.e., stem, root, fruit) have distinct Chinese names. Details on these traditional drugs can be found in the *Pharmacopoeia of the People's Republic of China* (Commission of the Ministry of Public Health, 2000), except where noted. This Pharmacopoeia includes the *Aristolochia* species presented in Table 1.1.

Table 1.1 *Aristolochia* species included in the *Pharmacopoeia of the People's Republic of China*

Aristolochia species	Part used	Pin Yin Name
Aristolochia fangchi	Root	Guang Fang Ji
Aristolochia manshuriensis	Stem	Guan Mu Tong
Aristolochia contorta	Fruit	Ma Dou Ling
Aristolochia debilis	Fruit	Ma Dou Ling
Aristolochia contorta	Herb	Tian Xian Teng
Aristolochia debilis	Herb	Tian Xian Teng
Aristolochia debilis	Root	Qing Mu Xiang

In traditional Chinese medicine, *Aristolochia* species are also considered to be interchangeable with other commonly used herbal ingredients, and substitution of one plant species for another is established practice. Herbal ingredients are traded using their common Chinese Pin Yin name, and this can lead to confusion. For example, the name 'Fang Ji' can be used to describe the roots of *Aristolochia fangchi*, *Stephania tetrandra*, or *Cocculus* species (EMEA, 2000).

Similarly, the name 'Mu Tong' is used to describe *Aristolochia manshuriensis*, and certain *Clematis* or *Akebia* species. In some reports in

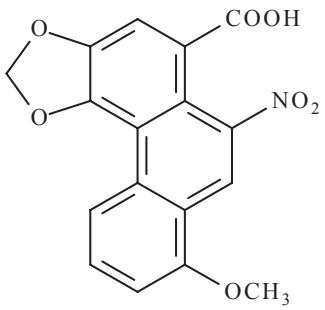
the Chinese literature, 'Mu Tong' is substituted with 'Ma Dou Ling' (EMEA, 2000).

Table 1.2 lists botanicals known or suspected to contain aristolochic acid.

1.1.1 Aristolochic acid I

Chem. Abstr. Serv. Reg. No.: 313-67-7
Chem. Abstr. Serv. Name: 8-Methoxy-6-nitrophenanthro[3,4-*d*]-1,3-dioxole-5-carboxylic acid
IUPAC Systematic Name: 8-Methoxy-6-nitronaphtho[2,1-*g*][1,3]benzodioxole-5-carboxylic acid
Synonyms: Aristinic acid; aristolochia yellow; aristolochic acid A; aristolochin; aristolochine; Descresept; isoaristolochic acid; 8-methoxy-3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid; 3,4-methylenedioxy-8-methoxy-10-nitro-1-phenanthrenecarboxylic acid
Description: Shiny brown leaflets (O'Neil, 2006)

(a) Structural and molecular formulae, and relative molecular mass

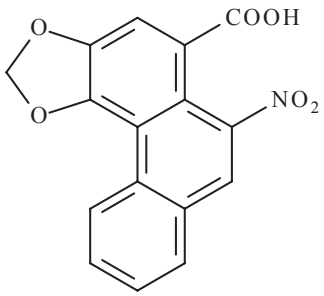


$C_{17}H_{11}NO_7$
Relative molecular mass: 341.27

1.1.2 Aristolochic acid II

Chem. Abstr. Serv. Reg. No.: 475-80-9
Chem. Abstr. Serv. Name: 6-Nitrophenanthro[3,4-*d*]-1,3-dioxole-5-carboxylic acid
IUPAC Systematic Name: 6-Nitronaphtho[2,1-*g*][1,3]benzodioxole-5-carboxylic acid
Synonyms: Aristolochic acid B; 3,4-methylenedioxy-10-nitrophenanthrene-1-carboxylic acid

(a) Structural and molecular formula, and relative molecular mass



$C_{16}H_9NO_6$
Relative molecular mass: 311.25

1.2 Use of the agent

Several *Aristolochia* species (notably *A. contorta*, *A. debilis*, *A. fangchi*, and *A. manshuriensis*) have been used in traditional Chinese medicine as anti-inflammatory agents, diuretics, and in the treatment of oedema (IARC, 2002). In addition, Chinese herbal remedies labelled 'Fang Ji' were sold in Europe as slimming agents. The ingredient *S. tetrandra* was substituted with *A. fangchi* in the preparation distributed in Belgium during the period 1990–92 (Nortier *et al.*, 2000).

The aristolochic acid occurring in *Aristolochia* species used in traditional herbal medicines has been reported to function as a phospholipase A_2 inhibitor, and as an antineoplastic, antiseptic, anti-inflammatory, and bactericidal agent (Buckingham, 2001; Cosyns, 2003).

Table 1.2 Botanicals known or suspected to contain aristolochic acid

Botanical name	Common or other names
Aristolochia spp.	Aristolochia Guan Mu Tong Guang Mu Tong
Aristolochia acuminata Lam. Syn. *Aristolochia tagala* Champ.	Oval leaf Dutchman's pipe
Aristolochia argentina Griseb.	
Aristolochia baetica Linn. Syn. *Aristolochia bracteolata* Lam.	
Aristolochia bracteata Retz.	Ukulwe
Aristolochia chilensis Bridges in Lindl.	
Aristolochia cinnabarina C.Y. Cheng & J.L. Wu	
Aristolochia clematitis L.	Birthwort
Aristolochia contorta Bunge	Ma Dou Ling Tian Xian Teng
Aristolochia cymbifera Mart. & Zucc.	Mil homens
Aristolochia debilis Siebold & Zucc. Syn. *Aristolochia longa* Thunb. Syn. *Aristolochia recurvilabra* Hance Syn. *Aristolochia sinarum* Lindl.	Ma Dou Ling Tian Xian Teng Qing Mu Xiang Sei-mokkou (Japanese) Birthwort Long birthwort
Aristolochia elegans Mast. Syn. *Aristolochia hassleriana* Chodat	
Aristolochia esperanzae Kuntze	
Aristolochia fangchi Y.C. Wu ex L.D. Chow & S.M. Hwang	Guang Fang Ji Fang Ji Mokuboi (Japanese) Kwangbanggi (Korean) Fang Chi Kou-boui (Japanese)
Aristolochia fimbriata Cham.	
Aristolochia indica L.	Indian birthwort

Table 1.2 (continued)

Botanical name	Common or other names
Aristolochia kaempferi Willd. Syn. *Aristolochia chrysops* (Stapf) E.H. Wilson ex Rehder Syn. *Aristolochia feddei* H. Lév. Syn. *Aristolochia heterophylla* Hemsl. Syn. *Aristolochia mollis* Dunn Syn. *Aristolochia setchuenensis* Franch. Syn. *Aristolochia shimadai* Hayata Syn. *Aristolochia thibetica* Franch. Syn. *Isotrema chrysops* Stapf Syn. *Isotrema heterophylla* (Hemsl.) Stapf Syn. *Isotrema lasiops* Stapf	Yellowmouth Dutchman's pipe
Aristolochia kwangsiensis Chun & F.C. How Syn. *Aristolochia austroszechuanica* C. B. Chien & C. Y. Cheng	
Aristolochia macrophylla Lam. Syn. *Aristolochia sipho* L'Hér.	Dutchman's pipe
Aristolochia manshuriensis Kom. Syn. *Hocquartia manshuriensis* (Kom.) Nakai Syn. *Isotrema manshuriensis* (Kom.) H. Huber	Manchurian birthwort Manchurian Dutchman's pipe Guang Mu Tong Kan-Mokutsu (Japanese) Mokuboi (Japanese) Kwangbanggi (Korean)
Aristolochia maurorum L.	
Aristolochia maxima Jacq. Syn. *Aristolochia maxima* var. *angustifolia* Duchartre in DC. Syn. *Howardia hoffmannii* Klotzsch	
Aristolochia mollissima Hance	
Aristolochia pistolochia L.	
Aristolochia rigida Duch.	
Aristolochia rotunda Linn.	
Aristolochia serpentaria L. Syn. *Aristolochia serpentaria* var. *hastata* (Nutt.) Duch.	Virginia snakeroot Serpentaria Virginia serpentary
Aristolochia watsoni Wooton & Standley or ***Aristolochia watsonii*** Wooton & Standley Syn. *Aristolochia porphyrophylla* Pfeifer	
Aristolochia westlandii Hemsl. or ***Aristolochia westlandi*** Hemsl.	

Table 1.2 (continued)

Botanical name	Common or other names
Aristolochia zollingeriana Miq. Syn. *Aristolochia kankauensis* Sasaki Syn. *Aristolochia roxburghiana* subsp. *kankauensis* (Sasaki) Kitam. Syn. *Hocquartia kankauensis* (Sasaki) Nakai ex Masam. Syn. *Aristolochia tagala* var. *kankauensis* (Sasaki) T. Yamaz.	
Asarum canadense Linn. Syn. *Asarum acuminatum* (Ashe) E.P. Bicknell Syn. *Asarum ambiguum* (E.P. Bicknell) Daniels Syn. *Asarum canadense* var. *ambiguum* (E.P. Bicknell) Farw. Syn. *Asarum canadense* var. *reflexum* (E.P. Bicknell) B.L. Rob. Syn. *Asarum furcatum* Raf. Syn. *Asarum medium* Raf. Syn. *Asarum parvifolium* Raf. Syn. *Asarum reflexum* E.P. Bicknell Syn. *Asarum rubrocinctum* Peattie	Wild ginger Indian ginger Canada snakeroot False coltsfoot Colic root Heart snakeroot Vermont snakeroot Southern snakeroot
Asarum himalaicum Hook. f. & Thomson ex Klotzsch or ***Asarum himalaycum*** Hook. f. & Thomson ex Klotzsch	Tanyou-saishin (Japanese)
Asarum splendens (F. Maek.) C.Y. Cheng & C.S. Yang	Do-saishin (Japanese)
Bragantia wallichii R.Br. Specimen exists at New York Botanical Gardens. Tropicos does not list this species as a synonym for any *Thottea* species. Kew Gardens Herbarium does not recognize the genera Bragantia. Until additional information is obtained the name used is as cited in J. Nat. Products 45:657–666 (1982)	

From FDA (2001)

2. Cancer in Humans

The previous evaluation of herbal remedies containing plant species of the genus *Aristolochia* was based on four case reports and two ecological studies (IARC, 2002). These studies as well as more recent publications are presented below.

2.1 Case reports

Since the early 1990s, several case reports from various countries have raised the possibility of a link between the consumption of Chinese herbal products containing *Aristolochia* species and human nephropathy (Chinese herb nephropathy, subsequently called aristolochic acid nephropathy), and urothelial cancer (Cosyns *et al.*, 1994; Vanherweghem *et al.*, 1995; Yang *et al.*, 2000; Lord *et al.*, 2001, 2004; Arlt *et al.*, 2004).

2.2 Aristolochic acid nephropathy

Cosyns *et al.* (1999) examined 19 kidneys and ureters removed prophylactically during and/or after renal transplantation from ten patients treated for aristolochic acid nephropathy in one urology unit in Belgium. [The Working Group noted that no further specification of the formulation of the herbs was given.] The patients were all women with a mean age of 40 years (range 27–59 years). Multifocal high-grade carcinoma *in situ* was observed in four patients (40%).

Nortier *et al.* (2000) examined 77 kidneys and 78 ureters removed prophylactically from 39 patients treated for aristolochic acid nephropathy in another urology unit in Belgium. The period of use among these patients was closely related to the period of distribution in Belgium of pills containing *A. fangchi* (from 1990–92). Except for a 60-year-old man, all patients were women (aged 54 ± 7 years). Eighteen cases of urothelial carcinoma were found (prevalence, 46%; 95%CI, 29–62%). Except for one case of bladder cancer, all the carcinomas were located in the upper urinary tract and were almost equally distributed between the pelvis and the ureter. Mild-to-moderate dysplasia of the urothelium was found in 19 of the 21 patients without urothelial carcinoma. Among 24 patients who reported a cumulative consumption of less than 200 g of herbs containing *A. fangchi*, eight cases of urothelial cancer were recorded, and among the 15 patients who had ingested more than 200 g, ten cases of urothelial cancer were observed ($P = 0.05$).

[The Working Group noted that there were no control groups in either studies for nephropathy or cancer; however, the use of Chinese herbs by all women, the absence of other common exposure, the presumed low prevalence of malignant disease in this age group compared to the high prevalence observed, and the strong temporal association led the Working Group to the conclusion that there is a causal association between use of the herb and nephropathy/urothelial cancer.]

3. Cancer in Experimental Animals

3.1 Aristolochic acid

Aristolochic acid, tested for carcinogenicity mainly by oral administration in several studies in rats, one study in mice, and by intraperitoneal injection in one study in rabbits, induced tumours at multiple sites. In most studies, the animals were administered a mixture of aristolochic acid I and II (see Table 3.1). However, carcinogenic effects were also observed with aristolochic acid I alone (Schmeiser *et al.*, 1990; Cui *et al.*, 2005).

In female NMRI mice, a mixture of aristolochic acid I and II given orally at a dose of 5 mg/kg body weight for 3 weeks increased the incidence of forestomach tumours, kidney adenomas, and lung carcinomas (Mengs, 1988).

Table 3.1 Studies of cancer in experimental animals exposed to aristolochic acid

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, NMRI (F) up to 56 wk Mengs (1988)	Oral 5 mg/kg bw daily for 3 wk 39, 11 (controls)	Forestomach: 25/39 (including 10/39 squamous cell carcinomas) Stomach (adenocarcinomas): 1/39 Kidney (adenomas): 11/39 Lung (carcinomas): 13/39 Uterus (haemangiomas): 3/39 Malignant lymphomas: 10/39 No tumours observed in controls	[$P < 0.0001$], [NS][a] (carcinomas) [NS] [$P = 0.04$] [$P = 0.02$] [NS] [NS]	Mixture (77.2% AAI, 21.2% AAII) Mice were kept for 56 wk with interim sacrifice at 3, 9, 18, 26, 37, and 48 wk First tumours at 26 wk; by Week 56 all remaining mice had tumours
Rat, Wistar (M, F) up to 9 mo Mengs et al. (1982)	Oral 0 or 10 mg/kg bw daily for 3 mo 30/sex	Forestomach (squamous cell carcinomas): M–13/18 killed at 6 mo F–8/13 killed at 6 mo; F–4/4 killed at 9 mo Controls– M 0/10 killed at 6 mo F 0/10 killed at 6 mo	6 mo: [$P = 0.0002$][a] (M); [$P = 0.0003$] (F)	Mixture (77.2% AAI, 21.2% AAII) No controls at 9 mo
		Renal pelvis (carcinomas): M–8/18 killed at 6 mo F–2/13 killed at 6 mo Controls– M 0/10 killed at 6 mo F 0/10 killed at 6 mo	[$P = 0.01$] (M); [NS] (F)	
		Urinary bladder (carcinomas): M–3/18 killed at 6 mo F–1/13 killed at 6 mo Controls– M 0/10 killed at 6 mo F 0/10 killed at 6 mo	[NS] (for either sex)	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Wistar (M, F) up to 9 mo Mengs et al. (1982)	Oral 1.0 mg/kg bw daily for 3 mo 30/sex	Forestomach (squamous cell carcinomas): M–3/11 killed at 6 mo M–0/10 Controls– M 6/9 killed at 6 mo F 2/11 killed at 9 mo Forestomach: M–9/11 killed at 6 mo Controls– M 0/10 killed at 6 mo	[NS][a] [$P = 0.0002$]	Mixture (77.2% AAI, 21.2% AAII) No controls at 9 mo
Rat, Wistar (M, F) 12 mo Mengs et al. (1982)	Oral 0.1 mg/kg bw daily for 3 mo 30/sex	Forestomach (squamous cell carcinomas): M–2/7 killed at 12 mo Controls–0/6	[NS][a]	Mixture (77.2% AAI, 21.2% AAII)
Rat, Wistar (M, F) 16 mo Mengs et al. (1982)	Oral 0.1 mg/kg bw daily for 12 mo 30/sex	Forestomach (squamous cell carcinomas): M–4/4 killed at 16 mo F–1/5 killed at 16 mo Controls– M 0/6 F 0/4	[$P = 0.0048$][a] (M); [NS] (F)	Mixture (77.2% AAI, 21.2% AAII)
Rat, Wistar (M) up to 6 mo Mengs (1983)	Oral 10 mg/kg bw daily for up to 6 mo 108 killed sequentially; 37 (controls)	Forestomach (papillomas): 8/8 killed at 1 mo Forestomach (invasive squamous cell carcinomas): 13/18 killed at 6 mo		Mixture (77.2% AAI, 21.2% AAII) Study on histopathogenesis of forestomach carcinoma Controls: tumour data NR

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Wistar (M) up to 7 mo Schmeiser et al. (1990)	Oral 10 mg/kg bw daily for 3 mo 40; 8 (controls)	Forestomach (squamous cell carcinomas): 15/40	[$P = 0.04$][a]	AAI
		Ear duct (squamous cell carcinomas): 7/40	[NS]	
		Intestine (adenocarcinomas or sarcomas): 23/40	[$P = 0.003$]	
		Kidney (adenocarcinomas): 1/40	[NS]	
		Pancreas (squamous cell carcinomas): 3/40	[NS]	
		Lung (squamous cell carcinomas metastasis): 1/40	[NS]	
		Haematopoietic system (lymphomas): 1/40	[NS]	
		Controls–0/8 for each tumour site		
Rat, Sprague-Dawley (F) 6 mo Cui et al. (2005)	Oral 50 mg/kg bw daily for 3 d 14; 10 controls	Kidney (tumours): 4/14	[NS][a]	AAI
		Breast (mammary duct carcinomas): 1/14	[NS]	
		Controls–0/10 for each tumour site		
Rat, BD-6 (M) 46 wk Hadjiolov et al. (1993)	Oral 10 mg/kg bw twice weekly for 12 wk 20	Forestomach (squamous cell carcinomas): 9/20 Urinary bladder (carcinomas): 1/20 Thymus (thymomas): 2/20		Mixture NR No control group
Rat, Wistar (M, F) 6 mo Cosyns et al. (1998)	Oral 10 mg/kg bw daily for 3 mo 8/sex; 6/sex (controls)	Forestomach (squamous cell carcinomas): M–3/6	[NS][a]	Mixture (44% AAI, 56% AAII) no fibrosis detected Small number of animals
		Kidney (malignant tumours): F–2/6	[NS]	
		Bladder (carcinomas): M–1/6	[NS]	
		Controls (for each tumour site)– M 0/6 F 0/6		

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Wistar (M) 105 d Debelle et al. (2002)	s.c. 1 or 10 mg/kg bw daily for 35 d 24; 18 (controls)	Urothelial carcinomas: high-dose–3/24 Fibrohistiocytic sarcomas at the injection site: high-dose–7/11 low-dose–2/6		Mixture (40% AAI, 60% AAII) together with single i.p. injection of furosemide and were remained on a low-salt diet Controls: tumour incidence NR
Rat, Sprague-Dawley (M, F) 90 d Hwang et al. (2006)	Oral 5 mg/kg bw daily for 90 d 10; 10 (controls)	Forestomach (carcinomas): M–10/10 F–1/10 Forestomach (papillomas): M–9/10 F–9/10 Kidney (carcinomas): M–1/10 Controls–0/10 for each tumour site	[$P < 0.0001$][a] (M); [NS] (F); [$P < 0.0001$] (M); [$P < 0.0001$] (F) [NS]	Mixture (44% AAI, 56% AAII)
Rabbit, New Zealand (F) 21 mo Cosyns et al. (2001)	i.p. 0.1 mg/kg 5 d per wk for 17–21 mo 12; 10 (controls)	Kidney (one carcinoma and one adenoma): 2/12 Ureter (carcinomas): 1/12 Peritoneum (mesotheliomas): 1/12 Controls–0/10 for each tumour site	[NS][a] [NS] [NS]	Mixture (44% AAI, 56% AAII)

[a] Fisher Exact test, Working Group analysis

AA, aristolochic acid; bw, body weight; d, day or days; F, female; i.p., intraperitoneal; M, male; mo, month or months; NR, not reported; NS, not significant; s.c., subcutaneous; wk, week or weeks

Table 3.2 Studies of cancer in experimental animals exposed to extracts from *Aristolochia* species

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Sprague-Dawley (F) 6 mo Qiu et al. (2000)	Oral 20 g/kg bw daily for 15 d 30 or 50 g/kg daily for 7 d 30, 30, 40; 30 (controls)	Kidney (stromal renal tumours): 20 g/kg bw–0% 30 g/kg bw–25% 50 g/kg bw–42.8% Controls–0%	NR	Decoctions of *A.manshuriensis* with amount of aristolochic acids not determined
Rat, Sprague-Dawley (M, F) 90 d Hwang et al. (2006)	Oral 0 (control) or 2135 mg/kg bw daily for 90 d (equivalent to 5 mg/kg bw aristolochic acid) 10, 10	Forestomach (carcinomas): M–3/10 F–2/10 Forestomach (papillomas): M–7/10 F–18/10	[NS][a] (M, F); [$P < 0.001$] (F)	Aqueous extract of *A.fructus* containing aristolochic acids
		Kidney (carcinomas): M–2/10 Controls–0/10 for each tumour site	[NS]	

[a] Fisher Exact test, Working Group analysis
bw, body weight; d, day or days; F, female; M, male; mo, month or months; NS, not significant

Oral administration of aristolochic acid to rats caused a dose- and time-dependent tumour response. Exposure to 50 mg/kg body weight aristolochic acid I for 3 days resulted in neoplastic lesions of the kidney after 6 months (Cui *et al.*, 2005). Rats exposed to lower doses by gavage over a longer period (1–10 mg/kg body weight for 3–6 months or 0.1 mg/kg body weight for 12 months) developed a variety of benign or malignant tumours, including those of the forestomach, kidney, renal pelvis, urinary bladder, ear duct, thymus, small intestine, and pancreas. Single cases of haematopoietic system, lung, mammary gland, and peritoneal tumours were also reported (Mengs *et al.*, 1982; Mengs, 1983; Schmeiser *et al.*, 1990; Hadjiolov *et al.*, 1993; Cosyns *et al.*, 1998).

Subcutaneous injection of 10 mg/kg body weight aristolochic acid into rats for 35 days induced a low incidence of urothelial carcinomas and fibrohistiocytic sarcomas at the injection site (Debelle *et al.*, 2002). A single intraperitoneal injection of aristolochic acid at 10 mg/kg body weight increased the incidence of liver neoplastic nodules in male F344 rats when coupled with the liver tumour promoter orotic acid (Rossiello *et al.*, 1993).

Rabbits given intraperitoneal injections of aristolochic acid at 0.1 mg/kg body weight for 17–21 months developed tumours of the kidney, ureter, and of the peritoneal cavity (Cosyns *et al.*, 2001).

3.2 Extracts from Aristolochia species

Decoctions from *A. manshuriensis* and an aqueous extract of *A. fructus*, when administered orally to rats, induced tumours of the forestomach and the kidney (Qiu *et al.*, 2000; Hwang *et al.*, 2006).

See Table 3.2

Table 3.3 Studies of cancer in experimental animals exposed to a herbal weight-loss regimen containing aristolochic acid

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, Wistar (M/F) 14 mo Cosyns et al. (1998)	Oral 0 or 70 mg/kg bw herbal powder containing 0.15 mg/kg bw aristolochic acids daily for 3 mo 8, 8	Forestomach (squamous cell carcinomas): M–2/4 Controls– M 0/7	[NS][a]	Included a mixture of various herbs and other treatments to mimic the weight-loss regimen prescribed at a Belgian clinic in the early 1990s

[a] Fisher Exact test, Working Group analysis
bw, body weight; F, female; M, male; mo, month or months; NS, not significant

3.3 Herbal remedy containing aristolochic acids

Squamous cell carcinomas of the forestomach were found in male rats treated with a weight-loss regimen of herbal ingredients that contained aristolochic acids (Cosyns *et al.*, 1998).

See Table 3.3

4. Other Relevant Data

4.1 Absorption, distribution, metabolism, and excretion

Aristolochic acid is absorbed from the gastrointestinal tract and distributed unchanged and/or in metabolized form throughout the body. Several structurally defined metabolites (mainly nitroreduction products) were identified following the oral administration of aristolochic acid I and aristolochic acid II to rats and mice (Krumbiegel *et al.*, 1987; Chan *et al.* 2006, 2007). Fewer metabolites were observed in beagle dogs, rabbits, guinea-pigs, and humans than in rats or mice (Krumbiegel *et al.*, 1987). The major metabolites of aristolochic acid are products derived from nitroreduction, *O*-demethylation, and denitration. In humans, the reduction products aristolactam I and II are the only metabolites detected in urine (Krumbiegel *et al.*, 1987), although full metabolic profiles have not been reported. Phase II metabolites of aristolochic acids have been identified in the urine of rats, and include *N*- and *O*-glucuronides, and acetate and sulfate esters (Chan *et al.*, 2007).

4.2 Toxic effects

The toxic effects of aristolochic acids I and II have been inferred from effects seen in patients diagnosed with kidney nephropathy as a result of consuming herbal mixtures containing *Aristolochia* species, leading to rapidly progressive fibrosing interstitial nephritis (Nortier *et al.*, 2000). In experimental animals, high doses of aristolochic acids administered either orally or intravenously caused severe necrosis of the renal tubules, atrophy of the spleen and thymus, and ulceration of the forestomach, followed by hyperplasia and hyperkeratosis of the squamous epithelium (IARC, 2002; Cosyns, 2003).

4.3 Genotoxic effects

Aristolochic acids are consistently active in genotoxicity tests *in vivo* and *in vitro* (Arlt *et al.*, 2002a; IARC, 2002). The major activation

pathway involves reduction of the nitrogroup, and is catalysed by several human cytosolic and microsomal enzymes such as hepatic and renal cytosolic NAD(P)H:quinone oxidoreductase (NQO1), hepatic microsomal cytochrome P450 (CYP)1A2 and renal microsomal NADPH:CYP reductase – NQO1 being the most important (Stiborová *et al.*, 2008). During reductive activation, aristolochic acids form an electrophilic cyclic *N*-acylnitrenium ion that reacts with purine bases to form DNA adducts. These aristolochic-acid-specific DNA adducts have been identified and detected in experimental animals exposed to aristolochic acid or botanical products containing aristolochic acid, and in urothelial tissues from aristolochic acid nephropathy patients (Arlt *et al.*, 2002a, b). In addition, Grollman *et al.* (2007) detected DNA adducts derived from aristolochic acids in formalin-fixed renal cortical tissues embedded in paraffin blocks from four patients with a verified Balkan endemic nephropathy and in tumour tissue from three long-term residents of endemic villages who had upper urinary tract cancer. No such adducts were detected in five control patients with common forms of chronic renal disease or in five control patients with upper urinary tract transitional cell cancers who resided in a non-endemic region in Croatia. In rodent tumours, the major DNA adduct formed by aristolochic acid (7-[deoxyadenosin-N^6-yl]aristolactam I) has been associated with the activation of *RAS* oncogenes through a specific CAA→CTA transversion mutation in codon 61 (Schmeiser *et al.*, 1990; Cheng *et al.*, 2006). Such A:T→T:A transversions were the predominant mutation type in studies using transgenic Muta™mice (Kohara *et al.*, 2002), Big Blue transgenic rats (Chen *et al.*, 2006; Mei *et al.*, 2006), and in human *TP53* knock-in mouse fibroblasts treated with aristolochic acid (Liu *et al.*, 2004; Feldmeyer *et al.*, 2006). In humans, A:T→T:A transversion mutations in codon 139 of exon 5 of the *TP53* gene were identified in an urothelial tumour from an aristolochic acid nephropathy patient (Lord *et al.*, 2004), and in several patients having Balkan endemic nephropathy, along with aristolochic-acid-specific DNA adducts (Lord *et al.*, 2004; Grollman & Jelaković, 2007; Grollman *et al.*, 2007).

4.4 Synthesis

Key steps in the mechanism by which aristolochic acid causes tumours in experimental animals have been identified (Arlt *et al.* 2002a), and are consistent with events occurring in patients with urothelial cancers associated with aristolochic acid nephropathy and Balkan endemic nephropathy. The same DNA adducts identified in humans are also found in experimental animals (Arlt *et al.* 2002a, b) exposed to the natural mixture or the pure major components. A:T→T:A transversions in the *TP53* gene in urothelial tumours of aristolochic acid nephropathy and Balkan endemic nephropathy patients were the predominant mutations found in human *TP53* knock-in mouse fibroblasts treated with aristolochic acid (Liu *et al.*, 2004; Feldmeyer *et al.*, 2006; Arlt *et al.*, 2007). Collectively, these data support the strong mechanistic evidence of the carcinogenicity of aristolochic acid – a mixture of aristolochic acids I and II – in humans.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of plants containing aristolochic acid. Plants containing aristolochic acid cause cancer of the renal pelvis, and of the ureter.

There is *sufficient evidence* in experimental animals for the carcinogenicity of extracts of plants containing aristolochic acid.

There is *limited evidence* in humans for the carcinogenicity of aristolochic acid.

There is *sufficient evidence* in experimental animals for the carcinogenicity of aristolochic acid.

Plants containing aristolochic acid are *carcinogenic to humans (Group 1).*

Aristolochic acid is *carcinogenic to humans (Group 1).*

In making the overall evaluation of aristolochic acid, the Working Group took into consideration that:

- Aristolochic-acid-specific DNA adducts identified in experimental animals exposed to aristolochic acid or herbal products containing aristolochic acid were found in urothelial tissue of aristolochic acid nephropathy patients, in renal tissue from Balkan endemic nephropathy patients, and in tumour tissue from residents of endemic villages.
- A:T→T:A transversions were found in the *TP*53 gene of urothelial tumours from aristolochic acid nephropathy patients and Balkan endemic nephropathy patients. The same type of mutation predominated in human *TP*53 knock-in mouse fibroblasts treated with aristolochic acid.

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METHOXSALEN PLUS ULTRAVIOLET A RADIATION

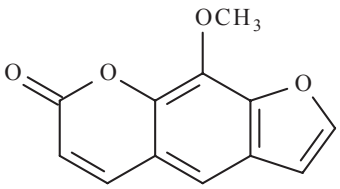
Methoxsalen plus ultraviolet A radiation was considered by previous IARC Working Groups in 1980 and 1987 (IARC, 1980, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 298-81-7
Chem. Abstr. Name: 9-Methoxy-7*H*-furo[3,2-*g*][1]benzopyran-7-one
IUPAC Systematic Name: 9-Methoxyfuro[3,2-*g*]chromen-7-one
Synonyms: 5-Benzofuranacrylic acid, 6-hydroxy-7-methoxy-, δ-lactone; Meladinine; 8-methoxy-6,7-furanocoumarin; 8-methoxypsoralen; 8-methoxy[furano-3′,2′:6,7-coumarin]; 8-MOP; 8-MP; Oxsoralen; Puvasoralen; Uvadex
Description: Silky needles or long rhombic prisms; odourless with a bitter taste followed by a tingling sensation (O'Neil, 2006)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_{12}H_8O_4$
Relative molecular mass: 216.19

1.2 Use of the agent

Information for Section 1.2 is taken from McEvoy (2007), Thomson Healthcare (2007), and Sweetman (2008)

Methoxsalen is a psoralen produced naturally by various plants (e.g. celery, parsnips, limes, figs, and others) found in both temperate and tropical regions (Ashwood-Smith *et al.*, 1985; NTP, 2005). It is also a constituent of the seeds of the *Ammi majus* plant, and of the roots of *Heracleum candicans*. It is a photosensitizer, which markedly increases skin reactivity to long wavelength ultraviolet radiation (UVA: 320–400 nm), an effect used in photochemotherapy or PUVA

[psoralen (P) and high-intensity long wavelength (UVA) irradiation].

1.2.1 Indications

(a) Psoriasis

Oral methoxsalen is used in conjunction with controlled exposure to UVA radiation for the symptomatic treatment of severe, recalcitrant, disabling psoriasis that is refractory to other forms of therapy, and when the diagnosis has been confirmed by biopsy. Psoralens have been used topically in conjunction with UVA irradiation for the treatment of psoriasis, but the use of topical methoxsalen has largely been abandoned because it produces a greater incidence of adverse effects, and is cosmetically less acceptable than oral psoralens.

(b) Cutaneous T-cell lymphoma

Oral methoxsalen is used in conjunction with photopheresis for the palliative treatment of the skin manifestations of cutaneous T-cell lymphoma (e.g. mycosis fungoides, Sézary syndrome).

(c) Idiopathic vitiligo

Methoxsalen has been used orally or topically in conjunction with controlled exposure to UVA or sunlight to repigment vitiliginous skin in patients with idiopathic vitiligo. To retain new pigment, periodical treatment with the drug and some form of UVA irradiation is often required.

(d) Other uses

Methoxsalen used in conjunction with UVA irradiation has been shown to be effective in the treatment of selected diseases mediated by T cells, rejection after solid organ transplantation, and chronic graft-versus-host disease (Greinix *et al.*, 2000).

1.2.2 Dosage

For the treatment of psoriasis, oral methoxsalen therapy is accompanied by some form of UVA irradiation. Methoxsalen is usually administered with milk or food 1.5–2 hours before exposure to high-intensity UVA light, two or three times weekly. The initial dose of methoxsalen is based on the patient's weight; from 10 mg for a patient weighing less than 30 kg, up to 70 mg for a patient weighing more than 115 kg.

For the treatment of cutaneous T-cell lymphoma, methoxsalen is usually administered orally. Oral methoxsalen may be administered as a single dose with food or in two divided doses approximately 30 minutes apart, to minimize adverse gastrointestinal effects (McEvoy, 2007).

To repigment vitiliginous areas, methoxsalen is usually given in a dose of up to 600 µg/kg (20 mg daily) orally 2–4 hours before measured periods of exposure to UVA radiation twice a week, at least 48 hours apart.

Methoxsalen may also be applied topically in the form of a 1% lotion; occasionally, the lotion may be diluted 10- or 100-fold to avoid adverse reactions.

Methoxsalen is available as 10 mg capsules and liquid-filled capsules for oral administration; it is also available as a 1% lotion for topical administration.

1.2.3 Trends in use

No information was available to the Working Group.

2. Cancer in Humans

2.1 Cohort studies

Cohort studies have been used to examine the association between PUVA treatment and skin cancer. Most studies reviewed in Table 2.1

(available at http://monographs.iarc.fr/ENG/Monographs/vol100A/100A-19-Table2.1.pdf) have investigated cohorts of patients with psoriasis, although a few studies also included subgroups of patients treated with PUVA for other skin disorders. In all studies available to the Working Group, most of the psoriatic patients had also been exposed to other antipsoriatic agents, some of which known or suspected to be carcinogenic, including ionizing radiation, arsenics, methotrexate, UVB, and topical tars. Although several of the studies adjusted for such previous or concurrent exposures, missing or incomplete information may have led to insufficient adjustment and consequently, an overestimation of the carcinogenic risk of PUVA. All studies included in this review presented a risk analysis of squamous cell carcinoma of the skin, while only a subgroup of studies evaluated the risk for basal cell carcinoma, and skin melanoma. One study further included selected extracutaneous tumour sites in the analysis. The studies compared the incidence of cancer in the cohorts with expected numbers based on general population rates, and some papers also made internal comparisons, by level of exposure to PUVA.

2.1.1 Squamous cell carcinoma of the skin

The relative risk of squamous cell carcinoma of the skin in PUVA-treated patients has been assessed in three large multicentre studies from the United States of America and Sweden, in one countrywide study from Finland, and in four single centre studies from Austria, Scotland, the Netherlands, and the USA.

The cohort studies used a variety of methods, but all found significant increases in the incidence of squamous cell carcinoma of the skin in people treated with PUVA compared to general population rates, with relative risks in the range of 5–10 (Forman *et al.*, 1989; Perkins *et al.*, 1990; Bruynzeel *et al.*, 1991; Chuang *et al.*, 1992; Stern & Laird, 1994; Maier *et al.*, 1996; Hannuksela-Svahn *et al.*, 1999, 2000; Lindelöf *et al.*, 1999). Much higher relative risks (more than 100-fold) were found in two studies of men (Stern, 1990; Stern *et al.*, 2002).

The studies that undertook analyses by level of exposure to PUVA found dose-related increases in the incidence of squamous cell carcinoma. Dose was assessed by frequency of exposure in one study (Stern & Laird, 1994), and by cumulative dose of UVA in J/cm^3 in three others (Forman *et al.*, 1989; Chuang *et al.*, 1992; Lindelöf *et al.*, 1999).

2.1.2 Basal cell carcinoma of the skin

The two multicentre studies from the USA also included data on incident cases of basal cell carcinoma among PUVA-treated patients. Although the study by Forman *et al.* (1989) observed a significantly increased relative risk of basal cell carcinoma, the relative risk was reduced to a non-significant level in the analysis of the subgroup of patients with no previous exposure to ionizing radiation or arsenics, and there was no clear relationship with the standard morbidity and cumulative dosage of UVA. In the multicentre study by Stern and Laird (1994), the risk of basal cell carcinoma in patients with high-intensity exposure to PUVA was increased 1.7-fold (95%CI: 1.1–2.5) compared to that of patients with low-intensity exposure (see Table 2.1 online). The investigators of the Dutch study judged the risk of basal cell carcinoma to be increased 5-fold over that of the general population (Bruynzeel *et al.*, 1991), but there was no correlation between the development of basal cell carcinoma and the cumulative dosage of UVA. [The Working Group noted that the reference rates of basal cell carcinoma applied in the analysis were derived from the routine registration of a regional cancer registry of the country; substantial underreporting of this tumour type is likely, more so than for squamous cell carcinoma, and it may explain the increases in the standard morbidity ratio observed in these

studies, which ascertained cases in the psoriasis cohorts in a manner that differed from the ascertainment in routine cancer registration.]

2.1.3 Malignant melanoma of the skin

In the study by Lindelöf *et al.* (1999), the standard morbidity ratios were estimated to be slightly, but non-significantly increased. No increase was noted for the subgroup of patients followed for 15 years or more since first treatment with PUVA. In the study from the USA (Stern *et al.*, 1997; Stern, 2001), patients with at least 200 PUVA treatments were not at significantly higher risk than patients who received less than 200 treatments in multivariate analyses, taking into account other antipsoriatic treatments.

3. Cancer in Experimental Animals

3.1 Methoxsalen and UVA

Many of the earlier studies on methoxsalen were several short-duration experiments that found no statistically significant increase in the incidence of skin tumours or tumours of internal organs in either sex of mice of various strains when given methoxsalen orally alone or in combination with ultraviolet (250–400 nm) irradiation (IARC, 1980, 1987a).

Methoxsalen administered in the feed to groups of 40 Swiss mice at 500 ppm for 12 months and exposed to UVA for 2 hours daily for 3 months caused skin tumours in 35% of the mice; when given for 30 minutes daily for 3 months, caused skin tumours in 25% of the mice; and when given for 10 minutes daily for 6 weeks, caused skin tumours in 20% of the mice. No skin tumours were seen in three groups of 40 control mice that received UVA only (Griffin, 1959; IARC, 1980, 1987a). A second feed study exposed groups of 36 male and 36 female HRA/Skh hairless mice to methoxsalen at 0, 9, 21 or 80 mg/kg body weight/week for 52 weeks, followed by exposure to UVA half an hour after feeding. Surviving animals were kept for study for an additional 28 weeks without treatment. An increased incidence in squamous cell carcinoma was observed in the mid- and high- dose groups in females, and in the high-dose group in males (Dunnick *et al.*, 1991). Another oral study involved exposing two groups of 16 female Tg.AC mice to methoxsalen in corn oil by gavage at 8 mg/kg body weight for 5 days over a 2-week period for 10 weeks, followed by weekly administration of the chemical for an additional 6 weeks. One exposure group was also exposed to UVA one hour after dosing with methoxsalen. A third group of eight animals served as a control, and were treated with corn oil only followed by UVA exposure. The incidence of skin papillomas in the PUVA group was significantly higher than that of the other two groups (Chignell *et al.*, 2003).

Methoxsalen has been studied in mice by other routes of administration including skin application or intraperitoneal injection in combination with exposure to UVA. Skin application induced epidermal and dermal skin tumours in five studies. These included carcinomas, squamous cell carcinomas, fibrosarcomas and basal cell carcinomas. Tumours of the eye and ear regions (epidermal fibrosarcomas and squamous carcinomas) were observed in one study following the intraperitoneal administration of methoxsalen to female mice (IARC, 1980, 1987a).

See Table 3.1.

3.2 Methoxsalen alone

Groups of 50 male and 50 female Fischer 344 rats were administered methoxsalen by gavage in corn oil at concentrations of 0, 37.5 or 75 mg/kg 5 days/week for 103 weeks. These animals were not exposed to UVA radiation. Mortality was significantly affected by treatment of the male but not the female rats. In males, Increased incidences of tubular cell adenomas and/or adenocarcinomas

Table 3.1 Studies of cancer in experimental animals exposed to PUVA

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, Swiss (F) 100 d Griffin (1959)	Feed 0 or 500 mg/kg methoxsalen for 100 d and exposed to UVA for 2 h daily for 3 mo, 30 min daily for 3 mo, or for 10 min daily for 6 wk 40/group	Skin tumours: 35% (UVA for 2 h/d), 25% (UVA for 30 min/d), and 20% (UVA for 10 min/d) No skin tumours in corresponding control mice that were exposed to UVA only	NR	Wood's type ultraviolet lamp (Black-Ray Model XX15 Long Wave Ultraviolet) [wavelength presumed to be > 320nm]
Mouse, HRA/Skh (M, F) 80 wk Dunnick et al. (1991)	Feed 0, 9, 21 or 80 mg methoxsalen/kg bw/wk 30 min before exposure to 0 or 2 J/cm^2 UVA (320–400 nm) for 5 min Exposure for 52 wk 36/group Animals observed for additional 28 wk without exposure	Skin (squamous cell carcinomas): M–control, 0%; 9 mg/kg bw, 0%; 21 mg/kg bw, 6%; 80 mg/kg bw, 39% F–control, 0%; 9 mg/kg bw, 6%; 21 mg/kg bw, 17%; 80 mg/kg bw, 47%	NR [$P < 0.0001$][a] (high-dose) [$P < 0.05$] (mid-dose); [$P < 0.0001$] (high-dose)	
Mouse, Tg.AC (F) 17 wk Chignell et al. (2003)	Gavage 0, 8, or 8 mg methoxsalen/kg bw in corn oil 5×/2 wk for 10 wk followed by once/wk for 6 wk. The corn oil control and one dosed group also exposed to UVA (2 J/cm^2) 1 h after dosing 8, 16, 16; (all 20-wk-old)	Skin (papillomas): 11/16 (UVA), 13/14 (PUVA), 4/8 (methoxsalen only) Mean of maximum tumours/tumour-bearing animal: 7.4 (PUVA) vs 1.5 (UVA alone) ($P < 0.01$, Mann–Whitney U-test) Mean maximum tumours/animal: 6.9 (PUVA) vs 1.1 for other groups ($P < 0.01$, Mann–Whitney U-test)	$P < 0.05$[a] for PUVA vs methoxsalen only	
Mouse, SKH:hairless (sex NR) 30 wk Forbes & Urbach (1975)	Skin painting Daily skin applications of 40 µL of either methanol or 0.01% methoxsalen in methanol 30–60 min before a 10-min whole-body UVA exposure (300–400 nm) on 5 d/wk for 30 and 14 wk, respectively 30/group	Skin tumours: 15/30 at 14 wk for the PUVA group 12/24 at 30 wk for the methanol + UVA group	NR	Most of the observed skin tumours developed at the site of application were squamous cell carcinomas (≈90%). Study was poorly reported Age NR

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, SKH:hairless (sex NR) 38 wk Forbes et al., (1976)	Skin painting Skin applications of 40 µL of either methanol or 0.1% methoxsalen in methanol before a 2-h whole body UVA exposure (300–400 nm) on 5 d/wk for 30 and 14 wk, respectively 24/group	Skin tumours: 50% at 20 wk for the PUVA group 50% at 27 wk in the methanol + UVA group ($P < 0.01$)	From 18 to 28 wk, the numbers of tumour-bearing animals and of tumours per animal were significantly higher in the PUVA group than in the methanol + UVA group ($P < 0.05$ and $P < 0.01$, respectively)	In both groups, ≈45/50 histologically analysed tumours were squamous cell carcinomas Age NR
Mouse, SKH:hairless-1 and HRS/J/AN1 (F) 90 wk Grube et al. (1977)	Skin painting Skin applications of 250 µg methoxsalen in ethanol 60 min before whole body UVA exposure to either 300–400 nm, 320–400 nm or 365 nm on 5 d/wk for 24 wk 20–25 animals per strain	SKH:hairless-1, respective to the 3 wavelengths: Squamous cell or basal cell carcinomas of the skin–17/20, 15/19, and 8/19 Fibrosarcomas–6/20, 5/19, and 2/19 HRS/J/An1, respective to the 3 wavelengths: Squamous cell or basal cell carcinomas of the skin–0/25, 4/23, and 9/23 Fibrosarcomas–5/25, 1/23, and 4/23	NR	Lack of information on controls and inadequate reporting of experimental details
Mouse, Swiss (F) 60 wk Santamaria et al., (1979)	Skin painting Skin applications of 0 ($n = 5$/group) or 5 ($n = 25$/group) µg of methoxsalen in 0.05 mL ethanol 60 min before UVA exposure (300–400 nm) twice/wk for 15, 30, 45 or 60 min respectively for 60 wk 8 groups	Subcutaneous malignant tumours: 43% incidence in treated animals from all four groups combined 15% incidence in the four control groups of mice exposed to UVA alone	NR	Tumours reported to be mammary adenocarcinomas, skin carcinomas and carcino-mixo-sarcomas. Inadequate reporting of use of controls and of survival times. The pooling of various experimental groups and tumour types make the study difficult to evaluate Age unspecified

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, Swiss (F) 11 mo Griffin et al. (1958)	Intraperitoneal 0.4 mg methoxsalen 6×/wk for 10 mo with exposure to UVA for either 30 min daily for 3 mo, or for 10 min daily for 6 wk 20/group Two control groups (n = 20) only exposed to UVA	Epidermal tumours: > 50% in mice exposed to UVA for 30 min/d 100% in mice exposed to UVA for 10 min/d No tumours in corresponding controls	NR	Wood's type ultraviolet lamp (Black-Ray Model XX15 Long Wave Ultraviolet; wavelength > 320 nm). The tumours reported were fibrosarcomas and squamous cell carcinomas of the ears and the eye region
Mouse, SKH: hairless-1 (F) 40 wk Nagayo et al. (1983)	Skin painting Daily skin applications of 40 µL of either methanol or 0.1% methoxsalen in methanol before a 15-min whole-body UVA exposure (320–420 nm) 5 d/wk for 40 wk 20/group	Skin tumours: Methanol + UVA, 0/18; PUVA, 20/20	[$P < 0.0001$][a]	6/58 skin tumours were squamous cell carcinomas

[a] Fisher Exact test

bw, body weight ; d, day or days; F, female; M, male; min, minute or minutes; mo, month or months; PUVA, methoxsalen with UVA; vs, versus; wk, week or weeks

Table 3.2 Studies of cancer in experimental animals exposed to methoxsalen

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, F344 (M, F) 103 wk NTP (1989)	Gavage 0, 37.5 or 75 mg methoxsalen/kg bw in corn oil 5 ×/wk for 103 wk 50/group	**Males** Kidney tumours: Tubular cell adenomas or adenocarcinomas–1/50 (control), 12/50 (low), 11/49 (high) Tubular cell adenocarcinomas–0/50 (control), 1/50 (low), 3/49 (high)	$P < 0.001$ (Life Table)	Study was negative in females
		Zymbal gland: Carcinomas or squamous cell carcinomas–1/50, 7/50, 4/49	$P = 0.008$ for low dose (Life Table)	
		Fibromas, subcutaneous–1/50, 5/50, 7/49	$P = 0.004$ (Life Table)	
		Lung: Alveolar/bronchiolar adenomas–4/50, 9/50, 9/49	$P = 0.015$ (Life Table)	

bw, body weight; F, female; M, male; wk, week or weeks

of the kidney and carcinomas of the Zymbal gland were observed. This was also true for subcutaneous tissue fibromas and alveolar/bronchiolar adenomas of the lung. There were no significant increased incidences of neoplasms observed in dosed female rats (NTP, 1989).

See Table 3.2.

4. Other Relevant Data

4.1 Absorption, distribution, metabolism and excretion

4.1.1 Humans

Methoxsalen is rapidly absorbed after oral treatment, with peak plasma concentrations reached after 2–6 hours, 50% as unchanged drug (Schalla *et al.*, 1976; Gazith & Schaefer, 1977; Steiner *et al.*, 1977; Busch *et al.*, 1978). Following topical application, methoxsalen penetrates rapidly into the epidermis and dermis, and the high concentrations reached remain constant over a period of 16 hours (Kammerau *et al.*, 1976).

Methoxsalen is extensively metabolized, and no unchanged drug is excreted in the urine (Schalla *et al.*, 1976; Busch *et al.*, 1978). Approximately 80% of a dose is excreted in the urine within 8 hours as hydroxylated and glucoronide derivatives (Pathak *et al.*, 1974).

4.1.2 Experimental systems

After oral administration of ^{14}C-methoxsalen to dogs and rats, absorption is rapid, with peak plasma radioactivity levels occurring within 2 hours after dosing in rats, and within 30 minutes in dogs (Busch *et al.*, 1978). After oral and intravenous administration of ^{14}C-methoxsalen to rats, radioactivity is found in the liver, kidneys, and the cortic part of the adrenal gland. Skin concentrations of radioactivity are comparable with blood levels and are similar in albino and pigmented rats; ultraviolet light increases the subcutaneous concentrations of radioactivity (Wulf & Hart, 1979).

After oral administration to rats, ^{14}C-methoxsalen is excreted in trace amounts together with eight polar metabolites, some of them conjugated (Busch *et al.*, 1978).

After intravenous administration of ^{14}C-methoxsalen to dogs, radioactivity disappears rapidly from plasma, although small levels of radioactivity are observed to persist for 5 weeks after administration, suggesting that the persistent plasma radioactivity is due to a metabolite bound to plasma protein. Elimination occurs in both urine and bile; 45% of the dose appearing in the urine, and 40% in the faeces within 72 hours of administration. Methoxsalen is extensively metabolized, and less than 2% of the drug is excreted unchanged in the urine. Two metabolites resulting from the opening of the furan ring are 7-hydroxy-8-methoxy-2-oxo-2*H*-1-benzopyran-6-acetic acid, and *α*,7-dihydroxy-8-methoxy-2-oxo-2*H*-1-benzopyran-6-acetic acid, and one resulting from the opening of the pyrone ring is an unknown conjugate of (*Z*)-3-(6-hydroxy-7-methoxybenzofuran-5-yl)-2-propenoic acid (Kolis *et al.*, 1979).

4.2 Mechanisms of carcinogenesis

4.2.1 Genotoxic effect

Evidence from a large number of studies indicates that PUVA causes DNA damage in a variety of prokaryotic and eukaryotic cells. The events mentioned in the previous *IARC Monograph* included the induction of chromosomal aberrations, sister chromatid exchange, mutations, DNA damage, and DNA crosslinks in human cells *in vitro*. The treatment was also reported to transform mouse C3H10T1/2 cells, and to induce chromosomal aberrations, micronuclei, sister chromatid exchange, mutation, unscheduled DNA synthesis, and DNA crosslinks in rodent cells in culture. In addition, mitotic recombination and mutation were found in fungi, and mutation and DNA damage in bacteria. The treatment was also reported to induce sister chromatid exchange in epithelial cells of cheek pouches of hamsters treated *in vivo*; by contrast, negative results were mentioned for the induction of sister chromatid exchange in patients treated with PUVA (IARC, 1987b).

In the absence of UVA light, methoxsalen was found to induce mutation in bacteria, but the evidence was considered inconclusive with respect to chromosomal aberrations and sister chromatid exchange in human cells *in vitro*, gene mutation and DNA damage in rodent cells *in vitro*, and mutation in yeast (IARC, 1987b).

4.2.2 DNA adduct formation

The major photochemical reactions of methoxsalen in the presence of DNA were described several decades ago (Cole, 1971), and are briefly summarized below.

Methoxsalen undergoes DNA intercalation, with a preference for 5′-TpA sites (Tessman *et al.*, 1985), and subsequently alkylates DNA upon photo-activation. The major reaction mechanism involves [2+2] cycloaddition to the 5,6-double bond of a thymidine upon absorption of the first photon, which generates two types of cyclobutane mono-adducts, depending on whether the addition occurs at the 4′,5′-double bond of the furan ring or the 3,4-double bond of the pyrone ring of the psoralen. The absorption of a second photon by the furan mono-adduct then leads to cycloaddition of the 5,6-double bond of the pyrone to a flanking thymidine in the complementary strand, generating an interstrand cross-link (Johnston & Hearst, 1981). Other types of photoproducts have been reported, including adducts to the sugar moiety of deoxyadenosine (Cadet *et al.*, 1988).

4.2.3 Mutagenic effects

The mutagenic effects of PUVA in mammalian cells have been extensively investigated.

Treatment of Chinese hamster ovary cells with PUVA (for analysis of the mutation spectrum at the adenine phosphoribosyl transferase (*Aprt*) locus) suggests that bi-adducts are likely to be the major PUVA-induced pre-mutagenic lesions in mammalian cells (Sage *et al.*, 1993). Likewise, the spectrum of mutations induced at the hypoxanthine (guanine) phosphoribosyl transferase (*HPRT*) gene of human fibroblasts exposed to a split-dose protocol (two UVA doses separated in time, the first one producing mainly mono-adducts) indicates that most of the mutations are observed at crosslinked sites; however, a significant level of mutation induction is also detected after the first dose of UVA, where a higher proportion of mono-adducts would be expected (Yang *et al.*, 1994). This study suggests that both mono-adducts and crosslinks have mutagenic properties.

There has also been extensive use of the *supF* gene for the analysis of PUVA-induced mutations. In one representative study, the spectrum of mutations was characterized after treatment of fibroblasts from transgenic mice containing chromosomally integrated lambda phage with the *supF* gene as a mutation reporter gene (Gunther *et al.*, 1995); a significant feature of the mutation spectrum was the predominant occurrence of mutations at 5′TpA sites, in concordance with the sequence specificity observed *in vitro* in reactions with isolated DNA (Sage & Bredberg, 1991).

Whereas the formation of psoralen photoproducts at 5′ApT:5′TpA sites is closely correlated with the *Tp53* mutation spectra obtained *in vitro* and in studies of PUVA treatment of animals (Nataraj *et al.*, 1996; Besaratinia & Pfeifer, 2004; Lambertini *et al.*, 2005), such correlations do not hold for *TP53* mutations in PUVA-related human squamous cell carcinoma (Nataraj *et al.*, 1997; Wang *et al.*, 1997; Gasparro *et al.*, 1998; Monti *et al.*, 2000). PUVA-induced DNA adducts have recently been shown to be substrates for the base-excision repair pathway in human cells (Couvé-Privat *et al.*, 2007) but the efficiency of the repair mechanism remains to be established. The peculiarity of the *TP53* mutation spectrum in PUVA-induced human squamous cell carcinoma has been interpreted as indicating that other types of DNA damage may contribute to the carcinogenicity and mutagenicity of PUVA. In addition to the direct interaction of photo-activated methoxsalen with DNA, reactive oxygen species (including singlet oxygen and superoxide) have been suggested as having a role in PUVA-induced cytotoxicity (Foote, 1991; Liu *et al.*, 1999). The formation of increased levels of the 8-hydroxy-2′-deoxyguanosine biomarker following PUVA treatment of either calf thymus DNA or cultured human epidermoid carcinoma cells (compared with those obtained after treatment with methoxsalen or UVA alone) (Liu *et al.*, 1999) is consistent with this interpretation.

4.3 Synthesis

Methoxsalen in combination with UVA is carcinogenic via a genotoxic mechanism that involves photo-activation.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of methoxsalen plus with UVA radiation. Methoxsalen in combination with UVA radiation causes cancer of the skin (squamous cell carcinoma).

There is *sufficient evidence* in experimental animals for the carcinogenicity of methoxsalen plus UVA radiation.

There is *limited evidence* in experimental animals for the carcinogenicity of methoxsalen.

Methoxsalen plus UVA radiation is *carcinogenic to humans (Group 1).*

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PHENACETIN

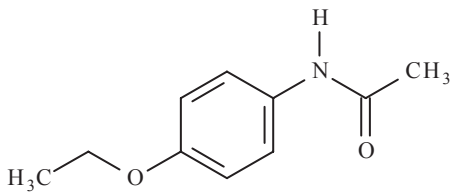
Phenacetin was considered by previous IARC Working Groups in 1976 and 1980 (IARC, 1977, 1980). Analgesic mixtures containing phenacetin were considered by a previous IARC Working Group in 1987 (IARC, 1987a). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agent

Chem. Abstr. Serv. Reg. No.: 62-44-2
Chem. Abstr. Name: Acetamide, *N*-(4-ethoxyphenyl)-
IUPAC Systematic Name: *N*-(4-Ethoxyphenyl)acetamide
Synonyms: Aceto-4-phenetidine; acetophenetidin; acetophenetidine; acetophenetin; acetphenetidin; 4-(acetylamino)phenetole; *N*-acetyl-4-ethoxyaniline; *N*-acetyl-*p*-ethoxyaniline; *N*-acetyl-*p*-phenetidine; 4-ethoxyacetanilide; 4′-ethoxyacetanilide; *p*-ethoxyacetanilide; *N*-(4-ethoxyphenyl) acetamide; Mironal
Description: Slightly bitter, crystalline scales or powder (O'Neil, 2006)

1.1.1 Structural and molecular formulae, and relative molecular mass



$C_{10}H_{13}NO_2$
Relative molecular mass: 179.22

1.2 Use of the agent

1.2.1 Indications

Phenacetin was used as an analgesic and fever-reducing drug in both human and veterinary medicine for many years. It was introduced into therapy in 1887 and was extensively used in analgesic mixtures until it was implicated in kidney disease (nephropathy) due to abuse of analgesics. Phenacetin also was once used as a stabilizer for hydrogen peroxide in hair-bleaching preparations (IARC, 1980; Nugent & Hall, 2000).

1.2.2 Dosage

Analgesic mixtures containing phenacetin were previously marketed as tablets or capsules containing between 150 and 300 mg phenacetin. Common combinations were: 150 mg phenacetin, 230 mg aspirin, and 15 or 30 mg caffeine; or 150 mg phenacetin, 230 mg aspirin, 30 mg caffeine, and 8, 15, 30, or 60 mg codeine phosphate. Phenacetin alone was also available in 250 and 300 mg doses as tablets, and up to 500 mg doses as powder. The usual dose was 300 mg 4–6 times per day, and the daily dose was not to exceed 2 g (IARC, 1977, 1980).

1.2.3 Trends in use

Phenacetin was withdrawn from the market in Canada in 1978, in the United Kingdom in 1980 (IARC, 1980), and in the Unites States of America in 1983 (FDA, 1999).

Over-the-counter sales of phenacetin-containing analgesics have been legally prohibited in most countries. For example, in Australia, analgesic mixtures containing phenacetin were legally banned in 1977 (Michielsen & de Schepper, 2001), in Belgium in 1987 (Michielsen & de Schepper, 2001), in Germany in 1986 (Schwarz *et al.*, 1999), and in Denmark in 1985 (Nørgaard & Jensen, 1990). In the Czech Republic, analgesic mixtures containing phenacetin were recently removed from the market. They are still available in Hungary under the trade names Antineuralgica and Dolor (Sweetman, 2008). Phenacetin was withdrawn from many analgesic mixtures long before the legal ban in several countries.

2. Cancer in Humans

2.1 Case reports

Many case reports of renal pelvic and other urothelial tumours in patients who used large amounts of phenacetin-containing analgesics have been recorded (IARC, 1987a).

2.2 Case–control studies

Case–control studies have been used almost exclusively to examine the association between consumption of analgesics and various cancers of the urinary tract. In all of the studies available to the Working Group, the cumulative use of groups of pharmaceuticals was assessed by asking study subjects about their retrospective use. In most epidemiological studies reviewed, it was rather difficult to estimate the effect of phenacetin separately from the effect of other analgesics, as various pain-relieving substances are often combined in the same pharmaceutical product.

See Table 2.1

2.2.1 Cancer of the renal pelvis and ureter

Small-to-medium-sized case–control studies from Australia (McCredie *et al.*, 1982, 1983a, b; McCredie & Stewart, 1988), Denmark (Jensen *et al.*, 1989) and Germany (Pommer *et al.*, 1999) all suggested a moderate-to-strong association between the regular use of analgesics containing phenacetin and tumours of the renal pelvis (relative risk, 4.2–6.0).

This relationship was also tested in three larger case–control studies, one from Australia (McCredie *et al.*, 1993), and two from the USA (Ross *et al.*, 1989; Linet *et al.*, 1995), each of which included some 150–500 patients with cancers of the renal pelvis and ureter. The Australian study, which included information on subjects' cumulative intake of phenacetin before 1987, showe a

Table 2.1 Case–control studies of cancer of the urinary tract and consumption of analgesic mixtures containing phenacetin

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Cancer of the renal pelvis and ureter									
McCredie et al. (1982) New South Wales, Australia, 1977–80	Renal pelvis (ICD-8: 189.1)	27 men, 40 women from the New South Wales Cancer Registry and from 3 Sydney hospitals; cases confirmed histologically	180 volunteers, friends and relatives	In-person structured interview undertaken at hospital or at home	*Lifetime consumption of phenacetin-containing analgesics (kg) in women* No latency: No use (< 0.1) ≥ 0.1 5-year latency: No use (< 0.1) ≥ 0.1	 NR NR NR NR	 1 (ref) 2.4 (1.0–6.0) 1 (ref) 4.1 (1.8–9.2)	Age and use of other types of analgesics. Not adjusted for smoking	Response rates not given, rates of renal pelvis cancer in men not given
McCredie et al. (1988) New South Wales, Australia, 1980–82	Renal pelvis (ICD-8: 189.1)	73 (31 men, 42 women) from the New South Wales Central Cancer Registry	688 (307 men, 381 women) population-based controls 50-yr-old or above selected at random from the New South Wales election rolls 1980–81; response rate 72%	Standardized questionnaire mailed to participants	*Lifetime consumption of phenacetin (kg)* No use ≥ 0.1 ≥ 1.0	 [37] NC [24]	 1 (ref) 5.7 (3.2–10.0) 7.9 (4.6–13.8)	Sex, paracetamol use, smoking	Response rate for cases not given. No use defined as lifetime consumption < 0.1 kg up till the date of diagnosis or the equivalent date for control. Phenacetin always in preparations containing aspirin and either caffeine or codeine

Table 2.1 (continued)

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
McCredie et al. (1983a; 1983b) 1980–82	Ureter (ICD-8: 189.2)	36 males from the New South Wales Central Cancer Registry	307 male population-based controls 50-yr-old or above selected at random from the New South Wales election rolls 1980–81; response rate 72%		No use	NR	1 (ref)		Re-analysis of the original data
					≥ 0.1	NR	0.7 (0.3–2.2)		
					≥ 1.0	NR	1.2 (0.5–3.0)		
McLaughlin et al. (1983) Minneapolis-St. Paul metropolitan area, USA, 1974–79	Renal pelvis (ICD-8: 189.1)	74 (50 men, 24 women) from the Minneapolis-St. Paul metropolitan area 30–79-yr-old; cases confirmed histologically; 95% response rate	697 (428 men, 269 women) selected at random from telephone listings, from files of the Health Care Financing Administration and from death certificate listings; response rate 94%	In-person structured interview of participants or next-of-kin undertaken at home	*Use of phenacetin- or acetaminophen-containing analgesics*			Age and cigarette smoking	No use defined as: never used phenacetin- or paracetamol-containing drugs regularly for ≥ 1 mo. Of 7 cases who were long-term users, 1 used paracetamol compounds only
					Men:				
					No use	23	1 (ref)		
					Non-regular use	21	0.9 (0.4–1.8)		
					Regular use, ≤ 36 mo	2	0.7 (0.1–3.9)		
					Regular use, > 36 mo	4	3.9 (0.7–20.4)		
					Women:				
					No use	9	1 (ref)		
					Non-regular use	10	1.4 (0.5–4.2)		
					Regular use, ≤ 36 mo	2	2.3 (0.2–19.7)		
					Regular use, > 36 mo	3	3.7 (0.5–24.7)		

Table 2.1 (continued)

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Jensen et al. (1989) Copenhagen, Denmark, 1979–82	Renal pelvis and ureter	96 (60 men, 36 women) notified directly from 27 hospital units of Eastern Denmark; response rate 99%	288 (180 men, 108 women) selected at random from medical or surgical wards matched for hospital, sex and age in 5-yr age groups; patients with urinary-tract- and smoking-related diseases were not eligible; response rate 99%	In-person structured interview undertaken at hospital	*Lifetime consumption of phenacetin (g)*			Age	Use of analgesics measured up until 5 yr before interview. The 'no use' level not clearly defined. The influence of phenacetin and aspirin could not be separated in the analysis. Incomplete adjustment for smoking habits (< 10 vs ≥ 10 pack-yr)
					Men				
					Never used analgesics	31	1 (ref)		
					Ever used phenacetin	13	3.9 (1.7–9.1)		
					1–749	6	3.1 (1.0–9.6)		
					≥750	5	9.1 (2.2–38)		
					Women				
					Never use of analgesics	9	1 (ref)		
					Ever used phenacetin	17	6.9 (2.7–18)		
					1–749	4	6.1 (1.5–26)		
					≥750	7	6.1 (1.9–20)		
					Ever used phenacetin			Age, tobacco smoking, 'high risk' occupations and aspirin	
					Men	12	2.4 (0.9–6.8)		
					Women	15	4.2 (1.5–12)		
Pommer et al. (1999) Berlin Germany 1990–94	Renal pelvis and ureter (ICD-9: 189.1–2)	76 (37 men, 39 women) diagnosed in 1 of 8 urological departments; response rate 85%	76 subjects selected at random from the central inhabitant registry of the former West Berlin area individually matched to case subject by sex and age (± 2 yr); response rate 70%	In-person structured interview undertaken at hospital (cases) or at participants' home (controls)	No or rare intake of analgesics	31	1 (ref)	Current smoking, former smoking, socioeconomic status, laxative intake	Rare intake defined as intake of less than 1 analgesic dose/mo
					Lifetime consumption of phenacetin (kg)				
					≥ 1	7	1.8 (0.2–14)		

Table 2.1 (continued)

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
McCredie et al. (1993) New South Wales Australia, 1989–90	Renal pelvis (ICD-9: 189.1)	147 (58 men, 89 women) in age range 20–79-yr at diagnosis from a rapid-ascertainment system and the New South Wales Central Cancer Registry; patients had to be listed in telephone directory and able to speak English to be included; response rate 89%	523 (231 men, 292 women) frequency-match to cases on age and sex and selected at random from the New South Wales electoral rolls; controls had to be listed in telephone directory and able to speak English to be selected; response rate 74%	Structured interview undertaken at participants' home or conducted over the telephone	*Phenacetin-containing analgesics* Non-consumers Any *Total use pre-1987 (kg)* ≤ 2.04 2.04–6.87 ≥ 6.88	 76 70 12 16 42	 1 (ref) 12.2 (6.8–22) 5.2 (2.2–12) 8.3 (3.4–21) 18.5 (8.7–39) Test for trend: $P = 0.019$	Age, sex, cigarette smoking, education level, method of interview Age, sex, cigarette smoking, education level, method of interview, paracetamol in any form	The non-consumer category defined as persons reporting taking analgesics less than 20 times in their lifetime. Intake of analgesics included in analysis up till end of 1986. Phenacetin always in preparations containing aspirin plus either caffeine or codeine
Ross et al. (1989) Los Angeles County USA, 1978–82	Renal pelvis and ureter	187 (127 men, 60 women) selected from the Los Angeles County Cancer Registry, diagnosed under age 75; response rate 80%	187 neighbourhood controls selected at random and matched individually to cases by sex, date of birth (± 5 yr), and race	Structured interview over the telephone	*Phenacetin-containing analgesics* > 30 d/yr > 30 consecutive d	 11 7	 1.1 ($P = 0.83$) 1.4 ($P = 0.56$)	Age, sex, race	Response rate not given for control subjects. Use of analgesic measured up until date of diagnosis or equivalent date for control. Reference category defined as 'no use or use fewer than 30 times in a yr'. Non-prescription compounds in focus of the study

Table 2.1 (continued)

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Linet et al. (1995) Multicentre study USA, 1983–86	Renal pelvis and ureter	502 (331 men, 171 women) Caucasians in age range 20–79-yr-old at diagnosis selected from population-based cancer registries in New Jersey, Iowa and Los Angeles; response rate 72%; cases confirmed histologically	496 (315 men, 115 women) frequency-matched to cases by sex and 5-yr age groups and selected using random-digit dialling (under age 65) or taken from the files of Health Care Financing Administration (65-yr-old or above); response rate 66%	In-person structured interview in subjects' home	None or no regular use of analgesics	385	1 (ref)	Age, sex, geographic area, cigarette smoking	Regular use defined as at least 2 or more times/wk for at least 1 mo. Use of analgesics measured up until 5 yr before interview. Non-prescriptive as well as prescriptive analgesics included. Low proportion of regular analgesic users and lack of "phenacetin abusers." Phenacetin rarely available as a single agent
					Regular use of phenacetin-containing analgesics	30	0.8 (0.5–1.4)		
					Duration of use of phenacetin-containing analgesics (yr)				
					≤ 4	12	0.7 (0.3–1.6)		
					5–9	6	0.8 (0.3–2.7)		
					≥ 10	11	0.9 (0.4–2.2)		
					Lifetime consumption of phenacetin (kg)				
					≤ 1.0	21	0.8 (0.4–1.6)		
					> 1.0	9	0.8 (0.3–2.1)		
Cancer of the kidney									
Mc Laughlin et al. (1992) Shanghai, China, 1987–89	Renal cell carcinoma (ICD-9: 189.0)	154 (90 men, 64 women) in age range 35–74-yr-old from a population-based cancer registry covering urban Shanghai; response rate 87%; cases confirmed histologically	157 (91 men, 66 women) selected at random from the Shanghai Resident Registry and frequency matched on sex and age; response rate 100%	In-person structured interview of participants at home	No or non-regular use of analgesics	NR	1 (ref)	Age, sex, education, BMI, smoking	Regular consumption of analgesics defined as at least twice/wk for 2 wk or longer; use of analgesics uncommon in study area
					Use of phenacetin-containing analgesics				
					Regular use	NR	2.3 (0.7–7.0)		

Table 2.1 (continued)

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Kreiger et al. (1993) Province of Ontario, Canada, 1986–87	Renal cell carcinoma (ICD-9: 189.0)	513 (312 men, 201 women) in age range 25–69-yr-old from the Ontario Cancer Registry; response rate 81%; cases confirmed histologically	1381 (664 men, 705 women) selected at random from the 1986 and 1987 Enumeration Records of Ontario and frequency-matched to case group on age, sex and geographic region of residence	Standardized questionnaire mailed to participants	*Use of phenacetin or phenacetin-containing analgesics* Men No phenacetin or paracetamol Phenacetin only Any phenacetin Women No phenacetin or paracetamol Phenacetin only Any phenacetin	 265 2 5 166 3 3	 1 (ref) 2.5 (0.3–18.5) 1.7 (0.5–5.9) 1 (ref) 1.8 (0.5–7.3) 0.8 (0.2–2.7)	Age, smoking, BMI	Exposure to analgesics was defined as use at least every other day for one month or more before 1980; very few individuals were classified as analgesic users; only 5 male cases and 3 female cases ever exposed
Chow et al. (1994) Minnesota USA, 1988–90	Renal cell carcinoma (ICD-9: 189.0)	440 in age range 20–79-yr-old from the Minnesota state cancer surveillance system; response rate 87%; cases confirmed histologically	707 frequency-matched to cases by sex and 5-yr age groups and selected using random-digit dialling (under age 65) or the files of Health Care Financing Administration (65-yr-old or above); response rate ≈85%	In-person structured interview of participants or next-of-kin (for deceased patients) at their homes	*Lifetime consumption of phenacetin (kg)* No or non-regular use of analgesics Men < 0.1 0.1–1 > 1 No or non-regular use of analgesics Women < 0.1 0.1–1 > 1	195 2 1 3 101 2 2 2	1 (ref) 0.4 (0.1–1.9) 0.1 (0.0–0.8) 0.6 (0.2–2.6) 1 (ref) 0.9 (0.1–5.8) 0.6 (0.1–3.5) 0.4 (0.1–2.3)	Age, smoking, quartile of BMI	Regular use of analgesics were defined as at least 2 or more times/wk for 1 mo before 1987. Overlapping at least in part with the international study described below

Table 2.1 (continued)

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
McCredie et al. (1995) International multicentre study: Australia, Denmark, Germany, Sweden and USA, 1989–91	Renal cell carcinoma	1732 in age range 20–79-yr-old (20–75 in German centre) recruited through a nationally modified rapid ascertainment system (all study centres) in combination with the files of population-based cancer registries (in Australia, Denmark, Sweden and the USA); overall response rate (including non-response due to death) 72.3%; cases confirmed histologically	2309 frequency-matched to cases by age and sex and selected by centre-specific methods from the same study base as the case group; response rate 74.7%	In-person structured interview at participants' home	*Lifetime consumption of phenacetin (kg)*			Centre, age, sex (where appropriate), BMI, pack-yr of tobacco	Reference group was composed of subjects who – before 1987 – never took analgesics, were not regular takers (less than twice/wk), or took a lifetime total of < 0.1 kg of all types of analgesics combined; lack of association not altered by restricting analgesic use to that which occurred 5 or 10 yr before cutoff data (1987)
					Men and women				
					Reference group	1313	1 (ref)		
					< 0.1	31	0.8 (0.5–1.3)		
					≥ 0.1	97	1.1 (0.8–1.4)		
					0.1–1.0	51	0.9 (0.6–1.3)		
					1.1–5.0	36	1.6 (0.9–2.6)		
					> 5.0	10	0.9 (0.4–2.1)		
					Men				
					< 0.1	14	0.6 (0.3–1.2)		
					≥ 0.1	46	0.9 (0.6–1.4)		
					0.1–1.0	25	0.7 (0.4–1.2)		
					1.1–5.0	16	1.3 (0.6–2.7)		
					> 5.0	5	2.6 (0.5–14.2)		
					Women				
					< 0.1	17	1.1 (0.6–2.3)		
					≥ 0.1	51	1.4 (0.9–2.1)		
					0.1–1.0	26	1.3 (0.7–2.3)		
					1.1–5.0	20	2.1 (1.0–4.4)		
					> 5.0	5	0.6 (0.2–1.8)		

Table 2.1 (continued)

Reference, study location and period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Gago-Dominguez et al. (1999), Los Angeles, USA, 1986–94	Renal cell cancer	1204 (781 men, 423 women) non-Asians in age range 25–74-yr-old from the Los Angeles County Cancer Registry; response rate (including non-response due to death) 74%; cases confirmed histologically	1204 non-Asians neighbourhood controls selected at random and matched individually to cases by sex, date of birth (± 5 yr), race; response rate not given	In-person structured interview at participants' home	No or irregular use of analgesics	616	1 (ref)	Level of education, obesity, history of hypertension, smoking (yes/no), regular use of amfetamines	Exposure information requested up to 2 yr before the date of diagnosis for cases and at the equivalent date for the matched control. No use was defined as an intake less than 20 times in a lifetime; irregular use was defined as intake of analgesics less than twice/wk. Equally increased risks were seen for other types of analgesics, i.e. aspirin, non-aspirin NSAIDs, and paracetamol
					Regular use of phenacetin	86	1.9 (1.3–2.7)		
					Maximum weekly dose (g)				
					< 2	41	1.3 (0.8–2.2)		
					2– < 4	22	4.1 (1.5–10.8)		
					4– < 8	20	2.3 (1.0–5.0)		

BMI, body mass index; d, day or days; mo, month or months; NR, not reported; NSAID, non-steroidal anti-inflammatory drug; ref., reference; vs, versus; wk, week or weeks; yr, year or years

highly increased relative risk of cancers of the renal pelvis and ureter associated with regular use of phenacetin-containing preparations, and a strong, statistically significant dose–response relationship of increasing risk with increasing consumption of phenacetin (McCredie *et al.*, 1993). In the study area (New South Wales), phenacetin was banned in 1979 from all preparations but was commonly used as an analgesic before that year. The Californian cancer-registry-based study (Ross *et al.*, 1989), including 187 patients with tumours of the renal pelvis and ureter, found a non-significant increased risk for these tumour types associated with heavy use of phenacetin-containing preparations. In the second study from the USA, Linet *et al.*, (1995) did not find a positive association between the use of phenacetin and tumours of the renal pelvis and ureter, and no indication of a positive trend in risk estimates with increasing duration of use or increasing cumulative dose of phenacetin-containing preparations. This apparent lack of association was ascribed by the authors to the fact that phenacetin products had been off the US market for a decade or more when the patients were diagnosed during 1983–87, and that the study revealed a low prevalence of regular users (6–7%) with an apparent lack of any phenacetin abusers.

In the only study in which tumours of the ureter were analysed separately (McCredie & Stewart, 1988), the use of phenacetin was not associated with an increased incidence of tumours of the ureter. [The Working Group noted that the statistical power of the study was limited.]

2.2.2 Cancer of the kidney parenchyma

Two early case–control studies from the USA and Australia, which included kidney cancer patients diagnosed in the late 1970s and early 1980s, i.e. in the decade following the withdrawal of phenacetin from these markets, reported an increased risk of kidney cancer among regular users of phenacetin-containing preparations (McLaughlin *et al.*, 1984; McCredie *et al.*, 1988). However, the US study findings were not statistically significant, and the Australian study did not show any positive trend in risk by increasing cumulative intake of phenacetin.

Three further case–control studies from the USA, Canada and the People's Republic of China with recruitment of patients with renal cell cancer diagnosed in the late 1980s did not report significant associations with use of phenacetin, even in cumulative doses of more than 1 kg (McLaughlin *et al.*, 1992; Kreiger *et al.*, 1993; Chow *et al.*, 1994). Similarly, a large international multicentre case–control study from six well defined geographic areas in Australia, Denmark, Germany, Sweden, and the USA did not link renal cell cancer with consumption of phenacetin-containing preparations (McCredie *et al.*, 1995).

In a large case–control study from California, a statistically significant elevation in risk of renal cell cancer was associated with use of each of four chemical classes of analgesics included in the analysis (Gago-Dominguez *et al.*, 1999). However, no clear increase in risk was observed even with an increase in maximum weekly intake doses of phenacetin.

2.2.3 Cancer of the urinary bladder

Two small case–control studies from Australia (McCredie *et al.*, 1988) and one study from the USA (Piper *et al.*, 1986), with recruitment of bladder cancer patients during the late 1970s and early 1980s, reported a positive association with use of phenacetin-containing analgesics. In the US study, heavy use, defined as daily use of phenacetin-containing analgesics, was associated with a non-significant increased risk of bladder cancer; in the Australian study, lifetime use of phenacetin of 0.1 kg or more was associated with a significant 2-fold increased risk.

In a large study from California (Castelao *et al.*, 2000), any use of phenacetin-containing analgesics was related to a non-significant increase in bladder cancer risk, however, it was associated with a significant increase in relative risk estimates by an increasing cumulative lifetime consumption of phenacetin. Furthermore, in a cancer-registry-based case–control study from the USA, risks of bladder cancer were evaluated in association with use of analgesics and anti-inflammatory drugs (Fortuny *et al.*, 2007). Based on information obtained through an in-person interview, the investigators found a significantly increased risk of bladder cancer in association with use of phenacetin-containing analgesics. A positive, statistically significant trend was observed with reported increasing duration of use.

These findings were not corroborated in a medium-sized German case–control study, in which no elevation of the relative risk estimate for bladder cancer was found in subjects with a lifetime consumption of phenacetin of 1 kg or more (Pommer *et al.*, 1999). [The Working Group noted that phenacetin was banned in 1986 from the pharmaceutical market of the Federal Republic of Germany before reunification, and patients included in the study were diagnosed during 1990–94, i.e. less than 10 years after the ban.]

In another large case–control study from Spain (Fortuny *et al.*, 2006), ever use of phenacetin was slightly, but non-significantly, more prevalent among cases than among controls. [The Working Group noted that in this study, most cases and controls were interviewed more than 10 years after phenacetin-containing preparations were withdrawn from the Spanish market, and few participants only reported use of this compound.]

3. Cancer in Experimental Animals

3.1 Analgesic mixtures containing phenacetin

Phenacetin has been tested in mice and rats by oral administration, alone and in combination with aspirin, caffeine, and/or phenazone. In a study in male and female mice and rats, a mixture of aspirin (50%), phenacetin (46%), and caffeine (4%) was administered in the diet (0.7% or 1.4%) for up to 78 weeks. There was no observed increase in tumour incidence in mice. There was a significant increase in pituitary adenomas and carcinomas, and adrenal pheochromocytomas in treated males, and a small increase (not statistically significant) in the incidence of urinary tract tumours in female rats (NCI, 1978; IARC, 1980, 1987b). Male rats treated with phenacetin, phenazone, and caffeine combined developed liver tumours (hepatomas), while phenacetin-alone or in combination with phenazone with or without caffeine slightly increased the incidence of renal cell and renal pelvic tumours combined (Johansson, 1981; IARC, 1987b).

Phenacetin-alone given orally at doses ranging from 0.5–2.5% in the diet caused benign and malignant tumours of the urinary tract in mice and rats of both sexes (Isaka *et al.* 1979; Nakanishi *et al.*, 1982; Muradian, 1986; IARC, 1987b), and of the nasal cavity in rats of both sexes (Isaka *et al.* 1979; IARC, 1980, 1987b). In male rats, phenacetin at doses of 2.5% also enhanced the incidence of urinary bladder tumours induced by *N*-nitrosobutyl-*N*-(4-hydroxybutyl) amine (IARC 1980, 1987b).

See Table 3.1

Table 3.1 Studies of cancer in experimental animals exposed to phenacetin or analgesic mixtures containing phenacetin

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, B6C3F$_1$ (M, F) 94 wk NCI (1978)	Feed 0, 0.7 or 1.4% of a mixture of aspirin, phenacetin and caffeine (50:46:4) for up to 78 wk, observed for an additional 16 wk 50/group	**Males:**		
		Lung (alveolar/bronchiolar adenomas or carcinomas)– 6/48 in controls, 9/46 fed 0.7%, 12/47 fed 1.4%	NS	
		Haematopoietic system (leukaemias or malignant lymphomas)– 4/48 in controls, 7/48 fed 0.7%, 4/48 fed 1.4%	NS	
		Liver (hepatocellular carcinomas)– 7/48 in controls, 11/46 fed 0.7%, 6/47 fed 1.4%	NS	
		Females:		
		Lung (alveolar/bronchiolar adenomas or carcinomas)– 4/46 in controls, 7/45 fed 0.7%, 4/47 fed 1.4%	NS	
		Haematopoietic system (leukaemia or malignant lymphoma)– 5/48 in controls, 6/45 fed 0.7%, 5/47 fed 1.4%	NS	
		Liver (hepatocellular carcinomas)– 1/47 in controls, 2/45 fed 0.7%; 3/47 fed 1.4%	NS	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Mouse, C57BL/6 (M, F) 75–80 wk Macklin & Szot (1980)	Feed Diets containing aspirin, phenacetin and caffeine, single or in combination, for 75–80 wk. Group 1: 696 mg/kg bw/d aspirin-phenacetin-caffeine Group 2: 693 mg/kg bw/d aspirin-phenacetin Group 3: 321 mg/kg bw/d phenacetin-caffeine Group 4: 754 mg/kg bw/d phenacetin Group 5: 268 mg/kg bw/d phenacetin Group 6: none (controls) 40 males and 40 females/group	No tumours related to treatment reported		The study was limited by the limited extent of histological examination. Ratio of the components of the combinations was: aspirin, 7; phenacetin, 5; caffeine, 1
Rat, F344 (M, F) 113 wk NCI (1978)	Feed 0, 0.7 or 1.4% of a mixture of aspirin, phenacetin and caffeine (50:46:4) for up to 78 wk, observed for an additional 34–35 wk 50/group	**Males:** Pituitary gland (adenomas or carcinomas)–8/47 in controls, 18/47 fed 0.7%, 12/44 fed 1.4% Adrenal gland (pheochromocytomas)–7/47 in controls, 17/49 fed 0.7%, 9/48 fed 1.4% **Females:** One transitional cell carcinoma of the urinary bladder (low dose) One transitional cell carcinoma of the urinary bladder (high dose) One transitional cell papilloma of the urinary bladder (high dose) One tubular cell adenocarcinoma of the kidney (low dose) [also one in low-dose males] No urinary system tumours in control females	 $P = 0.018$ for low dose $P = 0.022$ for low dose NS NS NS NS	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours		Significance	Comments
Rat, Sprague-Dawley (M) 117 wk Johansson (1981)	Feed Group 1: 0.5% phenacetin Group 2: 0.5% phenazone Group 3: 0.1% caffeine Group 4: 0.5% phenacetin+ 0.5% phenazone+ 0.1% caffeine Group 5: 0.5% phenacetin+ 0.5% phenazone Group 6: 0.5% phenacetin 0.1% caffeine Group 7: acetaminophen Group 8: none (controls) 30/group	**Kidney**			
		Renal cell	*Renal pelvis*		
		Group 1: 4/29	1/29	[$P = 0.024$][a]	
		Group 2: 0/29	4/29		
		Group 3: 0/28	0/28		
		Group 4: 2/30	4/30	[$P = 0.012$][a]	
		Group 5: 7/29	3/29	[$P = 0.003$][a]	
		Group 6: 2/29	1/29		
		Group 7: 0/30	0/30		
		Group 8: 0/30	0/30		
		Urinary bladder			
		Group 1: 5/29			
		Group 2: 5/29			
		Group 3: 0/29			
		Group 4: 1/30			
		Group 5: 4/29			
		Group 6: 4/29			
		Group 7: 4/30			
		Group 8: 2/30			
		Liver (hepatomas)			
		Group 1: 0/29			
		Group 2: 0/29			
		Group 3: 0/29			
		Group 4: 14/30		[$P < 0.001$]	
		Group 5: 0/29			
		Group 6: 0/29			
		Group 7: 0/29			
		Group 8: 0/29			

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Phenacetin alone				
Mouse, $B6C3F_1$ (M, F) 110 wk Nakanishi et al. (1982)	Feed 1.25% or 0.6% phenacetin for 102 wk, observed for additional 8 wk 52/group 50/sex as untreated controls	**Kidney tumours:** *Adenomas*– M 32/48 (1.25% dose), 11/48 (0.6% dose), 0/48 (controls) F 0/48, 0/48, 0/48 *Carcinomas*– M 14/48, 1/48, 0/48 F 1/48, 0/48, 0/48 **Urinary bladder:** *Hyperplasia*– M 7/48, 1/48, 0/48 F 7/48, 0/48, 0/48 *Carcinomas*– M 0/48, 0/48, 0/48 F 2/48, 0/48, 0/48	 $P < 0.01$ (low and high dose) $P < 0.01$ (high dose) $P < 0.05$ (high dose) $P < 0.05$ (high dose)	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Route Dosing regimen Animals/group at start	Incidence of tumours	Significance	Comments
Rat, BDI and BDIII 31 mo Schmähl & Reiter (1954)	Feed 40–50 mg phenacetin for at least 770 d 30/treatment group	No tumours related to treatment observed		Information for controls and other experimental details lacking; sex NR
Rats, Sprague-Dawley (M, F), 9 wk Isaka et al. (1979)	Feed 2.5% or 1.25% phenacetin for 18 mo, observed for an additional 6 mo 50/sex/group 65/sex as untreated controls	**Nasal cavity tumours:** M–16/27 (high dose), 16/22 (low dose), 0/19 (controls) F–7/27, 6/25, 0/25 **Urinary tract tumours:** M–13/27, 1/22, 0/19 F–4/27, 0/25, 0/25	 [$P < 0.01$; $P < 0.01$] [$P < 0.01$; $P < 0.01$] [$P < 0.01$; NS] [NS; NS]	Tumours observed in the high dose groups were mainly malignant
Rats, Sprague-Dawley (F), 9 wk 110 wk Johansson & Angervall (1976)	Feed 0% or 0.6% phenacetin for 110 wk 30/group	Breast (adenocarcinomas): 5/30 in treated group, 1/30 in controls Ear duct (squamous cell carcinomas): 4/30 in treated group, 0/30 in controls	NS NS	

[a] whe n kidney tumours were combined

bw, body weight; d, day or days; F, female; mo, month or months; M, male; NR, not reported; NS, not significant; wk, week or weeks; yr, year or years

4. Other Relevant Data

4.1 Absorption, distribution, excretion, and metabolism

4.1.1 Humans

After oral administration of phenacetin, *N*-acetyl-*p*-aminophenol, either conjugated or free, is the major metabolite found in urine. Small amounts of 2-hydroxyphenacetin, 3-[(5-acetamido-2-hydroxyphenyl)thio]alanine, *S*-(1-acetamido-4-hydroxyphenyl)cysteine, 3-methyl-thio-4-hydroxy-acetanilide, and *N*-hydroxyphenacetin are also detected.

Urinary excretion of 2-hydroxyphenacetin, *N*-acetyl-*p*-aminophenol and their conjugates is decreased when phenacetin is administered in combination with aspirin, caffeine, and codeine (Gault *et al.*, 1972)

4.1.2 Experimental systems

Metabolic pathways for phenacetin involve de-ethylation, *N*-deacetylation, and ring hydroxylation. The main route, as shown in rats, is oxidative de-ethylation, forming *N*-acetyl-*p*-aminophenol, which is excreted in the urine as the sulfate or as the glucuronide (Dubach & Raaflaub, 1969). Metabolism by the second pathway, *N*-deacetylation, is greatest in rats (21% of the dose), and least in guinea-pigs and rabbits (7 and 4% of the dose, respectively) (Smith & Timbrell, 1974). *p*-Phenetidine, resulting from *N*-deacetylation, can be converted to 2-hydroxy-*para*-phenetidine, which in rats is excreted as the sulfate in increasing amounts with increasing doses of phenacetin (Dubach & Raaflaub, 1969).

Other metabolites identified in the urine of experimental animals are 2-hydroxyphenacetin, 3-[(5-acetamido-2-hydroxyphenyl)thio] alanine, 3-methylthio-4-hydroxyacetanilide, 2-hydroxyacetophenetidine glucuronide, 4-acetaminophenoxyacetic acid, 4-hydroxy-3-methylthioacetanilide, and *N*-hydroxyphenacetin (IARC, 1980). Intestinal microflora in rats have been shown to deconjugate the metabolite *N*-acetyl-*p*-aminophenyl glucuronide, excreted partly in bile, to *N*-acetyl-*p*-aminophenol (Smith & Griffiths, 1976).

Evidence that phenacetin is *N*-hydroxylated by a cytochrome P-450 mono-oxygenase-catalysed reaction has been demonstrated *in vitro* with hamster and rabbit liver microsomes (Hinson & Mitchell, 1976; Fischbach *et al.*, 1977).

Using rat liver preparations, Mulder *et al.* (1977) demonstrated the formation of *N*-*O*-glucuronide and *N*-*O*-sulfate conjugates of *N*-hydroxyphenacetin.

In rats treated with 3-methylcholanthrene or benzo[*a*]pyrene or exposed to cigarette smoke, an increased rate of *O*-de-ethylation of phenacetin to *N*-acetyl-*p*-aminophenol in the lung and intestine was observed (Welch *et al.*, 1972; Kuntzman *et al.*, 1977).

4.2 Genetic and related effects

4.2.1 Humans

In 29 *TP53* mutations found in 89 renal pelvic carcinomas, the incidence and type of mutations did not differ significantly between patients with a history of phenacetin abuse, smoking or neither of these habits, and thus do not reflect a mutagenic effect of exposure to phenacetin and/or smoking in the renal pelvis (Bringuier *et al.*, 1998). No other data were available to the Working Group on the genetic and related effects of phenacetin in humans.

4.2.2 Experimental systems

The previous *IARC Monograph* states that the results of studies on the induction of chromosomal aberrations, sister chromatid exchange and micronuclei in rodents treated with phenacetin

in vivo were equivocal (IARC, 1987a). More recently, several studies have provided additional evidence that phenacetin can induce chromosomal alterations or DNA damage in both target and non-target tissues. Treatment of mice either orally or via intraperitoneal injection with high doses of phenacetin results in increases in micronuclei in the bone-marrow erythrocytes (Hayashi *et al.*, 1989; Sutou *et al.*, 1990). Similarly in rats, daily oral phenacetin treatment for 2 or 14 days increases the frequency of micronuclei in bone-marrow cells, and in peripheral blood (Asanami *et al.*, 1995). In rodents treated with phenacetin, increased DNA damage is detected in the kidney of mice 2–3 hours after treatment, and in the urinary bladder of rats after 20 hours of exposure (Sasaki *et al.*, 1997; Sekihashi *et al.*, 2001; Robbiano *et al.*, 2002). Phenacetin induces cell proliferation in the urothelium of the kidney, the bladder, the renal pelvis (Johansson *et al.*, 1989), and DNA synthesis in the nasal respiratory and olfactory mucosa of rats (Bogdanffy *et al.*, 1989). Phenacetin also induces chromosomal aberrations in Chinese hamster cells *in vitro* and DNA strand breaks in rat and human cells from the urinary bladder *in vitro* but not in rat hepatocytes after exposure *in vivo* (IARC, 1987a; Robbiano *et al.*, 2002). In rat kidney cells, a mixture of aspirin, phenacetin and caffeine as well as phenacetin-alone did not induce micronuclei, but the metabolite *N*-hydroxyphenacetin was found to induce micronuclei (Dunn *et al.*, 1987). Phenacetin does not induce sex-linked recessive lethal mutations in *Drosophila*, and is not mutagenic in mouse embryo cells, but was found to induce a small number of transformed foci (IARC, 1987a; Patierno *et al.*, 1989).

Phenacetin is mutagenic to bacteria when tested in the presence of a metabolic system derived from hamster or rat liver but not mouse (IARC, 1987a; Nohmi *et al.*, 1987). The urine from phenacetin-treated Chinese hamsters, but not that from rats, is mutagenic to bacteria (IARC, 1987a). Administration of phenacetin (0.75%) mixed in the feed of mice deficient in nucleotide-excision repair results in an observed increased mutation frequency in a *Lac Z* reporter gene in the kidney (Luijten *et al.*, 2006).

While there is evidence of genetic damage caused by phenacetin in various experimental systems, similar data are not available in humans.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of analgesic mixtures containing phenacetin. Analgesic mixtures containing phenacetin cause cancer of the renal pelvis, and of the ureter.

There is *limited evidence* in experimental animals for the carcinogenicity of analgesic mixtures containing phenacetin.

There is *sufficient evidence* in humans for the carcinogenicity of phenacetin. Phenacetin causes cancer of the renal pelvis, and of the ureter.

There is *sufficient evidence* in experimental animals for the carcinogenicity of phenacetin.

Analgesic mixtures containing phenacetin are *carcinogenic to humans (Group 1).*

Phenacetin is *carcinogenic to humans (Group 1).*

For the overall evaluation of phenacetin, the Working Group took into consideration that tumours of the renal pelvis and ureter are not known to result from the other components of the analgesic mixtures used in most countries; namely, aspirin, codeine phosphate, and caffeine.

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LIST OF ABBREVIATIONS

ABVD	Adriamycin, bleomycin, vinblastine, dacarbazine
ACS	American Cancer Society
ACT	Alternatives to carcinogenicity testing
AGT	O6-alkylguanine-DNA alkyltransferase
ALL-1	Acute lymphoblastic leukaemia-1 gene
AML	Acute myeloid leukaemia
Aprt	Adenine phosphoribosyl transferase
BCRP	Breast cancer resistance protein
BEACOPP	Bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone
BEP	Bleomycin, etoposide, and cisplatin
BMI	Body mass index
BN	Brown Norway rats
CAE	Cyclophosphamide, adriamycin, and etoposide
CASH	Cancer and steroid hormone
CAV	Cyclophosphamide, adriamycin, and vincristine
CCA	Clear cell adenocarcinoma
CHL	Chinese hamster lung
ChlVPP	Chlorambucil, vincristine, procarbazine, prednisone
CHO	Chinese hamster ovary
CI	Confidence interval
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CnA	Heterodimeric calcineurin
COP	Copenhagen rats
CPS	Cancer Prevention Study
CYP	Cytochrome P450
CYP3A4	Cytochrome P450 3A4
DESAD	US National Cooperative Diethylstilbestrol Adenosis
DMBA	7,12-Dimethylbenz[a]anthracene
DMSO	Dimethylsulfoxide
dthioGTP	6-Thioguanine deoxynucleoside triphosphate
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
EPT	Estrogen-progestogen trial
ER	Estrogen receptor
ESR1	ERα gene
FVB	Her-2/neu transgenic mouse

GSH	Glutathione
GST	Glutathione S-transferase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HERS	Heart and Estrogen/progestin Replacement Study
HIV	Human immunodeficiency virus
HPRT	Hypoxanthine(guanine)phosphoribosyl transferase
HPV	Human papilloma virus
HR	Hazard ratio
HRX	Human trithorax gene
HTRX-1	Human homologue of Drosophila thrithorax gene
IBD	Inflammatory bowel disease
IL-2	Interleukin-2
ILSI	International Life Sciences Institute
IRR	Incidence rate ratio
LC/MS-MS	Liquid chromatography-tandem mass spectrometry
MDS	Myelodysplastic syndromes
me6-TG	S-Methylthioguanine
meTIMP	Methylthioinosine monophosphate
MGMT	O6-Methylguanine-DNA methyl transferase
MLL	Mixed lineage leukaemia gene
MMMTs	Malignant mixed mullerian tumours
MMP	Matrix metalloproteinases
MMR	Mismatch repair
MNU	N-Methyl-N-nitrosourea
MOPP	Mechlorethamine, oncovin, procarbazine, prednisone
MPA	Medroxyprogesterone acetate
mPTEN	Murine PTEN
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NCI	US National Cancer Institute
NDIC	Netherlands Diethylstilbestrol Information Centre
NFAT	Nuclear factor of activated T cells
NHL	Non-Hodgkin lymphoma
nor-G	N-(2-Chloroethyl)-N-[2-(7-guaninyl)ethyl]amine
NQO1	NAD(P)H:quinone oxidoreductase
Nsbp1	Nucleosomal binding protein-1
PP2B	Protein phosphatase 2B
PR	Progesterone receptor
PR+	Progesterone receptor-positive
PrR	Prevalence ratio
PUVA	Psoralen (P) and high-intensity long wavelength (UVA) irradiation
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
RR	Relative risk
S. cerevisiae	Saccharomyces cerevisiae
SEER	Surveillance, Epidemiology and End Results
SIR	Standardized incidence ratio
SNP	Single-nucleotide polymorphism
SMADs	Small mothers against decapentaplegic proteins
S. typhimurium	Salmonella typhimurium

SULT2A1	Hydroxysteroid sulfotransferase 2A1
T	Thymine
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
TGN	Thioguanine nucleotide
TIMP	Thioinosine monophosphate
TPMT	Thiopurine methyltransferase
UDP	Uridine 5′-diphosphate
UDS	Unscheduled DNA synthesis
USP	United States Pharmacopoeia
UVA	Ultraviolet A
VAC	Vincristine, actinomycin D, and cyclophosphamide
VBMCP	Vincristine, carmustine, melphalan, cyclophosphamide, and prednisone
WHI	Women's Health Initiative
WHI-EPT	Women's Health Initiative Estrogen-Progestogen Trial
WHI-ET	Women's Health Initiative Estrogen-only Trial
WHS	Women's Health Study
WISH	Woman's Interview Study of Health
βERKO	Estrogen receptor β knockout

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Volume 100 of the *IARC Monographs*, A Review of Human Carcinogens, covers all agents previously classified by IARC as *carcinogenic to humans (Group 1)* and was developed by six separate Working Groups: Pharmaceuticals; Biological Agents; Arsenic, Metals, Fibres, and Dusts; Radiation; Personal Habits and Indoor Combustions; Chemical Agents and Related Occupations.

This Volume 100A covers Pharmaceuticals, specifically Busulfan, Chlorambucil, Methyl-CCNU, Cyclophosphamide, Etoposide in combination with Cisplatin and Bleomycin, Melphalan, MOPP, Tamoxifen, Thiotepa, Treosulfan, Diethylstilbestrol, Estrogen-only Menopausal Therapy, Combined Estrogen–Progestogen Menopausal Therapy, Combined Estrogen–Progestogen Contraceptives, Azathioprine, Chlornaphazine, Ciclosporin, Plants containing Aristolochic Acid, Methoxsalen plus Ultraviolet A Radiation, and Phenacetin.

Because the scope of Volume 100 is so broad, its *Monographs* are focused on key information. Each *Monograph* presents a description of a carcinogenic agent and how people are exposed, critical overviews of the epidemiological studies and animal cancer bioassays, and a concise review of the agent's toxicokinetics, plausible mechanisms of carcinogenesis, and potentially susceptible populations, and life-stages. Details of the design and results of individual epidemiological studies and animal cancer bioassays are summarized in tables. Short tables that highlight key results are printed in Volume 100, and more extensive tables that include all studies appear on the *Monographs* programme website (http://monographs.iarc.fr).

It is hoped that this volume, by compiling the knowledge accumulated through several decades of cancer research, will stimulate cancer prevention activities worldwide, and will be a valued resource for future research to identify other agents suspected of causing cancer in humans.

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