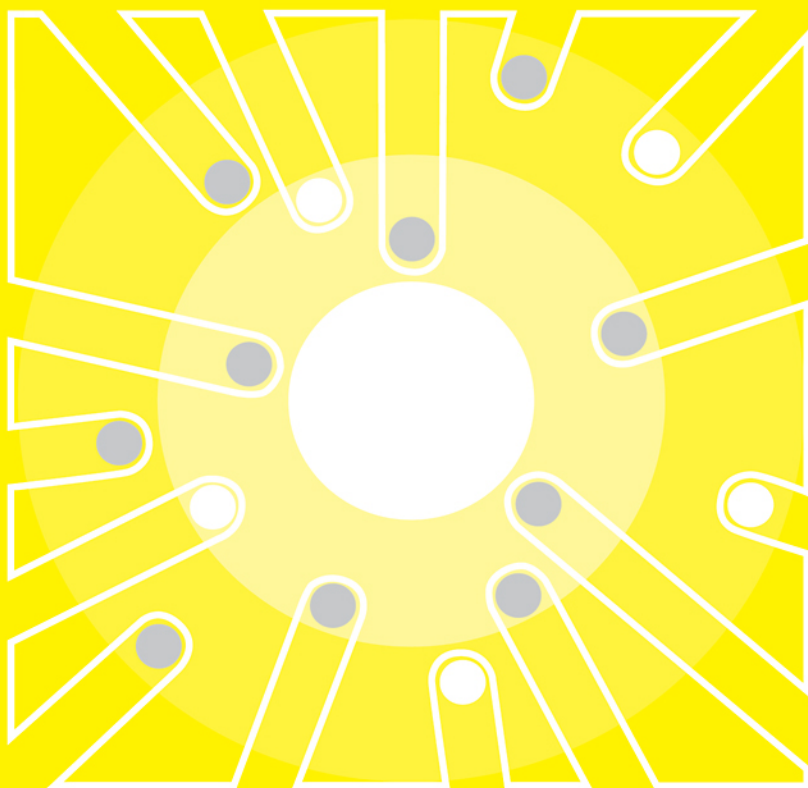
WHO FOOD ADDITIVES SERIES: 61

Toxicological evaluation of certain veterinary drug residues in food

Prepared by the
Seventieth meeting of the Joint FAO/WHO
Expert Committee on Food Additives (JECFA)

Food and Agriculture Organization of the United Nations

World Health Organization



IPCS

International Programme on Chemical Safety

World Health Organization

WHO FOOD ADDITIVES SERIES: 61

Toxicological evaluation of certain veterinary drug residues in food

Prepared by the
Seventieth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)

The summaries and evaluations contained in this book are, in most cases, based on unpublished proprietary data submitted for the purpose of the JECFA assessment. A registration authority should not grant a registration on the basis of an evaluation unless it has first received authorization for such use from the owner who submitted the data for JECFA review or has received the data on which the summaries are based, either from the owner of the data or from a second party that has obtained permission from the owner of the data for this purpose.

World Health Organization, Geneva, 2009

IPCS—International Programme on Chemical Safety

WHO Library Cataloguing-in-Publication Data

Toxicological evaluation of certain veterinary drug residues in food / prepared by the seventieth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

(WHO food additives series ; 61)

1.Drug residues - toxicity. 2.Veterinary drugs - adverse effects. 3.Food contamination. 4.Risk assessment. 5.Tylosin - toxicity. I.Joint FAO/WHO Expert Committee on Food Additives. Meeting (70th : 2008 : Geneva, Switzerland). II.International Programme on Chemical Safety. III.Series.

ISBN 978 92 4 166061 7 (NLM classification: WA 701)
ISSN 0300-0923

© World Health Organization 2009

All rights reserved. Publications of the World Health Organization can be obtained from WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland (tel.: +41 22 791 3264; fax: +41 22 791 4857; e-mail: bookorders@who.int). Requests for permission to reproduce or translate WHO publications – whether for sale or for noncommercial distribution – should be addressed to WHO Press, at the above address (fax: +41 22 791 4806; e-mail: permissions@who.int).

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

All reasonable precautions have been taken by the World Health Organization to verify the information contained in this publication. However, the published material is being distributed without warranty of any kind, either expressed or implied. The responsibility for the interpretation and use of the material lies with the reader. In no event shall the World Health Organization be liable for damages arising from its use.

This publication contains the collective views of an international group of experts on Food Additives and does not necessarily represent the decisions or the policies of the World Health Organization.

Typeset in India
Printed in India

CONTENTS

Preface v

Residues of veterinary drugs

- Avilamycin 3
- Malachite green 37
- Melengestrol acetate (addendum) 69
- Monensin 93
- Narasin 133
- Tylosin 183

Annexes

- Annex 1 Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives 219
- Annex 2 Abbreviations used in the monographs 231
- Annex 3 Participants in the seventieth meeting of the Joint FAO/WHO Expert Committee on Food Additives 235
- Annex 4 Recommendations on compounds on the agenda and further information required 237

This publication is a contribution to the International Programme on Chemical Safety.

The **International Programme on Chemical Safety (IPCS)**, established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), the International Labour Organization (ILO) and the World Health Organization (WHO). The overall objectives of the IPCS are to establish the scientific basis for assessing the risk to human health and the environment from exposure to chemicals, through international peer review processes, as a prerequisite for the promotion of chemical safety, and to provide technical assistance in strengthening national capacities for the sound management of chemicals.

The Inter-Organization Programme for the Sound Management of Chemicals (IOMC) was established in 1995 by UNEP, ILO, the Food and Agriculture Organization of the United Nations, WHO, the United Nations Industrial Development Organization, the United Nations Institute for Training and Research and the Organisation for Economic Co-operation and Development (Participating Organizations), following recommendations made by the 1992 UN Conference on Environment and Development to strengthen cooperation and increase coordination in the field of chemical safety. The purpose of the IOMC is to promote coordination of the policies and activities pursued by the Participating Organizations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

PREFACE

The monographs contained in this volume were prepared at the seventieth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at WHO headquarters in Geneva, Switzerland, on 21–29 October 2008. These monographs summarize the data on the safety of residues in food of selected veterinary drugs reviewed by the Committee.

The seventieth report of JECFA has been published by WHO as WHO Technical Report No. 954. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication; a summary of the conclusions of the Committee is given in Annex 4. Some of the substances listed in Annex 4 were considered at the meeting for residue evaluation only.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The toxicological monographs contained in this volume are based on working papers that were prepared by temporary advisers. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by M. Sheffer, Ottawa, Canada.

Many unpublished proprietary reports are unreferenced. These were voluntarily submitted to the Committee by various producers of the veterinary drugs under review and in many cases represent the only data available on those substances. The temporary advisers based the working papers they wrote on all the data that were submitted, and all these reports were available to the Committee when it made its evaluations.

The preparation and editing of the monographs included in this volume were made possible through the technical and financial contributions of the Participating Organizations of the International Programme on Chemical Safety (IPCS), which supports the activities of JECFA.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the organizations participating in the IPCS concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the organizations in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of the compounds evaluated in this publication should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Expert Committee on Food Additives, International Programme on Chemical Safety, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

RESIDUES OF VETERINARY DRUGS

USE OF JECFA REPORTS AND EVALUATIONS BY REGISTRATION AUTHORITIES

The summaries and evaluations contained in this book are, in most cases, based on unpublished proprietary data submitted for the purpose of the JECFA assessment. A registration authority should not grant a registration on the basis of an evaluation unless it has first received authorization for such use from the owner who submitted the data for JECFA review or has received the data on which the summaries are based, either from the owner of the data or from a second party that has obtained permission from the owner of the data for this purpose.

AVILAMYCIN

First draft prepared by

Dr Sang-Hee Jeong,[1] Dr Carl Cerniglia[2] and Dr Kevin Greenlees[3]

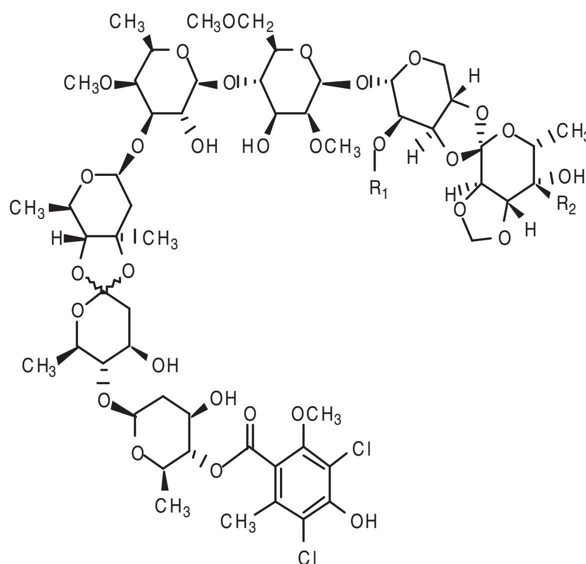
[1] National Veterinary Research and Quarantine Service, Ministry for Food, Agriculture, Forestry and Fisheries, Anyang City, Republic of Korea
[2] National Center for Toxicological Research, Food and Drug Administration, Department of Health and Human Services, Jefferson, AR, United States of America (USA)
[3] Center for Veterinary Medicine, Food and Drug Administration, Department of Health and Human Services, Rockville, MD, USA

Explanation 3
Biological data 5
  Biochemical aspects 5
    Absorption, distribution, excretion and metabolism 5
    Biotransformation 8
  Toxicological studies 8
    Acute toxicity 8
    Short-term studies of toxicity 8
    Long-term studies of toxicity and carcinogenicity 13
    Genotoxicity 14
    Reproductive and developmental toxicity 15
    Special studies 20
  Observations in humans 25
Comments 25
  Biochemical data 25
  Toxicological data 26
  Microbiological data 29
Evaluation 30
References 30

1. EXPLANATION

Avilamycin is an antibiotic of the orthosomycin family, produced by the fermentation of *Streptomyces viridochromogenes*. It is a mixture of oligo saccharides of orthosomycins, with a linear heptasaccharide chain linked to a terminal dichloroisoeverninic acid (DIA) (Boll et al., 2006). Avilamycin blocks the formation of the 70S initiation complexes in bacterial protein synthesis by inhibiting the binding of formylmethionine transfer ribonucleic acid (tRNA) to the 30S or 50S ribosomal subunit (Wolf, 1973; McNicholas et al., 2000). Avilamycin is composed of a mixture of avilamycin A (≥60%), avilamycin B (<18%) and 14 minor factors: avilamycin A′, C, D1, D2, E, F, G, H, I, J, K, L, M and N. Avilamycin A plus B constitute at least 70% of avilamycin, whereas the other single factors represent less than 6% each (Figure 1) (Mertz et al., 1986).

Figure 1. Structural formula of avilamycin



Avilamycin	Molecular formula	Relative molecular mass	R_1	R_2
A	$C_{61}H_{88}Cl_2O_{32}$	1403	$COCH(CH_3)_2$	$COCH_3$
B	$C_{59}H_{84}Cl_2O_{32}$	1375	$COCH_3$	$COCH_3$

Avilamycin is mainly active against Gram-positive bacteria, including *Bacillus* spp., *Clostridium* spp., *Corynebacterium bovis*, *Enterococcus* spp., *Lactobacillus* spp., *Listeria monocytogenes*, *Micrococcus luteus*, *Staphylococcus aureus* and *Streptococcus* spp. Avilamycin is intended for use as a veterinary medicine in chickens, turkeys, pigs and rabbits to control bacterial enteric infections. It is intended to be administered at a dose of 100 mg/kg in feed to chickens, turkeys and pigs for 21 days. In rabbits, it is intended to be administered at a dose of 80 mg/kg in feed for 28 days. The typical avilamycin content of the fermentation product is 260 mg activity/g.

Avilamycin has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee evaluated avilamycin to establish an acceptable daily intake (ADI) and to recommend maximum residue limits (MRLs) in relevant species at the request of the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods (Codex Alimentarius Commission, 2007).

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 *Absorption, distribution, excretion and metabolism*

(a) Rats

In a study that was conducted according to Good Laboratory Practice (GLP), three male and three female Sprague-Dawley rats weighing 248–265 and 214–222 g, respectively, were dosed by gavage with [^{14}C]avilamycin at levels equivalent to 100 mg/kg body weight (bw) for 3 consecutive days. Urine and faeces were collected separately from each animal at 24-h intervals following the initial dose for the assay of total radioactivity. In addition, faeces were collected during the 24-h period following the third dose for the assay of avilamycin and its metabolites. Avilamycin was rapidly excreted following oral administration, with over 90% of the administered radioactivity recovered in faeces within 24 h after the third dose. Less than 0.25% of the administered radioactivity was eliminated via the urine. The neutral fraction of faeces contained 85–87% of the total sample of radioactivity, whereas the acidic fraction contained 12–14%. Avilamycins A and B represented 40–60% of the radioactivity in the neutral fraction (Magnussen, 1985a).

In a study that was conducted in accordance with GLP, six rats (three males and three females) were fed a ration containing uniformly radiolabelled [^{14}C]-avilamycin at a concentration of 550 mg/kg diet for 4.5 days. Urine and faeces were collected during the dosing period, and livers were collected at zero withdrawal. Avilamycin A constituted approximately 19% of the faecal radioactivity. There were three major metabolites derived from the oligosaccharide and eurekanate portion of avilamycin in faecal samples. The most abundant metabolite in faeces was flambic acid (metabolite B). Flambic acid was relatively unstable and readily converted to flambalactone (metabolite A) (Donoho & Magnussen, 1987).

(b) Pigs

In an experiment that complied with GLP, two crossbred female pigs weighing approximately 40 kg each received 0.9 kg of feed containing unlabelled avilamycin at 60 mg activity/kg in the diet twice daily for 7 days. After being fed the unlabelled drug, each pig received a one-time dose of 120 mg of [^{14}C]avilamycin (9.3 kBq/mg) incorporated into 450 g of diet. After the consumption of the diet containing [^{14}C]avilamycin, the animals were given an additional 450 g of unmedicated diet. The female pigs were then fed twice daily with 0.9 kg of unmedicated feed for the duration of the experiment. Most of the ^{14}C residues in both pigs were excreted in the first 4 days, with over 91% eliminated on days 2 and 3. The peak excretion of ^{14}C residues in urine occurred in the first 24-h collection period, with 2.75% and 3.30% recovery for the two animals. During the 9-day collection period, the two pigs excreted 96.9% and 99.0%, respectively, of the total dose administered. An average of 93.4% of the excreted dose was found in the faeces, and 4.54% was found in the urine (Dalidowicz et al., 1983).

Seven female and four to five male starter pigs weighing 7–12 kg were fed standard diets containing 20 mg avilamycin/kg in three different product forms (crystalline, micronized and non-micronized) for 6 days. The faeces collected from pigs that were fed crystalline, micronized and non-micronized product had microbiologically active residues that represented 2.0%, 4.5% and 15.0%, respectively, of the residues of avilamycin and its degradation products, as determined by gas chromatographic assays. The faeces contained an average of 0.94, 2.28 and 8.45 µg of microbiologically active residues per gram for pigs fed crystalline, micronized and non-micronized avilamycin, respectively. The gas chromatographic assay, which determined the total residues of avilamycin plus any degradation products that hydrolyse to DIA, indicated that the faeces contained 43.3, 40.1 and 43.4 µg/g for pigs fed the crystalline, micronized and non-micronized product forms, respectively (West & Anderson, 1984).

In a GLP-compliant experiment, nine crossbred pigs (five males and four females) weighing approximately 44 kg each were fed a ration containing 76.19 mg [^{14}C]avilamycin/kg in the diet (equivalent to 80 mg avilamycin activity/kg in the diet) at 12-h intervals for 4, 7 or 10 days. This resulted in a daily dose of approximately 134 mg of [^{14}C]avilamycin per animal (equivalent to 3 mg avilamycin activity/kg bw). All animals were sacrificed at 6 h after the final feeding. Muscle, liver, kidney, fat, bile and excreta were assayed for unmetabolized parent avilamycin and for residues containing the DIA moiety. After 10 days of dosing, total mean radioactive residues in liver, fat and kidney, expressed as avilamycin equivalents, were 0.22, 0.12 and 0.10 µg/g, respectively. Residues in muscle were less than 0.025 µg/g. Steady-state concentrations of radioactivity were attained in muscle, liver and kidney within 4 days after the initiation of dosing. Radioactivity found in fat was ^{14}C residues of avilamycin that represented carbon breakdown products synthesized into cellular components, the fatty acid portion of triglycerides. Approximately 7% of the average daily dose was excreted each day in the bile, which indicates that biliary excretion is not a major route of elimination of avilamycin in pigs and reflects the fact that avilamycin is not well absorbed. No residues of unmetabolized parent avilamycin were detected in either kidney or fat, whereas only a trace amount (less than 0.05 µg/g) was found in liver. Both liver and kidney contained detectable quantities of DIA-related residues, with the levels in liver representing 50% or more of the total radioactive residue. No DIA-related residues were detected in fat. Avilamycin A and B represented less than 5% of the total radioactive residue in urine and faeces. One major metabolite observed in the extracts of both liver and excreta was flambic acid, which was formed as a result of cleavage of the ortho ester linking the C and D rings of avilamycin. Flambic acid represented 40–50% of the total radioactive residue in urine and faeces and 15–20% of the residue in liver (Magnussen et al., 1984, 1991; Dalidowicz, 1985; Magnussen, 1985b).

In a GLP-compliant experiment, six crossbred pigs (four males and two females) weighing approximately 44 kg each were fed a ration containing 60 mg [^{14}C]avilamycin/kg diet at 12-h intervals for 10 or 14 days. Muscle, liver, kidney and fat were collected for radiochemical analysis after 10 and 14 days of treatment. The mean concentrations in muscle, liver and kidney were not statistically different between the 10- and 14-day treatments. The mean concentration in fat was

significantly higher after 14 days of dosing than after 10 days of dosing. However, the residue in fat was radioactivity that was incorporated into the natural fatty acids and was not related to avilamycin residues. Non-extractable liver residues were 33–37% of total liver residues and were not different between the 10- and 14-day treatment groups. Extractable liver radioactivity consisted of several minor metabolites (<0.1 μg/g). The most abundant metabolite was flambic acid (metabolite B), which was present at concentrations up to 0.06 μg/g. Parent [^{14}C]avilamycin concentrations in liver were less than 0.05 μg/g. Radioactivity in kidney had a pattern qualitatively similar to that in liver. Approximately 92% of the recovered dose was in the faeces, and 8% was in the urine. The concentration of radioactivity ([^{14}C]avilamycin equivalents) in faeces was approximately 120 μg/g (Magnussen et al., 1987).

(c) Chickens

In an experiment that complied with GLP, two female and two male broiler chickens were fed, ad libitum, a ration containing unlabelled avilamycin (20 mg avilamycin activity/kg) in the diet for 7 days. Each chicken was then dosed once with a capsule containing 4.0 mg of [^{14}C]avilamycin (15 kBq/mg). During the 13-day collection period, the four birds excreted 84–99% of the total dose administered. The bulk (84–96%) of the ^{14}C residues were excreted in the first 4 days, with 50–78% being eliminated during the first 24-h collection period (Dalidowicz et al., 1984).

In a non-GLP-compliant experiment, four broiler chickens were fed diets containing 22 mg avilamycin/kg for 25 days. Both a microbiological method and a gas chromatographic method were utilized to measure the total residue of avilamycin and some of its potential degradation products in blood. No residues of avilamycin or its potential degradation products were detected in the blood of broiler chickens in this experiment (West et al., 1982).

In a GLP-compliant experiment, six male and six female 7-week-old broiler chickens (Hubbard-White Mountain Cross) were fed a standard broiler finishing ration containing 14.16 mg [^{14}C]avilamycin/kg diet (equivalent to 15 mg avilamycin activity/kg in the diet) for 4, 7 or 10 days. Medicated ration was provided ad libitum throughout the dosing phase. At the end of each dosing period, two birds of each sex were deprived of food and water for 6 h, and then samples of muscle, liver, abdominal fat, kidney and skin with subcutaneous fat were collected for radiochemical analysis. Radioactive residue levels in muscle and kidney were lower than the detection limits of 0.008 and 0.024 μg/g, respectively, at all sampling times. The mean peak level of 0.039 μg/g was attained in liver after 7 days of dosing. After 10 days of dosing, the mean total radioactive residues in skin, liver and fat, expressed as avilamycin equivalents, were 0.018, 0.022 and 0.024 μg/g, respectively. Steady-state concentrations of radioactivity were attained in all tissues within 4–7 days after the initiation of dosing (Magnussen et al., 1986).

2.1.2 Biotransformation

Avilamycin is poorly absorbed and is extensively metabolized in the gut of pigs. Only about 8% of total radioactivity in pig faeces was attributable to parent avilamycin. Metabolites were found in liver, whereas they were not detected in other tissues. The primary metabolite is flambic acid, representing 40–50% of the total radioactive residue in urine and faeces and 15–20% of the residue in liver. No microbiologically active residues were detected in liver. Avilamycin is unlikely to be persistent in the environment following excretion from treated animals, as it is highly metabolized or degraded in animals (Magnussen et al., 1991; Burnett, 2005).

2.2 Toxicological studies

2.2.1 Acute toxicity

The acute toxicity of avilamycin has been evaluated using various routes of administration in mice, rats and rabbits (Sachsse & Bathe, 1977, 1978a,b; Williams & Quarles, 1983, 1984a,b; Williams et al., 1983a,b,c,d,e; Inui, 1984a,b; Ito, 1986, 1988). Most of the studies of acute toxicity were conducted in compliance with GLP. The oral median lethal dose (LD_{50}) of the dried avilamycin products was generally higher than 5000 mg/kg bw in mice and rats. In terms of potency, the oral LD_{50} was higher than 390 or 745 mg avilamycin activity/kg bw in both mice and rats. The LD_{50} by the dermal route in rabbits was also higher than 298 mg avilamycin activity/kg bw. The acute intraperitoneal toxicity of avilamycin was more severe than its oral or dermal toxicity. However, the deaths observed after intraperitoneal administration were mainly due to the inflammatory reactions to the unabsorbed avilamycin in the abdominal cavity rather than to the toxicity of avilamycin itself. The LD_{50} by the intraperitoneal route ranged from 1200 to 3400 mg/kg bw in mice and from 680 to 3100 mg/kg bw in rats. In terms of potency, the intraperitoneal LD_{50} in mice and rats ranged from 264 to 337 mg avilamycin activity/kg bw and from 101 to 1020 mg avilamycin activity/kg bw, respectively (Table 1). Deaths induced by the intraperitoneal dosing were preceded by lethargy, with hunchback position, distended abdomen, piloerection and decreased locomotor activity.

2.2.2 Short-term studies of toxicity

The results of studies of short-term toxicity are summarized in Table 2.

(a) Mice

The short-term toxicity studies conducted in mice may not have been conducted in accordance with GLP.

Twenty mice (10 males and 10 females) approximately 4 weeks of age were fed avilamycin incorporated into the pelleted diet at levels of 0, 30, 300 or 3000 mg avilamycin activity/kg diet continuously for 28 days. These doses were equivalent to 0, 4.5, 45 and 450 mg avilamycin activity/kg bw per day. There was a slight increase in feed intake and body weight of the males given 450 mg avilamycin activity/kg bw per day. No mortality or toxic signs were observed (Suter & Sachsee, 1977a). The no-observed-adverse-effect level (NOAEL) in this study was 450 mg avilamycin/kg bw per day, the highest dose administered.

Table 1. Results of studies of acute toxicity of avilamycin

Species	Sex	Route	LD_{50} (mg/kg bw)	Reference
Mouse	M & F	Oral	>6000	Sachsee & Bathe (1978a)
Mouse	M & F	Oral	>5000[a] (>745 mg activity)	Williams & Quarles (1984b)
Mouse	M & F	Oral	>5000[a] (>390 mg activity)	Williams et al. (1983b)
Mouse	M & F	Oral	>5000[b] (>390 mg activity)	Williams et al. (1983d)
Mouse	M & F	Oral	>12 000	Inui (1984a)
Mouse	M & F	Intraperitoneal	>157	Sachsee & Bathe (1978b)
Mouse	M	Intraperitoneal	3435.1	Inui (1984a)
	F	Intraperitoneal	1798.9	
Mouse	M	Intraperitoneal	1531[c] (337 mg activity)	Ito (1988)
	F	Intraperitoneal	1200[c] (264 mg activity)	
Rat	M & F	Oral	>4600	Sachsee & Bathe (1977)
Rat	M & F	Oral	>5000[a] (>745 mg activity)	Williams & Quarles (1984a)
Rat	M & F	Oral	>5000[a] (>390 mg activity)	Williams et al. (1983a)
Rat	M & F	Oral	>5000[b] (>390 mg activity)	Williams et al. (1983c)
Rat	M & F	Oral	>12 000	Inui (1984b)
Rat	M	Intraperitoneal	2319.3	Inui (1984b)
	F	Intraperitoneal	3114.5	
Rat	M	Intraperitoneal	1083[d] (1020 mg activity)	Ito (1986)
	M	Intraperitoneal	≈1875[c] (~255 mg activity)	
Rat	M	Intraperitoneal	676[a] (101 mg activity)	Williams & Quarles (1983)
	F	Intraperitoneal	944[a] (141 mg activity)	
Rat	M & F	Inhalation	>0.77[a,e] (>0.11 mg activity)	Williams et al. (1983e)
Rabbit	M & F	Dermal	>2000[a] (>298 mg activity)	Williams et al. (1983e)

F, female; M, male.
[a] Dried fermentation product.
[b] Micronized dried fermentation product.
[c] Dried mycelial product.
[d] Crystalline product.
[e] Median lethal concentration (LC_{50}), mg/l of air (total gravimetric).

Table 2. Results of studies of short-term toxicity of avilamycin

Species	Study duration	Sex	Route	NOAEL (mg avilamycin activity/kg bw)	References
Mouse	28 days	M & F	Oral in feed	450[a]	Suter & Sachsse (1977a)
Mouse	28 days	M & F	Oral in feed	4500[b]	Suter & Sachsse (1977b)
Rat	14 days	M & F	Oral in feed	1490[a]	Williams & Probst (1983a)
Rat	14 days	M & F	Oral in feed	6000[a]	Williams & Probst (1983b)
Rat	28 days	M & F	Oral in feed	300[a]	Sachsse et al. (1978a)
Rat	28 days	M & F	Oral in feed	3000[b]	Sachsse et al. (1978b)
Dog	6 months	M & F	Oral capsule	178[a]	Williams (1983)
Pig	21 weeks	M & F	Oral in feed	120[a]	Strittmatter & Somerville (1980)
Chicken	62 days	M & F	Oral in feed	375[a]	Strittmatter (1982)
Turkey	14 days	M & F	Oral in feed	5[b]	Cochrane & Thomson (1991)
Turkey	16 weeks	M & F	Oral in feed	12.5[a]	Redgrave et al. (1992)

F, female; M, male; NOAEL, no-observed-adverse-effect level.
[a] Highest dose administered.
[b] Only dose tested.

In another experiment, 10 male and 10 female mice approximately 4 weeks of age were fed avilamycin (mycelial form) incorporated into the pelleted diet at 0 or 30 000 mg avilamycin activity/kg diet continuously for 28 days. These doses were equivalent to 0 and 4500 mg avilamycin activity/kg bw per day. Weight gains and feed consumption were slightly increased in males administered avilamycin. No mortality or toxic signs related to avilamycin were observed (Suter & Sachsee, 1977b). The NOAEL was 4500 mg avilamycin activity/kg bw per day, the only dose tested.

(b) Rats

In an experiment conducted in compliance with GLP, five male and five female Fischer 344 rats 5–6 weeks of age received dietary doses of 0, 4, 6 or 10% dried fermentation product of avilamycin (14.9% avilamycin activity) for 2 weeks. These doses were equivalent to 0, 5960, 8940 and 14 900 mg avilamycin activity/kg in the diet or 0, 596, 894 and 1490 mg avilamycin activity/kg bw per day, respectively. All rats survived the 2-week treatment period. There were no toxicologically

significant effects on body weight, food consumption, efficiency of food conversion, haematological or clinical chemistry parameters or organ weights. No important gross or microscopic organ alterations were found. The only treatment-associated finding was brown to black discoloration of the waste trays by urine, although urine was yellow in the bladder or when freshly voided. Discoloration of the waste trays might have been caused by photoreaction of excreta (Williams & Probst, 1983a). The NOAEL was 1490 mg avilamycin activity/kg bw per day, the highest dose administered.

In another GLP-compliant experiment, five male and five female Fischer 344 rats 5–6 weeks of age were fed for 2 weeks with the crystalline form of avilamycin (100% activity), which was incorporated into the diet at doses of 0, 3000, 30 000 or 60 000 mg avilamycin activity/kg diet. These doses were equivalent to 0, 300, 3000 and 6000 mg avilamycin activity/kg bw per day. Brown to black discoloration of the waste trays by urine was observed. There were no toxicologically significant effects on body weight, food consumption, haematological or clinical chemistry parameters, organ weights or gross and histopathological findings, other than the increase of alanine aminotransferase (ALT) activity at 3000 and 6000 mg avilamycin activity/kg bw per day and the decrease of total bilirubin at all doses of avilamycin compared with the concurrent control, both with statistical significance in females. However, the level of total bilirubin in treated groups was in the normal range, and the induction of ALT was found only in females and was not accompanied by histopathological damage or changes in liver weight (Williams & Probst, 1983b). The NOAEL was 6000 mg avilamycin activity/kg bw per day, the highest dose administered.

In an experiment that may not have been conducted according to GLP, male and female rats approximately 4 weeks of age were placed into groups of 10 per sex per dose. Avilamycin was incorporated into the pelleted diet at levels of 0, 30, 300 or 3000 mg avilamycin activity/kg diet and fed continuously for 28 days. These doses were equivalent to 0, 3, 30 and 300 mg avilamycin activity/kg bw per day. No mortality or toxic signs were observed. Weight gains, haematology, blood chemistry and urinalysis parameters were not affected (Sachsee et al., 1978a). The NOAEL in this study was 300 mg avilamycin activity/kg bw per day, the highest dose administered.

In another experiment using the mycelial form of avilamycin (GLP compliance was not clear), 10 male and 10 female rats 4 weeks of age were fed for 28 days with the mycelial form of avilamycin incorporated into the pelleted diet at 0 or 30 000 mg avilamycin activity/kg diet. These doses are equivalent to 0 and 3000 mg avilamycin activity/kg bw per day. No mortality or toxic signs were observed. Weight gains, haematology, blood chemistry and urinalysis parameters were not affected (Sachsee et al., 1978b). The NOAEL was 3000 mg avilamycin activity/kg bw per day, the only dose administered.

(c) Dogs

In an experiment that was performed according to GLP, Beagle dogs aged 4–5 months (four per sex per dose) were given dried fermentation product of avilamycin (17.8% activity) orally by gelatine capsule once daily for a period of 6 months. The doses used were 0, 3.56, 35.6 and 178 mg avilamycin activity/kg bw

per day. There were no treatment-related mortalities or clinical signs of toxicity. Ocular and physical examinations revealed no effects of avilamycin treatment. No treatment-related gross or microscopic changes were observed. Haematology and urinalysis values were within normal ranges. Blood chemistry parameters were not different from control, except for serum ALT, which was slightly increased at 35.6 and 178 mg avilamycin activity/kg bw per day. This effect was statistically significant and dose dependent in male dogs 14 days after the initiation of administration, but the change recovered thereafter. In females, ALT was increased slightly, but significantly, at 178 mg avilamycin activity/kg bw per day 14 and 119 days after the initiation of the treatment. Increased ALT values were not associated with other parameters related to hepatotoxicity, including liver enzymes, liver weight changes and microscopic changes. In addition, the levels of ALT were in the historical control range. The dogs grew normally and tolerated daily oral doses of up to 178 mg avilamycin activity/kg bw per day for 6 months without any evidence of toxicity (Williams, 1983). The NOAEL was 178 mg avilamycin activity/kg bw per day, the highest dose administered.

(d) Pigs

In an experiment conducted according to GLP, avilamycin (mycelial cake form, activity 7.83%) was fed to Large White pigs at levels of 0, 30, 300 and 3000 mg avilamycin activity/kg in the diet for 21 weeks, followed by a 4-week withdrawal period. Each treatment group consisted of four castrated males and four females aged 8–9 weeks and weighing about 11–13 kg. The doses administered were equivalent to 0, 1.2, 12 and 120 mg avilamycin activity/kg bw per day. Observations included clinical signs, body weight changes, food consumption, haematological and blood chemistry parameters, urinalysis, necropsy findings and histopathology. Some blood biochemistry parameters, such as gamma glutamyl transferase (GGT), aspartate aminotransferase (AST), sodium and inorganic phosphorus, were changed from control values, but those changes were weak and in the normal range. There were no adverse treatment-related differences in the parameters examined (Strittmatter & Somerville, 1980). It was concluded from this experiment that the NOAEL was 120 mg avilamycin activity/kg bw per day, the highest dose administered.

(e) Chickens

Avilamycin (mycelial cake form, activity 7.83%) was fed continuously to broiler chickens at levels of 0, 30, 300 and 3000 mg avilamycin activity/kg in the diet for a period of 62 days. These doses were equivalent to 0, 3.75, 37.5 and 375 mg avilamycin activity/kg bw per day. Each treatment group consisted of 24 female and 24 male broiler chickens of the Hubbard strain. Observations included growth performance, clinical signs, gross pathology, organ weight, histopathology, haematology and clinical chemistry. There were no adverse treatment-related differences in any of the parameters (Strittmatter, 1982). The NOAEL was 375 mg avilamycin activity/kg bw per day, the highest dose administered.

(f) Turkeys

Avilamycin was fed to 8-week-old turkeys at 0 and 40 mg avilamycin activity/kg in the diet (equivalent to 0 and 5 mg avilamycin activity/kg bw per day) for 14 days. The treated group consisted of 10 males and 10 females. No differences in feed consumption, mean body weight gain or haematological and clinical biochemistry values were noted. No adverse clinical signs were observed (Cochrane & Thomson, 1991). The NOAEL was 5 mg avilamycin activity/kg bw per day, the only dose administered.

Avilamycin was also fed continuously to turkeys at levels of 0, 20 and 100 mg avilamycin activity/kg in the diet (equivalent to 0, 2.5 and 12.5 mg avilamycin activity/kg bw per day, respectively) for 16 weeks. Each treatment group consisted of 18 females and 18 males. Observations included clinical signs, body weight changes, food consumption and necropsy findings. No adverse treatment-related differences were observed for any of the parameters (Redgrave et al., 1992). The NOAEL was 12.5 mg avilamycin activity/kg bw per day, the highest dose administered.

2.2.3 Long-term studies of toxicity and carcinogenicity

The available toxicological data from long-term studies of toxicity and carcinogenicity are summarized in Table 3.

Table 3. Results of long-term studies of toxicity and carcinogenicity

Species	Study duration	Sex	Route	NOAEL (mg avilamycin activity/kg bw)	References
Mouse	104 weeks	M & F	Oral in feed	450[a]	Hunter et al. (1983b)
Rat	2 years	M & F	Oral in feed	150[a]	Hunter et al. (1983a)

F, female; M, male.
[a] Highest dose administered.

(a) Mice

In a GLP-compliant experiment, CD-1 mice approximately 6 weeks of age were allocated 60 per sex per dose and fed 0, 30, 300 or 3000 mg avilamycin activity/kg in the diet for 104 weeks, where avilamycin was derived from 7% raw material. These doses were equivalent to 0, 4.5, 45 and 450 mg avilamycin activity/kg bw per day. A further group of the same size was fed a diet containing purified material at 3000 mg avilamycin activity/kg diet. Mortality, clinical signs, growth, food consumption and behaviour were measured on all mice during the course of the study. The presence of masses and gross and microscopic pathological findings were also examined. No serum clinical chemistry end-points were measured. There were no differences in any of the parameters measured (Hunter et al., 1983b). The NOAEL in this study was 450 mg avilamycin activity/kg bw per day, the highest dose administered.

(b) Rats

In a GLP-compliant 2-year experiment, male and female Sprague-Dawley rat litters (80 per sex per dose) were fed avilamycin (derived from mycelial cake of 7% activity) at doses of 0, 30, 300 or 3000 mg avilamycin activity/kg in the diet and pure avilamycin at 3000 mg avilamycin activity/kg in the diet during the whole period of the study. These doses were equivalent to 0, 1.5, 15 and 150 mg avilamycin activity/kg bw per day. The litters were from parent rats fed avilamycin at the same doses of 0, 30, 300 or 3000 mg avilamycin activity/kg in the diet for 1 week and then mated and maintained on their treatments during gestation and lactation. Each group of 80 rats was further divided into a main group of 50 male and 50 female rats for tumorigenic evaluation and a satellite group (30 per sex per group) for blood and urine examination at intervals. Mortality, clinical signs, growth, food consumption, water consumption and the presence of palpable masses were investigated during the course of the study. In addition, urinalysis, haematology and blood chemistry were carried out on samples obtained from the satellite groups. Ten animals from each satellite group were killed at 52 weeks, and the remainder at 104 weeks. Terminal necropsy of the surviving rats in the main group was commenced from week 108 for females and from week 112 for males when survival approached 20%. Measurement of organ weights and gross and microscopic pathological examination were carried out at necropsy. The mortality was 58–78% for all groups, with no difference between treatments. Clotting times were significantly decreased, with dose dependency in males at 15 and 150 mg avilamycin activity/kg bw per day, where avilamycin was derived from mycelial cake, on weeks 13, 26, 52 and 78. However, they recovered at the last two sampling times (weeks 104 and 112). There was a non-statistically significant increase in pancreatic exocrine adenomas in male rats that were fed avilamycin derived from mycelial cake at 15 and 150 mg avilamycin activity/kg bw per day (incidence 2/59 and 4/60, respectively, compared with 0/59 for the control group). A higher incidence of thyroid parafollicular cell carcinoma was also observed in male rats treated with avilamycin derived from mycelial cake at 15 and 150 mg avilamycin activity/kg bw per day, without statistical significance (incidence 5/59 and 4/60, respectively, compared with 1/59 for the control group). No thyroid parafollicular cell carcinoma was found at 1.5 mg avilamycin activity/kg bw per day for the mycelial cake and 150 mg avilamycin activity/kg bw per day for the pure form of avilamycin. The incidences were in the historical control range (6.1–12%) for senescent rats (Quast et al., 1983; Attia, 1996; Pilling et al., 2007), and there were no increases in the two types of neoplastic lesions at 150 mg avilamycin activity/kg bw per day for the pure form of avilamycin. There were no treatment-related differences of toxicological significance in any of the parameters examined (Hunter et al., 1983a). The NOAEL in this study is 150 mg avilamycin activity/kg bw per day, the highest dose administered.

2.2.4 Genotoxicity

In assays performed in compliance with GLP, the genotoxic effects of avilamycin were investigated using a range of genotoxic end-points in vitro and in vivo (Table 4). Avilamycin was not mutagenic in the reverse mutation assay with *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538,

G46, C3076 and D3052 and *Escherichia coli* strains WP2 and WP2uvrA at 0.003 33–1000 μg/ml (or plate) both with and without S9 activation. Also, avilamycin did not present any genotoxicity in a deoxyribonucleic acid (DNA) repair assay using primary cultures of adult rat hepatocytes at 0.5–1400 μg/ml, in a forward mutation assay using L5178Y mouse lymphoma cells at 10–600 μg/ml and in a chromosomal aberration assay using Chinese hamster ovary cells at 125–375 μg/ml both with and without S9 activation. Avilamycin was not mutagenic in sister chromatid exchange assays in bone marrow of Chinese hamsters that were orally administered avilamycin at 200–500 mg/kg bw and in micronucleus tests with bone marrow of ICR mice orally administered avilamycin twice at 500–2000 mg/kg bw.

Thus, the weight of available evidence supports the conclusion that avilamycin is not genotoxic.

2.2.5 Reproductive and developmental toxicity

The results of available reproductive and developmental toxicity studies are summarized in Table 5.

(a) Multigeneration reproductive toxicity studies

(i) Rats

In a three-generation study that was compliant with GLP and investigated both reproductive and developmental toxicity, Sprague-Dawley (Crl:COBS CD (SD)) rats weighing 60–70 g were allocated to study groups (F_0 generation, 25 per sex per dose) and fed avilamycin at doses of 0, 30, 300 and 3000 mg avilamycin activity/kg in the diet (equivalent to 0, 1.5, 15 and 150 mg avilamycin activity/kg bw per day), where the avilamycin was derived from mycelial cake with 7% activity, and at another dose of 3000 mg avilamycin activity/kg in the diet, where the avilamycin originated from 100% pure active ingredient. Animals of the F_0 generation were maintained on their respective diets for at least 90 days prior to mating and throughout mating, gestation and lactation. Resulting litters (F_{1A}) reared to 21 days postpartum were examined morphologically. Animals of the F_0 generation maintained on the respective diets were remated approximately 10 days after the weaning of the F_{1A} generation. Three or four pregnant females per group were sacrificed on day 20 of gestation to examine the effects on late embryo or fetal development. The remaining dams were allowed to rear their young to 21 days postpartum, when 25 males and 25 females were selected to form the basis of the F_{1B} generation, which was treated in the same manner as the F_0 generation. At weaning, 12 male and 12 female F_{2A} pups were reared for 90 days on their respective diets and then subjected to gross examination and organ weight analysis. A further 12 male and 24 female F_{2A} pups per group were selected to form the basis of the next generation. Following the weaning of the F_{2A} pups, F_{1B} animals were remated, and then 9–12 females were sacrificed on gestation day 20 to determine fetal abnormalities. Remaining females were allowed to deliver and rear young pups to 21 days postpartum, and the parents (F_{1B}) and their offspring (F_{2B}) were sacrificed and subjected to macroscopic postmortem examination. The selected F_{2A} pups were maintained on their respective diets for 90 days and further during mating,

Table 4. Results of genotoxicity assays with avilamycin

Test system	Test object	Concentration	Results	References
In vitro				
Ames test[a]	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	1–25 µg/plate[b]	Negative	Williams & Rexroat (1983)
Ames test[a]	*S. typhimurium* TA98, TA100, TA1535, TA1537	0.003 33–10.0 µg/ml[c] (±S9 mix)	Negative	Mecchi (2004)
	E. coli WP2uvrA	0.1–100 µg/ml[d] (+S9 mix)	Negative	
		0.0333–33.3 µg/ml[e] (−S9 mix)	Negative	
Ames test[f]	*S. typhimurium* C3076, D3052, G46, TA98, TA100, TA1535, TA1537, TA1538	0.1–1000 µg/ml[g] (±S9 mix)	Negative	Williams & Thompson (1983a,b)
	E. coli WP2, WP2uvrA	0.1–1000 µg/ml[g] (±S9 mix)	Negative	
DNA repair assay	Primary cultures of adult rat hepatocytes	0.5–1000 µg/ml[h]	Negative	Williams et al. (1983f)
DNA repair assay	Primary cultures of adult rat hepatocytes	0.7–1400 µg/ml[h]	Negative	Williams et al. (1984)
Forward mutation assay[a]	L5178Y mouse lymphoma cells (thymidine kinase locus)	50–400 µg/ml[i]	Negative	Williams et al. (1983g)
Forward mutation assay[a]	L5178Y mouse lymphoma cells (thymidine kinase locus)	10–60 µg/ml (−S9 mix, 24 h)[j]	Negative	Cifone (2004)
		100–600 µg/ml (−S9 mix, 4 h)[j]		
		40–300 µg/ml (+S9 mix, 4 h)[k]		
Chromosomal aberration assay[a]	Chinese hamster ovary cells	125, 150 and 175 µg/ml (3 h)[l]	Negative	Murli (2004)
		225, 300 and 375 µg/ml (20 h)[l]		

Table 4 (contd)

Test system	Test object	Concentration	Results	References
In vivo				
Sister chromatid exchange assay	Chinese hamster bone marrow (F, 3 per dose)	200, 300, 400 and 500 mg/kg bw[m] (single oral)	Negative	Williams & Neal (1983)
Micronucleus assay	Mouse bone marrow (M & F, 5 per sex per dose)	500, 1000 and 2000 mg/kg bw, 2 times[m]	Negative	Murphy & Phelps (2004a,b)

F, female; M, male.

[a] Both with and without rat liver S9 fraction.

[b] *N*-Methyl-*N′*-nitro-*N*-nitrosoguanidine (−S9 mix), 2-nitrofluorene (−S9 mix), 9-aminoacridine (−S9 mix) and 2-aminoanthracene (+S9 mix) were used as positive controls.

[c] Benzo[*a*]pyrene (+S9 mix), 2-aminoanthracene (+S9 mix), 2-nitrofluorene (+S9 mix), sodium azide (+S9 mix) and ICR-191 (+S9 mix) were used as positive controls.

[d] 2-Aminoanthracene was used as a positive control.

[e] 4-Nitroquinoline-*N*-oxide was used as a positive control.

[f] Modified Ames test using a gradient plate. Both with and without rat liver S9 fraction.

[g] Streptozotocin (S9 mix), *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (−S9 mix), 2-acetylaminofluorene (+S9 mix) and 2-aminoanthracene (+S9 mix) were used as positive controls.

[h] *N*-Methyl-*N′*-nitro-*N*-nitrosoguanidine and 2-acetylaminofluorene were used as positive controls.

[i] Ethylmethanesulfonate (−S9 mix) and 3-methylcholanthrene (+S9 mix) were used as positive controls.

[j] Methylmethanesulfonate was used as a positive control.

[k] Methylcholanthrene was used as a positive control.

[l] Mitomycin C (−S9 mix) and cyclophosphamide (+S9 mix) were used as positive controls.

[m] Cyclophosphamide was used as a positive control.

Table 5. Results of studies of reproductive and developmental toxicity

Species	Study duration	Sex	Route	NOAEL (mg avilamycin activity/ kg bw)	References
Reproductive toxicity					
Rat	3 generations	M & F	Oral in feed	150[a]	Palmer et al. (1981)
Pig	21 weeks, 1 generation	M & F	Oral in feed	2.4[b]	Van Duyn et al. (1984)
Developmental toxicity					
Rat	GD 6–19	M & F	Oral in feed	528[a]	Lawler (2004a,b)
Rabbit	GD 6–18	M & F	Oral gavage	356[a]	Williams & Hagopian (1983)

F, female; GD, gestation day; M, male.
[a] Highest dose administered.
[b] Only dose tested.

gestation and lactation to produce the F_{3A} generation. F_{3A} pups were sacrificed and examined morphologically at weaning. The F_{2A} animals were then remated to produce the F_{3B} generation. At weaning, 10 males and 10 females of the F_{3B} generation per group were examined for organ weights and gross abnormalities. Remaining F_{3B} weanlings and F_{2A} adults were subjected to macroscopic or microscopic postmortem examination.

There were no signs or mortalities specifically attributable to avilamycin treatment over the three generations. Water consumption, food consumption and body weight differences were not consistently dosage related over the three generations. Mating performance, pregnancy rate, duration of gestation and total litter loss were generally comparable for all dietary concentrations. Macroscopic changes at terminal postmortem examination of mated animals were unrelated to treatment. Ocular changes, particularly opacities and encrustation, were found in reared young animals, but they were due to mild infections rather than to avilamycin treatment. Renal changes, such as renal pelvic dilatation or cortical surface cysts, were observed in reared young at weaning, but they were not different between groups. With respect to skeletal malformations, fetuses from F_0 and F_{1B} dams on gestation day 20 showed extra 14th ribs, with an incidence ratio of 5.0–11.4% of examined in avilamycin-treated groups (% incidence: 0 for control; 8.3–8.8 for 1.5 mg/kg bw per day; 7.4–8.7 for 15 mg/kg bw per day; 5.0–11.4 for 150 mg/kg bw per day for mycelial cake; 7.7 for 150 mg/kg bw per day for pure form). However, the absence of supernumerary ribbed fetuses among concurrent control groups was unusual. The incidences observed for avilamycin-treated groups were within the range of historical controls (approximately 14%) (Chernoff et al., 1991). Absolute and relative liver weights were slightly increased, with statistical significance in unmated F_{2A} adult females treated with 15 mg avilamycin activity/kg bw per day and both mycelial cake form and pure form at 150 mg avilamycin activity/kg bw per day (relative liver weight: 3.82–3.93% compared with 3.53% for the control group),

without histopathological changes. The small increase in absolute and relative liver weights was found only in F_2 females, not in males and not in other generations (Palmer et al., 1981). The NOAEL was 150 mg avilamycin activity/kg bw per day, the highest dose administered.

(ii) Pigs

In an experiment that was not conducted according to GLP, 50 crossbred female pigs, approximately 12 weeks old and weighing 35 kg, were fed 0 or 60 mg avilamycin activity/kg in complete diets for 21 weeks, including the periods of growing (0–8 weeks) and finishing (9–21 weeks). The doses were equivalent to 0 and 2.4 mg avilamycin activity/kg bw per day. Treatment was then withdrawn, and the female pigs were artificially inseminated. During the breeding, gestation and lactation periods, the female pigs were fed a non-medicated complete feed. Piglets were weaned at 3 weeks of age. The number of female pigs in heat, the number of pigs that conceived on first estrus or on second estrus, and the number of pigs that farrowed were examined as parameters of reproductive performance. The number of piglets born, the number of live pigs at birth and at weaning, and the weights of piglets at birth and at weaning were also measured. There was no statistically significant effect on growth performance during the growing or finishing phase. Treatment had no effect on any parameters of reproductive performance or on the number or weight of piglets at birth and at weaning. It was concluded that feeding of avilamycin to young female pigs did not adversely impact on their subsequent reproductive performance (Van Duyn et al., 1984). The NOAEL was 2.4 mg avilamycin activity/kg bw per day, the only dose tested.

(b) Developmental studies

(i) Rats

In a GLP-compliant experiment, 25 pregnant CD rats (Crl:CD (SD) IGS BR), aged 10 weeks, per group were administered granular avilamycin (26.4% activity) once daily at doses of 0, 500, 1000 or 2000 mg/kg bw (equivalent to 0, 132, 264 and 528 mg avilamycin activity/kg bw) by oral gavage during gestation days 6 through 19. Body weight, food consumption, and gross internal, uterine and ovarian examination were performed for maternal rats, and viability, sex, weight and gross external examination were performed for fetuses. Half of the fetuses were examined for visceral effects, whereas the other half were examined for skeletal effects. No treatment-related effects were observed on survival, clinical signs or maternal reproductive and fetal morphological development parameters. No adverse treatment-related effects on body weights or food consumption occurred (Lawler, 2004a,b). The NOAEL was 528 mg avilamycin activity/kg bw per day, the highest dose administered.

(ii) Rabbits

In an experiment compliant with GLP, 15 pregnant Dutch Belted rabbits per group were administered a dried fermentation product of avilamycin (17.8% activity) by oral gavage in daily doses of 0, 250, 716 and 2000 mg/kg bw (equivalent to 0, 44.5, 127.4 and 356 mg avilamycin activity/kg bw per day) for 13 days between

gestation days 6 and 18. Animals were observed daily for toxic signs. Body weights and daily food consumption were recorded. The animals were killed on gestation day 28 and examined for reproductive performance, and the fetuses were examined for abnormalities. The majority of animals from all treatment groups had orange-coloured urine on gestation days 9 through 20. Diarrhoea was observed at increased incidence in dosed rabbits (control, 0; low dose, 2; middle dose, 2; high dose, 4). Food consumption on gestation days 6 through 12 was significantly decreased in all treatment groups. Abortions occurred in two rabbits from the low dose group and one rabbit from both middle and high dose groups. All except one of these rabbits had diarrhoea or were anorectic prior to abortion. There were no effects related to avilamycin treatment on pregnancy rate, mean percentage of live fetuses, fetal weights or fetal external anomalies. The low incidence of abortions that occurred in all treatment groups was regarded as a secondary consequence of maternal toxicity (Williams & Hagopian, 1983). The NOAEL for prenatal toxicity and teratogenicity in rabbits was 356 mg avilamycin activity/kg bw per day, the highest dose administered.

2.2.6 Special studies

The results of available studies on the neurotoxicity, immunotoxicity and ocular toxicity of avilamycin are summarized in Table 6.

Table 6. Results of studies of neurotoxicity, immunotoxicity and ocular toxicity of avilamycin

Species	Study duration	Sex	Route	NOAEL	References
Neurotoxicity					
Mouse	Single dose		Oral	5000 mg/kg bw (activity not mentioned)[a]	Williams (1987)
Rabbit	Single dose		Oral	5000 mg/kg bw (activity not mentioned)[a]	Williams (1987)
Immunotoxicity					
Mouse	LLNA, 3 days	F	Topical	10%[a] w/v in acetone	Sire (2004)
Guinea-pig	3 times/week for 2 weeks + challenge dose in 3rd week	F	Topical	5%[b] w/w in petrolatum	Williams & Mattingly (1983)
Ocular irritation					
Rabbit	Single dose	M & F	Topical	10.3 mg activity[b]	Williams et al. (1983e)

F, female; LLNA, local lymph node assay; M, male; w/v, weight by volume; w/w, by weight.
[a] Highest dose administered.
[b] Only dose tested.

(a) Neurotoxicity

Neurobehavioural effects of avilamycin were evaluated using five mice and five rabbits per dose group. The study was not conducted according to GLP. Mice and rabbits were orally administered a single dose of avilamycin at 0, 1500 or 5000 mg/kg bw (activity was not mentioned). End-points of neurobehavioural, neurological and autonomic nervous system toxicity in treated mice were examined using the Irwin method. For rabbits, neurobehavioural clinical observations were recorded. There were no remarkable changes in any parameters at any doses tested in both mice and rabbits (Williams, 1987). The NOAEL was 5000 mg avilamycin/kg bw, the highest dose administered.

(b) Immune responses

The local lymph node assay was performed in compliance with GLP to investigate the skin sensitization potential of avilamycin. Twenty-eight female CBA/J mice approximately 9 weeks old and weighing 20 g were allocated to seven groups of four animals each: five treated groups, one positive control group and one negative control group. The treatment groups received 5, 10, 25, 50 or 100% of avilamycin dissolved in an acetone/olive oil (4:1 by volume [v/v]) mixture. As avilamycin is not soluble in the usual vehicles, an extract of granular avilamycin (purity 26.4%) at a concentration of 10% (weight by volume [w/v]) in acetone was used for treatment, and the concentrations of avilamycin tested were expressed as a percentage (by volume) of the extract in the vehicle. The negative control group received the acetone/olive oil (4:1, v/v) mixture only, and the positive control group received α-hexylcinnamaldehyde, a moderate sensitizer, dissolved in a mixture of acetone/olive oil (4:1, v/v) at a concentration of 25%. The test materials (25 μl) were applied over the ears for 3 consecutive days. Following 2 days of resting, ear thickness and the proliferation of lymph node cells in the lymph node draining the application site were measured. Clinical signs, morbidity, mortality and body weight were also examined during the study. No mortality and no clinical signs were observed during the study. No cutaneous reactions and no increase in ear thickness were observed in the animals of the treated groups. There was no lympho proliferation seen at any concentration, whereas significant lympho proliferation was observed with the positive control. Avilamycin did not induce delayed contact hypersensitivity in the murine local lymph node assay (Sire, 2004).

Avilamycin was evaluated for allergic contact sensitization in 18 female albino guinea-pigs 8–12 weeks of age and weighing approximately 430 g in an experiment conducted in compliance with GLP. Avilamycin, a 14.9% dried fermentation product, was applied at a concentration of 5% by weight (w/w) in petrolatum to the skin and held under occlusion for 6 h. During the induction phase, applications (0.2 ml) were given to 12 animals 3 times weekly for 2 weeks. A challenge application was performed 8 days after the final induction exposure. Six additional animals remained untreated during induction and received the avilamycin dose only at challenge. Dinitrochlorobenzene, 0.1% (w/v) dissolved in 70% ethanol, and undiluted petrolatum were tested as positive and vehicle controls, respectively. In the positive and vehicle control groups, 18 guinea-pigs each were allocated for challenge with or without induction. There was no evidence of sensitization or dermal irritation in any of the animals treated with 5% (w/w) avilamycin in petrolatum. Irritation and sensitization were observed only with the positive control,

dinitrochlorobenzene. Avilamycin did not elicit a contact sensitization response in guinea-pigs (Williams & Mattingly, 1983).

(c) Ocular irritation

In a GLP-compliant experiment, acute ocular irritation effects of avilamycin were examined in three female and three male rabbits (New Zealand White) approximately 12–18 weeks old and weighing 2.7–3.2 kg. One eye of each of six animals was treated once with 69 mg (equivalent in volume to 0.1 ml and in activity to 10.3 mg) of avilamycin dried fermentation product, and irritation signs were observed for 7 days. Corneal dullness, slight iritis and slight conjunctivitis developed in all treated eyes within 1 h after treatment, but the symptoms cleared within 7 days post-treatment. All treated eyes gave a negative response to sodium fluorescein dye 24 h after treatment, which indicates that there were no corneal lesions (Williams et al., 1983e).

(d) Microbiological effects

A JECFA decision tree approach that was adopted by the sixty-sixth meeting of the Committee (Annex 1, reference *181*) and complies with Guideline 36 of the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH GL36) (VICH, 2004) was used by the Committee to determine the need to establish a microbiological ADI for avilamycin. The decision tree approach initially seeks to determine if there may be microbiologically active avilamycin residues entering the human colon. If the answer is “no” to any of the first three steps, then no microbiological ADI is necessary. However, should such residues be present, then two end-points of public health concern are to be considered: 1) disruption of the colonization barrier and 2) increase of the population(s) of resistant bacteria. At Step 4 of the decision tree process, it is possible to provide scientific justification to eliminate testing (i.e. the need for a microbiological ADI) for either one or both end-points. Step 5 is where a microbiological ADI would be determined. Should a microbiological ADI not be necessary, then the toxicological or pharmacological ADI would be used.

The Committee evaluated minimum inhibitory concentration (MIC) susceptibility, faecal binding interaction and the biological activity of avilamycin residues and used the decision tree to answer the following questions in the assessment of avilamycin:

Step 1: Are residues of the drug, and (or) its metabolites, microbiologically active against representatives of the human intestinal flora?

Yes. Avilamycin is microbiologically active mainly against Gram-positive bacteria, including some bacterial genera and species representative of the human intestinal flora.

In a GLP-compliant study, the MIC of avilamycin was determined against 100 bacterial strains, comprising 10 isolates from each of 10 groups of genera representing the normal human intestinal microbiota (Pridmore, 2004a). All strains were sourced from the faecal microbiota of healthy unmedicated humans. The test system was standardized agar dilution MIC methodology using quality control

strains as described in the Clinical and Laboratory Standards Institute guidelines (CLSI, 2004). To assess the effect of bacterial density on avilamycin activity, each MIC was determined using high and low inoculum levels, 10^9 and 10^5 colony-forming units (cfu)/ml, respectively, for each strain. The avilamycin activity against each bacterial group is summarized in Table 7. MIC_{50}, MIC_{90}, geometric mean and MIC range were calculated for each bacterial group. In tests using the higher bacterial inoculum density, avilamycin exerted no measurable antibacterial activity against *Escherichia coli* (MIC_{50} >128 µg/ml). Against other strains, activity was variable, both within and between bacterial groups. Avilamycin exerted relatively poor activity against *Bacteroides fragilis* and other *Bacteroides* species (MIC_{50} = 8 µg/ml), *Lactobacillus* (MIC_{50} = 16 µg/ml) and *Bifidobacterium* spp. (MIC_{50} = 16 µg/ml). *Peptostreptococcus* (MIC_{50} = 0.25 µg/ml), *Eubacterium* (MIC_{50} = 0.5 µg/ml) and *Clostridium* spp. (MIC_{50} = 1 µg/ml) were the most susceptible groups. At the lower inoculum density, avilamycin was inactive against *E. coli* strains. *Enterococcus* and *Peptostreptococcus* MIC values were similar to those obtained using the higher inoculum density. Conversely, a large inoculum effect was seen in *Bifidobacterium*, *Eubacterium* and *Lactobacillus* spp.; in other words, avilamycin MICs were reduced by 3 or more doubling dilutions at the low inoculum level compared with those obtained using the high inoculum level.

Table 7. Susceptibility of representative human intestinal bacteria to avilamycin[a]

Bacterial group	Avilamycin MIC values (µg/ml)							
	High inoculum (1 × 10^9 cfu/ml)				Low inoculum (1 × 10^5 cfu/ml)			
	Range	MIC_{50}	MIC_{90}	Geometric mean	Range	MIC_{50}	MIC_{90}	Geometric mean
Bacteroides fragilis	4–128	8	>128	≥19.7	2–128	4	>128	≥9.8
Other *Bacteroides*	4–128	8	>128	≥16	2–128	8	>128	≥9.8
Bifidobacterium	2–128	16	>128	≥26	0.25–16	1	8	2.1
Clostridium	0.5–8	1	8	1.6	0.125–2	0.25	1	0.4
Enterococcus	2–4	2	4	2.5	1–2	1	2	1.1
Escherichia coli	All >128	>128	>128	>128	All >128	>128	>128	>128
Eubacterium	0.5–128	0.5	4	≥1.4	0.062–0.25	0.062	0.062	0.07
Fusobacterium	0.5–128	4	>128	≥8	0.5–128	1	32	3.5
Lactobacillus	8–128	16	>128	≥34	2–128	2	>128	≥12
Peptostreptococcus	0.062–2	0.25	2	0.35	0.062–2	0.125	2	0.25

[a] From Pridmore (2004a).

Step 2: Do residues enter the human colon?

Yes. A number of residue studies using two types of ^{14}C-radiolabelled avilamycin to detect total residues or analytical (liquid chromatography/tandem mass spectrometry) and bioautographic methods to detect parent avilamycin have been conducted in chickens, turkeys, pigs and rabbits. Muscle, kidney, fat and skin contain little or no avilamycin-derived residue, regardless of the period between withdrawal of medication and slaughter. However, residues may be present at low levels in the liver. Therefore, small concentrations of avilamycin-related residues could enter the colon of a person ingesting tissues from treated animals.

Step 3: Do the residues entering the human colon remain microbiologically active?

No. Avilamycin residue will be extensively metabolized, with very reduced microbiological activity, prior to entering the colon of the consumer; within the colon, it will become substantially bound (>95%) to faecal material. In addition, no microbiologically active residues were detected in edible tissues from chickens and pigs as determined by bioautographic methods.

To determine the effect of faecal binding on the antibacterial activity of avilamycin for time periods between 0 and 12 h, selected avilamycin concentrations of 0, 1, 2, 5, 10, 20, 50 and 100 µg/ml were incubated with increasing concentrations of sterilized human faeces (0, 10, 25 and 50% w/v in Mueller Hinton Broth), collected from three healthy individual donors who had not received antibiotic therapy 3 months prior to sample collection (Pridmore, 2004b). Avilamycin activity was determined using *Enterococcus faecalis* ATCC 29212 as an indicator organism, as it is susceptible to avilamycin. The antibacterial activity of the supernatant obtained from centrifugation of the incubation mixture was assessed for the presence or absence of *E. faecalis* growth before and after incubation with faeces and avilamycin. With solutions containing 10% and 25% faeces, the binding was variable (60–95%) and time dependent. All three faecal samples had 95–98% binding of avilamycin at 50% faecal concentration after 24 h of incubation. The 50% faecal concentration provided the closest representation of the in vivo situation with regard to binding of ingested avilamycin to intestinal contents. The results demonstrated the rapid, extensive and irreversible binding of avilamycin to human faeces. Based on this in vitro study, it can be estimated that the binding of avilamycin residues to undiluted faecal material would be highly likely to exceed 95%.

Avilamycin was extensively metabolized, rapidly excreted and converted to numerous metabolites by pigs, rats and poultry (Donoho & Magnussen, 1987; Magnussen et al., 1991). Flambalactone and flambic acid appear to be the major metabolites. These metabolites are formed as a result of cleavage of the ortho ester linking the C and D rings of avilamycin. The major residue detected in the liver of both rats and pigs was flambic acid. The level of microbiologically active residue detected in tissue was low.

Step 4: Is there any scientific justification to eliminate testing for either one or both end-points of concern, i.e. disruption of the colonization barrier or resistance development?

Yes. There is potential for adverse effects of human intestinal microbiota on the disruption of the colonization barrier, as avilamycin is most active against *Peptostreptococcus*, *Eubacterium* and *Clostridium* spp. However, the majority (>95%) of avilamycin residues in the colon are bound to faeces. In addition, avilamycin is extensively metabolized in pigs, and no microbiologically active residues could be detected in edible tissues of poultry and pigs. Therefore, avilamycin residues are unlikely to disrupt the colonization barrier of the human gastrointestinal tract.

A recent study by Delsol et al. (2005) assessed the effect of avilamycin on the emergence and persistence of resistance of enteric bacteria in pigs. Although avilamycin selects for resistance in the native enterococcal population of the pig, no resistant isolates were detected beyond 1 week post-treatment. This suggests that resistant isolates were unable to persist once selective pressure was removed and were outcompeted by sensitive microflora.

The only drug that so far shows a cross-resistance to avilamycin is the structurally related antibiotic evernimicin, developed for, but not introduced into, human medicine. Evernimicin shares a common mode of action with avilamycin. Avilamycin inhibits protein synthesis. The intrinsic resistance exhibited by most Gram-negative bacteria to avilamycin is attributed to their lower membrane permeability, which limits the accessibility of the target by avilamycin (McNicholas et al., 2000). Resistance to avilamycin in Gram-positive bacteria occurs exclusively by modification of the drug target—that is, alterations in the antibiotic-binding site of the ribosome decrease the binding affinity of the drug (Adrian et al., 2000a,b). This is incomplete cross-resistance, in that enterococci resistant to avilamycin exerted only decreased susceptibility, not complete resistance to evernimicin. In addition, the avilamycin class of antimicrobial compounds is not used in human medicine. Based on the existing information, the development of resistance to avilamycin and cross-resistance to a number of commonly used antimicrobials in veterinary and human medicine is not likely; however, further study should be considered to determine the emergence and transfer of resistant genes for this class of antimicrobial agents.

Based on the available data and scientific literature, there is no need to determine a microbiological ADI for disruption of the colonization barrier or resistance development for avilamycin.

2.3 Observations in humans

There are no data available regarding the effects of avilamycin in humans. Avilamycin is not known to be used for therapy in humans.

3. COMMENTS

3.1 Biochemical data

Avilamycin is poorly absorbed, extensively metabolized and rapidly excreted by pigs, rats and poultry. Orally administered avilamycin is excreted primarily in

faeces (over 90%), with less than 8% of the ingested drug eliminated in urine. Metabolites are formed as a result of cleavage of the ortho ester linking the C and D rings of avilamycin. The most abundant metabolite is flambic acid, which represents 40–50% of the total residue in urine and faeces and 15–20% of the residue in liver of pigs. No microbiologically active residues have been detected in tissues following avilamycin administration.

3.2 Toxicological data

Various forms of avilamycin (i.e. mycelial, crystalline and granular forms) were tested for toxicity. Doses of all forms of avilamycin in the toxicological studies were reported on the basis of the microbiological activity of avilamycin relative to that of the crystalline form.

The acute oral toxicity of avilamycin in mice and rats is low. The oral LD_{50} of the dried avilamycin products was higher than 390 or 745 mg avilamycin activity/kg bw in both mice and rats.

Groups of mice fed avilamycin at levels of 0, 30, 300 or 3000 mg avilamycin activity/kg in the diet (equivalent to 0, 4.5, 45 and 450 mg avilamycin activity/kg bw per day) continuously for 28 days showed a slight increase in feed intake and body weight of the males at 450 mg avilamycin activity/kg bw per day. No treatment-related mortality or toxicity was observed. The NOAEL was 450 mg avilamycin activity/kg bw per day, the highest dose administered. In another study, mice orally exposed to the mycelial form of avilamycin at 0 or 30 000 mg avilamycin activity/kg in the diet (equivalent to 0 or 4500 mg avilamycin activity/kg bw per day) for 28 days showed no mortality or toxicity. The NOAEL was 4500 mg avilamycin activity/kg bw per day, the only dose administered.

Male and female rats received a dried fermentation product of avilamycin at dietary doses of 0, 4, 6 or 10% (equivalent to 0, 596, 894 or 1490 mg avilamycin activity/kg bw per day) for 2 weeks. There were no toxicologically significant effects on body weight, feed consumption, feed conversion efficiency, haematology, clinical chemistry, organ weights or histopathology. The only treatment-associated finding was brown to black discoloration of the waste trays by urine, although urine was yellow in the bladder or when freshly voided. The NOAEL was 1490 mg avilamycin activity/kg bw per day, the highest dose administered.

In male and female rats fed for 2 weeks with crystalline avilamycin (100% activity) at doses of 0, 3000, 30 000 or 60 000 mg avilamycin activity/kg in the diet (equivalent to 0, 300, 3000 or 6000 mg avilamycin activity/kg bw per day), no toxicologically significant effects were found, other than increased ALT activity at 3000 and 6000 mg avilamycin activity/kg bw per day and decreased total bilirubin at all doses of avilamycin compared with the concurrent control, with statistical significance in females. However, the level of total bilirubin in treated groups was in the normal range. The induction of ALT was found only in females and was not accompanied by histopathological damage or changes in liver weight. The NOAEL was 6000 mg avilamycin activity/kg bw per day, the highest dose administered.

In male and female rats fed avilamycin at levels of 0, 30, 300 or 3000 mg avilamycin activity/kg in the diet (equivalent to 0, 3, 30 and 300 mg avilamycin

activity/kg bw per day) continuously for 28 days, no mortality or toxicity was observed. Weight gains, haematology, blood chemistry and urinalysis parameters were not affected. The NOAEL was 300 mg avilamycin activity/kg bw per day, the highest dose administered.

In another study, groups of rats fed mycelial avilamycin at 0 or 30 000 mg avilamycin activity/kg in the diet (equivalent to 0 or 3000 mg avilamycin activity/kg bw per day) showed no mortality or toxic signs. Weight gains, haematology, blood chemistry and urinalysis parameters were not affected. The NOAEL was 3000 mg avilamycin activity/kg bw per day, the only dose administered.

In dogs orally administered the dried fermentation product of avilamycin (17.8% activity) at 0, 3.56, 35.6 or 178 mg avilamycin activity/kg bw per day by gelatine capsule for 6 months, no treatment-related mortalities or clinical, haematological, biochemical or pathological signs of toxicity were observed, other than changes in serum ALT. Minor changes of ALT within the historical control range were not considered treatment related. The NOAEL for this study was 178 mg avilamycin activity/kg bw per day, the highest dose administered.

Groups of female and castrated male pigs were fed diets with the mycelial cake form of avilamycin at levels of 0, 30, 300 or 3000 mg avilamycin activity/kg (equivalent to 0, 1.2, 12 and 120 mg avilamycin activity/kg bw per day) for 21 weeks. There were no adverse treatment-related differences in clinical, haematological, biochemical or histopathological findings. The NOAEL was 120 mg avilamycin activity/kg bw per day, the highest dose administered.

Mice receiving avilamycin at concentrations of 0, 30, 300 or 3000 mg avilamycin activity/kg in the diet (equivalent to 0, 4.5, 45 and 450 mg avilamycin activity/kg bw per day) for 104 weeks showed no treatment-related toxicological or carcinogenic effects. The NOAEL was 450 mg avilamycin activity/kg bw per day, the highest dose administered.

In a carcinogenicity study, rats fed avilamycin at doses of 0, 30, 300 or 3000 mg avilamycin activity/kg diet for 1 week were mated and maintained on their treatments during gestation and lactation. The offspring were fed avilamycin derived from mycelial cake with 7% activity at doses of 0, 30, 300 or 3000 mg avilamycin activity/kg in the diet (equivalent to 0, 1.5, 15 and 150 mg avilamycin activity/kg bw per day) or pure avilamycin at 3000 mg avilamycin activity/kg in the diet (equivalent to 150 mg avilamycin activity/kg bw per day) for approximately 2 years. Clotting times were decreased in males receiving avilamycin derived from mycelial cake at 15 and 150 mg avilamycin activity/kg bw per day on weeks 13, 26, 52 and 78, but values recovered at the last two sampling times (weeks 104 and 112). A non-statistically significant increase in pancreatic exocrine adenomas was found in male rats that were fed avilamycin derived from mycelial cake at 15 and 150 mg/kg bw per day (incidence 2/59 and 4/60, respectively, compared with 0/59 for the control group). A higher, but not statistically significant, incidence of thyroid parafollicular cell carcinoma was also observed in male rats treated with avilamycin derived from mycelial cake at 15 and 150 mg/kg bw per day (incidence 5/59 and 4/60, respectively, compared with 1/59 for the control group). The tumour incidences were within the historical control range. There were no differences in any other

toxicological parameters. No neoplastic or non-neoplastic effects of pure avilamycin treatment were reported. Avilamycin was not found to cause cancer in this study, and the NOAEL was 150 mg avilamycin activity/kg bw per day, the highest dose administered.

Assays covering an adequate range of genotoxic end-points were conducted with avilamycin. Avilamycin was not genotoxic in any of the assays evaluated. The Committee concluded that avilamycin is not genotoxic.

A three-generation reproductive toxicity study was performed in rats with dietary avilamycin derived from mycelial cake or pure active ingredient. Rats received avilamycin at doses of 0, 30, 300 or 3000 mg avilamycin activity/kg in the diet (equivalent to 0, 1.5, 15 and 150 mg avilamycin activity/kg bw per day). Animals of each generation were maintained on their respective diets for at least 90 days prior to mating and throughout mating, gestation and lactation. Satellite groups of animals were subjected to gross and organ weight examination at 90 days of treatment. Three or four pregnant females of each generation were sacrificed on day 20 of gestation to examine the effects on fetal development. Resulting litters of each generation were reared to 21 days postpartum and examined morphologically. There were no signs or mortalities specifically attributable to avilamycin treatment over the three generations. Mating performance, pregnancy rate, duration of gestation and total litter loss were comparable for all dietary concentrations. Macroscopic changes at terminal postmortem examination of mated animals were unrelated to treatment. Absolute and relative liver weights were slightly, but statistically significantly, increased in unmated F_2 adult females treated with 15 or 150 mg avilamycin activity/kg bw per day. However, the increase was small and not supported by histopathological findings. The small increase in absolute and relative liver weights was found only in F_2 females, not in males and not in other generations. The NOAEL was 150 mg avilamycin activity/kg bw per day, the highest dose administered.

Female pigs receiving avilamycin at 0 or 60 mg avilamycin activity/kg in the diet (equivalent to 0 and 2.4 mg avilamycin activity/kg bw per day) for 21 weeks and then artificially inseminated showed no significant change in growth or reproductive performance. Treatment had no effect on any reproductive indices, including the number and weight of piglets at birth and at weaning. It was concluded that feeding of avilamycin to young pigs did not adversely impact on their subsequent reproductive performance. The NOAEL was 2.4 mg avilamycin activity/kg bw per day, the only dose tested.

Pregnant rats received avilamycin once daily at doses of 0, 500, 1000 or 2000 mg/kg bw (equivalent to 0, 132, 264 and 528 mg avilamycin activity/kg bw per day) by oral gavage during gestation days 6–19. No treatment-related effects were observed on survival, clinical signs or maternal reproductive and fetal morphological development parameters. The NOAEL was 528 mg avilamycin activity/kg bw per day, the highest dose administered.

Rabbits administered a dried fermentation product of avilamycin by oral gavage at daily doses of 0, 250, 716 or 2000 mg/kg bw (equivalent to 0, 44.5, 127.4 and 356 mg avilamycin activity/kg bw per day) during gestation days 6–18 showed

no treatment-related effects on pregnancy rate, mean percentage of live fetuses, fetal weights or fetal external anomalies. The NOAEL for maternal toxicity, prenatal toxicity and teratogenicity in rabbits was 356 mg avilamycin activity/kg bw per day, the highest dose administered.

Neurobehavioural effects of avilamycin were evaluated in mice and rabbits after a single administration of oral avilamycin at 0, 1500 or 5000 mg/kg bw. There were no significant changes in end-points of neurobehavioural, neurological or autonomic nervous system toxicity in treated mice or neurobehavioural clinical signs in rabbits at any of the doses tested.

Avilamycin has not been used for therapy in human medicine. There are no data available on the effects of avilamycin on human health.

3.3 Microbiological data

The Committee evaluated MIC susceptibility, faecal binding interaction and the biological activity of avilamycin residues and used the microbiological decision tree, adopted during the sixty-sixth JECFA (Annex 1, reference *181*) and compliant with VICH GL36 (VICH, 2004), to determine the impact of avilamycin on the human intestinal microbiota.

Avilamycin is microbiologically active mainly against Gram-positive bacteria, including some bacterial genera and species representative of the human intestinal flora. Avilamycin exerts no measurable antibacterial activity against *E. coli* (MIC_{50} >128 µg/ml) and relatively poor activity against *Bacteroides fragilis*, other *Bacteroides* spp., *Lactobacillus* spp. and *Bifidobacterium* spp. Avilamycin activity is clearly demonstrable against *Peptostreptococcus* (MIC_{50} = 0.25 µg/ml), *Eubacterium* (MIC_{50} = 0.5 µg/ml) and *Clostridium* spp. (MIC_{50} = 1 µg/ml).

Avilamycin residue is extensively metabolized to products with very low microbiological activity prior to entering the human colon. In addition, no microbiologically active residues are detected in edible tissues from chicken and pigs. Avilamycin binds to human faeces rapidly, extensively and irreversibly. Residues of avilamycin are substantially bound (>95%) to faecal material within the colon, further reducing their microbiological activity. Therefore, avilamycin residues are unlikely to disrupt the colonization barrier of the human gastrointestinal tract.

Although avilamycin selects for resistance in the native enterococcal population of pigs, no resistant isolates were detected beyond 1 week post-treatment. This suggests that resistant isolates are unable to persist once selective pressure is removed and are outcompeted by sensitive microflora. The only drug that shows cross-resistance to avilamycin is the structurally related antibiotic evernimicin, developed for, but not introduced into, human medicine. The intrinsic resistance exhibited by most Gram-negative bacteria to avilamycin is attributed to their lower membrane permeability, which limits the accessibility of the target by avilamycin. Resistance to avilamycin in Gram-positive bacteria occurs exclusively by modification of the drug target; in other words, alterations in the antibiotic-binding site of the ribosome decrease the binding affinity of the drug. This is incomplete cross-resistance, in that enterococci resistant to avilamycin exert only decreased

susceptibility and not complete resistance to evernimicin. In addition, the avilamycin class of antimicrobial compounds is not used in human medicine. Considering the existing information, the development of resistance to avilamycin and cross-resistance to a number of commonly used antimicrobials in veterinary and human medicine is not likely.

Based on the available data, a microbiological ADI for avilamycin is not needed.

4. EVALUATION

The most relevant animal studies for evaluation of the safety of residues of avilamycin in food are the 2-year toxicity study and the multigeneration reproductive study in rats. No significant adverse effects were observed following avilamycin administration, and the NOAEL was 150 mg avilamycin activity/kg bw per day, the highest dose administered in both studies. A toxicological ADI of 0–2 mg avilamycin activity/kg bw per day is established, using a safety factor of 100 and rounding to one significant figure.

5. REFERENCES

Adrian, P.V., Zhao, W., Black, T.A., Shaw, K.J., Hare, R.S. & Klugman, K.P. (2000a) Mutations in ribosomal protein L16 conferring reduced susceptibility to evernimicin (SCH 27899): Implications for mechanism of action. *Antimicrob. Agents Chemother.*, **44**, 732–738.

Adrian, P.V., Mendrick, C., Loebenberg, D., McNicholas, P., Shaw, K.J., Klugman, K.P., Hare, R.S. & Black, T.A. (2000b) Evernimicin (SCH 27899) inhibits a novel ribosome target site: Analysis of 23S ribosomal DNA mutants. *Antimicrob. Agents Chemother.*, **44**, 3101–3106.

Attia, M.A. (1996) Neoplastic and non-neoplastic lesions in the mammary gland, endocrine and genital organs in aging male and female Sprague-Dawley rats. *Arch. Toxicol.*, **70**, 461–473.

Boll, R., Hofmann, C., Heitmann, B., Hauser, G., Glaser, S., Koslowski, T., Friedrich, T. & Bechthold, A. (2006) The active conformation of avilamycin A is conferred by AviX12, a radical AdoMet enzyme. *J. Biol. Chem.*, **28**, 14756–14763.

Burnett, T.J. (2005) Avilamycin is poorly absorbed, is extensively metabolized by swine and is not persistent in the environment. *J. Mass Spectrom.*, **40**, 1505.

Chernoff, N., Rofers, J.M., Turner, C.I. & Francis, B.M. (1991) Significance of supernumerary ribs in rodent developmental toxicity studies: Postnatal persistence in rats and mice. *Fundam. Appl. Toxicol.*, **17**, 448–453.

Cifone, M.A. (2004) *Mutagenicity test on crystalline avilamycin in the L5178Y TK mouse lymphoma forward mutation assay*. Unpublished report No. 8180-721 from Covance Laboratories Inc., Vienna, VA, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

CLSI (2004) *Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard*, 6th ed. Wayne, PA, USA, Clinical and Laboratory Standards Institute (CLSI Document M11-A6).

Cochrane, R.L. & Thomson, T.D. (1991) *The safety of avilamycin to turkeys from a dietary source*. Unpublished report No. VX9009 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Codex Alimentarius Commission (2007) *Report of the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods, Breckenridge, CO, USA, 3–7 September*

2007. Rome, Italy, Food and Agriculture Organization of the United Nations (ALINORM 08/31/31; http://www.codexalimentarius.net/web/archives.jsp?year=08).

Dalidowicz, J.E. (1985) *Characterization of ^{14}C residues in fat from swine fed ^{14}C-avilamycin.* Unpublished report No. ABC-0307 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Dalidowicz, J.E., Thomson, T.D. & Herberg, R.J. (1983) *^{14}C-Avilamycin balance–excretion study in swine*. Unpublished report No. ABC-0229 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/ Elanco, Greenfield, IN, USA.

Dalidowicz, J.E., Thomson, T.D. & Herberg, R.J. (1984) *^{14}C-Avilamycin balance–excretion study in chickens*. Unpublished report No. ABC-0230 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/ Elanco, Greenfield, IN, USA.

Delsol, A.A., Randall, L., Cooles, S., Woodward, M.J., Sunderland, J. & Roe, J.M. (2005) Effect of avilamycin on antimicrobial resistance in enteric bacteria in the pig. *J. Appl. Microbiol.*, **98**, 564–571.

Donoho, A.L. & Magnussen, J.D. (1987) *Comparative metabolism of ^{14}C-avilamycin in swine and rats.* Unpublished report No. ABC-0371 from Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Hunter, B., Berryman, L., Heywood, R., Street, A.E., Prentice, D.E., Gibson, W., Harling, S., Abbott, D. & Gopinath, C. (1983a) *CGA 59327 (avilamycin) long-term feeding study in rats following in utero exposure.* Unpublished report No. CBG 187/80979 from Huntingdon Research Centre, Huntingdon, Cambridgeshire, England. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Hunter, B., Graham, C., Heywood, R., Prentice, D.E., Gibson, W.A. & Lewis, D. (1983b) *CGA 59327 (avilamycin) potential tumorigenic effects in prolonged dietary administration to mice*. Unpublished report No. CBG/186-G/80641 from Huntingdon Research Centre, Huntingdon, Cambridgeshire, England. Submitted to WHO by Eli Lilly and Company/ Elanco, Greenfield, IN, USA.

Inui, S. (1984a) *Acute toxicity study of EL-750 in mice—oral and intraperitoneal administration*. Unpublished report from Research Institute for Animal Science in Biochemistry and Toxicology, Sagamihara-Shi, Kanagawa, Japan. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Inui, S. (1984b) *Acute toxicity study with EL-750 in rats—oral and intraperitoneal administration*. Unpublished report from Research Institute for Animal Science in Biochemistry and Toxicology, Sagamihara-Shi, Kanagawa, Japan. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Ito, Y. (1986) *Comparative acute intraperitoneal toxicity study of crystalline form and dried mycelial form of EL-750 in rats.* Unpublished report No. 86-046 from Research Institute for Animal Science in Biochemistry and Toxicology, Sagamihara-Shi, Kanagawa, Japan. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Ito, Y. (1988) *Acute intraperitoneal toxicity study with EL-750 dried mycelial product in mice.* Unpublished report No. 86-040 from Research Institute for Animal Science in Biochemistry and Toxicology, Sagamihara-Shi, Kanagawa, Japan. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Lawler, N.J. (2004a) *A pilot embryo-fetal development study in female CD rats given granular avilamycin (compound 048740) daily by gavage*. Unpublished report No. R00173 from Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/ Elanco, Greenfield, IN, USA.

Lawler, N.J. (2004b) *An embryo-fetal development study in female CD rats given granular avilamycin daily by gavage.* Unpublished report No. R00228 from Eli Lilly and Company,

Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Magnussen, J.D. (1985a) *^{14}C-Avilamycin rat metabolism study*. Unpublished report No. ABC-0311 from Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Magnussen, J.D. (1985b) *Characterization of ^{14}C avilamycin residues in swine liver and excreta*. Unpublished report No. ABC-0309 from Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Magnussen, J.D., Herberg, R.J. & Thomson, T.D. (1984) *^{14}C avilamycin steady-state tissue residue study in swine.* Unpublished report No. ABC 0287 from Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Magnussen, J.D., Herberg, R.J. & Thomson, T.D. (1986) *^{14}C avilamycin steady-state tissue residue study in broilers.* Unpublished report No. ABC-0329 from Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Magnussen, J.D., Donoho, A.L., Herberg, R.J. & Thomson, T.D. (1987) *A steady-state tissue residue study in swine dosed with uniformly labeled ^{14}C-avilamycin.* Unpublished report No. ABC-0360 from Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Magnussen, J.D., Dalidowicz, J.E., Thomson, T.D. & Donoho, A.L. (1991) Tissue residues and metabolism of avilamycin in swine and rats. *J. Agric. Food Chem.*, **39**, 306–310.

McNicholas, P.M., Najarian, D.J., Mann, P.A., Hesk, D., Hare, R.S., Shaw, K.J. & Black, T.A. (2000) Evernimicin binds exclusively to the 50S ribosomal subunit and inhibits translation in cell-free systems derived from both Gram-positive and Gram-negative bacteria. *Antimicrob. Agents Chemother.*, **44**, 1121–1126.

Mecchi, M.S. (2004) Salmonella–Escherichia coli*/mammalian-microsome reverse mutation assay with a confirmatory assay with crystalline avilamycin.* Unpublished report No. 6180-742 from Covance Laboratories Inc., Vienna, VA, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Mertz, J.L., Peloso, J.S., Barker, B.J., Babbitt, G.E., Occolowitz, J.L., Simson, V.L. & Kline, R.M. (1986) Isolation and structural identification of nine avilamycins. *J. Antibiotics*, **39**, 877–887.

Murli, H. (2004) *Chromosomal aberrations in Chinese hamster ovary (CHO) cells with crystalline avilamycin.* Unpublished report No. 6180-738 from Covance Laboratories Inc., Vienna, VA, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Murphy, G.P. & Phelps, J.B. (2004a) *The effect of crystalline avilamycin given orally by gavage for 2 consecutive days on the induction of micronuclei in bone marrow of ICR mice*. Unpublished report No. M00054 from Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Murphy, G.P. & Phelps, J.B. (2004b) *The effect of granular avilamycin given orally by gavage for 2 consecutive days on the induction of micronuclei in bone marrow of ICR mice*. Unpublished report No. M00052 from Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Palmer, A.K., Bottomley, A.M., Leeming, N.M., Clark, R., Offer, J.M. & Gibson, W.A. (1981) *Effect of CGA 59327 (avilamycin) on reproductive function of multiple generations in the rat.* Unpublished report No. CBG/188/80780 from Huntingdon Research Centre, Huntingdon, Cambridgeshire, England. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Pilling, A.M., Jones, S.A., Endersby-Wood, H.J., McCormark, N.A.M. & Turton, J.A. (2007) Expression of thyroglobulin and calcitonin in spontaneous thyroid gland tumors in the Han Wistar rat. *Toxicol. Pathol.*, **35**, 348–355.

Pridmore, A. (2004a) *Activity of avilamycin against bacterial strains representing the normal human intestinal microbiota: Determination of minimum inhibitory concentration (MIC).* DWS Report No. DWS/026/04, Elanco Reference: T4EAUKO4O1. Submitted to FAO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pridmore, A. (2004b) *Effect of faecal binding on antibacterial activity of avilamycin.* DWS Report No. DWS/027/04, Elanco Reference: T4EAUTCO4O2. Submitted to FAO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Quast, J.F., Humiston, C.G., Wade, C.E., Ballard, W.J., Beyer, J.E., Schwetz, R.W. & Norris, J.M. (1983) A chronic toxicity and oncogenicity study in rats and subchronic toxicity study in dogs on ingested vinylidene chloride. *Fundam. Appl. Toxicol.*, **3**, 55–62.

Redgrave, V.A., Cameron, D.M., Gopinath, C., Gregson, R.L. & Ruckman, S.M. (1992) *Avilamycin target animal safety study in turkeys*. Unpublished report No. LLY31/920430 from Huntingdon Research Centre, Huntingdon, Cambridgeshire, England. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Sachsse, K. & Bathe, R. (1977) *Acute oral LD_{50} in the rat of CGA 59327*. Unpublished report No. Siss 5972 from Ciba-Geigy Ltd, Basel, Switzerland. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Sachsse, K. & Bathe, R. (1978a) *Acute oral LD_{50} in the mouse of technical CGA 59327.* Unpublished report No. Siss 6480 from Ciba-Geigy Ltd, Basel, Switzerland. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Sachsse, K. & Bathe, R. (1978b) *Acute intraperitoneal LD_{50} in the mouse of technical CGA 59327.* Unpublished report No. Siss 6480 from Ciba-Geigy Ltd, Basel, Switzerland. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Sachsse, K., Suter, P. & Luetkemeier, H. (1978a) *CGA 59327 28-day oral toxicity study in rats.* Unpublished report No. Siss 6265 from Ciba-Geigy Ltd, Basel, Switzerland. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Sachsse, K., Suter, P. & Luetkemeier, H. (1978b) *CGA 59327 (avilamycin-mycel) 28-day oral toxicity study in rats.* Unpublished report No. Siss 6354 from Ciba-Geigy Ltd, Basel, Switzerland. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Sire, G. (2004) *Avilamycin, evaluation of skin sensitization potential in mice using the local lymph node assay (LLNA).* Unpublished report No. 28382 TSS from CIT, Evreux, France. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Strittmatter, J. (1982) *CGA 59327 tolerability study—8 weeks continuous feeding in chicken.* Unpublished report No. CRA 78/36 from Ciba-Geigy Ltd, Basel, Switzerland. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Strittmatter, J. & Somerville, J.M. (1980) *CGA 59327 tolerability study—21 weeks continuous feeding in pigs.* Unpublished report No. CRA 78/40 from Ciba-Geigy Ltd, Basel, Switzerland. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Suter, P. & Sachsse, K. (1977a) *28-day oral toxicity study in mice with CGA 59327 (avilamycin)*. Unpublished report No. Siss 6265 from Ciba-Geigy Ltd, Basel, Switzerland. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Suter, P. & Sachsse, K. (1977b) *CGA 59327 (avilamycin-mycel) 28-day oral toxicity study in mice.* Unpublished report No. Siss 6354 from Ciba-Geigy Ltd, Basel, Switzerland. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Van Duyn, R.L., Tonkinson, L.V. & Waitt, W.P. (1984) *Summary of two time-replicated studies of the reproductive safety of feeding avilamycin to gilts during the growing–finishing phase*. Unpublished report No. T4E758201/T4E758202 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/ Elanco, Greenfield, IN, USA.

VICH (2004) *Studies to evaluate the safety of residues of veterinary drugs in human food: General approach to establish a microbiological ADI.* Brussels, Belgium, International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH Guideline 36; http://www.vichsec.org/pdf/05_2004/Gl36_st7_F_rev.pdf).

West, S.D. & Anderson, D.B. (1984) *Determination of residues in the feces of swine fed diets containing avilamycin*. Unpublished report Nos I-EWD-81-13 and I-EWD-81-15 from Lilly Research Laboratories, Eli Lilly Co., Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

West, S.D., Poole, G.M. & Wellenreiter, R.J. (1982) *Determination of residues in the blood of broilers fed diets containing avilamycin*. Unpublished report from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. (1983) *A chronic toxicity study of avilamycin (compound 48740, EL-750) administered orally to dogs for six months*. Unpublished report No. D03782 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. (1987) *General pharmacology testing, test substance: avilamycin*. Unpublished report from Biological Research Center for Protection of Environment, Kouka-Gun, Shiga, Japan. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Hagopian, G.S. (1983) *A teratology study of avilamycin (compound 48740, EL-750) administered orally to Dutch Belted rabbits.* Unpublished report No. B03482 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Mattingly, C.L. (1983) *Guinea pig sensitization study of avilamycin compound number 48740 (EL-750).* Unpublished report No. G00283 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Neal, S.B. (1983) *The effect of crystalline avilamycin (compound 48740, EL-750) on the in vivo induction of sister chromatid exchange in bone marrow of Chinese hamsters.* Unpublished report No. 830810SCE1602 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Probst, K.S. (1983a) *A toxicity study of avilamycin dried fermentation product given in the diet to Fischer 344 rats for two weeks.* Unpublished report No. R04983 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Probst, K.S. (1983b) *A toxicity study of crystalline avilamycin given in the diet to Fischer 344 rats for two weeks*. Unpublished report No. R05083 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Quarles, J.P. (1983) *The acute intraperitoneal toxicity of avilamycin (compound 48740, EL-750) in the Fischer 344 rat.* Unpublished report Nos R-P-13-83 and R-P-14-83 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Quarles, J.P. (1984a) *The acute oral toxicity of avilamycin (compound 48740, EL-750) in the ICR mouse*. Unpublished report Nos M00183 and M00283 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Quarles, J.P. (1984b) *The acute oral toxicity of avilamycin (compound 48740, EL-750) in the Fischer 344 rat*. Unpublished report Nos R00783 and R00883 from Lilly

Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Rexroat, M.A. (1983) *The effect of crystalline avilamycin (compound 48740, EL-750) on the induction of reverse mutations in* Salmonella typhimurium *using the Ames test*. Unpublished report No. 831003AMS1602 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Thompson, C.Z. (1983a) *The effect of crystalline avilamycin (compound 48740, EL-750) on the induction of bacterial mutation using a modification of the Ames test*. Unpublished report No. 810803GPA1602 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D. & Thompson, C.Z. (1983b) *The effect of crystalline avilamycin (compound 48740, EL-750) on the induction of bacterial mutation using a modification of the Ames test*. Unpublished report No. 830808GPA1602 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D., Lake, S.G. & Quarles, J.P. (1983a) *The acute oral toxicity of non-micronized avilamycin (compound 48740, EL-750) in the ICR mouse*. Unpublished report Nos M027381 and M027081 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D., Lake, S.G. & Quarles, J.P. (1983b) *The acute oral toxicity of micronized avilamycin (compound 48740, EL-750) in the ICR mouse.* Unpublished report Nos M027281 and M027181 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D., Lake, S.G. & Quarles, J.P. (1983c) *The acute oral toxicity of non-micronized avilamycin (compound 48740, EL-750) in the Fischer 344 rat.* Unpublished report Nos R023381 and R023281 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D., Lake, S.G. & Quarles, J.P. (1983d) *The acute oral toxicity of micronized avilamycin (compound 48740, EL-750) in the Fischer 344 rat.* Unpublished report Nos R023181 and R023081 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D., Arthur, B.H., Gomez, S.R. & Negilski, D.S. (1983e) *The acute dermal, ocular and inhalation toxicity of avilamycin (compound 48740, EL-750), a dried fermentation product*. Unpublished report Nos B-D-24-83, B-E-30-83 and R-H-10-83 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D., Hill, L.E. & Probst, G.S. (1983f) *The effect of crystalline avilamycin (compound 48740, EL-750) on the induction of DNA repair synthesis in primary cultures of adult rat hepatocytes.* Unpublished report Nos 830927UDS1602 and 831005UDS1602 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D., Oberly, T.J. & Bewsey, B.J. (1983g) *The effect of avilamycin (Lilly compound 48740, EL-750) on the induction of forward mutation at the thymidine kinase locus of L5178Y mouse lymphoma cells.* Unpublished report No. 830809MLA1602 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Williams, G.D., Probst, G.S. & Hill, L.E. (1984) *The effect of crystalline avilamycin (Lilly compound 48740, EL-750) on the induction of DNA repair synthesis in primary cultures of adult rat hepatocytes.* Unpublished report No. 810630UDS1602 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Eli Lilly and Company/Elanco, Greenfield, IN, USA.

Wolf, H. (1973) Avilamycin, an inhibitor of the 30 S ribosomal subunits function. *FEBS Lett.*, **36**,181–186.

MALACHITE GREEN

First draft prepared by

Dr Donald Grant,[1] Dr John Christian Larsen,[2] Dr Rajinder Sharma,[3] Mr Mark Feeley[4] and Dr Dieter Arnold[5]

[1] Consultant, D.L. Grant & Associates, Ottawa, Ontario, Canada
[2] National Food Institute, Technical University of Denmark, Søborg, Denmark
[3] Veterinary Drugs Directorate, Health Canada, Ottawa, Ontario, Canada
[4] Food Directorate, Health Canada, Ottawa, Ontario, Canada
[5] Consultant, Berlin, Germany

Explanation 37
Biological data 38
Biochemical aspects 38
Absorption, distribution, excretion and metabolism 38
Toxicological studies 40
Acute toxicity 40
Short-term studies of toxicity of MG 40
Short-term studies of toxicity of LMG 42
Carcinogenicity of MG 44
Carcinogenicity of LMG 45
Genotoxicity 46
Reproductive toxicity 50
Special studies 50
Observations in humans 54
Dietary exposure 54
Comments 54
Biochemical data 54
Toxicological data 55
Malachite green 55
Leucomalachite green 57
Consideration of mode of action for MG and LMG 59
Preliminary dietary exposure considerations 60
Evaluation 62
References 66

1. EXPLANATION

Malachite green (MG) is an *N*-methylated triphenylmethane dye that is used mainly industrially for leather, wool, cotton, silk, jute, paper and certain fibres. For such purposes, large quantities of extremely variable composition have been produced. About 10–15% of all dyes are lost directly to wastewater in the dyeing process.

The chemical has been used routinely in some countries in aquaculture since the early 1930s and is considered by many in the fish industry to be an effective antifungal and antiprotozoal agent in fish, fish eggs and crayfish. It is currently

registered in some countries for use as a veterinary drug in ornamental fish, to which it is applied as a topical antiseptic or to treat parasites, fungal infections and bacterial infections in fish and fish eggs. It is not permitted in aquaculture of fish destined for human consumption. Reported types of treatment of fish include dip treatment, flush treatment, sustained culture treatment and application in feed. Extremely wide ranges of concentrations and exposure times have been used.

MG in water originating from contamination as a result of its industrial applications or from its illegal use in aquaculture is efficiently taken up from the water by fish and distributed to all tissues. MG is metabolically reduced by fish to the persistent colourless metabolite, leucomalachite green (LMG), and possibly other, as yet unidentified, degradation products (Plakas et al., 1996). The rate of excretion of MG (as LMG) from fish is dependent on the fat content of the fish, with more LMG being retained in fatty fish than in lean fish. Therefore, when fish that have been exposed to MG reach the consumer, the amount of LMG present in the fish is expected to be higher than that of MG, because of its longer elimination half-life. As LMG is the predominant residue found in fish tissues following exposure to MG, it is the residue of primary concern from a safety point of view.

MG has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). It was placed on the agenda of the current meeting at the request of the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods (Codex Alimentarius Commission, 2007), which requested JECFA to consider a literature review and advise if this substance could be supported for use in food-producing animals (as the available data were probably not sufficient to derive an acceptable daily intake [ADI] and maximum residue limits [MRLs]).

Two risk assessments were provided by national authorities. In addition, a comprehensive literature search was performed.

2. *BIOLOGICAL DATA*

2.1 *Biochemical aspects*

2.1.1 *Absorption, distribution, excretion and metabolism*

(a) Bacteria

Henderson et al. (1997) investigated the reduction of MG to LMG by intestinal bacteria from humans, rats, mice and rhesus monkeys and 14 pure cultures of anaerobic bacteria representative of those found in the human gastrointestinal tract. The bacterial cultures were incubated with 300 µg of MG in 5 ml of brain–heart infusion broth for 24–48 h under anaerobic conditions. Virtually all of the MG was converted to its LMG derivative by the intestinal microflora. The pure bacterial cultures converted 7.3–99.3% of the MG to LMG. These results indicate the importance of the gastrointestinal tract microflora in the conversion of MG to LMG.

(b) Mice and rats

Three male and three female rats weighing about 300–325 g were given a single dose (2 mg/kg body weight [bw]) of ^{14}C-labelled MG in water by gavage. Urine and faeces were collected daily after MG administration. One week after MG administration, the liver, kidney, muscle, skin and blood were removed from the rats and stored at −20 °C prior to analysis. About 96.3% ± 5.9% (mean ± standard deviation) of the orally administered dose was excreted in the urine and faeces of rats over the 7-day study period, with more than 80% appearing in the faeces. Since the rat tissues showed only low levels of ^{14}C, they were not investigated further (Law, 1994).

In short-term feeding studies, Culp et al. (1999) showed that MG is sequentially *N*-demethylated to secondary and primary aromatic amines in rats and mice both before and after reduction to LMG. Female mice (eight per dose group; B6C3F1:Nctr BR (C57BL/6N × C3H/HeN MTV−)) as well as male rats (eight per dose group; F344:N Nctr BR) were fed 0, 100 or 600 mg MG/kg diet (equivalent to 0, 15 and 90 mg MG/kg bw per day in mice and 0, 10 and 60 mg MG/kg bw per day in rats) (as the chloride salt, ≥94% purity) or 0, 96 or 580 mg LMG/kg diet (equivalent to 0, 13.7 and 72 mg LMG/kg bw per day in mice and 0, 9.6 and 58 mg LMG/kg bw per day in rats) (≥98% purity) for 28 days. Liver extracts from the mice and rats were analysed by high-performance liquid chromatography in combination with atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI/MS).

Analysis of liver extracts from rats treated with MG detected the molecular ions for MG, its mono-, di-, tri- and tetradesmethyl derivatives and MG *N*-oxide. A small, but measurable, amount of LMG was also detected. Concentrations of MG and metabolites increased with increasing dose. Similarly, in liver extracts from rats treated with LMG, primarily protonated LMG, protonated demethylated derivatives and the molecular ions of MG *N*-oxide and demethylated *N*-oxide derivatives were seen. A small, but measurable, amount of MG was also found. A dose-related increase in LMG and metabolites was observed in both rat and mouse liver extracts.

The demethylated metabolites observed in the livers of rats fed MG or LMG are presented in Figure 1 (Culp et al., 1999).

Figure 1. Demethylated metabolites in livers of rats fed MG or LMG

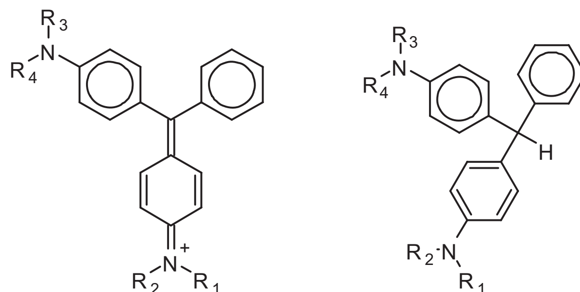


Figure 1 (contd)

Structures of MG, LMG and demethylated derivatives	Malachite green				Leucomalachite green			
	R_1	R_2	R_3	R_4	R_1	R_2	R_3	R_4
Parent molecule	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3
Desmethyl-	CH_3	CH_3	CH_3	H	CH_3	CH_3	CH_3	H
Didesmethyl- (symmetric)	CH_3	H	CH_3	H	CH_3	H	CH_3	H
Tridesmethyl-	CH_3	H	H	H	CH_3	H	H	H
Tetradesmethyl-	H	H	H	H	H	H	H	H

(c) Humans

No studies on the absorption, distribution, metabolism or excretion of MG or LMG in humans were found in a review of the literature (NTP, 2005).

2.2 Toxicological studies

2.2.1 Acute toxicity

Results of studies of the acute toxicity of MG are summarized in Table 1.

Table 1. Results of studies of the acute toxicity of MG

Species (sex)	Route	LD_{50} (mg/kg bw)	Reference
NMRI mice	Gavage	50	Clemmensen et al. (1984)
Wistar rats (M & F)	Gavage	275	Clemmensen et al. (1984)
Wistar rats (M & F)	Dermal	>2000	Clemmensen et al. (1984)
SD rats (F)	Oral	520	Meyer & Jorgenson (1983)

F, female; LD_{50}, median lethal dose; M, male.

The acute effects observed in rats were reduced motor activity, diarrhoea and piloerection, but only for the 1st day. The major findings were hyperaemia and atonia of the intestinal walls, often in conjunction with dilatation of the gastrointestinal tract as far as the substance had reached before the death of the animal. Survivors were free of symptoms after 2 days (Clemmensen et al., 1984).

2.2.2 Short-term studies of toxicity of MG

(a) Mice

In a study compliant with Good Laboratory Practice (GLP), groups of eight female and eight male mice (B6C3F1:Nctr BR (C57BL/6N × C3H/HeN MTV−))

(6–7 weeks old) were given MG (as the chloride salt, purity >94%) at concentrations of 0, 25, 100, 300, 600 or 1200 mg/kg feed (equivalent to 0, 3.75, 15, 45, 90 and 180 mg MG/kg bw per day) for 28 days. Haematology measurements included leukocyte count, erythrocyte count, haemoglobin, haematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count. Clinical chemistry measurements included total protein, bile acids, blood urea nitrogen, creatinine, alanine aminotransferase (ALT) and alkaline phosphatase.

Female mice fed 1200 mg MG/kg diet had significantly lower body weights (91–92% of control group) at weeks 3 ($P < 0.02$) and 4 ($P < 0.03$). The body weights of male mice were not significantly affected at any of the dose levels of MG. The female mice fed 600 or 1200 mg MG/kg diet had significant decreases in the erythrocyte count and haemoglobin and haematocrit levels as compared with the control group; in male mice, significant decreases in these parameters were observed only in the 1200 mg MG/kg diet dose group. The mean erythrocyte volume was increased ($P < 0.05$) in female mice fed 300, 600 or 1200 mg MG/kg diet as compared with the control group. There was also a 1.4- to 1.9-fold increase in reticulocytes in these groups ($P < 0.05$). Male mice fed 1200 mg MG/kg diet showed a 1.6-fold increase in reticulocytes ($P < 0.05$). There were no significant histopathological changes observed in the mice fed MG (Culp et al., 1999). The no-observed-adverse-effect level (NOAEL) was 100 mg/kg feed, equivalent to 15 mg MG/kg bw per day.

(b) Rats

In a non-GLP-compliant study, groups of eight male and eight female Wistar rats were given <0, 10, 100 or 1000 mg MG/kg feed as MG oxalate (purity >90%) (equivalent to 0, 1, 10 and 100 mg MG/kg bw per day) for 28 days. Blood samples were taken after 3 weeks and analysed for alkaline phosphatase, aspartate aminotransferase (AST), urea, creatinine, glucose and methaemoglobin. The numbers of red blood cells, white blood cells and reticulocytes were counted. Differential counts of white blood cells were performed on smears and packed cell volume. All animals were autopsied, and tissues from liver, kidney, adrenals and testis were weighted and prepared for microscopy. No clinical effect of MG was observed in the treated animals, apart from apparent hyperactive behaviour in the rats in the highest dose group. These animals also had a significant reduction in weight gain and a reduced food intake. In females in the 1000 mg/kg diet group, an increase in lymphocytes and a concomitant decrease in neutrophils and a slight but significant decrease in packed cell volume were observed. The males in the 1000 mg/kg diet group showed a significant increase in plasma urea (Clemmensen et al., 1984). The NOAEL was 100 mg MG/kg feed, equivalent to 10 mg MG/kg bw per day.

In a GLP-compliant study, groups of eight female and eight male rats (F344/N Nctr BR) (6–7 weeks of age) were given MG (as the chloride salt, purity >94%) at dietary concentrations of 0, 25, 100, 300, 600 or 1200 mg/kg feed (equivalent to 0, 2.5, 10, 30, 60 or 120 mg MG/kg bw per day) for 28 days. Haematology

measurements included leukocyte count, erythrocyte count, haemoglobin, haematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count. Clinical chemistry measurements included total protein and bile acids, blood urea nitrogen, creatinine, ALT, alkaline phosphatase, AST, glucose, cholesterol, triglycerides, gamma-glutamyl transferase (GGT), albumin, sorbitol dehydrogenase, creatine kinase, sodium, potassium, chloride, calcium and phosphorus.

In female rats, there were significant decreases in the mean body weights in the 1200 mg MG/kg diet dose group for weeks 1–4, with the animals weighing 80–83% of the control rats. Although the male rats fed 1200 mg MG/kg diet tended to have lower body weights (82–87%), compared with the control group, the differences were not significant. The female rats of the 300, 600 and 1200 mg MG/kg diet groups had significantly increased ratios of liver weights to body weights. The ratio of liver weight to body weight was significantly increased in the male rats fed 600 and 1200 mg MG/kg diet. In both sexes, there was a significant linear increasing trend in the levels of GGT, with the value in females in the 1200 mg MG/kg diet dose group being 4.2-fold greater ($P < 0.0005$) than that in the controls. Blood haematology measurements in female rats showed slight (<7%), but significant, decreases in the 1200 mg MG/kg diet dose group in erythrocyte count, haemoglobin, haematocrit, mean erythrocyte haemoglobin and mean erythrocyte haemoglobin concentration. Male rats had slight (<3%), but significant, decreases in mean erythrocyte haemoglobin in the 300, 600 and 1200 mg MG/kg diet dose groups. Seven out of eight female rats fed 1200 mg MG/kg diet had minimal to mild hepatocyte vacuolization ($P < 0.01$). The same lesion, primarily midzonal in location, was observed in one and four male rats fed 600 and 1200 mg MG/kg diet, respectively (Culp et al., 1999). The NOAEL was 100 mg MG/kg feed, equivalent to 10 mg MG/kg bw per day.

2.2.3 Short-term studies of toxicity of LMG

(a) Mice

In a GLP-compliant study, groups of eight female mice (B6C3F1/Nctr BR (C57BL/6N × C3H/HeN MTV−)) were given LMG (>98% pure) at 0, 290, 580 or 1160 mg/kg diet (equivalent to 0, 43.5, 87 and 174 mg/kg bw per day) for 28 days. Haematology measurements included leukocyte count, erythrocyte count, haemoglobin, haematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count. Clinical chemistry measurements included total protein and bile acids, blood urea nitrogen, creatinine, ALT and alkaline phosphatase.

The female mice fed 1160 mg LMG/kg diet had significantly lower body weights (93% of the control group) at week 4. A marginally significant decrease in body weight ($P < 0.01$) from the control group was also observed in the female mice fed 580 mg LMG/kg diet at week 4. In addition, there were statistically significant linear dose trends for week 3 ($P < 0.02$) and week 4 ($P < 0.002$). All female mice

fed 1160 mg LMG/kg diet had scattered dead or degenerate cells in the transitional epithelium of the urinary bladder ($P < 0.001$). Many of the cells lacked nuclei; when visible, the nuclei were condensed or fragmented, which the authors thought suggested apoptosis. Examination of thin sections revealed that many apparent apoptotic cells were contained within phagocytic vacuoles inside viable epithelial cells. The in situ end labelling technique for detecting deoxyribonucleic acid (DNA) fragmentation showed that the cytoplasm of apparently apoptotic cells was moderately positive for the presence of DNA fragments, and condensed nuclei stained intensely for DNA fragmentation. Individual cell necrosis was not accompanied by inflammatory changes. Similar apoptosis was not seen in transitional epithelium of the bladders of female mice fed 0, 290 or 580 mg LMG/kg diet (Culp et al., 1999). The NOAEL was 290 mg LMG/kg diet, equivalent to 43.5 mg LMG/kg bw per day.

(b) Rats

In a GLP-compliant study, groups of eight male rats (F344/N Nctr BR) (6–7 weeks of age) were given 0, 290, 580 or 1160 mg LMG/kg diet (equivalent to 0, 29, 58 and 116 mg LMG/kg bw per day) for 28 days. Haematology measurements included leukocyte count, erythrocyte count, haemoglobin, haematocrit, mean erythrocyte volume, mean erythrocyte haemoglobin, mean erythrocyte haemoglobin concentration, platelet count, segmented neutrophils, lymphocytes, monocytes, eosinophils and reticulocyte count. Clinical chemistry measurements included total protein and bile acids, blood urea nitrogen, creatinine, ALT, alkaline phosphatase, AST, glucose, cholesterol, triglycerides, GGT, albumin, sorbitol dehydrogenase, creatine kinase, sodium, potassium, chloride, calcium and phosphorus.

Male rats fed 1160 mg LMG/kg diet had significantly lower body weights (91–92% of the control group) at weeks 2, 3 and 4. There were also significant decreases in body weights in the 580 mg LMG/kg diet group at weeks 3 and 4 (94% of the control group). The ratio of liver weights to body weights was significantly increased for all three dose groups as compared with the control group. GGT levels were 2.2-fold higher ($P < 0.05$) and phosphorus levels were slightly increased (10%; $P < 0.05$) in rats fed 1160 mg LMG/kg diet. In addition, erythrocyte count, haemoglobin and haematocrit levels showed slight (<6%), but significant, decreases from the controls in the 1160 mg/kg diet dose group. Hepatocyte vacuolization, primarily midzonal and centrilobular in location, was seen in seven rats fed 1160 mg LMG/kg diet ($P < 0.005$), five rats fed 580 mg LMG/kg diet ($P < 0.04$) and two rats fed 290 mg LMG/kg diet, a significant dose trend ($P < 0.0004$). Two rats fed 1160 mg LMG/kg diet and two rats fed 580 mg LMG/kg diet had apoptotic follicular epithelial cells in the thyroid gland. Morphological changes consisted of sloughed follicular cells with condensed nuclei located within the follicles. An inflammatory reaction was not present. There was evidence of follicular epithelium regeneration, since even the most severely affected follicles were still lined by viable epithelium (Culp et al., 1999). A NOAEL was not established in this study.

2.2.4 Carcinogenicity of MG

(a) Mice

In a GLP-compliant study, groups of 48 female B6C3F1/Nctr Br (C57BL/6N × C3H/HeN MTV) mice (approximately 6 weeks old) were fed 0, 100, 225 or 450 mg MG/kg diet (as the chloride, 87% pure) (equal to 0, 15, 33 and 67 mg MG/kg bw per day) for 104 weeks. NIH-31 meal and Millipore-filtered tap water were available ad libitum throughout the study, and the animals were maintained on a 12-h light–dark cycle. Homogeneity and stability tests were conducted on the diets to ensure the integrity of the test materials. Food consumption and individual body weights were recorded weekly for the first 12 weeks and approximately every 4 weeks thereafter. Complete necropsies were performed on all mice, including those that died or became moribund. All major tissues were fixed and preserved in 10% neutral buffered formalin. Tissues were processed, trimmed, embedded and stained with haematoxylin and eosin for microscopic examination. There were no treatment-related effects upon food consumption, body weights, survival or incidence of neoplasms in the female mice (Culp et al., 2006).

(b) Rats

In a GLP-compliant study, groups of 48 female F344/N Nctr Br rats were fed 0, 100, 300 or 600 mg MG/kg diet (as the chloride, 87% pure) (equal to 0, 7, 21 and 43 mg MG/kg bw per day) for 104 weeks. NIH-31 meal and Millipore-filtered tap water were available ad libitum throughout the study, and the animals were maintained on a 12-h light–dark cycle. Homogeneity and stability tests were conducted on the diets to ensure the integrity of the test materials. Food consumption and individual body weights were recorded weekly for the first 12 weeks and approximately every 4 weeks thereafter. Complete necropsies were performed on all rats, including those that died or became moribund. All major tissues were fixed and preserved in 10% neutral buffered formalin. Tissues were processed and trimmed, sectioned, embedded and stained with haematoxylin and eosin for microscopic examination.

Food consumption was not affected by treatment. The mean body weights were statistically decreased compared with controls beginning at 16, 44 and 76 weeks in rats fed 600, 300 and 100 mg MG/kg diet. These final body weights were approximately 88%, 90% and 98%, respectively, of control group weight. The survival of female rats was not affected by feeding MG. Female rats fed MG had an increasing trend (0/46, 0/48, 3/47 and 2/46) in the incidence of thyroid gland follicular cell adenoma or carcinoma, with the increase being significant only at 300 mg MG/kg diet. Hepatocellular adenomas were minimally (1/48, 1/48, 3/48 and 4/48), but not statistically significantly, increased at the two highest dose levels. In addition, a non-significant trend (2/48, 2/48, 1/48 and 5/48) in mammary gland carcinoma was observed. There was also a dose-related decreasing trend in the incidence of mononuclear cell leukaemia, with the decrease being significant in all dose groups except for the 100 mg/kg diet group rats (Culp et al., 2006). The NOAEL for non-cancer effects was 100 mg MG/kg diet, equal to 7 mg MG/kg bw per day.

2.2.5 Carcinogenicity of LMG

(a) Mice

In a GLP-compliant study, groups of 48 female B6C3F1/Nctr Br (C57BL/6N × C3H/HeN MTV) mice (approximately 6 weeks old) were fed 0, 91, 204 or 408 mg LMG/kg diet (99% pure) (equal to 0, 15, 31 or 63 mg LMG/kg bw per day) for 104 weeks. NIH-31 meal and Millipore-filtered tap water were available ad libitum throughout the study, and the animals were maintained on a 12-h light–dark cycle. Homogeneity and stability tests were conducted on the diets to ensure the integrity of the test materials. Food consumption and individual body weights were recorded weekly for the first 12 weeks and approximately every 4 weeks thereafter. Complete necropsies were performed on all mice, including those that died or became moribund. All major tissues were fixed and preserved in 10% neutral buffered formalin. Tissues were processed and trimmed, embedded, sectioned and stained with haematoxylin and eosin for microscopic examination.

There were no treatment-related effects upon food consumption, body weights or survival. A dose-related increasing trend (3/47, 6/48, 6/47 and 11/47) in the incidence of hepatocellular adenoma or carcinoma was noted, with the incidence being significant in the highest dose group (Culp et al., 2006).

(b) Rats

In a GLP-compliant study, groups of 48 female and 48 male F344/N Nctr Br rats were fed LMG (99% pure) at 0, 91, 272 or 543 mg/kg diet (equal to 0, 6, 17 and 35 mg LMG/kg bw per day for females and 0, 5, 15 and 30 mg LMG/kg bw per day for males) for 104 weeks. NIH-31 meal and Millipore-filtered tap water were available ad libitum throughout the study, and the animals were maintained on a 12-h light–dark cycle. Homogeneity and stability tests were conducted on the diets to ensure the integrity of the test materials. Food consumption and individual body weights were recorded weekly for the first 12 weeks and approximately every 4 weeks thereafter. Complete necropsies were performed on all rats, including those that died or became moribund. All major tissues were fixed and preserved in 10% neutral buffered formalin. Tissues were processed and trimmed, embedded, sectioned and stained with haematoxylin and eosin for microscopic examination.

Female and male rats fed 543 mg LMG/kg diet consumed less food, intermittently, than control rats; the same observation was noted with female rats fed 272 mg LMG/kg diet. Statistically significant body weight decreases were observed beginning at 8, 16 and 52 weeks in female rats fed 543, 272 or 91 mg LMG/kg diet, with the final body weights being approximately 77%, 90% and 95% of the control group weight, respectively. LMG had a less severe effect on the male rats. Statistically significant decreases were observed beginning at 16, 20 and 88 weeks in male rats fed 543, 272 and 91 mg LMG/kg diet, respectively, with the final body weights being approximately 89%, 93% and 99% of the control group weight, respectively. The survival of female rats was not affected by feeding LMG. The survival of male rats was also not affected by LMG, except for the 272 mg LMG/kg diet group, which had an increased survival. Female and male rats exposed to

0, 91, 272 and 543 mg LMG/kg diet also had a low incidence (0/46, 1/46, 2/47 and 1/48 and 0/47, 2/47, 1/48 and 3/46, respectively) (2–7%) of thyroid gland follicular cell adenoma or carcinoma. Although the increase was not statistically significant, this neoplasm was not detected in control rats. The mammary gland adenoma or carcinoma tumour incidence in the 0, 91, 272 and 543 mg LMG/kg diet group female rats was 0/48, 2/48, 3/48 and 4/48, respectively. Female and male rats had a dose-related decreasing trend in the incidence of mononuclear cell leukaemia, with the decrease being significant in all dose groups. With male rats only, there was a decreasing trend in pituitary gland adenoma, with the decrease being significant at all doses (Culp et al., 2006). The NOAEL for non-cancer end-points was 91 mg LMG/kg diet, equal to 5 mg LMG/kg bw per day.

2.2.6 Genotoxicity

In assays performed in compliance with GLP, the genotoxic and mutagenic effects of MG (Table 2) and LMG (Table 3) were investigated. In vitro tests for genotoxicity of MG are complicated by its strong cytotoxicity towards bacterial and mammalian cells in culture. In one of two experiments, MG (as the oxalate salt) caused mutations in *Salmonella typhimurium* strain TA98 with metabolic activation (S9 mix), but not in any other strains tested or in TA98 without metabolic activation (Clemmensen et al., 1984). MG did not induce mutations in Chinese hamster ovary cells (CHO-K1) and produced DNA damage in the comet assay in CHO-K1 cells only at cytotoxic concentrations (Fessard et al., 1999). MG (as the oxalate salt) did not produce clastogenic effects in the in vivo mouse bone marrow micronucleus test at the maximum tolerated dose of 37.5 mg/kg bw (Clemmensen et al., 1984). However, it was not known whether the test compound had reached the bone marrow. MG did not increase the occurrence of recessive spots when tested in the mammalian spot test (in mice) at doses up to 40 mg/kg bw (Jensen, 1984). MG did not induce micronuclei in erythrocytes, *Hprt* mutations in lymphocytes or (in contrast to LMG) *cII* mutations in liver cells of female Big Blue B6C3F1 transgenic mice administered 450 mg/kg diet (equal to 67.5 mg MG/kg bw per day) for up to 16 weeks (Mittelstaedt et al., 2004). MG gave rise to a single DNA adduct species, the level of which increased linearly with dose in the mouse and rat (Culp et al., 1999).

LMG is much less cytotoxic than MG to bacterial and mammalian cells in vitro and could therefore be tested for mutagenicity at higher concentrations. LMG did not induce mutations in any of the tested *S. typhimurium* strains or in Chinese hamster ovary cells (CHO-K1) and was negative in the comet assay in CHO-K1 cells (Fessard et al., 1999).

LMG did not produce any significant increase in the *lacI* mutation frequencies and changes in the mutation spectrum of *lacI* mutants in female Big Blue rats administered dietary doses of 0, 0.9, 2.7, 9.1, 27.2 or 54.3 mg LMG/kg bw per day for 4, 16 or 32 weeks (Culp et al., 2002). In addition, no effect was observed in the liver *cII* mutation frequency (Mittelstaedt et al., 2004), the *Hprt* lymphocyte mutant assay and the bone marrow micronucleus assay in these rats (Manjanatha et al., 2004). LMG gave rise to a single DNA adduct species, the level of which increased linearly with the dose (Culp et al., 2002).

Table 2. Results of tests for genotoxicity and mutagenicity with MG

Test system	Test object	Concentration	Results	References
In vitro				
Ames test[a]	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0.05–160[b] μg/plate	Negative[c] except for TA98 with activation	Clemmensen et al. (1984)
Ames test[d]	*S. typhimurium* TA97, TA98, TA100 and TA102	0.01–10[b] μg/plate	Negative[e]	Fessard et al. (1999)
Hprt assay[f]	CHO-K1 cells	0.001–1 μg/ml and 1–20 μg/ml[g]	Negative[h]	Fessard et al. (1999)
SCGE (comet) assay	CHO-K1 cells	1–20[i] μg/ml	DNA damage at cytotoxic doses of >3 μg (no activation)[j] and 15 μg (activation)[k]	Fessard et al. (1999)
In vivo				
Micronucleus assay	Mice bone marrow	37.5 mg/kg bw	Negative	Clemmensen et al. (1984)
Mammalian spot test	Mice[l]	10, 20 or 40 mg/kg bw	Negative	Jensen (1984)
Micronucleus	Big Blue B6C3F1 (F) mice[m] peripheral blood	450 mg/kg diet for 4 or 16 weeks	Negative	Mittelstaedt et al. (2004)
Lymphocyte *Hprt* mutant frequency	Big Blue B6C3F1 (F) mice[m] spleen	450 mg/kg diet for 4 or 16 weeks	Negative	Mittelstaedt et al. (2004)
cII mutant frequency	Big Blue B6C3F1 (F) mice[m] liver	450 mg/kg diet for 16 weeks	Negative	Mittelstaedt et al. (2004)
DNA adduct formation	Mice[n]	0, 100 or 600 mg/kg diet for 28 days	Positive, a single DNA adduct formed[o]	Culp et al.(1999)
DNA adduct formation	Rat[p]	0, 100 or 600 mg/kg diet for 28 days	Positive[q]	Culp et al. (1999)

F, female; SCGE, single-cell gel electrophoresis.

[a] Organisation for Economic Co-operation and Development (OECD) Test Guideline, with and without metabolic activation.

Table 2 (contd)

[b] Each dose tested in triplicate, and all assays in duplicate.
[c] Cell toxicity was usually encountered at 1.28 µg/plate unless S9 was added.
[d] OECD Test Guideline 471, with and without metabolic activation.
[e] Cell toxicity above 0.5 µg/plate.
[f] OECD Test Guideline 476, with and without activation.
[g] 1–10 µg/plate without activation and 1–20 µg/plate with activation.
[h] MG was very cytotoxic and could be evaluated only for concentrations up to 0.05 µg/ml.
[i] Each dose was tested in duplicate, and at least two independent assays were performed.
[j] There was a fair relationship between cytotoxicity and DNA lesions.
[k] Cell viability decreased by <20%.
[l] Pregnant C57B1/6J Han mice were treated by gavage with 10, 20 or 40 mg MG/kg bw on days 8, 9 and 10.
[m] Twelve per group.
[n] B6C3F1:Nctr BR (C57BL/6N × C3H/HeN MTV−) female mice, eight per group.
[o] A single adduct or co-eluting adducts with a dose-related response. The mice fed the 600 mg/kg diet had a significantly higher adduct level than the corresponding (580 mg/kg diet) group fed LMG.
[p] F344:N Nctr BR male rats, eight per group.
[q] A single adduct or co-eluting adducts with a dose-related response.

Table 3. Results of tests for genotoxicity and mutagenicity with LMG

Test system	Test object	Concentration	Results	References
In vitro				
Ames test[a]	*S. typhimurium* TA97, TA98, TA100 and TA102	10–2000[b] µg/plate	Negative	Fessard et al. (1999)
Hprt assay[c]	CHO cells	5–100 µg/ml	Negative	Fessard et al. (1999)
SCGE (comet) assay	CHO cells	5–500,[d] 25–300[e] µg/ml	Negative	Fessard et al. (1999)
In vivo				
DNA adduct formation	Mice (F)[f]	0, 96 or 580 mg/kg diet for 28 days	Positive[g]	Culp et al. (1999)
Micronucleus	Big Blue B6C3F1 mice (F)[h] peripheral blood	0, 204 or 408 mg/kg diet for 4 or 16 weeks	Negative	Mittelstaedt et al. (2004)
Lymphocyte *Hprt* mutant frequency	Big Blue B6C3F1 mice (F)[h] spleen	0, 204 or 408 mg/kg diet for 4 or 16 weeks	Negative	Mittelstaedt et al. (2004)
cII mutant frequency	Big Blue B6C3F1 mice (F)[h] liver	0, 204 or 408 mg/kg diet for 16 weeks	Positive[i]	Mittelstaedt et al. (2004)
DNA adduct formation	Rat (M)[f]	0, 96 or 580 mg/kg diet for 28 days	Positive[g]	Culp et al. (1999)

Table 3 (contd)

Test system	Test object	Concentration	Results	References
lacI mutant assay[j]	Big Blue rats (F)[k]	0, 9, 27, 91, 272 or 543 mg/kg diet[l]	Increased mutant frequency only at 16 weeks for 543 mg/kg diet group[m]	Culp et al. (2002)
DNA adduct formation	Big Blue rats (F)[n]	0, 9, 27, 91, 272 or 543 mg/kg diet for 28 days	Positive at ≥91 mg/kg diet[g]	Culp et al. (2002)
Hprt lymphocyte mutant assay[o]	Big Blue rats (F)[k]	0, 9, 27, 91, 272 or 543 mg/kg diet[l]	Negative[p]	Manjanatha et al. (2004)
Bone marrow micronucleus assay	Big Blue rats (F)[k]	0, 9, 27, 91, 272 or 543 mg/kg diet[l]	Negative[q]	Manjanatha et al. (2004)
cII mutant frequency	Big Blue rats (F)[r]	0 or 543 mg/kg diet for 16 weeks	Negative[s]	Mittelstaedt et al. (2004)

F, female; M, male; SCGE, single-cell gel electrophoresis.

[a] OECD Test Guideline 471, with and without metabolic activation.

[b] Precipitated at concentrations of or higher than 500 μg/plate.

[c] OECD Test Guideline 476, with and without activation.

[d] No activation. Each dose was tested in duplicate, and at least two independent assays were performed.

[e] Activation.

[f] Eight per group.

[g] A single adduct or co-eluting adducts with a dose-related increase. The mice fed the 580 mg LMG/kg diet had a significantly lower adduct level than the corresponding group fed the 600 mg MG/kg diet.

[h] Twelve mice per group.

[I] The mutation spectrum in LMG-treated mice revealed an increase of G → T and A → T transversions, the types of mutations typical of those produced by bulky arylamine carcinogens.

[j] This assay measures mutations from base pair substitutions, frameshifts and small deletions.

[k] Six rats per group.

[l] Six-week-old rats were fed the diets for 4, 16 or 32 weeks.

[m] When corrected for clonality, the 16-week *lacI* mutation frequency was not significantly different from the clonally corrected control (Manjanatha et al., 2004).

[n] Four rats per group.

[o] The lymphocytes were obtained from the spleens of the treated animals.

[p] The mutant frequency in the lymphocytes of rats fed the control diet ranged from 3×10^{-6} to 12×10^{-6}, whereas the mutant frequency in rats fed LMG ranged from 2×10^{-6} to 11×10^{-6}.

[q] None of the LMG doses or time points showed a significant increase in Hprt over the appropriate control.

[r] Six per group.

[s] There was no increase in either cell mutant or mutation frequency in Big Blue female rats treated with 543 mg LMG/kg diet.

LMG did not induce micronuclei in erythrocytes or mutations in lymphocytes of female Big Blue B6C3F1 transgenic mice administered 30.6 or 61.2 mg LMG/kg bw per day for 16 weeks. However, LMG induced *cII* mutations with an increased frequency of guanine to thymine (G → T) and adenine to thymine (A → T) transversions in the liver cells of the female mice at the highest dose level (Mittelstaedt et al., 2004).

2.2.7 Reproductive toxicity

In a non-GLP-compliant study, Meyer & Jorgenson (1983) gavaged pregnant New Zealand White rabbits (20 per dose group) with MG (as the oxalate salt, technical grade) at doses of 0, 5, 10 or 20 mg/kg bw per day or with 150 mg thalidomide/kg bw per day from days 6 through 18 of gestation. Improper gavaging resulted in the loss of two rabbits in the 5 and 20 mg MG/kg bw per day groups. All rabbits were observed daily, and body weights were recorded on days 0, 6, 9, 12, 15, 18 and 29, when they were killed and progeny were delivered by caesarean section. Resorption sites were recorded, and all young were examined, weighed and incubated for 24 h. During incubation, pups were examined hourly for viability during the first 4 h and then after 24 h. After 24 h, all progeny were killed, sexed and examined for gross developmental anomalies. Approximately one third were dissected to check for visceral anomalies, and the remainder were examined for skeletal anomalies after staining with alizarin red S.

The rabbits treated with MG consumed less food and had lower body weights than the controls. In the three MG-treated groups, there were significant increases in pre-implantation losses and in the ratio of dead implants and decreases in the number of living fetuses. The mean body weights of the fetuses of the MG-treated groups were less than those of the control group. Viability after 24 h was not consistently affected by the MG treatment. A variety of developmental anomalies were observed in the MG-treated groups; although a dose–response was not evident, they were approximately twice those of the negative control. A NOAEL was not established. The thalidomide positive control group had decreased body weight gain for the does, number of live fetuses, body weight of fetuses and viability, along with an increase in developmental anomalies.

2.2.8 Special studies

(a) Thyroid status parameters

(i) MG

In a GLP-compliant study, groups of eight male and eight female rats (F344/N Nctr BR) (6–7 weeks of age) were fed 0 or 1200 mg MG/kg diet (as the chloride salt; equivalent to 0 or 120 mg MG/kg bw per day) for 4 or 21 days. Blood was collected for triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) analyses. The T_3 levels were significantly higher in female rats fed 1200 mg MG/kg diet as compared with the control group on day 21. The T_4 levels were significantly lower on both days 4 and 21 in the female rats in the 1200 mg MG/kg diet group as compared with the respective control groups. There were no significant changes in T_3 or T_4 levels in males or in the TSH levels in either sex (Culp et al., 1999).

(ii) LMG

Doerge et al. (1998) conducted in vitro studies on the ability of LMG to inhibit thyroid peroxidase (TPO), the enzyme that catalyses the iodination and coupling reactions required for thyroid hormone synthesis. These studies consisted of LMG inhibition of TPO-catalysed tyrosine iodination; LMG inhibition of iodination and coupling in goitre thyroglobulin; and LMG inhibition of TPO-catalysed coupling in preiodinated goitre thyroglobulin. The authors concluded that on chronic exposure, this inhibitory effect could cause thyroid follicular cell tumours through a hormonal mechanism. The authors further speculated that the TPO-catalysed oxidative demethylation of LMG to a primary arylamine could be the basis for a genotoxic mechanism for tumour formation.

In a GLP-compliant study, groups of eight male rats (F344/N Nctr BR) (6–7 weeks of age) were fed 0 or 1160 mg LMG/kg diet (equivalent to 0 or 116 mg LMG/kg bw per day) for 4 or 21 days, and blood was collected for T_3, T_4 and TSH analyses. There was no effect on T_3, but a significant decrease in T_4 and an increase in TSH levels on days 4 and 21 as compared with the respective control groups were noted (Culp et al., 1999).

(b) Cytotoxicity

(i) MG

Stammati et al. (2005) studied the cytotoxicity of MG to two tumour cell lines (Caco-2 and Hep-2). Total protein content and neutral red uptake (NRU) assays were used to assess Hep-2 viability. Proliferation capability was measured by the colony-forming ability test. Dose-dependent results were obtained with both viability assays, with 50% inhibitory concentration (IC_{50}) values of 2.03 μmol/l and 2.19 μmol/l for NRU and total protein content, respectively. From the colony-forming ability assay results, an IC_{50} value of 2.06 μmol/l was calculated. The conversion of yellow tetrazolium salt to the coloured formazan (MTT assay) and the release of cytoplasmic lactate dehydrogenase (LDH leakage) and NRU were used with the Caco-2 cells to assess cytotoxicity. The relative IC_{50} values obtained were 16.2 μmol/l, 18.4 μmol/l and 13.8 μmol/l for MTT, LDH and NRU, respectively.

(ii) LMG

Stammati et al. (2005) studied the cytotoxicity of LMG to two tumour cell lines (Caco-2 and Hep-2). Total protein content and NRU assays were used to assess Hep-2 viability. Proliferation capability was measured by the colony-forming ability test. Only a slight viability inhibition at the highest concentration tested (610 μmol/l) was observed. The results for the colony-forming ability assay were negative at all concentrations tested. The conversion of yellow tetrazolium salt to the coloured formazan (MTT assay) and the release of cytoplasmic LDH (LDH leakage) and NRU were used with the Caco-2 cells to assess cytotoxicity. No signs of cytotoxicity were observed with any of the tests.

(c) Cell transformation

Panandiker et al. (1992) assessed the cytotoxicity and morphological alterations of MG on Syrian hamster embryo (SHE) cells. Cytotoxicity was studied by determining the relative colony-forming efficiency of the MG-treated groups and the controls using logarithmically growing SHE cells. To assess enzyme activities, SHE cells were cultured and treated with MG (0.025–0.1 μg/ml) for 24 h. The cells were then collected, resuspended, sonicated and centrifuged, and the supernatant was used to determine enzyme activities. MG treatment resulted in induction of the mono-oxygenase system (aryl hydrocarbon hydroxylase and aminopyrene-*N*-demethylase activities), lipid peroxidation (superoxide dismutase activity) and catalase activity in a dose-dependent manner. These results were seen by the authors as an indication of the involvement of free radicals in the cytotoxic action of MG.

In continuing their research, Panandiker et al. (1993) used electron spin resonance analysis to show that reactive free radicals were formed during the in vitro (SHE cells) metabolism of MG.

Panandiker et al. (1994) used SHE cells to investigate the effect of MG on free radical formation, lipid peroxidation and DNA damage. SHE cells were cultured with “appropriate concentrations of MG”, and procedures were applied to assess these parameters. A dose–response increase was reported for all three parameters. In addition, through the use of antioxidant enzymes (catalase and glutathione peroxidase), the authors concluded that there was an involvement of reactive free radicals in the results observed and that this could be the explanation for the genotoxicity of MG to SHE cells.

The effects of MG on cell cycle phase distribution of normal and MG-transformed SHE cells in asynchronous and synchronous cell populations were investigated by Rao et al. (1998). Treatment with MG induced a dose-dependent G2/M arrest in normal cells, but no such accumulation of cells at the G2/M phase of the cell cycle was observed in the malignantly transformed cells.

Rao et al. (2000) extended this research to show that MG-transformed SHE cells had decreased sensitivity to apoptosis compared with control cells and that an overexpression of altered p53 and bcl-2 may be conferring resistance to MG-induced apoptosis.

Fernandes et al. (1991) compared the effects of MG and phenobarbitone (PB) on the development of preneoplastic lesions during *N*-nitrosodiethylamine (DEN)-induced hepatocarcinogenesis in male Wistar rats. Sixty rats, approximately 3 months old, were randomized and placed in six groups. Group 1 served as control, whereas groups 2, 4 and 6 were given 200 mg DEN/l of tap water for 4 weeks and then DEN-free water for 2 weeks. Groups 4 and 6 were then given water containing 25 mg MG/l (equivalent to 1.88 mg MG/kg bw per day) and 500 mg PB/l tap water, respectively, for 2.5 months. Groups 3 and 5 were given tap water for the first 6 weeks and then 25 mg MG/l tap water and 500 mg PB/l tap water, respectively, for 2.5 months. The effects were monitored on the basis of the morphological appearance of the liver, histological pattern, GGT-positive foci, total GGT activity

and the induction of glycogen-deficient islands. MG and PB were found to enhance liver carcinogenesis to a significant extent when compared with either the corresponding controls or animals given DEN alone. The enhancing effect of MG at 25 mg/l of tap water is comparable with that of PB at 500 mg/l water.

Rao & Fernandes (1996) conducted a second study (10 groups with 10 rats per group) on the dose-dependent tumour promoter effects of MG in Wistar male rats (2 months of age) that were pretreated with DEN. Group 1 was the untreated control, whereas groups 2, 4, 6, 8 and 10 were given tap water containing 200 mg DEN/l for 4 weeks. A 2-week recovery period in which rats were given DEN-free water followed. Groups 4, 6, 8 and 10 were then given drinking-water containing 25, 50 or 100 mg MG/l (equivalent to 1.88, 3.75 and 7.5 mg MG/kg bw per day) or 500 mg PB/l tap water, respectively, for 22 weeks. Groups 3, 5, 7 and 9 were given tap water for an initial 6 weeks and then tap water containing 25, 50 or 100 mg MG/l (equivalent to 1.88, 3.75 or 7.5 mg/kg bw per day) or 500 mg PB/l, respectively, for the remaining 22 weeks. The rats were all killed after 28 weeks, and livers were weighed, pictures were taken for morphology, and samples were prepared for histology, GGT activity measurement and hepatocyte isolation. The isolated hepatocytes were used to measure the rate of hepatocyte DNA synthesis and LDH activity.

The body and liver weights were not affected when only MG or PB was administered. Body weights were lower in the rats in the DEN and 100 mg MG/l or 500 mg PB/l water groups. Body weights of the other dose groups were not affected. Liver to body weight ratios were increased in those rats receiving DEN, DEN and MG, and DEN and PB. Livers from all treated rats exhibited varying degrees of structural and cytological change from the controls. The livers of the DEN-treated rats showed the development of basophilic foci distinguished by a compression of the surrounding parenchyma. The livers of rats treated with only MG showed a dose-dependent incidence of mixed abnormal cells with multiple nuclei and mitotic figures. Liver hyperplasia was observed in the rats treated with PB only. MG promoted DEN-induced preneoplastic lesions to hepatocellular carcinomas in a dose-related manner. Rats treated with DEN as well as PB developed hepatocellular carcinomas. GGT-positive foci were not observed in untreated controls or in those rats treated with MG only. A dose-dependent increase in the size of the GGT-positive foci was observed in the livers from rats treated with DEN plus MG. A significant increase in GGT activity was observed in those rats treated with DEN and MG or PB. MG inhibited DNA synthesis at all concentrations tested. The increase in LDH release from the MG-treated hepatocytes into the culture medium was directly proportional to the inhibitory effect of MG on DNA synthesis.

Further studies on the tumour promotional activity of MG on DEN-induced liver tumours were conducted in the rat by Gupta et al. (2003). In order to understand the mechanism of tumour promotion, hepatic levels of proliferating cell nuclear antigen (a marker of cell proliferation) and the cell cycle regulatory proteins cyclin D1 (and its associated kinase, cdk4) and cyclin B1 (and its associated kinase, cdc2) were measured. The authors concluded that the results obtained provide strong evidence for a link between dysregulation of the two critical checkpoints of the cell cycle as one of the possible mechanisms involved during tumour promotion by MG.

2.3 Observations in humans

A healthy 3-year-old girl weighing 17.3 kg ingested about 57 g of an aquarium product containing 0.075% MG (45 mg). The child was discovered by her father with blue lips and blue nail beds. On arrival at the hospital emergency department, she was awake and crying, with generalized cyanosis, including blue head, hands, arms, feet and legs. Initial vital signs were as follows: heart rate, 115 beats per minute; respirations, 30 per minute; and temperature, 36.8 °C. An initial arterial blood gas was reported as pH 7.43; carbon dioxide partial pressure, 22.9 mmHg (3.1 kPa); oxygen partial pressure, 57.7 mmHg (7.7 kPa); bicarbonate, 15 mmol/l; oxygen saturation, 47.4%; and methaemoglobin, 50.6%. An infusion of methylene blue was begun at 2 mg/kg bw, and the child responded rapidly by becoming pink. The methaemoglobin decreased to 6.5% after 2.5 h. The child was transferred to a paediatric intensive care unit and observed for 20 h, without return of symptoms. The authors suggested that the quantity (45 mg) of MG ingested may have exceeded the capacity of the intestinal flora to reduce MG to LMG, thus leaving sufficient MG to be absorbed in its more active state (Spiller et al., 2008).

3. DIETARY EXPOSURE

Two different sets of residue data were available. The first set consisted of a number of reports on monitoring and surveillance data for MG and LMG. The concentrations found may have resulted from environmental contamination or from illegal uses. Of 3277 samples selected from these reports, 222 samples were reported positive for MG in the range from 0.2 to about 600 μg/kg fish muscle. For many of the results, it is not defined what “malachite green” means (MG, LMG or the sum of both, because the method was inadequate). However, it is possible that some national authorities have more detailed data. The details of these reports, including references, are given in the residue monograph on MG published by the Food and Agriculture Organization of the United Nations (FAO) (Annex 1, reference *195*). Estimates of dietary exposure from these data are given in section 4.3.

In the open literature, well conducted residue studies suitable to predict the concentration–time course of residues of MG in fish are available for only two species, the rainbow trout and the channel catfish. Only for trout were sufficient individual animal data available to perform a statistical evaluation. The second set of data was taken from a well conducted study using MG under realistic conditions of use in trout of a size ready for human consumption (Law, 1994). The data and the results of the assessment are described in section 4.3. A discussion of the individual studies, including references, is provided in the residue monograph on MG published by FAO (Annex 1, reference *195*).

4. COMMENTS

4.1 Biochemical data

There is only limited information available on the absorption, distribution, metabolism and excretion of MG in mammalian species, including humans. In male

and female rats given an oral dose of 2 mg ^{14}C-labelled MG/kg bw, more than 95% of the radioactivity was excreted within 7 days, with more than 80% appearing in the faeces. No attempt was made to identify any metabolites. In vitro studies have shown that MG is readily converted into LMG under anaerobic conditions by a multitude of bacterial species present in the intestinal microflora from mice, rats, rhesus monkeys and humans. Therefore, MG that is ingested can be converted to LMG by the intestinal microflora.

In short-term feeding studies, it was shown that MG is sequentially *N*-demethylated to secondary and primary aromatic amines in rats and mice both before and after reduction to LMG. Analysis of liver extracts from rats treated with MG by HPLC-APCI/MS identified MG, its mono-, di-, tri- and tetradesmethyl derivatives and MG *N*-oxide. A small, but measurable, amount of LMG was also detected. Concentrations of MG and metabolites increased with increasing dose. Similarly, LMG, demethylated derivatives, MG *N*-oxide and demethylated *N*-oxide derivatives were detected in liver extracts from rats treated with LMG. A small, but measurable, amount of MG was also detected.

MG, in contrast to LMG, is highly cytotoxic to bacteria and mammalian cells in vitro. Studies using mammalian cells showed that MG treatment resulted in induction of the CYP mono-oxygenase system, lipid peroxidation and catalase activity in a concentration-dependent manner.

4.2 Toxicological data

4.2.1 Malachite green

In short-term (28 days) feeding studies in rats with doses ranging from 1 to 120 mg MG/kg bw per day, MG produced haematological changes (decreased erythrocyte count and haemoglobin and haematocrit values) and increased the relative liver weight in both sexes. This effect was accompanied by increased levels of GGT activity in the blood. Vacuolization of hepatocytes, primarily midzonal and centrilobular, was observed at the highest dose level tested. The NOAEL was 10 mg MG/kg bw per day based on haematological changes and effects on the liver, both of which were seen at 30 mg MG/kg bw per day and higher dose levels.

When rats were dosed with 120 mg MG/kg bw per day for 21 days, there was a significant increase in the T_3 levels and a significant decrease in the T_4 levels in the females. TSH was not affected, and no effects were seen in the males.

In a study using male Wistar rats pretreated for 4 weeks with DEN, it was shown that administration of MG in the drinking-water (resulting in daily doses ranging from 1.88 to 7.5 mg/kg bw per day) for 7 months dose-dependently enhanced the DEN-induced liver carcinogenicity to a significant extent. Increases in the relative liver weights and biochemical markers for hepatic preneoplastic lesions and cell cycle regulatory proteins were noted. A subsequent biochemical study confirmed that a number of markers for liver cell proliferation and cell cycle regulation were altered. The results provided evidence for dysregulation of checkpoints of the cell cycle as a possible mechanism during promotion of hepatic preneoplastic lesions by MG.

In a teratogenicity study in rabbits, MG (0, 5, 10 or 20 mg MG/kg bw per day from gestation days 6 to 18) was reported to produce a dose-related decrease in weight gain or marked weight loss in the dams and increased incidences of fetal anomalies (gross, visceral and skeletal) in all treated groups. A NOAEL could not be established. The lack of consistent dose–response relationships in most of the effects seen in the pups is noteworthy, but might be due to the very narrow dose range studied. The study was inadequately conducted and reported, and additional studies would be needed to properly address the potential of MG to produce reproductive and developmental toxicity.

Administration of MG and its major metabolite LMG at doses up to 60 and 58 mg/kg bw per day, respectively, for 28 days gave rise to a single liver DNA adduct species (or co-eluting adducts) in male F344 rats. Females were not studied. The adduct level increased significantly as a function of the dose and did not differ between groups administered equimolar doses of MG and LMG. In female B6C3F1 mice, doses up to 90 mg/kg bw per day (600 mg/kg diet) and 87 mg/kg bw per day (580 mg/kg diet) for 28 days for MG and LMG, respectively, also gave rise to a single DNA adduct species (or co-eluting adducts), the level of which increased as a function of the dose for MG, but only minimally and borderline for LMG. Thus, in the female mouse, MG produced much higher adduct levels than LMG at equimolar doses. In contrast, in the female Big Blue F344 transgenic rat, LMG at dose levels up to 54.3 mg/kg bw per day for up to 32 weeks gave rise to a single DNA adduct species, the level of which increased linearly with the dose.

MG did not produce mutations in the Ames test using *Salmonella typhimurium* strains or in Chinese hamster ovary cells (CHO-K1) and produced DNA damage in the comet assay in CHO-K1 cells only at cytotoxic concentrations. It did not produce a clastogenic effect in the in vivo mouse bone marrow micronucleus test and did not increase the occurrence of recessive mutations when tested in the mammalian spot test in mice. In addition, MG did not induce micronuclei in erythrocytes, mutations in lymphocytes or (in contrast to LMG) *cII* mutations in liver cells of female Big Blue B6C3F1 transgenic mice administered 67.5 mg MG/kg bw per day for 16 weeks.

It is concluded that although MG DNA adducts have been identified in the liver of male rats and female mice, the weight of evidence indicates that MG has no genotoxic potential in conventional in vitro and in vivo assays and did not produce mutations in the liver of transgenic female mice at the dose levels applied.

MG was tested for carcinogenicity in female F344 rats fed diets corresponding to daily intakes of 0, 7, 21 or 43 mg/kg bw per day for 2 years. A reduced body weight gain was observed at the two highest dose levels. There was a trend in increased occurrence of thyroid gland follicular cell adenoma or carcinoma, being statistically significant only at the middle dose level. Hepatocellular adenomas were minimally (but not statistically significantly) increased at the two highest dose levels, and there was a non-significant trend in the incidence of mammary gland carcinomas in the treated animals (see Table 4). The NOAEL for non-cancer effects was 7 mg MG/kg bw per day based on the reduced body weight gain at the two highest dose levels.

Table 4. Tumours in F344 female rats administered MG (0, 7, 21 or 43 mg/kg bw per day) for 2 years

Tumour type	Tissue toxicity	DNA adducts	Genotoxicity/ mutagenicity	Other effects
Liver adenoma 1/48, 1/48, 3/48, 4/48 Historical control: 1 adenoma in 6 studies	Eosinophilic foci 5/48, 10/48, 13/48, 14/48 Necrosis at highest dose Increased relative liver weight	A single liver DNA adduct species in male rats and female mice	Negative in conventional tests and transgenic female mice Positive in comet assay in CHO cells at cytotoxic concentrations	Promotor of DEN-initiated liver tumours Decreased body weight
Thyroid adenoma and carcinoma 0/46, 0/48, 3/47, 2/46 Historical control: NCTR 1.4% and NTP 0.9%	Cystic follicles 0/46, 1/48, 1/47, 3/46 Thyroid weight was not affected			120 mg/kg bw per day: T_3 ↑, T_4 ↓ Decreased body weight
Mammary carcinoma 2/48, 2/48, 1/48, 5/48 Historical control 0.7%				Decreased body weight

NCTR, National Center for Toxicological Research (USA); NTP, National Toxicology Program (USA).

MG was also tested for carcinogenicity in female mice. No increases in tumour incidences were seen in female mice fed diets corresponding to daily intakes of 15, 33 or 67 mg MG/kg bw per day for 104 weeks. No effects on mortality or body weight gain were observed.

4.2.2 Leucomalachite green

LMG was tested in short-term (28 days) feeding studies in female B6C3F1 mice at doses ranging from 43.5 to 174 mg/kg bw per day and in male F344 rats at doses ranging from 29 to 116 mg/kg bw per day. The NOAEL in female mice was 43.5 mg LMG/kg bw per day based on reduced body weights seen at higher dose levels. All females at the highest dose level had scattered dead or degenerate cells in the transitional epithelium of the urinary bladder. When LMG was tested in male rats, increased relative liver weights were reported for all dose groups, and the animals in the two highest dose groups had significantly lower body weights. At the highest dose level, GGT activity and phosphorus levels were significantly increased, whereas the erythrocyte count, haemoglobin and haematocrit levels were significantly decreased. Vacuolization of hepatocytes, primarily midzonal and

centrilobular, was observed at all dose levels. Apoptotic follicular epithelial cells in the thyroid gland were seen in some rats at the two highest dose levels. A NOAEL could not be established in this study.

In male rats given 116 mg LMG/kg bw per day for 21 days, there was a significant increase in the serum TSH levels and a significant decrease in the T_4 levels.

LMG did not induce mutations in any of the tested *S. typhimurium* strains and in Chinese hamster ovary cells (CHO-K1) and was negative for DNA damage in the comet assay in CHO-K1 cells. It did not produce any significant increase in the *lacI* mutation frequencies or changes in the mutation spectrum of *lacI* mutants in female Big Blue rats administered dietary doses up to 54.3 mg LMG/kg bw per day for up to 32 weeks. In addition, no effect was observed in the liver *cII* mutation frequency, the *Hprt* lymphocyte mutant assay or the bone marrow micronucleus assay in these rats.

LMG did not induce micronuclei in erythrocytes or mutations in lymphocytes of female Big Blue B6C3F1 transgenic mice administered 0, 30.6 or 61.2 mg LMG/kg bw per day (0, 204 and 408 mg/kg diet) for 16 weeks. However, LMG induced *cII* mutations with an increased frequency of G → T and A → T transversions in the liver cells of the female mice at the highest dose level tested (61.2 mg LMG/kg bw per day).

LMG was tested for carcinogenicity in male and female rats fed diets corresponding to dose levels of 0, 5, 15 or 30 mg/kg bw per day or 0, 6, 17 or 35 mg/kg bw per day, respectively, for 2 years. Mortality was not affected. Reduced body weight gains were observed at the two highest dose levels in both sexes. A low, not statistically significant increase in the incidence of thyroid gland follicular cell adenoma or carcinoma (2–7%) was seen in both sexes. There was no significant effect on the incidence of hepatocellular adenomas in the rats fed LMG. The mammary gland adenoma or carcinoma incidence in female rats was 0/48, 2/48, 3/48 and 4/48 at 0, 6, 17 and 35 mg/kg bw per day, respectively. The NOAEL for non-cancer effects was 5 mg/kg bw per day based on the reduced body weight gains seen at the higher dose levels.

In female mice fed diets corresponding to intakes of 0, 15, 31 and 63 mg LMG/kg bw per day for 104 weeks, the only finding was a dose-related trend in increased incidence of hepatocellular adenomas or carcinomas (3/47, 6/48, 6/47 and 11/47, respectively), with the incidence being statistically significant in the highest dose group (Table 5).

Table 5. Tumours in B6C3F1 female mice administered LMG (0, 15, 31 or 63 mg/kg bw per day) for 2 years

Tumour type	Tissue toxicity	DNA adducts	Genotoxicity/ mutagenicity
Liver adenoma and carcinoma 3/47, 6/48, 6/47, 11/47	No toxicity reported	Liver DNA adduct species only borderline in female mice	Negative in conventional in vitro and in vivo tests and in female transgenic rats

Table 5 (contd)

Tumour type	Tissue toxicity	DNA adducts	Genotoxicity/mutagenicity
		A single liver DNA adduct species seen in male and female rats	Induced *cII* mutations in transgenic female mice

4.2.3 Consideration of mode of action for MG and LMG

The incidences of tumours in female rats administered MG for 2 years and of tumours in female mice administered LMG for 2 years are shown in Table 4 and Table 5, respectively, together with findings related to the possible mode of action.

Both MG and LMG caused a weak, statistically non-significant increase in the occurrence of thyroid gland follicular cell adenoma and carcinoma in rats. Short-term (28 days) studies showed an increase in T_3 and a decrease in T_4 after MG treatment in female rats, and LMG treatment resulted in an increase in TSH and a decrease in T_4 levels. Mechanistic studies in vitro with LMG showed an inhibition in thyroid peroxidase–catalysed tyrosine iodination. This would support the view that chronic exposure to MG and LMG could result in thyroid gland follicular cell tumours through a hormonal, thresholded mechanism.

The tumour data supporting a treatment-related effect for MG and LMG in the induction of liver carcinogenicity in rats are not strong, with only the female rats treated with the two highest doses of MG having a minimally increased (not statistically significant) occurrence of hepatocellular adenomas. Although a dose-dependent formation of a DNA adduct species was demonstrated in the livers of F344 and Big Blue rats administered MG or LMG in the diet, analyses of liver *lacI* and *cII* mutants revealed that the mutant frequencies in LMG-treated rats were similar to those of control rats and that the majority of the independent mutations in treated rats were base pair substitutions, with a mutation spectrum similar to that found for control rats. In addition, MG did not induce *cII* mutations in female Big Blue mice. These data suggest that, in the rat, MG might be promoting spontaneous lesions in a manner similar to that reported in the studies on initiation/promotion of hepatic preneoplastic lesions and tumours in male Wistar rats using DEN as an initiator and MG as a promoter.

However, in female mice fed diets containing LMG for 104 weeks, a dose-related trend in increased incidence of hepatocellular adenomas or carcinomas was reported, with the incidence being statistically significant in the highest dose group. Although hepatocellular adenomas and carcinomas in mice often are not considered to originate from a DNA-reactive mechanism when they are the only induced tumour form, the induction of *cII* mutations in liver cells by a high dose of LMG in female transgenic mice indicates that a genotoxic mechanism cannot be ruled out. Importantly, the mutation spectrum of *cII* mutations was different from that of the control mice, with a notable increase in G → T and A → T transversions. A number of studies have shown that mutagenic aromatic amines produce predominantly such transversions in Big Blue rodents.

The Committee noted that LMG produced DNA adduct species, the levels of which increased significantly with dose, in the liver of male F344 and female Big Blue rats, but had no carcinogenic effect in the liver of male and female F344 rats. In contrast, in the liver of female B6C3F1 mice, where LMG induced mutations and tumours, the level of DNA adducts was borderline. This suggests that the DNA adduct species formed after treatment with MG and LMG is of low mutagenic and carcinogenic potential. In addition, the induction of *cII* mutations by LMG in the Big Blue mouse appears to be tissue specific, as tumours developed only in the liver of B6C3F1 mice.

4.3 Preliminary dietary exposure considerations

The Committee performed preliminary dietary exposure assessments on the basis of two different sets of residue data. The first set represented results of monitoring and surveillance data, and the concentrations found may have resulted from environmental contamination or from illegal uses. The second set of data was taken from a well conducted study using MG under realistic conditions of use in fish of a size ready for human consumption.

For the first approach, the Committee examined a number of reports on monitoring and surveillance data for MG and LMG. Of 3277 samples selected from these reports, 222 samples were reported positive for MG in the range from 0.2 to about 600 µg/kg fish muscle. For many of the results, it is not defined what “malachite green” means (MG, LMG or the sum of both), because the method was inadequate. However, it is possible that some national authorities have more detailed data. The details of these reports, including references, are given in the residue monograph on MG published by FAO (Annex 1, reference *195*).

Only a subset of the above data had been systematically collected. These were monitoring data (spanning from 1995 to 2006) published in the United Kingdom on the occurrence of MG and LMG in fish muscle. If both substances were found in a sample, the Committee calculated the sum. The Committee estimated the mean level in the positive samples to be 30.7 µg/kg fish muscle and the level at the 97.5th percentile to be 138 µg/kg. Assuming the daily consumption of fish to be 300 g/person, the daily exposure to the sum of MG and LMG can be calculated to be 9.2 and 41 µg/person at the mean and 97.5th percentile, respectively. For a 60-kg person, this would be equivalent to 0.15 µg/kg bw per day and 0.69 µg/kg bw per day, respectively.

In the open literature, well conducted residue studies suitable to predict the concentration–time course of residues of MG in fish are available for only two species, the rainbow trout and the channel catfish. Only for trout were sufficient individual animal data available to perform a statistical evaluation. The data and the results of the assessment are described below. A discussion of the individual studies, including references, is provided in the residue monograph on MG published by FAO (Annex 1, reference *195*).

A study investigating the metabolite profiles and residues of MG in trout tissues was conducted in trout kept in tanks under the following conditions: water temperature, 10 ± 2 °C; pH 6.0–7.0; hardness, 5–10 mg/l; and dissolved oxygen, 9 ± 2 mg/l. All experiments and analytical work were carried out under reduced-intensity room light. Concentrations in the exposure tanks were maintained at

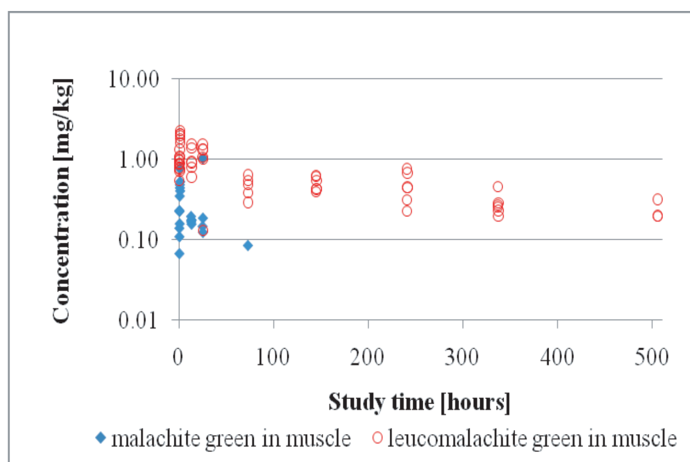
2 mg/l by a metering apparatus, using ^{14}C-labelled MG (radiochemical purity 98%) stock solution at 800 mg/l and delivering 10 ml/min of this solution to the tank.

Seventy-two randomly selected trout, each weighing about 350 g, were divided into three groups of 24 fish and put into three 200-l continuous-flow exposure tanks containing 2.0 mg ^{14}C-labelled MG/l (actual concentrations 1.84 ± 0.22 mg/l, 1.91 ± 0.29 mg/l and 1.88 ± 0.19 mg/l, respectively). A water sample (5 ml) was withdrawn from the exposure tanks every 15 min during the ^{14}C-labelled MG exposure period. After a 1-h exposure, the fish were removed to a depuration tank containing flowing, uncontaminated water. At specific time intervals during ^{14}C-labelled MG exposure and depuration, two to three trout were removed randomly from each group of fish and killed. The concentrations of total radioactive residue in tissue homogenates and the ratio of MG and LMG concentrations in an organic extract were determined. These data were used by the Committee to calculate the concentrations of MG and LMG in the tissues for the intake assessment.

The highest concentrations of residues were found in liver and kidney; however, for the intake estimates, muscle was used. In skin, there were also significant concentrations of residues; however, concentrations were not calculated for muscle and skin in natural proportions, since the concentrations in muscle were higher than those found in skin, and therefore the approach using the muscle data was slightly more conservative.

The kinetic data representing the concentrations of MG and LMG over the entire study time from the beginning of treatment until the end of the experiment at 505 h are given in Figure 2.

Figure 2. Concentration–time curves of MG and LMG in muscle of trout



The data representing the time period between the end of the treatment and 505 h were subjected to statistical analysis using one exponential term on the basis

Table 6. Parameters of the linear regression analysis of kinetic residue depletion data in trout muscle

Parameter	MG	LMG
Intercept	−0.99747	0.01994
Slope	−0.02461	−0.00352
Coefficient of correlation	−0.60012	−0.73361
Residual variance	0.58312	0.52876

of the natural logarithms of the residue contents. The parameters given in Table 6 were obtained by linear regression.

Depletion half-lives of 28 h for MG and 197 h for LMG were determined. The kinetic parameters, including the variance of the data, were used to calculate model intakes for every day of 80 years of a human lifespan, assuming daily consumption of 300 g of fish muscle. For this purpose, 29 220 approximately log-normally distributed random numbers were generated for each time point of interest, ranging from the predicted value of the regression line minus 4 times the residual variance to the same predicted value plus 4 times the residual variance. These calculations were repeated for a number of assumed slaughter times of the fish, ranging from 1 h (end of treatment) to 500 h. The results were expressed in mg MG (LMG)/kg of human body weight. The minima, maxima and several percentiles, including the median of these estimated daily intakes, were calculated. The median was used for an assessment of chronic intake. The median daily intake of LMG ranged from 7.3 μg/kg bw at 1 h to 0.87 μg/kg bw at 500 h (Table 7).

The Committee considered that the assumption of consumption of 300 g of fish contaminated with MG and LMG every day for a lifetime is a highly conservative assumption. In addition, it was assumed that the concentrations of MG and LMG would not change during cooking of the fish.

5. EVALUATION

The Committee first addressed the question of the use of MG for food-producing animals. There are no conventional studies available on the absorption, distribution, metabolism and excretion of MG in mammalian species. Although the available short- and long-term studies point to a NOAEL in the order of 10 mg/kg bw per day, the study on teratogenicity in rabbits, albeit of low quality, raises concern regarding the potential developmental toxicity of MG. Because a NOAEL could not be identified, additional studies would be needed to properly address the potential reproductive and developmental hazards of MG. In addition, following ingestion, MG is expected to be extensively reduced to LMG, primarily by the gastrointestinal microflora, before absorption, and it cannot be ruled out that LMG, the major metabolite of MG, induces hepatocellular adenomas and carcinomas in female mice via a mutagenic mode of action. Based on these considerations, the Committee considered it inappropriate to establish an ADI for MG. Therefore, the use of MG for food-producing animals cannot be supported.

Table 7. Results of an intake assessment for malachite green and leucomalachite green

	Intake (µg/kg bw per day) at various theoretical slaughter times of fish (h)														
	1.0	1.6	2.4	3.8	5.9	9.2	14.3	22.4	34.9	54.3	84.7	132.0	205.8	320.8	500.0
MG															
Lowest intake	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
Median intake	1.8	1.8	1.7	1.7	1.6	1.5	1.3	1.1	0.8	0.5	0.2	0.1	0.0	0.0	0.0
P90	3.8	3.8	3.7	3.6	3.4	3.1	2.8	2.3	1.6	1.0	0.5	0.2	0.0	0.0	0.0
P95	4.7	4.7	4.5	4.4	4.2	3.8	3.4	2.8	2.0	1.3	0.6	0.2	0.0	0.0	0.0
P97.5	5.6	5.6	5.4	5.2	5.0	4.6	4.0	3.3	2.4	1.5	0.7	0.2	0.0	0.0	0.0
P99	6.9	6.9	6.6	6.5	6.1	5.7	5.0	4.1	2.9	1.8	0.9	0.3	0.0	0.0	0.0
Highest intake	15.6	16.2	21.1	15.2	14.2	14.1	11.8	9.8	10.4	3.8	2.0	0.6	0.1	0.0	0.0
MOE for median intake	11 128	11 225	11 540	11 940	12 609	13 664	15 426	18 921	25 713	41 351	86 780	279 316	1 715 561	29 143 431	2 364 965 091
LMG															
Lowest intake	1.5	1.4	1.5	1.3	1.5	1.1	1.3	1.2	0.9	0.8	0.8	0.5	0.3	0.3	0.1
Median intake	7.3	7.3	7.2	7.1	6.9	6.7	6.4	6.0	5.5	4.8	4.0	3.3	2.5	1.7	0.9
P90	12.6	12.6	12.3	12.2	12.0	11.7	11.3	10.7	10.0	8.9	7.8	6.4	4.8	3.3	1.7
P95	14.7	14.7	14.5	14.6	14.1	13.8	13.3	12.6	11.7	10.7	9.3	7.7	5.8	4.0	2.1
P97.5	16.9	17.0	16.6	16.8	16.4	15.8	15.3	14.5	13.5	12.5	11.0	9.1	6.9	4.6	2.4

Table 7 (contd)

	Intake (µg/kg bw per day) at various theoretical slaughter times of fish (h)														
P99	19.7	19.5	19.6	19.7	19.1	18.9	18.3	17.3	16.0	15.0	13.4	10.9	8.3	5.6	3.0
Highest intake	48.5	42.4	36.0	39.7	36.2	34.6	38.0	38.9	31.5	32.0	30.3	22.6	16.6	14.3	6.4
MOE for median intake	2 750	2 746	2 792	2 832	2 902	2 976	3 108	3 337	3 653	4 159	4 958	6 071	8 076	12 095	22 891
Sum															
Lowest intake	1.7	1.6	1.7	1.4	1.7	1.3	1.4	1.3	1.0	0.8	0.8	0.5	0.3	0.3	0.1
Median intake	9.1	9.1	8.9	8.7	8.5	8.2	7.7	7.1	6.3	5.3	4.3	3.4	2.5	1.7	0.9
P90	16.4	16.4	16.0	15.8	15.4	14.8	14.1	12.9	11.6	9.9	8.3	6.6	4.9	3.3	1.7
P95	19.4	19.4	19.0	19.0	18.3	17.6	16.7	15.4	13.8	12.0	9.9	7.9	5.9	4.0	2.1
P97.5	22.5	22.5	22.0	22.0	21.4	20.4	19.3	17.8	15.9	14.0	11.7	9.3	7.0	4.6	2.4
P99	26.7	26.4	26.2	26.3	25.2	24.6	23.3	21.4	18.9	16.8	14.3	11.2	8.4	5.6	3.0
Highest intake	64.1	58.6	57.1	54.9	50.4	48.8	49.8	48.7	42.0	35.8	32.3	23.2	16.7	14.3	6.4
MOE for median intake	2 205	2 206	2 248	2 289	2 359	2 444	2 587	2 836	3 198	3 779	4 690	5 942	8 038	12 090	22 891

MOE, margin of exposure; P, percentile.

The Committee thereafter evaluated the safety of residues of MG and LMG in fish as a result of industrial use and other sources of contamination. The Committee considered whether it could establish a margin of exposure (MOE) for non-cancer end-points. In view of the deficient database, the Committee considered it inappropriate to derive an MOE for non-cancer end-points for MG and LMG.

In the evaluation of exposure to genotoxic and carcinogenic residues, the Committee considered the induction of hepatocellular adenomas or carcinomas in female mice treated with LMG to be the pivotal effect for the risk assessment of MG and LMG as contaminants in food. Because there is no information on the conversion rate of MG to LMG in food, the Committee considered it prudent to evaluate the sum of MG and LMG in food expressed as LMG.

For substances that are genotoxic and carcinogenic, JECFA (contaminants) has suggested using an MOE approach in the risk assessment of unintentional contaminants (Annex 1, reference *176*). The present Committee agreed to this approach. The MOE is the ratio between a defined reference point on the dose–response curve for the adverse effect and the human intake of the substance. As a reference point from the dose–response curve for the pivotal adverse effect, it was suggested that the $BMDL_{10}$, which is the lower limit of a one-sided 95% confidence interval on the benchmark dose (BMDL) calculated for a benchmark response of 10% incidence above the modelled background incidence, be used.

The United States Environmental Protection Agency's benchmark dose (BMD) software (BMDS) version 1.4.1 was used for modelling the liver tumour dose–response (hepatocellular adenomas and carcinomas combined) in the LMG-treated female mice. The following dose–response models were fitted to the dose–incidence data: gamma, logistic, log-logistic, multistage, probit, log probit, quantal linear and Weibull models (Table 8). The BMD and BMDL values for an extra 10% risk compared with the modelled background incidence (BMD_{10} and $BMDL_{10}$) were estimated by performing 250 iterations.

The BMD_{10} values from the accepted models ranged from 33.5 to 43.1 mg LMG/kg bw per day, and the $BMDL_{10}$ values ranged from 18.5 to 31.2 mg LMG/kg bw per day. In order to be prudent, the Committee decided to use the more conservative lower end of this range of values for the evaluation and, to simplify the calculation, chose a $BMDL_{10}$ value of 20 mg LMG/kg bw per day as the reference point for the MOE calculation.

Assuming a daily consumption of 300 g of fish contaminated with MG and LMG, the estimated exposure to the sum of MG and LMG for a 60-kg person was 0.15 µg/kg bw per day, expressed as LMG, for the average intake and 0.69 µg/kg bw per day for the high (97.5th percentile) intake. Comparison of these mean and high-level exposures with the $BMDL_{10}$ of 20 mg/kg bw per day indicates MOEs of about 130 000 and 30 000, respectively. JECFA has previously, at its sixty-fourth meeting (Annex 1, reference *176*), considered MOEs of 10 000 or higher for unintended contaminants (polycyclic aromatic hydrocarbons and ethyl carbamate from food, excluding alcoholic beverages) to be of low concern for human health.

Table 8. BMD_{10} and $BMDL_{10}$ calculations for LMG based on the incidences of liver adenomas and carcinomas in female mice (Culp et al., 2006)

Model	Log likelihood (parameters)	*P*-value	AIC	Chi-square	*P*-value	Accept	BMD_{10} (mg/kg bw per day)	$BMDL_{10}$ (mg/kg bw per day)
Full model	−72.77 (4)							
Gamma multi-hit	−72.94 (3)	0.842	149.9	0.34	0.842	Yes	35.4	20.1
Logistic	−72.97 (2)	0.814	149.9	0.42	0.810	Yes	43.1	31.2
Log-logistic	−72.94 (3)	0.549	151.9	0.36	0.551	Yes	34.6	18.5
Multistage	−72.93 (3)	0.561	151.9	0.34	0.560	Yes	36.8	20.1
Probit	−72.96 (2)	0.882	149.9	0.40	0.818	Yes	41.9	29.5
Log-probit	−72.97 (3)	0.522	151.9	0.40	0.525	No	33.5	<0
Quantal linear	−72.94 (2)	0.842	149.9	0.34	0.842	Yes	35.4	20.1
Weibull	−72.94 (3)	0.558	151.9	0.34	0.560	No	34.8	<0
Reduced model	−75.70 (1)	0.118						

AIC, Akaike information criterion.

The Committee also performed preliminary estimates of the potential exposures in the case that MG had been used to treat fish in aquaculture and the fish had been subjected to various depletion times. For the median intake, the sum of MG and LMG was about 9 μg/kg bw per day at the end of the 1-h treatment period and about 1 μg/kg bw per day after a depletion time of 500 h. Comparison of these exposure levels with the $BMDL_{10}$ of 20 mg/kg bw per day indicates MOEs of about 2000 and 20 000, respectively. The 97.5th percentile intakes were about 23 and 2 μg/kg bw per day at the end of the 1-h treatment period and after a depletion time of 500 h, respectively, providing MOEs of about 900 and 10 000 (see Table 7).

The current Committee noted the conclusion at the sixty-fourth JECFA (Annex 1, reference *176*) and agreed that MOEs of less than 10 000 for genotoxic and carcinogenic contaminants indicate a health concern.

6. REFERENCES

Clemmensen, S., Jensen, J.C., Jensen, N.J., Meyer, O., Olsen, P. & Würtzen, G. (1984) Toxicological studies on malachite green: A triphenylmethane dye. *Arch. Toxicol.*, **56**, 43–45.

Codex Alimentarius Commission (2007) *Report of the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods, Breckenridge, CO, USA, 3–7 September*

2007. Rome, Italy, Food and Agriculture Organization of the United Nations (ALINORM 08/31/31; http://www.codexalimentarius.net/web/archives.jsp?year=08).

Culp, S.J., Blankenship, L.R., Kusewitt, D.F., Doerge, D.R., Mulligan, L.T. & Beland, F.A. (1999) Toxicity and metabolism of malachite green and leucomalachite green during short-term feeding to Fischer 344 rats and B6C3F1 mice. *Chem. Biol. Interact.*, **122**, 153–170.

Culp, S.J., Beland, F.A., Heflich, R.H., Benson, R.H., Blankenship, L.R., Webb, P.J., Mellick, P.W., Trotter, R.W., Shelton, S.D., Greenlees, K.J. & Manjanatha, M.G. (2002) Mutagenicity and carcinogenicity in relation to DNA adduct formation in rats fed leucomalachite green. *Mutat. Res.*, **506–507**, 55–63.

Culp, S.J., Mellick, P.W., Trotter, R.W., Greenlees, K.J., Kodell, R.L. & Beland, F.A. (2006) Carcinogenicity of malachite green chloride and leucomalachite green in B6C3F1 mice and F344 rats. *Food Chem. Toxicol.*, **44**, 1204–1212.

Doerge, D.R., Chang, H.C., Divi, R.L. & Churchwell, M.I. (1998) Mechanism for inhibition of thyroid peroxidase by leucomalachite green. *Chem. Res. Toxicol.*, **11**, 1098–1104.

Fernandes, C., Lalitha, V.S. & Rao, K.V.K (1991) Enhancing effect of malachite green on the development of hepatic pre-neoplastic lesions induced by *N*-nitrosodiethylamine in rats. *Carcinogenesis,* **12**, 839–845.

Fessard, V., Godard, T., Huet, S., Mourot, A. & Poul, J.M. (1999) Mutagenicity of malachite green and leucomalachite green in in vitro tests. *J. Appl. Toxicol.*, **19**, 421–430.

Gupta, S., Sundarrajan, M. & Rao, K.V.K. (2003) Tumor promotion by metanil yellow and malachite green during rat hepatocarcinogenesis is associated with dysregulated expression of cell cycle regulatory proteins. *Teratog. Carcinog. Mutagen.*, **1**(Suppl.), 301–312.

Henderson, A.L., Schmitt, T.C., Heinze, T.M. & Cerniglia, C.E. (1997) Reduction of malachite green to leucomalachite green by intestinal bacteria. *Appl. Environ. Microbiol.*, **63**, 4099–4101.

Jensen, N.J. (1984) Lack of mutagenic activity of malachite green in the mammalian spot test. *Mutat. Res.*, **130**, 248.

Law, F.C.P. (1994) *Total residues depletion and metabolic profile of selected drugs in trout.* Report prepared by Environmental Toxicology Program, Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., Canada, for Food and Drug Administration, United States Department of Health and Human Services, Washington, DC, USA (Contract No. 223-90-7016; OMB No. 0990-0115).

Manjanatha, M.G., Shelton, S.D., Bishop, M., Shaddock, J.G., Dorbrovolsky, V.N., Heflich, R.H., Webb, P.J., Blankenship, L.R., Beland, F.A., Greenlees, K.J. & Culp, S.J. (2004) Analysis of mutations of bone marrow micronuclei in Big Blue® rats fed leucomalachite green. *Mutat. Res.*, **547**, 5–18.

Meyer, F.P. & Jorgenson, T.A. (1983) Teratological and other effects of malachite green on development of rainbow trout and rabbits. *Trans. Am. Fish. Soc.*, **112**, 818–824.

Mittelstaedt, R.A., Mei, N., Webb, P.J., Shaddock, J.G., Dobrovolsky, V.N., McGarrity, L.J., Morris, S.M., Chen, T., Beland, F.A., Greenlees, K.J. & Heflich, R.H. (2004) Genotoxicity of malachite green and leucomalachite green in female Big Blue B6C3F1 mice. *Mutat. Res.*, **561**, 127–138.

NTP (2005) *Toxicology and carcinogenesis studies of malachite green chloride and leucomalachite green in F344/N rats and B6C3F1 mice*. Research Triangle Park, NC, USA, Department of Health and Human Services, National Institutes of Health, National Institute of Environmental Health Sciences, National Toxicology Program, 312 pp. (Technical Report Series 527).

Panandiker, A., Fernandes, C. & Rao, K.V.K. (1992) The cytotoxic properties of malachite green are associated with the increased demethylase, aryl hydrocarbon hydroxylase and lipid peroxidation in primary cultures of Syrian hamster embryo cells. *Cancer Lett.*, **67**, 93–101.

Panandiker, A., Fernandes, C., Rao, T.K.G. & Rao, K.V.K. (1993) Morphological transformation of Syrian hamster embryo cells in primary culture by malachite green correlates well with the evidence for formation of reactive free radicals. *Cancer Lett.*, **74**, 31–36.

Panandiker, A., Maru, G.B. & Rao, K.V.K. (1994) Dose–response effects of malachite green on free radical formation, lipid peroxidation and DNA damage in Syrian hamster embryo cells and their modulation by antioxidants. *Carcinogenesis,* **15**, 2445–2448.

Plakas, S.M., El Said, K.R., Stehly, G.R., Gingerich, W.H. & Allen, J.L. (1996) Uptake, tissue distribution, and metabolism of malachite green in the channel catfish (*Ictalurus punctatus*). *Can. J. Fish. Aquat. Sci.*, **53**, 1427–1433.

Rao, K.V.K. & Fernandes, C.L. (1996) Progressive effects of malachite green at varying concentrations on the development of *N*-nitrosodiethylamine induced hepatic preneoplastic lesions in rats. *Tumori*, **82**, 280–286.

Rao, K.V.K., Mahudawala, D.M. & Redkar, A.A. (1998) Malignant transformation of Syrian hamster embryo (SHE) cells in primary culture by malachite green: Transformation is associated with abrogation of G2/M checkpoint control. *Cell Biol. Int.*, **22**, 581–589.

Rao, K.V.K., Mahudawala, D.M. & Redkar, A.A. (2000) Malachite green induced malignant transformation of Syrian hamster embryo (SHE) cells in primary culture: Transformation is associated with enhanced expression of altered p53, Bcl-2 and decreased sensitivity to apoptosis. *J. Exp. Clin. Cancer Res.*, **19**, 89–98.

Spiller, H., Rodgers, G., Willias, D., Bosse, G. & Sullivan, J. (2008) Methemoglobinemia due to malachite green ingestion in a child. *Clin. Toxicol. (Phila.)*, **46**, 320–321.

Stammati, A., Nebbia, C., Angelis, I.D., Albo, A.G., Carletti, M., Rebecchi, C., Zampaglioni, F. & Dacasto, M. (2005) Effects of malachite green (MG) and its major metabolite, leucomalachite green (LMG), in two human cell lines. *Toxicol. In Vitro*, **19**, 853–858.

MELENGESTROL ACETATE (addendum)

First draft prepared by

Dr Susan Barlow,[1] Professor Alan R. Boobis[2] and Dr Richard Ellis [3]

[1] Consultant, Brighton, East Sussex, England
[2] Faculty of Medicine, Imperial College London, London, England
[3] Consultant, Myrtle Beach, SC, United States of America (USA)

Explanation 69
Biological data.. 71
Biochemical aspects.. 71
Absorption, distribution and excretion........................... 71
Biotransformation.. 72
Hormonal activities of MGA.. 73
Role of progesterone and progesterone receptors in human development and disease.. 75
Breast development and breast cancer......................... 75
Uterine (endometrial) cancer... 78
Ovarian cancer.. 79
Cervical cancer.. 79
Precocious puberty... 79
Gynaecomastia.. 79
Prenatal development, including the reproductive tract 80
Genotoxicity.. 81
Reproductive and developmental toxicity........................... 82
Immunotoxicity... 83
Comments.. 84
Evaluation... 87
References.. 89

1. EXPLANATION

Melengestrol acetate (17α-acetoxy-6-methyl-16-methylene-4,6-pregnadiene-3,20-dione; MGA) is a synthetic progestogen that is active after oral administration. It is administered as a feed additive to female beef cattle (heifers) to improve the efficiency of feed conversion, promote growth and suppress estrus. The range of approved doses is 0.25–0.50 mg/heifer daily, and it is fed for the duration of the fattening/finishing period, usually 90–150 days.

MGA has been reviewed previously by the Committee at its fifty-fourth, fifty-eighth, sixty-second and sixty-sixth meetings (Annex 1, references *146*, *157*, *169* and *181*). An acceptable daily intake (ADI) of 0–0.03 μg/kg body weight (bw) was established at the fifty-fourth meeting of the Committee. Maximum residue levels (MRLs) were initially recommended at the fifty-fourth meeting of the Committee and

subsequently reconsidered at the sixty-second and sixty-sixth meetings based on new metabolism studies that identified specific metabolites and determined their progestogenic activity relative to that of MGA. The sixty-sixth meeting recommended MRLs of 18 μg/kg in fat, 10 μg/kg in liver, 2 μg/kg in kidney and 1 μg/kg in muscle tissue of cattle. These MRLs are expressed as MGA equivalents and cover both MGA itself, as the marker residue, and its metabolites. The biological activity of the metabolites (non-MGA fraction of the tissue residues) was converted to MGA equivalents, taking into account their known, lower progestogenic activity and converting the marker residue to total residue, based on the fraction attributable to marker residue in each of the relevant tissues. The analytical methods were extensively reviewed at the fifty-eighth meeting of the Committee, at which time the Committee noted that the available high-performance liquid chromatography–mass spectrometry (HPLC-MS) method submitted by the sponsor was suitable to measure residues in food animal tissues.

At the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) (Codex Alimentarius Commission, 2007), CCRVDF recommended that MGA be reconsidered based on a commitment of new scientific data on toxicity and hormonal effects to be provided by the European Commission, including reference to the specific issues to be addressed. Scientific data have now been submitted by the European Commission, comprising a number of papers published in the scientific literature or submitted as unpublished original research reports to the European Commission between 1996 and 2007. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) was asked to reconsider MGA taking into account the more recent data on analytical methods, measurement of residues in tissues, toxicity and hormonal effects of MGA.

In the covering letter included in the European Commission submission, the Committee was asked in particular:

- To reconsider important end-points, such as the effects on pre-pubertal children (and other subpopulations that may be exposed) on the immune system, endocrinological effects etc.
- To discuss data indicating that hormones do not only act via hormone-mediated receptors but other mechanisms (e.g. direct and indirect genotoxic effects).
- To re-calculate the data from residues in meat from animals treated with these hormones for growth promotion purposes and to take into account data coming in particular from improved analytical capacities.
- To describe precisely what authorised use of *good practice of use of the veterinary drugs* it considers appropriate in the evaluation and their respective impact on potential exposure and risk estimate.

These issues were considered by the Committee at its present meeting, taking into account relevant earlier information as well as the submitted data. The Committee also searched the literature for any new publications on MGA since its last toxicological review in 2000 (Annex 1, reference *146*). The majority of the papers and reports submitted by the European Commission concerned actions of hormones or substances with hormone-like activity in general. There is only limited new or additional information on MGA itself, covering developments in analytical

methods for residue analysis, further information on plasma concentrations of MGA and MGA residues in tissues of heifers, the mode of action of MGA in accelerating onset of puberty in heifers, lack of effect of MGA in bulls, further in vitro studies on interactions of MGA with hormone receptors and downstream effects, pathological effects in zoo animals with long-term contraceptive MGA implants, and effects of prenatal, juvenile and adult exposure to MGA in rabbits.

The main focus of this re-evaluation is on MGA itself. The Committee did not consider it necessary to review the considerable amount of information that is now available from studies on experimental animals, wildlife and humans concerning the possible effects of a wide range of hormonally active chemicals on development, physiological function and disease (endocrine disrupter effects), as a number of general reviews have addressed these issues (see, for example, Damstra et al., 2002; Daston et al., 2003; Tabb & Blumberg, 2006; Hotchkiss et al., 2008; Phillips & Foster, 2008). This re-evaluation of MGA is predicated on an understanding that the present state of the science provides strong evidence for effects of endocrine disrupters in laboratory animals. However, while there are serious concerns for wildlife and human health effects, the causal links between exposure and endocrine disruption in these populations remain unclear. As others have pointed out, attempting to relate events during fetal and neonatal life to subsequent function and adult disease is an exceedingly difficult challenge for epidemiology (Vom Saal, 2007). The Committee also noted that there is still no consensus in the scientific community as to whether low-dose exposures to endocrine disrupters cause adverse effects in humans.

Since MGA has both progestogenic and glucocorticoid activity, the Committee gave specific consideration to background data on the possible role of progesterone and interactions of progestogens with progesterone receptors in development and disease and to the possible influence of glucocorticoids on immune function. The Committee noted that while MGA undoubtedly has been shown to have hormonal activity, as demonstrated by in vitro experiments and in vivo studies in laboratory animals and treated cattle, an important consideration in the risk assessment is whether human exposure to residues in meat at the upper bound of the ADI (0.03 μg/kg bw) would be expected to have any effect.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

MGA was administered to heifers at doses of 0, 0.5, 1.5 and 5 mg MGA/day (two heifers per dose group) for 8 weeks, at which time they were slaughtered and concentrations of MGA measured by enzyme immunoassay in plasma, liquid chromatography–mass spectrometry (LC-MS) in kidney and muscle, and gas chromatography–mass spectrometry (GC-MS) in perirenal fat. The results showed that MGA is lipophilic and accumulates in fat, with concentrations about 200-fold above plasma levels. Next highest concentrations were found in liver, about 20- to

40-fold above plasma levels, and lowest amounts in kidney and muscle, approximately 5-fold above plasma levels. Concentrations in perirenal fat were 6.5 and 8.4 μg/kg, 24.1 and 33.9 μg/kg, and 56.3[1] and 60.9 μg/kg after 0.5, 1.5 and 5 mg/day, respectively. Concentrations in liver were 0.8 and 1.0 μg/kg, 2.3 and 7.7 μg/kg, and 5.1 and 7.6 μg/kg after 0.5, 1.5 and 5 mg MGA/day, respectively. Concentrations in kidney and muscle were at or below 2 μg/kg in all three treated groups (Daxenberger et al., 1999). These results indicate considerable variability in tissue levels. Further information on these same tissue samples was reported by the same group in a later publication, for fat samples analysed by both enzyme immunoassay and GC-MS and for muscle samples analysed by both enzyme immunoassay and LC-MS. The results for each tissue/dose from the two analytical methods were in reasonable agreement, with mean coefficients of variation of 7.1% and 13.9% for fat and muscle, respectively (Hageleit et al., 2001). In each of the above studies, a further group of two heifers was given 0.5 mg MGA/day for 8 weeks, then subjected to a 48-h withdrawal period before slaughter. This made little difference to the amounts retained in fat. The authors pointed out that longer withdrawal periods cannot be used, because estrus would be triggered within 3–7 days of cessation of MGA treatment, and transporting heifers during estrus is not recommended (Daxenberger et al., 1999).

The passage of MGA across the placenta has been investigated in two rabbits given MGA orally in corn syrup at a dose of 0.5 mg/kg bw per day on gestation days 14–27. In the dams, MGA was detectable in the nanogram per millilitre range in plasma and was measured at 0.29 and 0.70 μg/kg in muscle, 190 and 160 μg/kg in liver, 2.80 and 2.50 μg/kg in kidney, and 28 and 72 μg/kg in fat (tissue origin not specified). Amounts in corresponding tissues in four fetuses were as follows: muscle, 0.88–0.10 μg/kg; liver, 5.10–7.10 μg/kg; kidney, 0.60–1.10 μg/kg; and fat, 3.10–7.10 μg/kg. Concentrations in the placenta ranged from 0.69 to 0.95 μg/kg. Concentrations in all tissues in controls were below the limit of detection. This study shows significant, but limited, passage of MGA across the placenta (Lange et al., 2002).

The data reviewed by the Committee in its previous evaluation indicate that MGA is extensively metabolized in the liver, with formation of numerous metabolites in most of the species studied, including humans (Annex 1, reference *147*). Progesterone itself is rapidly metabolized and cleared in humans and other species (Golub et al., 2006), and low doses administered by the oral route are almost completely metabolized in one pass through the gastrointestinal mucosa and liver (Simon, 1995).

2.1.2 Biotransformation

No new studies on the biotransformation of MGA have been identified since the last evaluation in 2004, in which the major monohydroxy and dihydroxy

[1] This figure is actually quoted as 6.3 in Daxenberger et al. (1999), but data on the same samples analysed by GC-MS were also quoted in Hageleit et al. (2001), and there the value is given as 56.3, which seems the more likely value.

metabolites were identified from in vitro studies using hepatic microsomes from heifers, rats and humans (Annex 1, reference *169*).

2.1.3 Hormonal activities of MGA

Early studies on the hormonal activities of MGA showed that it exerts both progestational and glucocorticoid activity (Lauderdale et al., 1977). In vitro, MGA shows strong progesterone receptor binding affinity, as measured by displacement of a progesterone analogue, 16α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]pregn-4-ene-3,20-dione, from bovine uterine progestin receptor in cell cytosol preparations; the relative binding affinity of MGA was 526% of that of progesterone, whereas the affinity of three MGA metabolites generated from bovine hepatocytes was between 25% and 85% of that of progesterone (Bauer et al., 2000). In a different assay using the MCF-7 human breast cancer cell line in which the test compounds are taken up into whole cells, MGA showed an 11-fold increase, compared with progesterone, in relative binding affinity to the human progesterone receptor, which has 90% homology with the bovine progesterone receptor (Perry et al., 2005). The progestational activity of MGA in vivo in cattle has been shown to be about 125 times that of progesterone, when both compounds are given parenterally and measured by estrous cycle inhibition (Lauderdale et al., 1977; Lauderdale, 1983).

The possibility that MGA possesses estrogenic activity has been investigated in vitro. Le Gueval & Padkel (2001) tested MGA in two different bioassays—a recombinant yeast assay expressing trout estrogen receptor, which is sensitive down to 0.1–1 nmol estradiol/l, and an assay that measures induction of vitellogenin gene expression (normally triggered by estradiol) in cultures of rainbow trout hepatocyte aggregates, which is sensitive down to about 10 nmol estradiol/l. In the recombinant yeast assay, MGA was inactive at 0.1 and 1 μmol/l (~40 000 and 400 000 pg/ml) but was active at 10 μmol/l (~4 000 000 pg/ml), whereas estrogenic steroids such as 17β- and 17α-estradiol, estrone, diethylstilbestrol, 17α-ethinylestradiol, zearalenone and related compounds were active at concentrations at least 2 orders of magnitude lower than MGA. MGA was inactive in the vitellogenin gene expression assay at 1 and 10 μmol/l (~400 000 and 4 000 000 pg/ml). In another in vitro bioassay utilizing cell proliferation in MCF-7 cells as a marker of estrogenic activity, MGA did not show any activity at picomole to nanomole per litre concentrations (10^{-11}–10^{-9} mol/l: ~4–400 pg/ml), but did show small, but statistically significant, increases in cell proliferation at higher (nanomole to micromole per litre) concentrations (10^{-8}–10^{-6} mol/l: ~4000–400 000 pg/ml), although it was again without activity at 10 μmol/l (~4 000 000 pg/ml) (Perry et al., 2005). The concentrations showing estrogenic activity in vitro are considerably higher than the plasma concentrations of MGA achieved in vivo of about 25–50 pg/ml in heifers treated with 0.5 mg MGA daily (Daxenberger et al., 1999; Hageleit et al., 2000; Pfaffl et al., 2002).

There are no data on plasma concentrations of MGA following human dietary exposure. However, in the studies in heifers reported above (Daxenberger et al., 1999; Hageleit et al., 2000; Pfaffl et al., 2002), the daily dose of MGA administered in the feed corresponds to approximately 1.6 μg/kg bw. Assuming similar absorption

and metabolism in cattle, rats, rabbits and humans, the plasma concentration in humans after ingestion of 0.03 μg MGA/kg bw (i.e. equivalent to the upper bound of the ADI) would be 0.5–1 pg/ml, some 4000-fold below the minimum concentration necessary to stimulate proliferation in MCF-7 cells. In addition, the study in rabbits reported above (Lange et al., 2002) found nanogram per millilitre concentrations of MGA in plasma following an oral dose of 0.5 mg/kg bw. This is in the same range as the minimum concentration necessary to produce effects on the estrogen receptor in MCF-7 cells. Given that the dose in this study is some 17 000-fold greater than the maximum dose that would be ingested, assuming consumption at the upper bound of the ADI, even allowing for the uncertainty introduced by species differences (rabbit versus human), it is not anticipated that dietary residues of MGA will have any estrogenic effects in humans consuming meat from animals treated with this drug.

MGA does not have antiestrogenic activity in MCF-7 cells (Perry et al., 2005). Nor does it have significant androgenic activity, as measured in assays of relative binding affinity to recombinant human androgen receptors or to human sex hormone binding globulin, when compared with a natural ligand such as dihydrotestosterone. In contrast, the active metabolite of the androgenic anabolic steroid trenbolone acetate, 17β-trenbolone, has a relative binding affinity to recombinant human androgen receptors similar to that of dihydrotestosterone (Bauer et al., 2000).

The anabolic mode of action of MGA in heifers is not firmly established, but is assumed to be by the stimulation of ovarian synthesis of endogenous estradiol, caused by follicular development; although there is follicular development, it is accompanied by inhibition of ovulation, likely due to blocking of the positive feed-back on gonadotrophin release that would normally be exerted by endogenous estrogen during an untreated estrous cycle (Henricks et al., 1997; Hageleit et al., 2000). The plasma concentrations of MGA that suppress estrus in heifers, when given at the recommended dose of 0.5 mg MGA/day in the feed, are in the range 25–50 pg/ml (Daxenberger et al., 1999; Hageleit et al., 2000; Pfaffl et al., 2002). Endogenous plasma estradiol concentrations increase from average values of about 1 pg/ml before treatment to about 5 pg/ml during treatment with 0.5 mg MGA/day (Henricks et al., 1997; Hageleit et al., 2000; Pfaffl et al., 2002). Mean plasma levels of luteinizing hormone (LH) increase slightly and the frequency of LH pulses increases during treatment with 0.5 mg MGA/day (Hageleit et al., 2000). If the amounts of MGA in the feed are increased to 1.5 or 5 mg MGA/day, which are 3 and 10 times, respectively, above the maximum recommended rate for addition to feed for growth promotion, there is a change in the profile of activity: there is negative feedback on pituitary gonadrotrophin secretion, with a fall in mean plasma LH concentration, absence of follicular development, low plasma estradiol concentrations of 1–2 pg/ml and no anabolic effect (Hageleit et al., 2000; Meyer, 2001; Pfaffl et al., 2002).

Pfaffl et al. (2002) investigated gene expression in heifers following hormone receptor activation by MGA in order to better define the mode of action of MGA in cattle. In heifers given 0, 0.5, 1.5 or 5 mg MGA/day for 8 weeks in the feed (two heifers per dose group), messenger ribonucleic acid (mRNA) expression for androgen, progesterone and estrogen (ERα and ERβ) receptors and for insulin-like

growth factor-1 (IGF-1) and its receptor was measured in liver and in neck and shoulder muscles using reverse transcription followed by real-time polymerase chain reaction (RT-PCR) amplification. IGF-1 is an important growth regulator in many tissues, and IGF-1 gene expression is known to be stimulated by estradiol. Plasma concentrations of MGA, IGF-1, estradiol and progesterone were also measured. In the lowest dose group of 0.5 mg MGA/day, corresponding to the maximum amount that is given for growth promotion in heifers, plasma estradiol and IGF-1 concentrations were significantly increased, whereas progesterone concentrations were significantly reduced compared with controls. At the higher doses of 1.5 and 5 mg/day, plasma estradiol and progesterone concentrations were reduced compared with controls. Linear regression analysis of expression data against increasing doses of MGA showed significant ($P < 0.05$) increases in androgen receptor and IGF-1 receptor expression in liver and in IGF-1 receptor expression in neck muscle. ERα in liver and neck muscle showed a dose-related trend of increasing expression, which achieved statistical significance only for liver. This study indicates that the anabolic properties of MGA may be mediated by IGF-1 and that MGA has some weak activity with respect to expression of the genes for androgen receptor and ERα in heifers in vivo.

2.2 Role of progesterone and progesterone receptors in human development and disease

The role of progesterone and progesterone receptors in human development and disease is relevant in considering exposures to MGA and its metabolites, given their progestogenic activity.

2.2.1 Breast development and breast cancer

The human mammary gland is not completely formed at birth but begins to develop in early puberty, when the primitive network of ductal structures enlarges and branches, forming more complex lobular structures at the ends of the terminal ducts, known as the terminal ductal lobular units (TDLUs) (Russo & Russo, 1987). The TDLUs are lined by a continuous layer of luminal epithelial cells, and most human breast tumours appear to be derived from TDLUs and have the morphological and biochemical characteristics of luminal epithelial cells. Human breast tumours also contain estrogen receptors and progesterone receptors that, in the normal breast, are expressed only in the luminal epithelial cells.

There is considerable clinical and epidemiological evidence concerning the role of endogenous estrogens in normal breast development (Laron et al., 1989; ESHRE Capri Workshop Group, 2004). There is also considerable evidence for the role of both endogenous estrogens and exogenous estrogens (in the form of oral contraceptives and hormone replacement therapy) as primary risk factors in the development of breast cancer in women (IARC, 1999; Liehr, 2001; ESHRE Capri Workshop Group, 2004; Rogan & Cavalieri, 2004), with estrogens causing proliferation of epithelial cells via estrogen receptor–mediated processes (Feigelson & Henderson, 1996).

The role of progesterone in breast development has been far less studied (Anderson, 2002). Studies on progesterone receptor knockout mice suggest that, while estradiol stimulates ductal elongation, progesterone induces lobuloalveolar development (Humphreys et al., 1997). It is assumed that progesterone plays a similar role in normal human breast development, stimulating TDLU formation and expansion during puberty and pregnancy (Anderson, 2002). It has also been shown that the normal luminal epithelial cell proliferation that occurs in the breast during the menstrual cycle is caused by progesterone (Schairer, 2002).

Progesterone has two receptor isoforms, PRA and PRB. Unlike estrogen receptors (ERα and ERβ), which are distinctly different receptors, in that the ERβ gene is smaller, has a different chromosomal location and encodes a shorter protein, PRA and PRB are transcribed from the same gene by alternative promoters. Both function as ligand-dependent nuclear transcription factors, and it is now thought that both can activate gene transcription, but can be differentiated in terms of the overlapping, but partially distinct, profile of genes that they can activate (Conneely et al., 2007). For example, in the mouse, PRB, but not PRA, mediates the effects of progesterone on mammary gland development (Conneely et al., 2002). In normal human breast tissue, progesterone receptor is present in 15–30% of luminal epithelial cells, but is not found elsewhere in the breast, and PRA and PRB are co-expressed at similar levels in the epithelial cells, suggesting that both proteins may be required to mediate physiological progesterone signalling (Mote et al., 2007). Cells with progesterone receptor also all contain ERα, and in normal tissue, estradiol stimulation of ERα expression also regulates the synthesis of progesterone receptor (Lee et al., 2006). Steroid receptor–expressing cells are separate from, but often adjacent to, dividing cells that contain markers of proliferation (Anderson, 2002; Clarke et al., 2004). Thus, it has been hypothesized that estradiol and/or progesterone control the proliferation of luminal epithelial cells indirectly by the receptor-containing cells acting as sensors and secreting positive or negative paracrine growth factors that influence the proliferative activity of nearby cells, according to the prevailing estradiol/progesterone concentrations (Anderson, 2002; Lee et al., 2006).

In normal breast tissue, ERα expression is inversely related to proliferation, whereas in the early stages of breast tumour development, there appears to be dysregulation, in that increasing ERα expression is accompanied by proliferation and progression towards malignancy (Anderson, 2002). In tumorigenesis, there is an increase in ERα expression at the early stages of ductal hyperplasia, with further increases in expression with increasing atypia. The role of progesterone and progesterone receptor is much less well understood (Wiebe, 2005), but human studies suggest that expression of progesterone receptor also increases with increasing atypia, and approximately 60% of invasive breast carcinomas express PRA and/or PRB (Allred et al., 2001). Enhanced ERα and progesterone receptor expression in premalignant epithelium would be expected to enhance the sensitivity to their respective ligands (Anderson, 2002).

There is evidence that human breast tissue is generated from stem cells and that it is the longevity of these stem cells that explains the extensive further development of the breast in puberty and pregnancy (Clarke et al., 2004; Lamb

et al., 2007). The pool of stem cells in breast tissue has also been suggested as a critical factor linking early life exposures with later development of cancer (Baik et al., 2005), the longevity of stem cells making them susceptible to genetic change and consequent transformation (Lamb et al., 2007). The pool of stem cells is known to contain some cells that are ERα/progesterone receptor positive, and they self-renew (Clarke et al., 2004). In normal tissue, progesterone acts by the secreted factor WNT4 to cause side branching in the mammary gland. WNT is also linked to self-renewal of adult stem cells; thus, it has been hypothesized that progesterone may regulate the mammary stem cell population (Lamb et al., 2007). The *WNT* family of genes is also associated with cellular responses such as carcinogenesis (IARC, 2007). In mammary tumorigenesis, it is hypothesized that accumulated mutations in stem cells lead to uncontrolled self-renewal of stem cells with aberrant differentiation (Dontu et al., 2004). Thus, progesterone as well as estrogen may play a role by increasing the number and rate of cell divisions, which in turn will increase the likelihood of mutational damage, or, after mutational change has occurred, by promoting the growth and survival of tumorigenic cells in progesterone receptor–positive phenotypes (ESHRE Capri Workshop Group, 2004).

Concerning the development of breast cancer in women, epidemiological evidence that has emerged during the last decade suggests that exogenous progestogens (medroxyprogesterone acetate [MPA], norethisterone or levonorgestrel), used together with estrogens in combined hormone replacement therapy, increase the risk of postmenopausal breast cancer to a greater extent than estrogen replacement therapy alone, with significant increases in relative risk for exposures both less than 5 years and more than 5 years (Ross et al., 2000; Schairer et al., 2000; Beral et al., 2003). In the most recent review of these data, the International Agency for Research on Cancer (IARC) commented that there are consistent epidemiological observations that the addition of a progestogen to estrogen in hormone replacement therapy for menopause confers a small, but significant, increase in the risk of breast cancer, which is greater than that in users of estrogen alone (IARC, 2007). The increase in risk in menopausal women is largely confined to current or recent users. The available evidence was considered by IARC to be inadequate to evaluate whether or not the risk for breast cancer varies according to the type of progestogen, its dose or the number of days each month it is added to the estrogen therapy. IARC concluded that there is sufficient evidence in humans for the carcinogenicity of combined estrogen–progestogen menopausal therapy in the breast. IARC also concluded that there is sufficient evidence in humans for the carcinogenicity of combined oral estrogen–progestogen contraceptives in the breast among current and recent users. IARC classified both types of preparation as “carcinogenic to humans (Group 1)” (IARC, 2007).

Some epidemiological studies have also identified an increase in the risk of breast cancer in association with red meat consumption, although the studies overall are inconsistent (Missmer et al., 2002; Cho et al., 2006), and it is notable that the reports of the World Cancer Research Fund, which has twice reviewed the evidence on the association between food categories and cancer at various sites, while identifying red meat as possibly associated with breast cancer in its 1997 report, did not do so in its 2007 report (WCRF, 1997, 2007). In a 12-year prospective

investigation, with follow-up to 2003 of over 90 000 premenopausal women enrolled in the United States Nurses Health Study II, greater red meat intake was strongly associated with an elevated risk of breast cancers that were estrogen receptor– or progesterone receptor–positive (512 such cancers out of a total of 1021 cases of invasive breast carcinoma), but not those that were estrogen receptor– or progesterone receptor–negative (Cho et al., 2006). The authors discussed the possible components of (cooked) red meat that might be carcinogenic, including not only residues of growth hormones in meat, but also heterocyclic amines, *N*-nitroso compounds, polycyclic aromatic hydrocarbons, haem iron and fat. Estrogenic mechanisms are mentioned for some of these components.

In established breast cancer, the results of use of progestogens in treatment regimens have been conflicting, with some reports showing no effects and others showing either stimulation or inhibition in human tumours or in human breast cancer cell lines (Wiebe, 2005). This is presumably a consequence of the heterogeneity of human breast tumours with respect to estrogen receptor and progesterone receptor responsiveness. In vitro metabolism studies in both normal tissue and human breast tumour tissue and in non-tumorigenic and tumorigenic breast cell lines have shown that they all convert progesterone into two groups of metabolites, the 5α-reduced and the 4-ene metabolites, which have marked breast cancer modulating activities. The 5α-reduced group stimulates cell proliferation, and the 4-ene group suppresses cell proliferation. In tumour tissue and tumorigenic cell lines, there is a marked change in the ratio of the metabolites towards increased 5α-reductase activity and lower production of 4-ene metabolites (Wiebe, 2005). The possible effects of metabolites of exogenous progestogens have not been investigated, but the studies on progesterone suggest that metabolites may influence tumour progression.

Animal models using progesterone receptor knockout mice also suggest that the progesterone receptor has a role in growth stimulation and tumour promotion in mammary carcinogenesis (Schairer, 2002; Conneely et al., 2003). Zoo felids are at high risk for mammary cancer, but the risk is further increased by the use of MGA in the form of contraceptive implants, and it has been noted that in most felid mammary tumours, estrogen receptor expression is low, but progesterone receptor expression persists (Munson & Moresco, 2007).

2.2.2 Uterine (endometrial) cancer

A number of reports on captive animals in zoos have described uterine endometrial pathology, including cancer, induced by MGA given as a contraceptive implant over several years in species belonging to the families Felidae (cats), Canidae (dogs) and Viverridae (civets) (Chittick et al., 2001; Munson et al., 2002). However, in women, the addition of a progestogen to estrogen for menopausal therapy prevents the development of endometrial hyperplasia and reduces the rate of endometrial cell proliferation induced by treatment with estrogen alone (IARC, 2007). The addition of a progestogen to estrogen therapy is also associated with a reduced incidence of uterine cancer compared with the elevated risk from use of estrogen alone, with five out of eight studies in which progestogen was added every day showing relative risks below unity, compared with never users of hormone replacement therapy, irrespective of the specific type or dose of progestogen used

(IARC, 2007). Taking all the results of combined estrogen–progestogen menopausal therapy together, IARC (2007) concluded that they “are consistent with the view that the addition of progestagens to estrogen therapy lessens the risk associated with the use of estrogens alone, and that the greater the number of days per month that progestagens are added, the greater is the reduction in risk”. The most commonly used progestogens in combined menopausal therapy are MPA, norethisterone and levonorgestrel; of the three, MPA is the closest to MGA in chemical structure and hormonal activity profile. IARC concluded that there is convincing evidence in humans that combined oral estrogen–progestogen contraceptives also have a protective effect against carcinogenicity in the endometrium (IARC, 2007).

2.2.3 Ovarian cancer

Use of combined estrogen–progestogen oral contraceptives reduces the risk of ovarian cancer compared with never users, with the reduction in risk persisting for at least 20 years after cessation of use. The data on combined estrogen–progestogen hormone replacement therapy and ovarian cancer are inadequate for evaluation (IARC, 2007).

2.2.4 Cervical cancer

The risk for cervical cancer is increased with increasing duration of use of combined estrogen–progestogen oral contraceptives. The data on combined estrogen–progestogen hormone replacement therapy and cervical cancer are inadequate for evaluation (IARC, 2007).

2.2.5 Precocious puberty

Available data do not allow evaluation of the possible influence of environmental exposures, including endocrine disrupters, on the timing of onset of puberty in humans (Lee et al., 2001), but speculation about any such influence has concerned exposure to xenoestrogens and not progestogens (Partsch & Sippell, 2001). However, even in the case of estrogens, it has been commented that published data suggest that the composition of the diet is a less important determinant of pubertal events than is attainment of a certain body size or fat mass (de Muinich Keizer & Mul, 2001).

2.2.6 Gynaecomastia

Two outbreaks of gynaecomastia possibly associated with food contaminated with growth promoters have been described in the literature. An epidemic of gynaecomastia in 1977 in prepubertal boys and girls in a private school in Milan, Italy, has been described (Fara et al., 1979). Although estrogenic contamination of meat in the school canteen was suspected as the cause of the outbreak, it was never proven (Chiumello et al., 2001). In a follow-up case–control study of the affected children 20 years after the outbreak (Chiumello, 1997), a slightly earlier onset of puberty in girls and a slightly later onset of puberty in boys were found, when case groups were compared with controls; in boys, testicular

volume was slightly reduced and testicular hypoplasia more frequent in the case group compared with controls. However, none of these changes were statistically significant at the $P = 0.05$ level. Fertility as measured by paternity was significantly reduced in the boys who were aged 11–14 years at the time of the outbreak. Chiumelo et al. (2001) also described the reports from Puerto Rico in the 1980s of high incidences of gynaecomastia, premature thelarche, precocious puberty and polycystic ovaries found in a large number of children, said to be in excess of 10 000. The effects were said to be associated with estrogen contamination of poultry and with environmental pollution from factories making estrogen–progestogen medicines (Chiumello et al., 2001). There have been no reports of gynaecomastia in association with progestogenic contamination of meat.

2.2.7 Prenatal development, including the reproductive tract

The effects of progesterone and, to a limited extent, progestogens on fetal development, including fetal sexual development, have been reviewed by Golub et al. (2006). A Cochrane review is also available that describes a meta-analysis of 15 randomized controlled trials of the outcome of progestogen therapy to prevent spontaneous abortion during the first 20 weeks of pregnancy (Haas & Ramsey, 2008). The information in this section is drawn from these reviews.

Only limited work has been done on progesterone receptor expression in the embryo. In human fetuses, progesterone receptor expression (mainly PRB) is seen in weeks 11–21 of gestation in a wide variety of reproductive and non-reproductive tissues. Later in gestation, expression is limited to reproductive organs, pancreas and intestinal cells.

Concern about adverse prenatal effects of exposure to progestogens has emerged from the use of progestogens as a therapeutic agent during pregnancy. Early case-reports of hypospadias in progestogen-treated pregnancies triggered subsequent epidemiological investigations, but reports of an association between exposure to progestogens during gestation and hypospadias in male offspring have been conflicting, and meta-analyses and reviews have not been able to confirm an association. Laboratory animal studies on the influence of progesterone and other progestogens on anogenital distance have produced conflicting findings (Golub et al., 2006).

Virilizing effects on female genitalia of prenatal exposure to some progestogens have been reported in both animals and humans, but the progestogens concerned were those known to also have androgenic activity. MGA is devoid of androgenic activity.

The Cochrane review assessed miscarriage, preterm birth, stillbirth, neonatal death, low birth weight, fetal genital anomalies (including virilization and hypospadias), teratogenic effects and admissions to special care units. There was no significant effect of progestogen treatment on any of these adverse outcomes. The progestogens used in the trials were MPA, cyclopentyl enyl ether of progesterone, dydrogesterone, hydroxyprogesterone caproate, allylestrenol and progesterone, given orally, intramuscularly or by vaginal suppository (Haas & Ramsey, 2008).

Northen et al. (2007) examined surviving children of mothers participating in a multicentre, placebo-controlled trial of intramuscular 17α-hydroxyprogesterone caproate for prevention of recurrent miscarriage (mean age at follow-up, 48 months). The children were screened for general health by a questionnaire to the guardian and given a physical examination and a developmental screen, and gender-specific roles were assessed. There were no significant differences between progestogen-exposed children (n = 194) and the placebo group (n = 84). 17α-Hydroxyprogesterone is one of the major circulating metabolites of natural progesterone in women.

Laboratory animal studies on the effect of exposure to high doses of progesterone either prenatally or between birth and weaning on male sexual development suggest that it can suppress androgen production and alter male sexual development.

2.3 Genotoxicity

The genotoxicity of substances used as growth promoters became a high-profile issue during the last decade following publication of experimental studies showing that natural estrogenic hormones, such as estradiol, estrone and estriol, can damage deoxyribonucleic acid (DNA). It has now been established that the genotoxic agents formed by these estrogens are quinones, which form DNA adducts. The quinones are derived by aromatic hydroxylation at the C-2 and C-4 positions by cytochrome P450 enzymes, followed by peroxidation to form catechol estrogens, then oxidation to the corresponding 2,3- and 3,4-quinones (Cavalieri & Rogan, 2004; IARC, 2007).

The genotoxicity of hormonal steroids used in human medicinal drugs has been reviewed by Joosten et al. (2004). Progestogens were considered in several separate groups, depending on their chemical structures—progesterone itself, the norethisterone group, the norgestrel group, the MPA group and the spironolactone group. MGA was not among those considered, but it is most closely related to the MPA group in terms of its structure and hormonal profile, although it should be noted that several steroids in the MPA group, including MPA itself and cyproterone acetate, have hormonal profiles that differ somewhat from that of MGA, in that they are also anti-androgenic. Several steroids in the MPA group, but not MPA itself, have genotoxic potential. Cyproterone acetate, a member of the MPA group, was negative in vitro in tests for gene mutation in bacteria (Ames tests) and in hamster V79 cells, but was positive for gene mutation in vivo in liver of the Big Blue rat. Cyproterone acetate was also positive in several indicator tests for DNA damage using human or rat hepatocytes, including measurement of DNA adducts using the ^{32}P-postlabelling technique, unscheduled DNA synthesis and formation of DNA single strand breaks. Joosten et al. (2004) considered that the genotoxicity is likely to be attributable to the highly reactive carbonium ion metabolite that is formed from the unstable sulfonated 3-hydroxy metabolite of cyproterone acetate; the carbonium ion is short-lived and so active only when formed within a target cell. DNA adduct formation in rat liver in vivo and in human hepatocytes in vitro has also been shown with other members of the MPA group, chlormadinone and megestrol acetate (MA) (the latter being the member of this MPA group structurally most closely related to

MGA). In tests for chromosome breakage, cyproterone acetate gave an equivocal response in cultured rat hepatocytes but was negative in cultures of human lymphocytes; in vivo, cyproterone acetate was negative in a mouse bone marrow micronucleus test but positive in rat liver. Similarly, chlormadinone and MA were negative for chromosomal abnormalities in cultured human lymphocytes but positive for micronucleus induction in rat liver in vivo. The more recent review of estrogens and progestogens by IARC (2007) did not reveal any additional tests on steroids from the MPA group.

Since the previous evaluation by the Committee (Annex 1, reference *147*), additional in vitro studies on the genotoxicity of MGA have been conducted by Metzler & Pfeiffer (2001), although few details of the studies were given. They reported that MGA did not induce *HPRT* gene mutations in V79 cells at concentrations between 50 and 100 μmol/l, micronuclei in V79 cells at 20–100 μmol/l or *lacI* mutations in *Escherichia coli* bacteria at 400 μmol/l. It was also noted that there were clear signs of apoptosis in the nuclei of the V79 cells at concentrations of 75 and 100 μmol/l. Subsequent DNA gel electrophoresis confirmed the typical DNA ladder of apoptotic cells. When the commercial sample of MGA used was subjected to HPLC analysis, it was revealed that there were a number of impurities that had mass spectra identical to those of some of the MGA metabolites. Separation of these impurities and retesting showed that pure MGA was devoid of apoptotic activity, whereas the sample of the impurities induced apoptosis.

2.4 Reproductive and developmental toxicity

The developmental effects of exposure to 0.5 mg MGA/kg bw per day given orally in corn syrup to rabbits have been investigated following administration during gestation and lactation (gestation day 15 through to postnatal week 4), the "adolescent" period (postnatal weeks 4–12) or adulthood (12 weeks of exposure between the 1st and 2nd years of life). Comparable groups were given trenbolone acetate or zeranol subcutaneously. The numbers of male rabbits examined per dose group were 10, 10 and 8 for the gestation/lactation, adolescent and adulthood periods of exposure, respectively, but the numbers of dams from which the males were derived in the gestation/lactation study were not stated. No data were available in the report on the variance in the measurements. In the gestation/lactation and adolescent studies, the offspring were killed at 25 weeks of age. The objective of the study was to examine testicular function and sexual capacity at maturity. Body and organ weights were significantly increased in males exposed to MGA during adolescence, compared with controls. Significantly smaller testis weight was seen in males exposed to MGA during gestation/lactation. Cryptorchidism was not seen in the MGA-treated groups, but there were four cases in animals exposed to trenbolone acetate or zeranol during gestation/lactation or adolescence. Males exposed to MGA during adulthood showed a low degree of germinal epithelium loss in the testis. Baseline concentrations of follicle stimulating hormone and LH were lower at 6 weeks of age, but not at 12 weeks of age, in those exposed to MGA during adolescence, and estrone was significantly elevated at 6 and 12 weeks. Cauda epididymal sperm reserves were reduced in those exposed to MGA in adolescence, but this was an isolated finding among no changes in semen volume, sperm

concentration or sperm motility in ejaculate or in daily sperm production (Rajpert-De Meyts et al., 2001).

A study has examined the semen quality in 387 male partners of pregnant women in five cities in the USA between 1999 and 2005, in relation to the amount of beef their mothers reported consuming during pregnancy. Regression analysis showed that sperm quality was inversely related to the number of beef meals per week that their mothers reported consuming during pregnancy. In sons of high consumers (>7 beef meals per week), sperm concentration was 24.3% lower (43.1×10^6/ml versus 56.9×10^6/ml), and the proportion of sons with sperm concentrations below 20×10^6/ml (the World Health Organization threshold for subfertility) was 3 times higher (17.7% versus 5.7%) than in sons whose mothers ate less beef. Both these differences were statistically significant. A history of previous subfertility was also significantly higher in sons of mothers who were high consumers of beef. Sperm concentrations were not significantly related to the mothers' consumption of other types of meat or to the consumption of meats of any type by the sons themselves. Sperm motility and morphology were not affected by mothers' beef consumption. The authors commented that the results may be subject to recall errors but that the mothers did not know their sons' sperm quality at the time of completing the questionnaire about food consumption and would be unlikely to suspect subfertility in their sons, given that the sons' partners were pregnant. The authors speculated that the reductions in sperm concentration observed may be due to prenatal exposure to anabolic steroid residues and/or to other xenobiotics in beef (Swan et al., 2007).

2.5 Immunotoxicity

The immunotoxicity of MGA was considered by the Committee in its evaluation of 2000 (Annex 1, reference *147*), which can be summarized as follows. In the mammalian repeat-dose studies, immune system parameters, such as serum cortisol, leukocyte counts, and adrenal, thymus and spleen weights, were affected by MGA only at doses of ≥50 μg/kg bw per day. Similarly, in the three special studies on immunotoxicity in the rat, MGA showed immunosuppressive activity only at doses of ≥5 mg/kg bw per day. In long-term clinical trials of MGA in humans, no adverse effects associated with immunosuppression were reported. In a human study, a dose of 20 mg/person reduced plasma cortisol concentrations to about 20% of pretreatment values, but there was no reduction in plasma cortisol at a dose of 10 mg/person; MGA was reported to have a potency of only 1/40th of that of dexamethasone with respect to suppression of plasma cortisol levels.

In its previous evaluation (Annex 1, reference *147*), the Committee also considered reports of effects on the immune system during long-term treatment of cattle. Heifers given 0.45 mg MGA/day for 2.5 months showed a 50% reduction in plasma cortisol levels compared with untreated controls, together with significant reductions in adrenal weight and in the width of the zona fasciculata. (Note that there is an error in Annex 1, reference *147:* the dose was reported by JECFA as 0.2 mg/kg bw per day, whereas the dose used and reported by Purchas et al. [1971] was 0.45 mg/day.) MGA did not significantly alter the ability of the uterus to resist infection after infusion of *E. coli* (Lauderdale, 1971).

In a recent study, groups of 24 heifers were fed 0 or 0.5 mg MGA/day and after 14 days were inoculated with *Mannheimia haemolytica* (a bacterium causing bovine respiratory disease), which was described as a mild challenge. The animals were observed for clinical signs of respiratory disease and slaughtered at intervals up to 138 h after inoculation, and the lungs were examined. In those fed MGA, the response was increased, the lung lesions being of greater severity and present in a larger number of cattle at 138 h, compared with controls (Corrigan et al., 2007).

4. COMMENTS

The progestogenic activity of MGA in humans was reviewed by the Committee in 2000 (Annex 1, reference *147*). Steroid receptor specificity and relative binding activity of MGA and its four major metabolites were also reviewed by the Committee in 2004 (Annex 1, reference *169*), when it was concluded that MGA and its metabolites exert their biological action primarily as progestogens and secondarily as glucocorticoids, with no androgenic or estrogenic activity at relevant physiological concentrations. The Committee noted in 2004 that the most potent progestogenic metabolite of MGA, 2β-hydroxy-MGA, had a potency of only 12% compared with that of MGA itself. In the present evaluation, additional in vitro studies on progesterone receptor binding affinity were reviewed, and they are consistent with the previous data. Overall, the studies indicate that the relative progesterone receptor binding affinity of MGA is greater than that of progesterone by around 5- to 11-fold, depending on the assay, whereas the metabolites of MGA have lower affinities than progesterone.

The first area the Committee was asked to reconsider was the important toxicological end-points in relation to particular subpopulations that may be exposed. The Committee considered the hormonal activity profile of MGA in relation to prenatal and postnatal development, genotoxicity, cancer and immunotoxicity. Potential pharmacological and toxicological effects have been considered.

The Committee noted that many of the general concerns that have been expressed concerning the possible effects of endocrine disrupters on fetal and child development relate to estrogenic, androgenic and anti-androgenic activity of substances. MGA is not considered to show estrogenic activity in vivo and has estrogenic activity only at high concentrations (nanomoles to micromoles per litre) in vitro. In this regard, the Committee noted that many of the data submitted by the European Commission referred to the known or possible effects of estrogens, which are not considered relevant for MGA. Nor is MGA considered to have anti-estrogenic activity, from its lack of activity in the human breast cancer cell line, MCF-7 cells, or (anti-)androgenic activity, from its lack of binding to the androgen receptor.

Concerning progestogenic activity, one way of assessing whether exposure to residues of MGA and its metabolites in meat would have any effect in adult humans is to compare the pharmacological activity of progestogenic steroids that are structurally related to MGA, as was also done earlier by the Committee (Annex 1, reference *147*). MGA has not been used in human therapeutic medicine, but the closely related compounds MA and MPA are, or have been, used in human contraception and for the treatment of endometriosis and cancer (breast,

endometrium, ovary, testis). The amounts of MA or MPA used orally in endometriosis and cancer therapy are in the range of 30–800 mg daily. The amounts of MPA used orally for contraception are lower, in the range of 2.5–10 mg/day, whereas contraceptive doses of MA have been reported to be in the range of 0.35–0.5 mg/day. MGA has been estimated to be less potent than MA, based both on inhibition of menstruation in estrogen-primed women and on alteration of cervical mucus. The relative binding affinity of MPA to the bovine progesterone receptor, compared with that of progesterone, is 223%, whereas that of MGA is 526%. Data from humans and laboratory animals indicate that MGA is about 4 times more potent than MPA with respect to activity on the endometrium. This information, together with that on the lowest pharmacologically active doses of MPA in the range of 2.5–10 mg daily, suggests that amounts of orally ingested MGA in humans would need to be of the order of 0.5 mg/day (8 μg/kg bw for a 60-kg person) or more to exert any discernible pharmacological effect. This is supported by limited data showing that 7.5 or 10 mg MGA/day, but not 5 mg/day, delays the onset of menstruation in regularly ovulating women, and that 2.5 mg MGA/day induces withdrawal bleeding in estrogen-primed amenorrhoeic women. Ingestion of MGA at the level of the upper bound of the ADI (1.8 μg/day for a 60-kg person) would be around 300-fold below 0.5 mg/day, the estimate of the minimal or no-effect level for alteration of menstrual cycle hormones and endometrial effects of MGA in women. The ADI for MGA is derived by application of an uncertainty factor to the minimally effective progestogenic dose of 5 μg MGA/kg bw per day affecting the menstrual cycle in cynomolgus monkeys, which appear to be the most sensitive species.

Data on the role of progesterone during embryonic and fetal development and on development of the reproductive tract in particular are sparse in comparison with the information that is available on estrogenic, androgenic and anti-androgenic substances. Although there are laboratory animal studies and occasional human case-reports of adverse effects of prenatal or perinatal exposure to high doses of progesterone and other progestogens, data from human trials on the use of progestogens in pregnancy (e.g. to prevent spontaneous abortions) do not provide any evidence of adverse effects on the embryo, fetus or young child.

Data on the effects of MGA on the immune system are limited, and no new information was submitted. The information reviewed by the Committee in 2000 (Annex 1, reference *147*) indicated that there are measurable effects on adrenal gland size, cortisol secretion and susceptibility to infection in cattle given 0.5 mg MGA/day in the feed. Data from clinical trials in humans given MGA indicated that 10 mg/person (167 μg/kg bw) was without any effect on adrenal hormonal responsiveness, a no-observed-effect level (NOEL) that is at least 10 000 times higher than the exposure would be at the upper bound of the ADI (0.03 μg/kg bw). These exposure and dose considerations indicate that any indirect effect of MGA through glucocorticoid alteration of the immune system is unlikely. The Committee also noted that in the mammalian repeat-dose studies considered in its previous evaluation, immune system parameters, such as serum cortisol, leukocyte counts, and adrenal, thymus and spleen weights, were affected by MGA only at doses of 50 μg/kg bw per day or more. Similarly, in three special studies on immunotox-icity in the rat, also considered in the previous evaluation, MGA showed

immunosuppressive activity only at doses of 5 mg/kg bw per day or more. Overall, the data show that the effects of MGA on immune system parameters are apparent only at doses that are 6–600 times higher than the dose at which MGA is considered to have minimal progestogenic activity in humans (8 μg/kg bw).

The second area the Committee was asked to consider concerned the data indicating that hormones may act not only by interaction with hormone receptors and the consequent downstream effects, but also by other mechanisms, such as direct or indirect genotoxic activity. This is an important question, because it raises the possibility that adverse effects such as cancer might be incurred through toxic modes of action that are without a discernible threshold.

The Committee noted that in 2000 it had reviewed a comprehensive set of genotoxicity tests on MGA itself, including in vitro tests on gene mutation in bacteria and mammalian cells, unscheduled DNA synthesis in rat hepatocytes and single strand breaks in mammalian cells and an in vivo test for micronucleus formation in mouse bone marrow. The results were all negative, and the Committee had concluded that MGA was not genotoxic (Annex 1, reference *147*). New data on genotoxicity submitted for the present evaluation have shown that MGA does not induce gene mutations or micronuclei in mammalian cells in vitro, nor does it induce *lacI* mutations in *E. coli*. A review published in 2004 on the genotoxicity of hormonal steroids showed that the majority of progestogens are not genotoxic. However, some progestogens that are structurally related to MGA, sharing the 17-hydroxy-3-oxo-pregna-4,6-diene structure, do have genotoxic potential. Since the genotoxic profile of MGA itself is uniformly negative, it appears to differ from some of those with which it shares a structural similarity. The Committee reaffirmed its previous view that the data on MGA show that it is not genotoxic. Thus, there is no evidence that MGA would increase the risk of cancer by a genotoxic mechanism.

However, progesterone, after binding to progesterone receptors and triggering downstream gene expression, clearly plays a role in, for example, human breast development and progression of some types of breast cancer. The Committee also recalled that several long-term studies in mice, reviewed in its previous evaluation, showed dose-related increases in the incidence of mammary adenocarcinoma, but that mechanistic studies had shown that the effect was attributable to promotion caused by stimulation of prolactin secretion, with a NOEL for mammary tumorigenesis of 0.5 mg/kg bw per day.

Epidemiological evidence from both combined estrogen–progestogen contraceptive use and combined estrogen–progestogen hormone replacement therapy shows that the addition of the progestogen confers a small, but significant, increase in the risk of breast cancer, which is greater than that in users of estrogen alone. Since duration of exposure is a significant risk factor for breast cancer in women taking combined oral contraceptives or combined hormone replacement therapy and the increase in risk for breast cancer is largely confined to current or recent users, this also suggests that the progestogenic component is acting more as a promoter than as an initiator of cancer, which is consistent with a non-genotoxic mode of action. Although the increase in risk of breast cancer is seen in association with pharmacologically active doses of progestogen, the doses used are such that

the data do not allow no-effect levels for the increase in risk to be established. However, exposure to MGA from residues in meat is several orders of magnitude lower than the pharmacologically active doses of a progestogen that would elevate the risk of breast cancer. Given the involvement of progesterone receptors in the carcinogenic effects of progestogens, there should be no increase in the risk of cancer at exposure levels below those that activate progesterone receptors in vivo in humans. An additional consideration is that absorption of low concentrations of MGA present as residues in meat would be followed by extensive and rapid metabolism in the liver and that the metabolites of MGA have less binding affinity for progesterone receptors than progesterone itself. The Committee also noted that very young infants, in whom metabolizing capacity is not fully developed, would not be consuming meat or other animal tissues in which MGA residues may be present.

It can be concluded that progestogens do not have a carcinogenic effect on the uterus, since all the available clinical and epidemiological data show that progestogens oppose the hyperplastic effect of estrogens in the endometrium and reduce the likelihood of endometrial cancer in women also exposed to exogenous estrogens. Use of combined estrogen–progestogen oral contraceptives reduces the risk of ovarian cancer and increases the risk of cervical cancer, whereas the data from use of combined estrogen–progestogen hormone replacement therapy are inadequate for evaluation with respect to these two cancer sites. The influence of the progestogen component with respect to these two cancer sites has not been separately analysed.

The fourth area the Committee was asked to consider was the authorized uses according to good practice in the use of veterinary drugs that it considers appropriate in the evaluation and their respective impact on potential exposure and risk estimates. Good practice in the use of veterinary drugs is the official recommended or authorized usage, including withdrawal periods, approved by national authorities of veterinary drugs under practical conditions (Codex Alimentarius Commission, 2007b). In line with established practice in JECFA, when assessing exposure to residues and risk, the Committee considers only maximum residues from approved treatments. In the case of MGA, the Committee considered only the approved use in heifers at the recommended dose range of 0.25–0.5 mg/heifer per day for recommending MRLs. The Committee did note that in the case of MGA, which is added to the feed, if the level of addition increases to 3 times the maximum recommended dose of 0.5 mg/heifer per day, then the hormonal activity profile changes, and the desired effect (growth promotion) does not occur.

4. EVALUATION

In reaching its conclusions, the Committee considered not only the data on MGA from its previous toxicological evaluation in 2000 and the few new studies on MGA that have been published or submitted since then, but also more general information on the role of progestogens in human reproduction, prenatal and child development, and cancer. The issue of immunotoxicity was also revisited. The Committee noted previously that MGA has both progestogenic and glucocorticoid activity, and that both earlier and new data confirm these as the principal hormonal

activities of MGA, with only weak estrogenic activity shown at relatively high concentrations in vitro.

There are no data on plasma concentrations of MGA following human dietary exposure. However, based on the comparative data considered in the Committee's previous evaluations (Annex 1, references *146* and *169*) showing similarities in absorption and metabolism of MGA among cattle, rats, rabbits and humans, it can be estimated that the plasma concentration in humans after ingestion of 0.03 µg/kg bw (equivalent to the upper bound of the ADI) would be around 0.5–1 pg/ml. This is some 4000-fold below the minimum concentration necessary to stimulate proliferation in the human breast cancer cell line, MCF-7 cells, which is a sensitive indicator of estrogenic activity. In addition, a study in rabbits found concentrations of MGA in plasma in the low nanogram per millilitre range following an oral dose of 0.5 mg/kg bw. This is in the same range as the minimum concentration of MGA necessary to produce effects on the estrogen receptor in MCF-7 cells. Given that the dose in this study was some 17 000-fold greater than the maximum intake of humans exposed to residues in meat, assuming consumption at the upper bound of the ADI, even allowing for the uncertainty introduced by species differences (rabbit versus human), it is highly unlikely that dietary residues of MGA will have any estrogenic effects in humans consuming meat from animals treated with this drug. The Committee also noted that MGA is devoid of genotoxic activity both in vitro and in vivo; thus, non-thresholded mechanisms of carcinogenicity are unlikely to play any role.

Concerning progestogenic activity, there is a small, but significant, increase in risk of breast cancer in humans exposed to progestogens in the form of combined estrogen–progestogen oral contraceptives or hormone replacement therapy, and the evidence suggests that the progestogenic component is acting more as a promoter than as an initiator of cancer. On the basis of comparative estimates of progestogenic activity, the exposure to MGA and its metabolites from residues in meat consumed at the upper bound of the ADI is 200–300 times lower than these pharmacologically active doses and below a dose that would produce any measurable effect on progesterone receptors. Although MGA causes mammary tumorigenesis in the mouse, this is attributable to stimulation of prolactin secretion having a promoter activity, and there is a clear NOEL of 0.5 mg/kg bw per day for this effect. This NOEL is more than 15 000 times higher than the exposure would be at the upper bound of the ADI. The Committee therefore concluded that residues of MGA and its metabolites would be unlikely to have any influence on the development of breast cancer.

Concerning glucocorticoid activity and effects on the immune system, there is no new information. However, the Committee noted that the NOEL for adrenal hormonal responsiveness to MGA in humans is at least 10 000 times higher than the exposure would be at the upper bound of the ADI. Similarly, the NOEL for immunosuppressive activity is at least 1000 times higher than the exposure would be at the upper bound of the ADI.

The Committee concluded overall that the new data do not provide any basis to reconsider the ADI. Human exposure to residues of MGA and its metabolites in

meat, resulting from the use of MGA as a feed additive in cattle at a daily dose of 0.25–0.5 mg/heifer, would be unlikely to have any adverse effects on adults, children, the embryo or the fetus.

5. REFERENCES

Allred, D.C., Mohsin, S.K. & Fuqua, S.A.W. (2001) Histological and biological evolution of human premalignant breast disease. *Endocr. Relat. Cancer*, **8**, 47–61.

Anderson, E. (2002) The role of oestrogen and progesterone receptors in human mammary development and tumorigenesis. *Breast Cancer Res.*, **4**, 197–201.

Baik, I., DeVito, W.J., Ballen, K., Becker, P.S., Okulicz, W., Liu, Q., Delpapa, E., Lagiou, P., Sturgeon, S., Trichopoulos, D., Queensberry, P.J. & Hsieh, C.-C. (2005) Association of fetal hormone levels with stem cell potential: Evidence of early life roots of human cancer. *Cancer Res.*, **65**, 358–363.

Bauer, E.R.S., Daxenberger, A., Petri, T., Sauerwein, H. & Meyer, H.H.D. (2000) Characterisation of the affinity of different anabolics and synthetic hormones to the human androgen receptor, human sex hormone binding globulin and to the bovine progestin receptor. *APMIS*, **108**, 838–846.

Beral, V. & the Million Women Study collaborators (2003) Breast cancer and hormone replacement therapy. *Lancet*, **362**, 419–427.

Cavalieri, E. & Rogan, E. (2004) A unifying mechanism in the initiation of cancer and other diseases by catechol quinones. *Ann. N.Y. Acad. Sci.*, **1028**, 247–257.

Chittick, E., Rotstein, D., Brown, T. & Wolfe, B. (2001) Pyometra and uterine adenocarcinoma in a melengestrol acetate–implanted captive coati (*Nasua nasua*). *J. Zoo Wildlife Med.*, **32**, 245–251.

Chiumello, G. (1997) *Long-term effect in children of exposure to estrogen-contaminated meat: A retrospective group study.* Original research report submitted to WHO by the European Commission.

Chiumello, G., Guarneri, M.P., Russo, G., Stroppa, L. & Sgaramella, P. (2001) Accidental gynecomastia in children. *APMIS*, **109**, S203–209.

Cho, E., Chen, W.Y., Hunter, D.J., Stampfer, M.J., Colditz, G.A., Hankinson, S.E. & Willet, W.C. (2006) Red meat intake and risk of breast cancer in premenopausal women. *Arch. Intern. Med.*, **166**, 2253–2259.

Clarke, R.B., Spence, K., Anderson, E., Howell, A., Okano, H. & Potten, C.S. (2004) A putative human breast stem cell population is enriched for steroid receptor–positive cells. *Dev. Biol.*, **277**, 443–456.

Codex Alimentarius Commission (2007a) *Report of the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods, Breckenridge, CO, USA, 3–7 September 2007*. Rome, Italy, Food and Agriculture Organization of the United Nations (ALINORM 08/31/31; http://www.codexalimentarius.net/web/archives.jsp?year=08).

Codex Alimentarius Commission (2007b) *Procedural manual*, 17th ed. Rome, Italy, World Health Organization and Food and Agriculture Organization of the United Nations (http://www.fao.org/docrep/010/a1472e/a1472e00.htm).

Conneely, O.M., Mulac-Jericevic, B., DeMayo, F., Lydon, J.P. & O'Malley, B.W. (2002) Reproductive functions of progesterone receptors. *Rec. Prog. Horm. Res.*, **57**, 339–355.

Conneely, O.M., Mulac-Jericevic, B.M. & Lydon, J.P. (2003) Progesterone receptors in mammary gland development and tumorigenesis. *J. Mammary Gland Biol. Neoplasia*, **8**, 205–214.

Conneely, O.M., Mulac-Jericevic, B. & Arnett-Mansfield, R. (2007) Progesterone signalling in mammary gland development. *Ernst Schering Found. Symp. Proc.*, **2007**(1), 45–54.

Corrigan, M.E., Drouillard, J.S., Spire, M.F., Mosier, D.A., Minton, J.E., Higgins, J.J., Loe, E.R., Depenbusch, B.E. & Fox, J.T. (2007) Effect of melengestrol acetate on the inflammatory response in heifers challenged with *Mannheimia haemolytica*. *J. Anim. Sci.*, **85**, 1770–1779.

Damstra, T., Barlow, S., Bergman, A., Kavlock, R. & Van der Kraak, G., eds. (2002) *Global assessment of the state-of-the-science of endocrine disruptors*. Geneva, Switzerland, World Health Organization, International Programme on Chemical Safety (WHO/PCS/EDC/02.2).

Daston, G.P., Cook, J.C. & Kavlock, R.J. (2003) Uncertainties for endocrine disrupters: Our view on progress. *Toxicol. Sci.*, **74**, 245–252.

Daxenberger, A., Meyer, K., Hageleit, M. & Meyer, H.H.D. (1999) Detection of melengestrol acetate residues in plasma and edible tissues of heifers. *Vet. Q.*, **21**, 154–158.

de Muinich Keizer, S.M. & Mul, D. (2001) Trends in pubertal development in Europe. *Hum. Reprod. Update*, **7**, 287–291.

Dontu, G., El-Ashry, D. & Wicha, M.S. (2004) Breast cancer, stem/progenitor cells and the estrogen receptor. *Trends Endocrinol. Metab.*, **15**, 193–197.

ESHRE Capri Workshop Group (2004) Hormones and breast cancer. *Hum. Reprod. Update*, **10**, 281–293.

Fara, G.M., Del Corvo, G., Bernuzzi, S., Bigatello, A., Di Pietro, C., Scaglioni, S. & Chiumello, G. (1979) Epidemic of breast enlargement in an Italian school. *Lancet*, **2**, 295–297.

Feigelson, H.S. & Henderson, B.E. (1996) Estrogens and breast cancer. *Carcinogenesis*, **17**, 2279–2284.

Golub, M.S., Kaufman, F.L., Campbell, M.A., Li, L.-H. & Donald, J.M. (2006) "Natural" progesterone: Information on fetal effects. *Birth Defects Res. B Dev. Reprod. Toxicol.*, **77**, 455–470.

Haas, D.M. & Ramsey, P.S. (2008) Progestogen for preventing miscarriage. *Cochrane Database Syst. Rev.*, **2008**(2), CD003511.

Hageleit, M., Daxenberger, A., Kraetzl, W.-D., Kettler, A. & Meyer, H.H.D. (2000) Dose-dependent effects of melengestrol acetate (MGA) on plasma levels of estradiol, progesterone and luteinising hormone in cycling heifers and influences on estrogen residues in edible tissues. *APMIS*, **108**, 847–854.

Hageleit, M., Daxenberger, A. & Meyer, H.H.D. (2001) A sensitive immunoassay (EIA) for the determination of melengestrol acetate (MGA) in adipose and muscle tissues. *Food Addit. Contam.*, **18**, 285–291.

Henricks, D.M., Brandt, R.T., Jr, Titgemeyer, E.C. & Milton, C.T. (1997) Serum concentrations of trenbolone-17β and estradiol-17β and performance of heifers treated with trenbolone acetate, melengestrol acetate, or estradiol-17β. *J. Anim. Sci.*, **75**, 2627–2633.

Hotchkiss, A.K., Rider, C.V., Blystone, C.R., Wilson, V.S., Hartig, P.C., Ankley, G.T., Foster, P.M., Gray, C.L. & Gray, L.E. (2008) Fifteen years after "Wingspread"—Environmental endocrine disrupters and human and wildlife health: Where we are today and where we need to go. *Toxicol. Sci.*, **105**(2), 235–259.

Humphreys, R., Lydon, J., O'Malley, B. & Rosen, J. (1997) The use of PRKO mice to study the role of progesterone in mammary gland development. *J. Mammary Gland Biol. Neoplasia*, **2**, 343–354.

IARC (1999) *Hormonal contraception and postmenopausal hormone therapy*. Lyon, France, International Agency for Research on Cancer, pp. 474–530 (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 72).

IARC (2007) *Combined estrogen–progestogen contraceptives and combined estrogen–progestogen menopausal therapy*. Lyon, France, International Agency for Research on Cancer (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 91).

Joosten, H.F.P., van Acker, F.A.A., van den Dobbelsteen, D.J., Horbach, G.J.M.J. & Krajnc, E.I. (2004) Genotoxicity of hormonal steroids. *Toxicol. Lett.*, **151**,113–134.

Lamb, R., Harrison, H. & Clarke, R.B. (2007) Mammary development, carcinoma and progesterone: Role of Wnt signalling. *Ernst Schering Found. Symp. Proc.*, **2007**(1), 1–23.

Lange, I.G., Daxenberger, A., Meyer, H.H.D., Rajpert-De Meyts, E., Skakkebaek, N.E. & Veeramachaneni, D.N.R. (2002) Quantitative assessment of foetal exposure to trenbolone acetate, zeranol and melengestrol acetate, following maternal dosing in rabbits. *Xenobiotica*, **32**, 641–651.

Laron, Z., Pauli, R. & Pertzelan, A. (1989) Clinical evidence on the role of estrogens in the development of the breasts. *Proc. R. Soc. Edinb. B1*, **85**, 29–43.

Lauderdale, J.W. (1971) Influence of melengestrol acetate on acute responses of the bovine uterus inoculated with *Escherichia coli. Am. J. Vet. Res.*, **32**, 1033–1038.

Lauderdale, J.W. (1983) Use of MGA® (melengestrol acetate) in animal production. In: Meissonnier, E. & Mitchell-Vigneron, J., eds. *Proceedings of the Symposium on Anabolics in Animal Production: Public Health Aspects, Analytical Methods, and Regulation*, *15–17 February 1983, Paris*. Paris, France, Office International des Épizooties, pp. 193–212.

Lauderdale, J.W., Goyings, L.S., Krzeminski, L.F. & Zimbelman, R.G. (1977) Studies of a progestogen (MGA) as related to residues and human consumption. *J. Toxicol. Environ. Health*, **3**, 5–33.

Lee, P.A., Guo, S.S. & Kulin, H.E. (2001) Age of puberty: Data from the United States of America. *APMIS*, **109**, 81–88.

Lee, S., Mohsin, S.K., Mao, S., Hilsenbeck, S.G., Medina, D. & Allred, D.C. (2006) Hormones, receptors and growth in hyperplastic enlarged lobular units: Early potential precursors of breast cancer. *Breast Cancer Res.*, **8**, R6.

Le Gueval, R. & Padkel, R. (2001) Assessment of oestrogenic potency of chemicals used as growth promoter by in-vitro methods. *Hum. Reprod.*, **16**, 1030–1036.

Liehr, J.G. (2001) Genotoxicity of steroidal estrogens estrone and estradiol: Possible mechanism of uterine and mammary cancer development. *Hum. Reprod. Update*, **7**, 273–281.

Metzler, M. & Pfeiffer, E. (2001) Genotoxic potential of xenobiotics growth promoters and their metabolites. *APMIS*, **109**, 89–95.

Meyer, H.H.D. (2001) Biochemistry and physiology of anabolic hormones used for improvement of meat production. *APMIS*, **109**, 1–8.

Missmer, S.A., Smith-Warner, S.A., Spiegelman, D., Yaun, S.S., Adami, H.O., Beeson, W.L., van den Brandt, P.A., Fraser, G.E., Freudenheim, J.L., Goldbohm, R.A., Graham, S., Kushi, L.H., Miller, A.B., Potter, J.D., Rohan, T.E., Speizer, F.E., Toniolo, P., Willett, W.C., Wolk, A., Zeleniuch-Jacquotte, A. & Hunter, D.J. (2002) Meat and dairy food consumption and breast cancer: A pooled analysis of cohort studies. *Int. J. Epidemiol.*, **31**, 78–85.

Mote, P.A., Graham, J.D. & Clarke, C.L. (2007) Progesterone receptor isoforms in normal and malignant breast. *Ernst Schering Found. Symp. Proc.*, **2007**(1), 77–107.

Munson, L. & Moresco, A. (2007) Comparative pathology of mammary gland cancers in domestic and wild animals. *Breast Dis.*, **28**, 7–21.

Munson, L., Gardner, I.A., Mason, R.J., Chassy, L.M. & Seal, U.S. (2002) Endometrial hyperplasia and mineralisation in zoo felids treated with melengestrol acetate contraceptives. *Vet. Pathol.*, **39**, 419–427.

Northen, A.T., Norman, G.S., Anderson, K., Moseley, L., Divito, M., Cotroneo, M., Swain, M., Bousleiman, S., Johnson, F., Dorman, K., Milluzzi, C., Tillinghast, J.A., Kerr, M., Mallett, G., Thom, E., Pagliaro, S. & Anderson, G.D.; National Institute of Child Health and Human Development (NICHD) Maternal-Fetal Medicine Units (MFMU) Network (2007) Follow-up of children exposed in utero to 17 alpha-hydroxyprogesterone caproate compared with placebo. *Obstet. Gynecol.*, **110**, 865–872.

Partsch, C.-J. & Sippell, W.G. (2001) Pathogenesis and epidemiology of precocious puberty. Effects of exogenous oestrogens. *Hum. Reprod. Update*, **7**, 292–302.

Perry, G.A., Welshons, W.V., Bott, R.C. & Smith, M.F. (2005) Basis of melengestrol acetate action as a progestin. *Domest. Anim. Endocrinol.*, **28**, 147–161.

Pfaffl, M.W., Daxenberger, A., Hageleit, M. & Meyer, H.H.D. (2002) Effects of synthetic progestagens on the mRNA expression of androgen receptor, progesterone receptor, estrogen receptor α and β, insulin-like growth factor-1 (IGF-1) and IGF-1 receptor in heifer tissues. *J. Vet. Med. A*, **49**, 57–64.

Phillips, K.P. & Foster, W.G. (2008) Key developments in endocrine disrupter research and human health. *J. Toxicol. Environ. Health B*, **11**, 233–244.

Purchas, R.W., Pearson, A.M., Pritchard, D.E., Hafs, H.D. & Tucker, H.A. (1971) Some carcass quality and endocrine criteria of Holstein heifers fed melengestrol acetate. *J. Anim. Sci.*, **32**, 629–635.

Rajpert-De Meyts, E., Veeramacheneni, D.N.R., Andersson, A.-M. & Skakkebaek, N.E. (2001) *Reproductive sequelae of developmental exposure to zeranol, trenbolone acetate and melengestrol acetate with special emphasis upon differentiation and neoplastic transformation of germ cells.* Final report to the European Commission (FAIR CT 98-4753, 31 December 2001).

Rogan, E.G. & Cavalieri, E.L. (2004) Estrogen metabolites, conjugates and DNA adducts: Possible biomarkers for risk of breast, prostate and other human cancers. *Adv. Clin. Chem.*, **38**, 135–149.

Ross, R.K., Paganini-Hill, A., Wan, P.C. & Pike, M.C. (2000) Effect of hormone replacement therapy on breast cancer risk: Estrogen versus estrogen plus progestin. *J. Natl. Cancer Inst.*, **92**, 328–332.

Russo, J. & Russo, I.H. (1987). Development of the human mammary gland. In: Neville, M. & Daniel, C.W., eds. *The mammary gland, development, regulation and function*. New York, NY, USA, Plenum Press, pp. 67–93.

Schairer, C. (2002) Progesterone receptors—animal models and cell signalling in breast cancer. Implications for breast cancer of inclusion of progestins in hormone replacement therapies. *Breast Cancer Res.*, **4**, 244–248.

Schairer, C., Lubin, J., Troisi, R., Sturgeon, S., Brinton, L. & Hoover, R. (2000) Menopausal estrogen and estrogen–progestin replacement therapy and breast cancer risk. *JAMA*, **283**, 485–491.

Simon, J.A. (1995) Micronised progesterone: Vaginal and oral uses. *Clin. Obstet. Gynecol.*, **38**, 902–914.

Swan, S.H., Liu, F., Overstreet, J.W., Brazil, C. & Skakkebaek, N.E. (2007) Semen quality of fertile US males in relation to their mothers' beef consumption during pregnancy. *Hum. Reprod.*, **22**, 1497–1502.

Tabb, M.M. & Blumberg, B. (2006) New modes of action for endocrine-disrupting chemicals. *Mol. Endocrinol.*, **20**, 475–482.

Vom Saal, F. (2007) Could hormone residues be involved? *Hum. Reprod.,* **22**, 1503–1505.

WCRF (1997) *Food, nutrition and the prevention of cancer: A global perspective.* Washington, DC, USA, American Institute for Cancer Research, World Cancer Research Fund.

WCRF (2007) *Food, nutrition, physical activity and the prevention of cancer: A global perspective*. Washington, DC, USA, World Cancer Research Fund, American Institute for Cancer Research (http://www.dietandcancerreport.org/).

Wiebe, J.P. (2005) Role of progesterone metabolites in mammary cancer. *J. Dairy Res.*, **72** (Special Issue), 51–57.

MONENSIN

First draft prepared by

Professor Leonard Ritter,[1] Ms Kathleen Nichol,[2] Professor Gordon Kirby,[2] Dr Carl Cerniglia,[3] Ir Astrid Bulder[4] and Professor Arturo Anadón[5]

[1] Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada
[2] Department of Biomedical Sciences, University of Guelph, Guelph, Ontario, Canada
[3] Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Department of Health and Human Services, Jefferson, AR, United States of America (USA)
[4] RIKILT Institute of Food Safety – Wageningen UR, Wageningen, Netherlands
[5] Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, Madrid, Spain

Explanation........ 93
Biological data........ 95
Biochemical aspects........ 95
Absorption, distribution and excretion........ 95
Biotransformation........ 99
Toxicological studies........ 100
Acute toxicity........ 100
Short-term studies of toxicity........ 101
Long-term studies of toxicity and carcinogenicity........ 105
Genotoxicity........ 106
Reproductive and developmental toxicity........ 106
Special studies........ 109
Observations in humans........ 118
Comments........ 118
Biochemical data........ 118
Toxicological data........ 119
Microbiological data........ 124
Evaluation........ 125
References........ 125

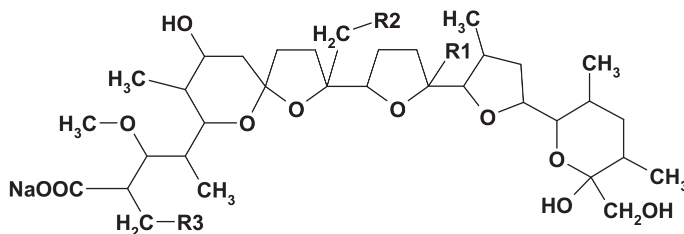
1. EXPLANATION

Monensin is a polyether carboxylic ionophore antibiotic produced by *Streptomyces cinnamonensis* ATCC15413. It is used for the treatment of coccidiosis in poultry (chickens, turkeys and quail) and ruminants (cattle, sheep and goats) (Shumard & Callender, 1967; Anderson et al., 1976; Calhoun, 1986; USFDA, 1986). Monensin is also used to control ketosis and bloat in cattle (Shumard & Callender, 1967; Anderson et al., 1976; Calhoun, 1986; USFDA, 1986) and as a growth promoter feed additive in cattle and sheep (Goodrich et al., 1984). Monensin

is mainly effective against Gram-positive bacteria (Haney & Hoehn, 1967). Monensin is not used in human medicine and was therefore not classified as a critically important antibiotic for humans by the 2007 World Health Organization (WHO) expert meeting on categorization of critically important antimicrobials for human medicine for the development of risk management strategies to contain antimicrobial resistance due to non-human antimicrobial use (WHO, 2007). Monensin acts by interfering with ion flux across bacterial membranes, causing reallocation of bacterial energy resources to maintaining cellular pH and ion balance rather than growth and reproduction (Haney & Hoehn, 1967; Pressman & Fahim, 1982; Russell, 1987). Effects on feed conversion efficiency may arise from the ability of monensin to shift rumen fermentation towards the more energetically efficient propionate pathway, reduce methane production and increase nitrogen retention by reducing dietary protein deamination and urinary ammonia excretion (Van Nevel & Demeyer, 1977; Russell, 1987; Russell & Strobel, 1989).

Monensin (2-(5-ethyltetrahydro-5-(tetrahydro-3-methyl-5-(tetrahydro-6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-2H-pyran-2-yl)-2-furyl)-2-furyl)9-hydroxy-β-methoxy-a,g,2,8-tetramethyl-1,6-diaoxaspiro(4,5)decane-7-butyric acid) (Chemical Abstracts Service No. 22373-78-0) is generally used as the sodium salt. Monensin is a mixture of four analogues, A, B, C and D, which are produced during fermentation, with monensin A being the major component (98%) (Haney & Hoehn, 1967). The chemical structures of monensin A, B and C are shown in Figure 1. Depending on the method of purification, monensin can exist in mycelial, crystalline and recrystallized forms.

Figure 1. Chemical structures of monensin A, B and C



Factor	R1	R2	R3
A	$-CH_2CH_3$	-H	-H
B	$-CH_3$	-H	-H
C	$-CH_2CH_3$	-H	$-CH_3$

Monensin has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Codex Committee on Residues of

Veterinary Drugs in Foods decided at its 17th Session (Codex Alimentarius Commission, 2007) to put monensin on the priority list for evaluation by JECFA.

The present Committee considered data on the pharmacokinetics (including metabolism), acute toxicity, short-term and long-term toxicity, carcinogenicity, genotoxicity, reproductive toxicity, immunotoxicity, cardiovascular and respiratory toxicity, epidemiological findings and microbiological effects of monensin. Additionally, residue depletion studies and analytical methods were reviewed. Many of the studies were conducted prior to the introduction of Good Laboratory Practice (GLP).

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Studies in laboratory and domestic animal species have demonstrated that orally administered monensin sodium is readily absorbed and extensively metabolized, mainly in liver, followed by biliary excretion and elimination in the faeces. Absorption is significantly greater in monogastric species than in poligastric species (cattle or sheep), which absorb only about 50% of the dose (Donoho, 1984).

(a) Rats

In a non-GLP-compliant study examining the absorption of monensin in Wistar rats, three animals per sex per dose with exteriorized bile duct cannulae were orally administered ^{14}C-labelled monensin at doses of 5 or 40 mg/kg body weight (bw) in males and 2 or 16 mg/kg bw in females. Recovery of the radioactivity in the bile within 72 h was independent of dose and ranged from 32.8% to 48.6% in males and from 30.7% to 53.2% in females (Howard & Lobb, 1981a).

In another study that was performed prior to the development of GLP regulations, Wistar rats were exposed to [^{14}C]monensin at 4 and 16 mg/kg bw (females) or 5 and 20 mg/kg bw (males) by oral gavage for 4 or 24 h, with five animals per sex per dose per exposure time. Following 4 h of exposure, radioactivity could be detected in all tissues examined from both sexes, with concentrations in the liver, duodenum, jejunum, ileum and colon more than 10-fold higher than serum concentrations. Females receiving 4 mg/kg bw also had elevated levels of radioactivity in the adrenal glands. The concentration of radioactivity in the serum and tissues significantly declined by 24 h, but a greater than 10-fold higher concentration was observed in the liver, ileum and colon of all animals relative to the serum. Greater than 10-fold higher [^{14}C]monensin concentrations were also observed at 24 h in the duodenum and jejunum of males given 20 mg/kg bw, the adrenal glands, pituitary gland, thyroid gland and jejunum of females given 4 mg/kg bw and the adrenal glands, duodenum and jejunum of females given 16 mg/kg bw (Howard & Lobb, 1981b). There was no indication that any tissue accumulated a large percentage of the dose.

Two studies provided evidence that monensin is primarily excreted in the faeces of rats. In the first non-GLP-compliant study, a male Harlan rat was administered 2.15 mg of [^{14}C]monensin by gavage. The single dose was preceded by 13 days of exposure to unlabelled monensin at 100 mg/kg in the diet (equivalent to 10 mg/kg bw per day), followed by 12 days of exposure to the dosed feed. Radioactivity was detected in the faeces for 3 days following exposure, with a recovery of 91.47% of the dose. Only 0.48% of the dose was recovered in the urine, and radioactivity in the urine was detectable only for 1 day following exposure (Herberg, 1973c).

In a second study that was conducted prior to GLP regulations, Wistar rats were administered a single oral dose of [^{14}C]monensin by gavage. Male rats received doses of 5, 10, 20 or 40 mg/kg bw, and females received 2, 4, 8 or 16 mg/kg bw, with five rats per treatment group. Excretion of radioactive monensin within 72 h was independent of dose, with 84.7–88.9% and 71.8–88.2% excreted in males and females, respectively. In males, 83.6–87.4% of the dose was excreted in the faeces and 1.0–1.6% in the urine, whereas females excreted 70.8–87.2% in the faeces and 1.0–1.3% in the urine. At 24- and 48-h time points, there was a significantly lower percentage of monensin excreted in males and females that received the two highest doses, which may have been due to toxicity observed at higher doses (Howard & Lobb, 1979).

(b) Dogs

Blood samples from a dog dosed orally with [^{14}C]monensin at 1 mg/kg bw were assayed by extraction with carbon tetrachloride and scintillation counting. [^{14}C]Monensin was rapidly absorbed, with the highest concentration of 0.056 mg [^{14}C]monensin/l detected in the blood 15 min after dosing. The radioactivity declined rapidly to less than 0.01 mg/l by 3 h. In a separate experiment, an unspecified dose of intravenously administered [^{14}C]monensin was recovered primarily in the faeces. Fractionation of faecal radioactivity indicated that approximately 6% was the parent compound monensin and the remainder had been metabolized, providing indirect evidence for rapid biliary excretion as a major route of elimination (Donoho, 1984).

(c) Chickens

Ten White Leghorn roosters and two White Leghorn hens were exposed orally to a single dose of [^{14}C]monensin in a gelatine capsule (dose range: 2.6–100 mg). Some birds were colostomized, whereas others had bile cannulae inserted. Absorption in the chickens ranged from 11% to 31% of the ingested [^{14}C]monensin. The primary route of excretion was in the faeces, with a small proportion excreted in the urine and by respiration (Davison, 1984).

In a non-GLP-compliant study, broiler chickens were administered [^{14}C]monensin sodium at a concentration of 120 mg/kg in the diet for 4 days (two males, three females) or 6 days (three males, three females). Six hours after withdrawal from the treated feed, radioactivity was detected in the liver, kidney, fat and skin, with the highest level detected in the liver (0.5 mg/kg liver). No radioactivity was detected in the muscle tissue (Donoho et al., 1980).

In a non-GLP-compliant study, six chickens were exposed to [^{3}H]monensin sodium at 121 mg/kg in the diet for 2 days. Only 52–73% of the radioactivity was recovered; of this, 97% was found in the faeces. The reason for poor radioactivity balance was unknown (Herberg, 1967). In a second non-GLP-compliant study, a broiler-type cockerel was exposed to unlabelled monensin sodium at 110 mg/kg in the diet for 15 days, followed by a single exposure to a capsule containing 7.4 mg of [^{14}C]monensin. Seventy-five per cent of the radioactivity was recovered in the excreta within 3 days, 90% within 6 days and 100% within 12 days of exposure (Herberg, 1973a). In an additional study, three Leghorn chickens were exposed to monensin sodium at 120 mg/kg in their feed for 35 days, receiving a single dose of [^{14}C]monensin on day 15 by capsules. The monensin capsule doses were equivalent to an average monensin feed concentration of 120 mg/kg. The radioactive dose was recovered in the faeces, with more than 75% recovered within 3 days and a total of 85–101% recovered by the end of the study (Herberg, 1975).

(d) Cattle

In a non-GLP-compliant study, two 3-month-old bile-cannulated calves (one male Shorthorn, one female Angus) were administered a single dose of [^{14}C]monensin sodium at 10 mg/kg bw orally in a gel capsule. Absorption in the calves was calculated to be 36–40% of the dose, and most of the absorbed ^{14}C was recovered in the bile. The primary route of ^{14}C excretion was in the faeces, with a small proportion excreted in the urine (Davison, 1984).

Serum levels of monensin remained low following oral administration in cattle. Administration of an intraruminal dose of 60 mg/kg bw in three fistulated animals resulted in plasma concentrations below 0.02 mg/l, when determined by semiquantitative autoradiography, even though all three animals eventually died from the dosing. Correspondingly, steers given an oral dose of 30 mg/kg bw had essentially no detectable monensin in the plasma at a limit of detection of approximately 0.05 mg/l. In addition, plasma concentrations of monensin rapidly declined when six steers received 0.15 mg monensin/kg bw intravenously. Monensin could not be detected in the serum 1 h after treatment (Donoho, 1984).

Low concentrations of monensin could be detected in the tissues of cattle shortly after monensin administration. In a non-GLP-compliant study, three animals were treated daily for 5 days with an oral capsule containing 330 mg of [^{14}C]monensin, and tissues were collected 12 h after the last capsule. The highest concentration of net radioactive residues was detected in the liver (0.2–0.4 mg/kg liver), whereas the muscle, fat, kidney and heart tissue had less than 0.021 mg/kg tissue (Herberg et al., 1978).

Additional experiments have demonstrated that the faeces are the primary route of excretion of monensin sodium in cattle. In a non-GLP-compliant study, a single 260-kg steer was fed 300 mg of unlabelled monensin for 15 days prior to administration of a capsule containing 299.8 mg [^{14}C]monensin by gavage. After administration of the capsule, the steer returned to the diet containing unlabelled monensin for 14 days while the excretion of radioactivity in the urine and faeces was monitored. Within 7 days, 93.7% of the radioactive dose was recovered in the

faeces, with no radioactivity detected in the urine (Herberg, 1973b). When this study was repeated using two additional Black Angus steers, 88–102% of the dose was recovered in the faeces within 8–11 days, with no radioactivity found in the urine (Herberg, 1974b). In a third non-GLP study using three Black Angus steers, 300 mg of unlabelled monensin was provided in the feed for 4 weeks, with a single 300 mg dose of [^{14}C]monensin in a capsule on day 14 of the study (equivalent to 1 mg/kg bw). Between 88% and 102% of the radioactivity was recovered in the faeces within 7–11 days after administration. No radioactivity was detectable in the urine (Herberg, 1974a).

(e) Sheep

In a non-GLP-compliant study to determine the rate and route of excretion of monensin, a wether lamb was exposed to unlabelled monensin at 50 mg/kg bw per day in the diet for 4 weeks, receiving 50 mg of [^{14}C]monensin in two oral capsules on day 14 of the study. Within 9 days, 101.96% of the radioactive dose was recovered in the faeces, whereas no radioactivity was detectable in the urine (Elanco Animal Health, 1998). In a separate study, groups of three finishing lambs were dosed daily for 3, 5 or 7 days with a gel capsule containing the equivalent of 16.5 mg [^{14}C]monensin/kg feed. Twelve hours after the last dose, radioactivity concentrations of 0.20–0.35 mg/kg tissue were detected in the liver; however, concentrations in the kidney, fat and muscle were less than 0.027 mg/kg tissue. The faeces were the primary route of excretion (Giera et al., 1984a).

(f) Pigs

In a non-GLP-compliant study, six pigs were exposed to [^{14}C]monensin at 110 mg/kg in the diet for 5 consecutive days. Six hours after the final feeding, the highest concentration of radioactivity was in the liver (2.5 mg/kg tissue), followed by the kidney (0.17 mg/kg tissue). Concentrations in the fat and muscle were less than 0.045 mg/kg tissue (Giera et al., 1984b).

Two non-GLP-compliant studies have been performed in pigs to examine the route and rate of excretion of monensin sodium (Donoho & Herberg, 1977; Herberg & Donoho, 1977). In the first, a 54.5-kg barrow was conditioned to a diet containing unlabelled monensin at 50 mg/kg for 2 weeks and was then administered a capsule containing 5.23 mg [^{14}C]monensin (equivalent to 0.1 mg/kg bw). Urine and faeces were collected for 13 days following the radioactive dose. Only 54.87% of the dose was recovered, with 53.89% in the faeces and 0.98% in the urine. Excretion occurred rapidly, with 92% of the ^{14}C in the faeces recovered in 3 days. The reason for the non-quantitative recovery of radioactivity in this experiment was unknown (Herberg & Donoho, 1977).

In the second study, a 50.5-kg barrow was conditioned to a diet containing monensin sodium at 50 mg/kg for an unspecified amount of time and then administered a capsule containing 10.4 mg [^{14}C]monensin (equivalent to 0.2 mg/kg bw). Urine and faeces were collected daily for 10 days following exposure to radioactive monensin. In 10 days, 78.14% of the dose was recovered, with 75.04% in the faeces and 3.10% in the urine. A majority of the ^{14}C in the urine was recovered

within the first 2.5 days after exposure, whereas most of the ^{14}C in the faeces was detected during the first 3.5 days (Donoho & Herberg, 1977).

2.1.2 Biotransformation

Monensin is extensively metabolized in the liver, producing more than 50 different metabolites that have been detected in the liver, bile and faeces of chickens, cattle, rats, pigs, dogs, turkeys, sheep and horses (Donoho et al., 1978, 1982a,b; Donoho, 1984, 1985; Grundy et al., 1998). In most species (chickens, rats, dogs, turkeys and pigs), less than 10% of monensin is excreted as the parent compound (Donoho, 1984), whereas a study in calves indicated that 50–68% of the ^{14}C identified in the faeces was unmetabolized monensin (Davison, 1984). This difference in amount of metabolized monensin may have been a result of differences in absorption of the molecule in different species (Donoho et al., 1978). Total microsomal monensin metabolism, estimated by measuring the rate of substrate disappearance by a high-performance liquid chromatographic (HPLC) analytical method, is highest in cattle, intermediate in rats, chickens and pigs, and lowest in horses (Nebbia et al., 2001). The pattern of metabolites is qualitatively similar between laboratory and non-laboratory animal species, although quantitative differences exist. No single metabolite dominates the metabolic profile.

Monensin metabolites result mainly from *O*-demethylation at the methoxylic group and/or hydroxylation at several places on the ionophore backbone (Donoho, 1984). To date, no metabolites representing fragmentation or conjugation of monensin have been identified. Although it is difficult to obtain sufficient monensin metabolites to test activity, four metabolites generated by rat liver microsomes, including a by-product of monensin production (*O*-desmethylmonensin), have been tested and have at least 10- to 20-fold less antibacterial, anticoccidial, cytotoxic, cardiotonic and ionophoric activity than the parent compound, indicating that metabolism eliminates most of the biological activity of monensin (Donoho et al., 1979; Donoho, 1984; Halstead et al., 2007).

The *O*-demethylation of monensin is greater in microsomes from phenobarbital-treated rats than in untreated rats and is dependent on reduced nicotinamide adenine dinucleotide phosphate (NADPH), suggesting that monensin is a cytochrome P450 (CYP) enzyme substrate (Ceppa et al., 1997). The oxidative metabolism of monensin appears to occur at least in part by CYP3A, since treatment of rat hepatic microsomes with chemical inducers of CYP3A significantly increased monensin *O*-demethylation (Nebbia et al., 1999). It has been speculated that competition between monensin and other CYP3A substrates may explain accidental poisonings that have occurred in several domestic species following co-administration of monensin and other chemotherapeutic agents, since monensin metabolism is significantly decreased in the presence of other CYP3A substrates in rats (Anadón & Reeve-Johnson, 1999; Nebbia et al., 1999).

The metabolism of monensin sodium in human liver microsomes has been compared with metabolism in the microsomes of horses and dogs (Herrera et al., 2005; Holmstrom, 2007). A pooled human microsomal sample from multiple donors (male and female, Caucasian, Hispanic and African American, 15–66 years old),

pooled dog microsome sample and equine microsomes from a single donor were incubated with 0.5, 1 and 10 µg monensin/ml in the presence or absence of NADPH. The metabolite profiles were examined at 0, 5, 10, 20, 40 and 60 min by liquid chromatography/mass spectrometry (LC-MS) analysis. Monensin was metabolized by first-order kinetics in all species, and metabolism was extensive (93–99% by 60 min). The turnover of monensin in humans was similar to that in dogs, whereas the turnover in horses was only 10% of that in dogs and humans (Herrera et al., 2005; Holmstrom, 2007).

2.2 Toxicological studies

2.2.1 Acute toxicity

The studies of acute toxicity were all conducted prior to the adoption of GLP. In all studies, monensin was administered as a single oral dose, the most relevant route of exposure for humans. The median lethal doses (LD_{50} values) for oral administration of monensin ranged from 22 to 96 mg/kg bw, with rats displaying more sensitivity than mice or rabbits and female rats being more sensitive than males (Table 1). Death was preceded by hypoactivity, ataxia, dyspnoea, ptosis, loss of righting reflex and muscle weakness in all species tested.

Table 1. Acute toxicity of a single dose of monensin sodium in laboratory species

Species	Strain	Sex	Route/form	LD_{50} (mg/kg bw)	Reference
Mouse	Cox ICR	M	Oral/mycelial	70	Broddle & Worth (1976)
Mouse	Cox ICR	F	Oral/mycelial	96	Broddle & Worth (1976)
Mouse	Not specified	Not specified	Oral/mycelial	44	Haney & Hoehn (1967)
Rat	Harlan Wistar	M	Oral/mycelial	40	Broddle & Worth (1976)
Rat	Harlan Wistar	F	Oral/mycelial	24	Broddle & Worth (1976)
Rat	Harlan Wistar	M	Oral/mycelial	50 (estimate)	Pierson (1981)
Rat	Harlan Wistar	F	Oral/mycelial	22	Pierson (1981)
Rat	Harlan Wistar	F	Oral/Coban premix	22	Arthur & Downs (1979)
Rabbit	New Zealand albino	M & F	Oral/crystalline	42	Arthur et al. (1976)

F, female; M, male.

Acute toxicity was also examined in a non-GLP-compliant study in mature rhesus monkeys. Pairs of monkeys were exposed to a single dose of 20, 40 or 60 mg monensin activity/kg bw by gavage and were monitored for 7 days. Thirty-four days later, five of the monkeys were dosed again by gavage with 80, 110 or 160 mg monensin activity/kg bw and were monitored for 7 days. No mortality was observed during the study. All animals developed diarrhoea within 24 h after dosing. Vomiting was observed in one animal receiving 110 mg monensin activity/kg bw and both animals receiving 160 mg/kg bw, and suppressed appetites were seen for one female monkey receiving 160 mg/kg bw and one receiving 110 mg/kg bw (Gossett et al., 1977a). The LD_{50} value for mycelial monensin in rhesus monkeys is greater than 160 mg/kg bw.

2.2.2 Short-term studies of toxicity

(a) Mice

In a GLP-compliant study, 5- to 6-week-old male and female B6C3F1 mice (15 mice per sex per dose) were fed diets containing 0, 37.5, 75, 150 or 300 mg mycelial monensin sodium/kg for 3 months (equivalent to 0, 5.6, 11.2, 22.5 and 45 mg/kg bw per day). Body and organ weight, haematology, clinical chemistry and histopathology were examined. A dose-dependent decrease in body weight gain occurred in all dose groups. At the end of the study, the decrease ranged from 27% and 21% in the lowest dose group in females and males, respectively, to 99% in the highest dose group in both sexes. Mean body weights also declined by 5% and 8% in the lowest dose group up to 29% and 35% in the highest dose group in females and males, respectively. Except for the declines in body weights and body weight gains of males in the lowest dose group, all changes were statistically significant. Doses of 75, 150 or 300 mg/kg in the diet also led to significant reductions in liver, kidney plus adrenal, and heart weight of both sexes, spleen and ovary weight of female mice, and testes weight of male mice, but this reduction in organ weight was considered to be due to the dose-dependent decrease in body weight. Decreased leukocyte counts were observed for female mice in all four treatment groups and in males receiving 75 mg/kg in the diet. Decreased erythrocytes, haemoglobin and haematocrit values were seen for all animals in the highest dose group, as well as many animals in the second highest dose group. Males in the 75 mg/kg dose group also had reduced leukocytes and per cent lymphocytes and increased per cent neutrophils. Differences in haematology were considered secondary to profound effects on growth, as were most observed changes in clinical chemistry. An increased incidence of elevated serum creatine kinase values for the males in the two highest dose groups and for the females receiving the highest dose was evident. Mild diffuse vacuolation of cardiac myofibres occurred in eight males and two females receiving 300 mg/kg in the diet and one male receiving 37.5 mg/kg in the diet. Since weight gain was affected in all dose groups, a no-observed-adverse-effect level (NOAEL) could not be identified (Howard, 1981a).

(b) Rats

In four parallel subchronic toxicity studies that were conducted according to GLP regulations, 15 Wistar rats per sex were exposed in their diet to crystalline, double-drum-dried, azeotrope or flash-dried mycelial monensin sodium at concentrations of 0, 50, 200 or 400 mg/kg (equivalent to 0, 2.5, 10 and 20 mg/kg bw per day) for 3 months. Mortality occurred in four females exposed in their diet to either double-drum-dried mycelial monensin at 400 mg/kg or azeotrope mycelial monensin at 400 mg/kg, one male exposed to flash-dried mycelial monensin at 400 mg/kg and one female exposed to azeotrope mycelial monensin at 200 mg/kg. The cause of death could not be determined; however, a relationship with the treatment could not be ruled out. Decreased weight gain was observed in all rats exposed to at least 200 mg monensin/kg in the diet and in female rats exposed to the mycelial forms of monensin at 50 mg/kg in the diet. Females consuming 200 or 400 mg mycelial monensin/kg in the diet consumed less food than females consuming the crystalline form, but weight gain was similar for both forms of monensin. Males receiving 200 or 400 mg mycelial monensin/kg in the diet consumed less food and gained less weight than males receiving crystalline monensin. Observed changes in haematology, clinical chemistry and organ weights were considered secondary to effects on growth. Histopathological examination identified focal interstitial myocarditis and myocardial degeneration; however, there was no difference in incidence between control animals and animals exposed to the various forms of monensin. Focal degeneration and interstitial myositis of the diaphragm and skeletal muscle occurred in higher incidence in female rats of the highest dose group than in controls; however, overall incidence and severity were low. Owing to a decreased body weight gain at the lowest dose, a NOAEL could not be identified (Howard & Young, 1981).

In a study conducted prior to the effective date of GLP regulations, the subchronic oral toxicity of mycelial monensin sodium was examined in Harlan Wistar rats. Fifteen 4- to 6-week-old animals per sex were exposed to mycelial monensin sodium at nominal concentrations of 0, 25, 50, 80 or 125 mg/kg in the diet for 3 months. Based on measured concentrations in the feed and feed consumption, this was equal to 0, 0.89–2.45, 1.83–4.63, 3.02–7.71 and 4.54–12.05 mg/kg bw per day in males and 0, 1.30–2.55, 2.75–5.83, 4.04–12.83 and 10.17–20.21 mg/kg bw per day in females. Physical and behavioural changes, growth, food consumption, terminal haematology, clinical chemistry, organ weights and histopathology were examined. All animals survived the study and were normal in appearance and behaviour. A transient dose-dependent decrease in mean body weight was observed in females receiving 50, 80 or 125 mg monensin/kg in their diet, and weight gain was reduced in these animals during the first 2 weeks of the study. Males in the highest dose group also had reduced weight gain during the first 2 weeks. During the 1st week of the study, decreased food consumption was seen in all animals in the highest dose group and in females exposed to 50 and 80 mg/kg in the diet, with the high-dose females also having decreased food consumption in the 2nd and 3rd weeks. No changes in haematology or clinical chemistry were attributed to monensin consumption. Minute lesions were observed in the heart and skeletal muscle of both control and treated animals, particularly in males, but the incidence

and severity of these lesions were not dose dependent. Based on the effects of mycelial monensin on body weight and food consumption, the NOAEL for this study was the nominal concentration of 25 mg/kg in the diet. An exact dose could not be determined owing to the wide range of measured concentrations of monensin in the feed (Howard, 1980a).

In order to better compare the toxic effects of crystalline and mycelial monensin, a non-GLP-compliant study comparing both forms of monensin was performed in Wistar barrier-reared 4- to 6-week-old rats. Twenty-five animals per sex were fed a control diet, and 15 animals per sex were exposed to crystalline or mycelial monensin sodium in their diet at concentrations of 50, 200 or 400 mg/kg (equivalent to 2.5, 10 and 20 mg/kg bw per day) for 3 months. Animals were examined for changes in physical condition, behaviour, body weight, haematology, clinical chemistry, organ weights, and gross and microscopic pathology. One control male and three high-dose females (one in the crystalline group, two in the mycelial group) died during the study. A severe reduction in body weight gain was observed for both formulations starting at 200 mg/kg in the diet. A slight, transient decrease in body weight gain was observed for females in the lowest dose groups for the first 2 weeks of the study. Decreases in organ weight were also observed in the highest and middle dose groups, but these appeared to be related to the decreased body weight gain. Haematology was normal in all animals except for white blood counts, which were decreased in males receiving either type of monensin at the highest dose. Analysis of clinical chemistry indicated an increase in total bilirubin and alkaline phosphatase levels and a decrease in mean serum glucose and creatinine levels in males and females receiving either preparation of monensin at 400 mg/kg in the diet. Similar changes were also observed in female rats receiving 200 mg monensin/kg in their diet. Female rats in all treatment groups also had decreased serum alanine aminotransferase. Initial histopathological examination revealed an incidence of scattered foci of a few myocardial fibres with degeneration, necrosis and infiltration of mononuclear cells, particularly in males, in all three dose groups for both forms of monensin. A second independent pathology evaluation concluded that the scattered myocardial lesions were not adverse and that the incidence was similar to the control incidence. A NOAEL could not be identified because of a slight, transient reduction in body weight gain in females in the lowest dose group, which became severe and non-transient in the next higher dose group (Gossett et al., 1977b).

(c) Dogs

In a study that was not compliant with GLP, mongrel dogs (two per sex per dose, age unknown) were administered monensin sodium orally in capsules at daily doses of 0, 2.5, 5, 11 or 25 mg/kg bw for 90 days. Deaths attributed to treatment with monensin occurred in one female of the second highest dose group and two males of the highest dose group. Females of the highest dose group developed ataxia, tremors, loss of muscular control and slight relaxation of the nictitating membrane; therefore, treatment was stopped after 5 days. There were no signs of toxicity in surviving male and female dogs receiving 11 and 5 mg/kg bw per day or less, respectively. Haematology, clinical chemistry, urinalysis, organ weights and

gross pathology of all animals were normal, with the exception of transiently elevated serum alanine aminotransferase levels in the second highest dose group. The NOAEL was 5 mg/kg bw per day (Worth et al., 1967).

In a second non-GLP-compliant study, mycelial monensin sodium manufactured using a new method was administered to Beagle dogs in gel capsules. Two 12- to 18-month-old dogs of each sex were administered 0, 5, 15 or 50 mg monensin activity/kg bw per day for 91 days. Two males in the highest dose group died, and one male in the middle dose group was sacrificed within the first 2 weeks of the study. Necropsy on these animals revealed myopathy of the heart with degeneration of the muscle fibre, macrophage infiltration and visceral congestion. Animals dosed with 15 and 50 mg/kg bw per day vomited more frequently, lost body weight and developed muscular weakness, ataxia, arrhythmias, convulsions and mydriasis. Haematology, urinalysis and clinical chemistry were normal for all animals, with the exception of transiently increased serum lactate dehydrogenase and alanine aminotransferase in animals in the two highest dose groups. Pathology results indicated that middle- and high-dose males and high-dose females had degenerative changes in striated muscle, including diffuse degeneration of muscle fibres and infiltration of histiocytes, at the end of the study. A slight loss of body weight was observed in dogs treated in all dose groups, but no other effects were seen. Since toxic effects were seen at the lowest dose, a NOAEL could not be identified (Gibson et al., 1974).

In a GLP-compliant study, mycelial monensin sodium was orally administered as an equally divided dose in gel capsules twice daily to Beagle dogs (5–6 months old, four per sex per dose) for 1 year at doses of 0, 1.25, 2.5, 5 or 7.5 mg/kg bw per day. The animals were observed for changes in physical appearance, behaviour, body and organ weights, ophthalmology, haematology, clinical chemistry, urinalysis, and gross and microscopic pathology. No data on feed intake were reported. Two dogs receiving 5 mg/kg bw per day and four dogs receiving 7.5 mg/kg bw per day showed signs of toxicity, which included hypoactivity, muscle weakness (particularly legs and neck), stilted gait, difficulty standing and anorexia, but they recovered within a few days. Increased alanine aminotransferase and creatine kinase levels were observed during the first 2 weeks of monensin administration in dogs receiving 5 and 7.5 mg/kg bw per day, and several dogs in these groups also had periodic increases in alanine aminotransferase and creatine phosphokinase throughout the entire study period. Decreased mean total protein in 5 mg/kg bw per day females and 7.5 mg/kg bw per day males during week 45 and elevated mean serum calcium in females receiving 5 or 7.5 mg/kg bw per day during weeks 45 and 52 may have been treatment related. No statistically significant decrease in weekly mean body weight was observed, although a decrease in body weight gain was seen in male dogs receiving 2.5, 5 or 7.5 mg/kg bw per day, which exceeded 10% for the highest dose. No changes were observed in ophthalmology, haematology, urinalysis or electrocardiography (ECG) results that could be directly attributed to monensin administration. Organ weights were unaltered by treatment, and no pathological changes were seen that were related to monensin exposure. Since body weight gain was decreased at the next higher dose, a NOAEL of 1.25 mg/kg per day was identified (Howard, 1980b).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In an experiment that complied with GLP, groups of 60 male and 60 female 5- to 6-week-old B6C3F1 mice received diets containing mycelial monensin at concentrations of 0, 10, 25, 75 or 150 mg/kg for 2 years, equal to 0, 1.2, 3.1, 10.2 and 22.6 mg/kg bw per day for males and 0, 1.4, 3.5, 11.7 and 25.6 mg/kg bw per day for females. Clinical signs and histopathological changes were examined. No substance-related deaths, physical signs or behavioural changes were observed. A statistically significant decrease in body weight and body weight gain occurred in mice receiving the three highest doses. Decreased body weight gain interfered with the development of meaningful conclusions regarding the significant effect of monensin on the weight of multiple organs. A statistically significant, dose-dependent decrease in leukocyte counts was observed in males receiving monensin at concentrations of 25, 75 or 150 mg/kg in their diet. Minimal increases in urea nitrogen, creatinine, bilirubin, alanine aminotransferase and creatine phosphokinase occurred with the highest dose. No evidence of carcinogenicity was observed at the gross or microscopic level that could be attributed to monensin. A NOAEL of 10 mg/kg in the diet (equal to 1.2 mg/kg bw per day) was identified, based on the effects of mycelial monensin on body weight gain and leukocyte counts (Howard & Usher, 1984).

(b) Rats

In a study conducted according to GLP guidelines, 5- to 6-week-old male and female Wistar rats (80 animals per sex per dose group) were maintained on a diet containing 25, 56 or 125 mg crystalline monensin sodium/kg, whereas control rats (120 per sex) received a normal diet for 2 years (Howard et al., 1981). The monensin concentrations in the diet were equal to a time-weighted average daily dose of 1.14, 2.57 and 5.91 mg/kg bw per day in males and 1.46, 3.43 and 8.68 mg/kg bw per day in females. All of the rats were monitored for changes in physical appearance, behaviour, body and organ weight, food and water consumption, efficiency of feed conversion, haematology, clinical chemistry, urinalysis and pathology. Survival was not adversely affected by crystalline monensin sodium administration. Body weight and weight gain were significantly decreased in animals receiving 125 mg monensin/kg in their diet and were transiently decreased during the first 4 months in rats in the middle dose group. Feed conversion efficiency was decreased in the animals receiving 56 or 125 mg monensin/kg in the diet, and mean feed consumption was decreased in animals in the highest dose group during the first 5 weeks of the trial. No physical signs of toxicity were observed that were attributable to monensin administration, and no differences in haematology or clinical chemistry values were observed at 6, 12, 18 or 24 months that were specific to monensin exposure. Urinalysis at 12 months was also normal, and absolute and relative organ weights were unaffected by treatment. Pathology revealed skeletal muscle degeneration and cardiomyopathy in control and treated animals, with no bias towards monensin-treated animals. Similarly, benign and malignant neoplasms were observed in treated and untreated animals, with no association between monensin

administration and neoplasm type or severity. It was concluded that lifetime exposure of rats to diets containing up to 125 mg crystalline monensin sodium/kg did not produce carcinogenicity. A NOAEL of 25 mg/kg (equal to 1.14 mg/kg bw per day) was identified based on the effects on body weight (Howard et al., 1981).

In a second GLP-compliant study conducted in Wistar rats, groups of 100 male and 100 female rats that were exposed to monensin in utero were further exposed to 0, 33, 50 or 80 mg mycelial monensin sodium/kg in their diet for 2 years. These monensin concentrations were equal to a time-weighted average daily dose of 0, 1.40, 2.18 and 3.60 mg/kg bw per day in males and 0, 1.72, 2.86 and 5.02 mg/kg bw per day in females. Rats were examined for changes in physical condition, body and organ weights, food consumption and efficiency of feed conversion, haematology, clinical chemistry, urinalysis results and pathology. Survival in both sexes increased following monensin exposure in a dose-dependent manner. A transient decrease in body weight was observed at the beginning of the study in all animals consuming 80 mg monensin/kg in the diet and in females receiving 50 mg/kg in the diet. Body weight gain was also significantly decreased during the 1st week in males receiving monensin in the diet at concentrations of 33 and 80 mg/kg and during the first 2 weeks in females receiving the highest dose. There was a statistically significant increase in feed intake in females in the highest dose group. As was observed for crystalline monensin (Howard et al., 1981), no differences in haematology, clinical chemistry, urinalysis or organ weights were observed that could be linked to monensin exposure, and no signs of physical toxicity occurred. Non-neoplastic lesions were observed in the muscle and cardiac tissues; however, the incidence and severity were not influenced by monensin exposure. As well, the latency and prevalence of benign and malignant neoplasms did not differ in treated and untreated rats. It was concluded that in utero exposure followed by 2 years of exposure to mycelial monensin sodium at doses up to 80 mg/kg in the diet did not lead to carcinogenicity. As the observed decrease in body weight gain was transient and restricted to the first few weeks of the 2-year study, the effect was not considered to be adverse. The NOAEL was identified to be the highest dose, 80 mg/kg in the diet, equal to 3.60 mg/kg bw per day (Howard, 1981b).

2.2.4 Genotoxicity

The genotoxic effects of monensin sodium were investigated in vitro and in vivo in several experiments that were conducted according to GLP. The results of these studies are summarized in Table 2. No evidence of genotoxicity was observed.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

(i) Rats

In a GLP-compliant multigeneration study, mycelial monensin was administered to three generations of Wistar-derived rats (25 of each sex) and their offspring at doses of 0, 33, 50 or 80 mg/kg in the diet (equivalent to 0, 1.6, 2.5 and

Table 2. Results of genotoxicity tests for crystalline monensin sodium

End-point	Test object	Concentration/ dose	Result	Reference
Reverse mutation[a,b]	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2uvrA	312.5–5000 μg/ plate	Negative	Garriott & Schwier (2001)
Chromosomal aberrations[a,c]	Chinese hamster ovary cells	25–100 μg/ml (4 h) 5–25 μg/ml (19 h)	Negative for chromosomal aberrations; increased diplo-chromosomes observed in 4-h studies	Garriott & Gilbert (2002)
In vivo micronucleus test[d]	Male and female ICR mice (5 per sex per group)	181.3, 362.5 and 725.0 mg/kg for 2 days by oral gavage	Negative	Phelps & Murphy (2002)

[a] Conducted with and without activation with S9 (9000 × *g* rat liver supernatant) fraction from Aroclor 1254–induced rat livers.
[b] Positive controls were *N*-methyl-*N*′-nitro-nitrosoguanidine for TA1535, TA100 and WP2uvrA, 2-nitrofluorene for TA98 and 9-aminoacridine for TA1537 in the absence of S9; 2-amino-anthracene for all strains in the presence of S9.
[c] Positive controls were mitomycin C for non-activated assays and cyclophosphamide for activated assays.
[d] Positive control was 50 mg cyclophosphamide/kg.

4 mg/kg bw per day). In male rats, a reduction in body weight gain during the growth phase was observed at all doses in the F_0 generation and at the middle and high doses in F_2 animals. In females, 80 mg monensin/kg in the diet reduced weight gain during the growth phase in F_0, F_1 and F_2 animals, whereas there was also reduced weight gain in F_2 females in the middle and highest dose groups. Mean body weight was reduced in pregnant and lactating females in the middle and highest dose groups, in F_1, F_2 and F_3 progeny in the highest dose groups and in F_2 progeny in the middle dose group. No statistically significant differences between the control and treatment groups could be found for the parameters of reproductive performance, including fertility, litter size, gestation length, parent and progeny survival, and sex distribution. No evidence of embryotoxicity or teratogenicity was observed. A NOAEL for parental and offspring toxicity could not be identified, owing to the reduction in body weight gain in both sexes in every generation and at all doses. The NOAEL for reproductive toxicity was 80 mg/kg feed, equivalent to 4 mg/kg bw per day, the highest dose tested (Adams, 1981).

(b) Developmental studies

(i) Rats

In a non-GLP-compliant study, the effects of exposure to monensin during development were studied using 28-day-old Wistar rats. Groups of 15, 14 and 12 female rats received powdered diets containing a non-specified preparation of monensin at concentrations of 0, 100 or 300 mg/kg (equivalent to 0, 5 or 15 mg/kg bw per day) until premating weights achieved 185 g and during pregnancy and lactation. Gestation length, maternal weight gain (difference in body weight from gestation days 3 to 10 and from gestation days 0 to 18), litter size, presence of external malformations, sex ratio and pup weight were examined. All pups were monitored for altered development, including time to development of primary coat of downy hair, ear unfolding, fur development, incisor eruption, ear opening and eye opening. The surface righting and negative geotaxis reflexes of all pups were also examined. Female body weight was significantly decreased in the highest dose group after 8 days of treatment and remained lower throughout the study. No significant differences in female fertility were observed. All females became pregnant, with the exception of two in the high dose group, which did not show vaginal opening and could not be mated. Weight gain by monensin-treated dams during pregnancy did not differ significantly from that of control dams. Gestation length, litter size and number of stillborn pups were also unaltered by monensin treatment. The body weights of male and female pups in the highest dose group were reduced from postnatal day 10 until postnatal day 21. Male offspring in the low dose group showed body weight reduction only on postnatal day 21. No external signs of malformation were detected in the pups. Females perinatally exposed to 100 mg monensin/kg in the diet showed a delay of incisor eruption; this effect was not seen in the high dose group. No other treatment-related effects were observed. Owing to effects on body weight in males in the low dose group on postnatal day 21, a NOAEL for developmental toxicity could not be identified (de Souza Spinosa et al., 1999).

(ii) Rabbits

In a study that was not conducted according to GLP, monensin sodium was administered by gavage to groups of 15 pregnant Dutch Belted rabbits at doses of 0.076, 0.38 or 0.76 mg/kg bw per day from gestation days 6 to 18, whereas 25 control rabbits received 5% vehicle. At gestation day 28, all animals were killed and examined for clinical condition and reproductive performance, and all progeny were weighed, sexed and examined for developmental defects. Maternal mean daily food consumption decreased in animals receiving 0.76 mg/kg bw per day during treatment only, but there was no effect on mean body weight. No differences were observed in litter size, corpora lutea number, implantation occurrence, fetal viability or resorption occurrence. In addition, sex distribution, progeny survival and mean fetal weights did not differ between groups. Fetal abnormalities occurred in low incidence and were unrelated to monensin treatment. The NOAEL for maternal toxicity was 0.76 mg/kg bw per day, the highest dose tested. The NOAEL for teratogenicity was 0.76 mg/kg bw per day, the highest dose tested (Gossett et al., 1974).

2.2.6 Special studies

(a) Dermal irritation

(i) Rabbits

In an experiment that was not GLP compliant, the exposed skin of three male and three female New Zealand albino rabbits was exposed to monensin in the feed premix Coban at a dose of 0.2 mg/kg bw and occluded for 24 h. Three rabbits had their skin abraded prior to application of the Coban. Toxicity was monitored for 2 weeks. Erythema occurred in only one animal. All animals lost between 50 and 1340 g bw during the study (Arthur & Downs, 1979). To confirm that the observed weight loss was due to dermal exposure and not due to oral ingestion, the study was repeated following the same protocol in six additional animals, which were collared to prevent licking of the exposure site. No dermal toxicity was observed, but weight losses still occurred and ranged from 20 to 370 g (Arthur & Downs, 1979). An additional study to ensure that weight loss was not due to procedural trauma confirmed that a very high dose of Coban placed on abraded skin of rabbits for 24 h leads to a transient loss in body weight (Arthur, 1980).

In another non-GLP-compliant dermal toxicity study, the fur of three male and three female New Zealand albino rabbits was clipped, and the skin was abraded in three rabbits. Mycelial monensin at 500 mg/kg (equivalent to 42 mg/kg bw) was applied to the abraded area, which was occluded for 24 h. Toxicity was evaluated for 2 weeks. Slight erythema was observed in a single rabbit 4 days after treatment. No other signs of toxicity were observed (Pierson, 1981).

(ii) Guinea-pigs

In a non-GLP-compliant study, groups of 12 albino guinea-pigs (males and females) were exposed on the anterior portion of their backs to mycelial monensin at 0 or 2 g/kg (equivalent to 0 and 220 mg/kg bw) for 4 h/day, 5 days/week, for a total of 15 treatments. The animals were examined throughout the study for signs of toxicity, including primary irritation, and six animals in each group underwent histopathological examination at the conclusion of the study. The remaining six animals in each group were held without treatment for 17 days and then were challenged with 2 g monensin/kg. No skin irritation was observed following initial treatments, and there was no evidence of contact sensitization following the challenge treatment. Four control and eight treated animals developed transient signs of lacrimation and eye irritation after 12 treatments. Body and organ weights and pathology were normal for all animals tested (Arthur, 1968).

(b) Ocular irritation

(i) Rabbits

In a non-GLP-compliant experiment, six New Zealand albino rabbits were treated in one eye with 53 mg of feed premix Coban (premix containing 9.9% monensin sodium). Corneal dullness, mild corneal opacity, marked iritis and

moderate conjunctivitis were observed within 1 h of treatment. Within 24 h, well defined to severe corneal opacity and severe conjunctivitis developed. Corneal changes appeared irreversible. Three additional rabbits were treated and then had their eyes rinsed after 2 min. Slight conjunctivitis developed in all animals, with corneal dullness and slight iritis observed in one animal. Ocular irritation was reversed within 48–72 h (Arthur & Downs, 1979).

In a non-GLP-compliant study, nine New Zealand albino rabbits were treated in one eye with 59 mg of monensin. Three rabbits had their eyes rinsed 2 min after treatment with 300 ml saline. One hour after treatment, slight corneal opacity, marked iritis and moderate conjunctivitis were observed in unrinsed eyes. In five rabbits, symptoms subsided by 7 days. Staphyloma with corneal perforation was observed in a single rabbit within 7 days. During healing, vascularization developed and involved 50% of the cornea by 21 days. Animals with rinsed eyes exhibited corneal dullness, moderate iritis and mild conjunctivitis. Evidence of irritation disappeared by 7 days (Pierson, 1981).

(c) Inhalational toxicity

(i) Rats

In a non-GLP-compliant inhalational exposure study, two groups of 10 male and 10 female 6- to 8-week-old Harlan Wistar SPF rats were exposed to either normal air or air containing particulate mycelial monensin sodium at a mean concentration of 79 mg/m^3 for 2 weeks (1 h/day, 5 days/week). All animals were observed for changes in physical appearance, behaviour, body weight, haematology, clinical chemistry and histopathology. All animals survived the study. Nine of 10 treated females became anorexic and lost weight during the 2nd week of the study. Several clinical chemistry values were altered by monensin treatment, including elevated creatinine levels in males, decreased serum alkaline phosphatase in females and increased serum alanine aminotransferase in females, but these changes were not considered biologically significant. Haematology was normal for all animals. Slight focal myositis of the skeletal muscle was seen in two males and two females but none of the controls. Multifocal myocardial changes, which were more pronounced than in the control group, were observed in four male rats treated with monensin; these changes included eosinophilic myocardial fibres, a few pyknotic nuclei and increased mononuclear cells (Arthur et al., 1976).

In two additional non-GLP-compliant inhalational exposure studies, the effects of mycelial and crystalline monensin dust were examined in groups of 10 male and 10 female Harlan Wistar SPF or barrier-derived rats. In one study, the animals were exposed (head only) to mycelial monensin sodium dust at concentrations of 0, 9.83, 18.14 or 33.33 mg/m^3 for 1 h/day, 5 days/week, for 2 weeks. In a second study, animals received mycelial monensin at 28.86 mg/m^3 or crystalline monensin dust at 0, 8.19, 12.83 or 23.93 mg/m^3 using the same dosing regimen. One male and two females receiving 33.33 mg mycelial monensin/m^3 and 30% of the females receiving 23.93 mg crystalline monensin/m^3 did not survive the study. Dose-dependent chromorhinorrhoea was observed, with a significantly greater incidence in males receiving 18.14 or 33.33 mg mycelial monensin/m^3, in

all females receiving mycelial monensin and in females receiving the high dose of crystalline monensin. A dose-dependent decrease in body weight was observed for females exposed to mycelial and crystalline monensin. Histological changes seen with both mycelial and crystalline monensin included scattered degeneration and regeneration of skeletal muscle fibres (particularly in females) and/or necrosis of myocardial fibres, although myocardial effects were infrequent. A no-observed-adverse-effect concentration (NOAEC) of 8.19 mg/m^3 was identified for inhalational exposure to crystalline monensin dust (Gossett et al., 1976).

In another inhalational exposure experiment that was not conducted using GLP, five female and five male Fischer 344 rats were exposed (head only) to a gravimetric concentration of 0.37 mg mycelial monensin sodium/l (8.88 mg monensin/l nominal) for 1 h/day for 14 days. Three animals showed signs of chromodacryorrhoea during the exposure period. Following exposure, all animals appeared normal. At necropsy, 9 of 10 animals had an enlarged caecum (Pierson, 1981).

(ii) Dogs

In a GLP-compliant subchronic inhalation study in Beagle dogs, males and females were exposed to a sub-80 sieve fraction of mycelial monensin sodium for 6 h/day, 5 days/week, for 90 days. Two animals per sex were exposed to gravimetric concentrations of 0, 0.23, 0.61 and 2.3 μg mycelial monensin sodium/l air (equivalent to 0, 0.08, 0.15 and 0.84 μg monensin activity/l). Animals in the highest exposure group were observed to have ocular irritation, bloody diarrhoea, excessive salivation and hypoactivity. These dogs also had elevated serum alanine aminotransferase, aspartate aminotransferase and creatine kinase (owing to skeletal muscle isoenzyme) and lactate dehydrogenase from study days 8 to 22 in males and from study days 8 to 29 in females. ECG effects, including tachycardia, R-wave suppression, altered T-waves and premature ventricular repolarization, were also observed in the high exposure group only. Elevated mean platelet counts in high-dose females were also observed on two sampling dates, but haematological parameters were otherwise normal for all animals tested. Myeloid:erythroid ratios calculated from examining terminal bone marrow samples were normal for all animals. No treatment-related effects were seen in organ weights, and there were no treatment-related pathological lesions. Since toxicity was observed only at 0.84 μg monensin activity/l, a NOAEL of 0.15 μg monensin activity/l (0.61 μg mycelial monensin sodium/l) was identified for both sexes (Dorato & Howard, 1983).

(d) Immunotoxicity

(i) Mice

In two separate GLP-compliant studies, the potential for delayed contact hypersensitivity was investigated in female CBA/J mice using the local lymph node assay (Griffon, 2002a,b). A 10% weight by volume (w/v) extract of monensin-containing feed premix (ELANCOBAN®200) in ethanol:water (50:50 by volume) was prepared, and groups of four mice were treated over the ears with 0.5, 1.0,

2.5, 5, 10, 25, 50 or 100% solutions of this extract, the positive control (25% α-hexylcinnamaldehyde) or vehicle alone for 3 consecutive days. After 2 days of resting, proliferation of cells in the lymph nodes draining the ears was determined using tritiated methyl thymidine and used to calculate stimulation indices. No cutaneous reactions were observed, and there was no significant change in ear thickness during either study. A dose-related increase in stimulation index was observed in the study using concentrations ranging from 5% to 100%, but not in the study with doses ranging from 0.5% to 10%. A significant increase in stimulation index occurred at doses greater than or equal to 5%. This increase was attributed to delayed contact hypersensitivity, and it was determined that feed premix ELANCOBAN®200 is a weak sensitizer (Griffon, 2002a,b).

(e) Cardiovascular and respiratory effects

(i) Dogs and pigs

The cardiovascular and respiratory effects of intravenous administration of monensin sodium in male mongrel dogs (conscious and anaesthetized, 11–23 kg) and pigs (anaesthetized, 19–27 kg) were investigated in a non-GLP-compliant study. Dogs were exposed to doses ranging from 0.69 μg/kg bw to 1.4 mg/kg bw, and pigs received between 0.0069 and 0.69 mg/kg bw. The exact dosing regime was not reported. In anaesthetized dogs, monensin significantly and dose-dependently increased left ventricular contractility (0.035 mg/kg bw), blood pressure (0.014 mg/kg bw), heart rate (0.035 mg/kg bw) and left anterior coronary artery blood flow (0.0069 mg/kg bw). Exposure to 0.035 mg/kg bw resulted in premature ventricular contractions and ventricular tachycardia. Respiration rate was also significantly increased in animals receiving at least 0.14 mg monensin/kg bw, and 50% of the animals receiving 1.4 mg monensin/kg bw died of respiratory arrest. The NOAEL for anaesthetized dogs was 0.0035 mg/kg bw (Holland, 1978).

To confirm that cardiovascular effects could be produced in conscious dogs, two mongrels were exposed intravenously to increasing doses of monensin. The exact dosing regime was not reported. A dose of 0.21 mg/kg bw or greater was required to cause premature ventricular contractions and ventricular tachycardia in these animals, with occasional premature contractions occurring up to 7 days following exposure. The dogs also became hyperactive, vomited, defecated and hyperventilated following administration of the highest doses of monensin. The NOAEL for conscious dogs was 0.0345 mg/kg bw (Holland, 1978), suggesting that concurrent administration of anaesthetics may potentiate the effects of monensin in dogs by a factor of 10.

Similar cardiovascular effects were observed in five anaesthetized pigs (7/8 Yorkshire, 1/8 Hapshire, 19–27 kg). The exact dosing regime was not reported. A monensin dose of 0.035 mg/kg bw administered intravenously caused increased left ventricular contractility, heart rate, coronary blood flow and premature ventricular contractions. Effects on left ventricular contractility were less pronounced than in dogs, whereas the effects on heart rate were greater in the pigs. The lowest effective dose in pigs was 0.0069 mg/kg bw, which significantly increased mean

blood pressure. Since this was the lowest dose used for the study, no intravenous NOAEL could be identified in pigs (Holland, 1978).

As acute overdose is more likely to occur through oral exposure than through intravenous exposure, a second non-GLP-compliant study was performed in conscious Beagle dogs to determine whether oral administration will have effects on cardiovascular and respiratory function similar to those observed following intravenous administration (Holland et al., 1981). The effects of oral exposure by gavage to 0, 0.138, 0.345, 0.690 or 1.38 mg monensin sodium/kg bw in 15 ml of 10% acacia were examined in four, four, four, six and four dogs, respectively, and compared with the effects in three male and three female dogs, weighing 8.5–15.2 kg, which were intravenously administered bolus doses of monensin sodium every 10 min to give cumulative doses of 0.0069, 0.0138, 0.0345, 0.069 and 0.138 mg/kg bw. In dogs that received monensin orally, coronary artery flow was significantly increased with 0.69 and 1.38 mg/kg bw, whereas heart rate and blood pressure remained unchanged. The elevation in coronary blood flow was maximal 13–17 min after dosing and returned to normal by 30 min. Intravenous doses of 0.069 and 0.138 mg/kg bw significantly increased coronary blood flow, and mean blood pressure increased with a dose of 0.138 mg/kg bw. No changes in heart rate were observed. When the dose required to cause 100% increase in coronary flow was estimated using log-linear interpolation, the intravenous route was approximately 11 times more active than the oral route in increasing coronary blood flow (Holland et al., 1981). The threshold for pharmacological effects on the heart following oral administration was 0.345 mg/kg bw, based on increased coronary blood flow at 0.690 and 1.38 mg/kg bw. The observed transient increase in coronary blood flow in dogs given a single oral dose of monensin was considered treatment related but not adverse, owing to the absence of effects on blood pressure or heart rate.

(f) Microbiological effects

A JECFA decision tree approach that was adopted at the sixty-sixth meeting of the Committee (Annex 1, reference *181*) and complies with Guideline 36 of the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH GL36) (VICH, 2004) was used by the Committee to determine the need to establish a microbiological acceptable daily intake (ADI) for impact of monensin residues on the intestinal microbiota. The decision tree approach initially seeks to determine if there may be microbiologically active monensin residues entering the human colon. If the answer is “no” to any of the first three steps, then no microbiological ADI is necessary. However, should such residues be present, then two end-points of public health concern are to be considered: 1) disruption of the colonization barrier and 2) increase of the population(s) of resistant bacteria. At Step 4 of the decision tree process, it is possible to provide scientific justification to eliminate testing (i.e. the need for a microbiological ADI) for either one or both end-points. Step 5 is where a microbiological ADI would be determined. Should a microbiological ADI not be necessary, then the toxicological or pharmacological ADI would be used. The Committee evaluated minimum inhibitory concentration (MIC) susceptibility, faecal

binding interaction and the biological activity of monensin metabolites and used the decision tree to answer the following questions in the assessment of monensin.

Step 1: Are residues of the drug, and (or) its metabolites, microbiologically active against representatives of the human intestinal flora?

Yes. Monensin is microbiologically active against some bacterial genera and species representative of the human intestinal flora.

The antimicrobial spectrum of activity for monensin was first reported by Haney & Hoehn (1967). Monensin is active against some Gram-positive bacteria and some Gram-negative anaerobes and has some limited activity against mycoplasma, fungi and viruses. Monensin is inactive against Gram-negative, aerobic enteric bacteria, including *Pseudomonas* spp., and Gram-negative, facultative anaerobic enteric bacteria, including Enterobacteriaceae (*Escherichia coli*, *Salmonella* spp.) and *Vibrio* spp. MICs of monensin against 68 strains of 18 species of *Clostridia* isolated from the digestive tract of cattle, poultry and pigs, as well as human strains of *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Bacteroides*, *Peptostreptococcus* and *Eubacterium*, have been determined (Dutta et al., 1983; Scott et al., 1999).

In a more recent GLP-compliant study, the MIC of monensin was determined against 100 bacterial strains, comprising 10 isolates from each of 10 groups of genera representing the normal human intestinal microbiota (Pridmore, 2004a). All strains were sourced from the faecal microbiota of healthy unmedicated humans. The test system was standardized agar dilution MIC methodology using quality control strains as described in the Clinical and Laboratory Standards Institute guidelines (CLSI, 2004). To assess the effect of bacterial density on monensin activity, each MIC was determined using two inoculum levels of 10^9 and 10^5 colony-forming units (cfu)/ml for each strain. Monensin activity against each bacterial group is summarized in Table 3. MIC_{50}, MIC_{90} and geometric mean MIC were calculated for each bacterial group. In tests using the higher bacterial inoculum density, monensin exerted little or no antibacterial activity against *Bacteroides fragilis*, other *Bacteroides* species or *E. coli*. This is consistent with the known spectrum of activity for this compound, which has low activity against Gram-negative bacteria. Monensin also exerted very weak activity against *Bifidobacterium* species. Monensin activity was clearly demonstrable against the other six bacterial groups tested at the higher inoculum density. *Peptostreptococcus* was the most susceptible group (MIC_{50} of 0.5 µg/ml), whereas *Fusobacterium* was the least susceptible. At the lower inoculum density, monensin MICs against *E. coli*, *Enterococcus* and *Peptostreptococcus* were similar to those obtained using the higher inoculum density. Thus, monensin activity against these organisms was not affected by bacterial density. Conversely, *B. fragilis*, other *Bacteroides* species and *Bifidobacterium* demonstrated a large "inoculum effect"; that is, susceptibility of these organisms to monensin was greatly enhanced when tested at a lower inoculum density. A moderate inoculum effect—i.e. monensin MICs were reduced by 1–3 doubling dilutions at the low inoculum level compared with those obtained using the high inoculum level—was seen in *Clostridium*, *Eubacterium*, *Fusobacterium* and *Lactobacillus*.

Table 3. Summary of monensin activity against bacterial groups representing the normal human intestinal microbiota[a]

Bacterial group	Summary of MIC parameters (µg/ml)							
	High inoculum density				Low inoculum density			
	MIC_{50}	MIC_{90}	Geometric mean MIC[b]	MIC range	MIC_{50}	MIC_{90}	Geometric mean MIC[b]	MIC range
Bacteroides fragilis	>128	>128	128	All >128	8	16	10.6	4–16
Other *Bacteroides* spp.	>128	>128	128	All >128	8	16	7.5	2–16
Bifidobacterium	128	>128	52	2–>128	2	4	1.9	0.5–4
Clostridium	1	4	1.6	0.5–>128	0.5	0.5	0.5	0.125–4
Enterococcus	8	8	7.5	4–8	8	8	6.5	4–8
Escherichia coli	>128	>128	128	128–>128	>128	>128	128	All >128
Eubacterium	2	4	2.3	1–4	0.5	1	0.7	0.5–1
Fusobacterium	16	128	19.7	0.5–>128	2	16	2	ND
Lactobacillus	8	>128	12.1	2–>128	2	>128	4	0.5–>128
Peptostrepto-coccus	0.5	2	0.6	0.25–4	0.25	4	0.5	0.125–4

ND, not determined (number of results <10).
[a] From Pridmore (2004b).
[b] To calculate the geometric mean, MIC results of >128 µg/ml were treated as being 128 µg/ml.

Step 2: Do residues enter the human colon?

Yes. A number of residue studies using ^{14}C radiolabelling to detect total residues or analytical methods to detect parent monensin have been conducted in chickens, turkeys, quail, pigs, ruminants, sheep, goats and milk, as described in section 2.1.1. Muscle contains little or no monensin-derived residue, regardless of the period between withdrawal of medication and slaughter. However, residues may be present at low levels in offal, fat and skin. Therefore, monensin-related residues could enter the colon of a person ingesting tissues or milk from treated animals.

Step 3: Do the residues entering the human colon remain microbiologically active?

No. Monensin residue will be extensively transformed to metabolites with very reduced activity prior to entering the colon of the consumer; within the colon, it will become substantially bound to faecal material.

To determine the effect of faecal binding on the antibacterial activity of monensin, selected monensin concentrations of 0, 1, 2, 5, 10, 20, 50 and 100 mg/ml were incubated with increasing concentrations of sterilized human faeces (0, 10, 20 and 50% w/v in Mueller Hinton Broth), collected from three individual donors (Pridmore, 2004b, 2007a). Monensin activity was determined using *Bacillus subtilis* ATCC 6633 as an indicator organism, as it is susceptible to monensin. All three faecal samples had maximal binding of monensin (>90% binding) at 50% concentration. The 50% faecal concentration provided the closest representation of the in vivo situation (Table 4). The results demonstrated the rapid and extensive binding of monensin to human faeces. Based on this in vitro study, it can be estimated that the binding of monensin residues to undiluted faecal material would be highly likely to exceed 90%. An additional faecal interaction study was conducted, incorporating the microbiological assay methodology and HPLC/MS chemical assays (Pridmore, 2007b). The mean proportion of monensin that became unavailable after 12 h interaction with faeces, as determined by the growth inhibition assay (n = 3) and chemical assay (n = 5), was 96.8% and between 94.3% and 98.6%, respectively. This confirmed the conclusions from the earlier study that the antibiotic activity of monensin in the colon would be reduced by >90% by contact with faecal material.

Table 4. Determination of monensin availability after interaction with faeces: definitive experiment with faecal sample 012/06/008 [a]

Interaction time (h)	Broth only (no faeces)		50% faeces by weight	
	Initial monensin concentration (μg/ml) required to inhibit growth ("a")	Percentage of monensin "unavailable" after interaction with faeces	Initial monensin concentration (μg/ml) required to inhibit growth ("b")	Percentage of monensin "unavailable" after interaction with faeces: [(b − a)/b] × 100
Incubation for 24 h				
0	10	0	100	90.0
1	10	0	100	90.0
2	10	0	100	90.0
4	10	0	100	90.0
6	10	0	120	91.7
8	10	0	120	91.7
12	10	0	120	91.7
Incubation for 48 h				
0	10	0	100	90.0
1	10	0	100	90.0
2	10	0	100	90.0

Table 4 (contd)

Interaction time (h)	Broth only (no faeces)		50% faeces by weight	
	Initial monensin concentration (μg/ml) required to inhibit growth ("a")	Percentage of monensin "unavailable" after interaction with faeces	Initial monensin concentration (μg/ml) required to inhibit growth ("b")	Percentage of monensin "unavailable" after interaction with faeces: [(b − a)/b] × 100
4	10	0	100	90.0
6	10	0	120	91.7
8	10	0	120	91.7
12	10	0	120	91.7

[a] From Pridmore (2007b).

Monensin is extensively metabolized and converted to numerous metabolites by cattle, pigs and rats. *O*-Demethylation and hydroxylation appear to be the major metabolic pathways. Antimicrobial activity of *O*-desmethylmonensin was determined by bioautography against *Bacillus subtilis* and by turbidimetric assay against *Streptococcus faecalis*. In these systems, *O*-desmethylmonensin was only 5% as active as monensin (Boder et al., 1979). Most of the monensin is metabolized to products without antimicrobial activity.

The antibiotic potency of monensin metabolites was further investigated by Pridmore (2007c). Based on a zone inhibition assay, the antibacterial activity of metabolite M1 (*O*-desmethylmonensin) was 19–26.6% of the activity of monensin. MIC values for metabolites M2 and M6 were 2–3 twofold dilutions higher than those for monensin, suggesting that their activity was 12.5–25% of the parent compound activity.

Step 4: Is there any scientific justification to eliminate testing for either one or both end-points of concern, i.e. disruption of the colonization barrier or resistance development?

Monensin does not appear to select for true acquired resistance in bacteria and is not a drug used in human medicine. Results from the microbiological studies suggest that the development of resistance to monensin and cross-resistance to a number of commonly used antimicrobials in veterinary and human medicine is unlikely (Callaway et al., 2003). Thus, the only potential adverse effect on human intestinal microbiota would be disruption of the colonization barrier as the end-point of concern for determining the microbiological ADI. However, since the majority of monensin residues in the colon are bound to faeces and are biologically inactive, the bioavailable concentration is below the lowest MIC_{50} of any of the representative human intestinal bacteria listed in Table 3. Therefore, monensin residues are unlikely to disrupt the colonization barrier of the human intestine. Consequently, there is no need to determine a microbiological ADI for monensin residues.

2.3 Observations in humans

No controlled studies have been performed in which humans have been intentionally exposed to monensin sodium. Two case-reports were found in the literature that described the effects of human monensin intoxication. In the first case, a 17-year-old boy ingested an unknown amount of monensin sodium (Kouyoumdjian et al., 2001); in the second, a 16-year-old boy consumed approximately 500 mg of monensin (Caldeira et al., 2001). In both cases, a similar pattern of toxicity was observed as has been seen previously during overdosing in domestic animal species. Early symptoms included nausea, loss of appetite and abdominal pain, followed by muscle weakness, severe pain, primarily in the lower limbs, and dark brown urine. Clinical chemistry results revealed highly elevated serum creatine kinase, lactate dehydrogenase and aspartate aminotransferase levels, and creatinine and potassium levels were also elevated. The haemogram revealed leukocytosis and a very high erythrocyte sedimentation rate. In both cases, monensin caused rhabdomyolysis, which led to acute kidney failure and in one case led to heart failure. Death occurred in both patients within 11 days of consumption. The primary targets of monensin overdose in humans appear to be skeletal and heart muscles.

The health effects of occupational exposure to monensin during production have also been documented (Twenty, 2001). During the 30-year period that was reviewed, irritant conjunctivitis was observed in several individuals who received a direct splash of monensin in the eye, and irritant contact dermatitis was also observed in one individual. Six employees developed an immunoglobulin E (IgE)-mediated allergic response to monensin, with symptoms including transient urticaria, swelling of the face or tongue, pruritus, chest congestion and chest tightness. These symptoms resolved upon the removal of the employees from the monensin manufacturing area (Twenty, 2001).

3. COMMENTS

3.1 Biochemical data

The absorption and excretion of monensin have been studied in a variety of species, including rats and dogs. The recovery of monensin in the bile was independent of dose (33–49% in male rats receiving 5 or 40 mg monensin/kg bw and 31–53% in female rats receiving receiving 2 or 16 mg monensin/kg bw).

Monensin rapidly disappears from the serum in rats and dogs, and the highest concentration of monensin is attained in the liver.

In rats and dogs, less than 10% of excreted monensin is the parent compound. Monensin is extensively metabolized in the liver in the majority of animals. In human microsomes, monensin sodium is extensively metabolized (93–99% by 60 min), and its turnover is similar to that in dogs.

Monensin metabolism occurs primarily through *O*-demethylation or hydroxylation of the ionophore backbone and is believed to occur at least in part by

CYP3A. Tested monensin metabolites have decreased antibacterial, anticoccidial, cytotoxic, cardiac and ionophoric activity.

The primary excretion route for monensin is the faeces. In rats, a majority of the administered dose (up to 10 mg/kg bw in males, up to 4 mg/kg bw in females) was excreted within 72 h (70–91%), and excretion was independent of dose. Higher doses in rats of both sexes led to decreased excretion of monensin.

3.2 Toxicological data

Crystalline monensin and several preparations of mycelial monensin were tested for toxicity. The Committee evaluated the toxicity of monensin on the basis of the specific form utilized in the studies.

The acute oral toxicity of monensin was moderate to high. The LD_{50} values in mice, rats and rabbits ranged from 22 to 96 mg/kg bw. Signs of toxicity included hypoactivity, ataxia, dyspnoea, ptosis, loss of righting reflex and muscle weakness. Pairs of monkeys given a single dose of monensin in a range from 20 to 160 mg/kg bw by gavage developed diarrhoea within 24 h at all doses and vomiting and appetite suppression starting at 110 mg/kg bw, but no mortality occurred. The LD_{50} value for mycelial monensin in monkeys is greater than 160 mg/kg bw.

In a GLP-compliant study, male and female mice fed diets containing 0, 37.5, 75, 150 or 300 mg mycelial monensin sodium/kg for 3 months (equivalent to 0, 5.6, 11.2, 22.5 or 45 mg/kg bw per day) showed a dose-dependent decrease in body weight gain at the end of the study, ranging from 27% and 21% in the lowest dose group in females and males, respectively, to 99% in the highest dose group in both sexes. Mean body weights also declined, with decreases ranging from 5% and 8% in the lowest dose group to 29% and 35% in the highest dose group in females and males, respectively. Except for the declines in body weights and body weight gains in the lowest-dose males, all changes were statistically significant. Elevated creatine phosphokinase values were observed for the males in the two highest dose groups and for the females receiving the highest dose. Mild diffuse vacuolation of cardiac myofibres occurred in the highest dose group. Since weight gain was affected in all dose groups, a NOAEL could not be identified from this study.

Several GLP-compliant and non-GLP-compliant oral subchronic toxicity studies were conducted in rats using mycelial or crystalline monensin. In a non-GLP-compliant study, rats were fed diets containing mycelial monensin sodium for 3 months at nominal concentrations of 0, 25, 50, 80 or 125 mg/kg (based on feed analysis, equal to 0, 0.89–2.45, 1.83–4.63, 3.02–7.71 and 4.54–12.05 mg/kg bw per day in males and 0, 1.30–2.55, 2.75–5.83, 4.04–12.83 and 10.17–20.21 mg/kg bw per day in females). Transient dose-dependent decreases in body weight gain, mean body weight and feed consumption were observed in females receiving 50, 80 or 125 mg/kg in the diet. Males receiving 125 mg/kg in the diet also had a transient decrease in body weight gain. The NOAEL for this study was the nominal concentration of 25 mg/kg in the diet. An exact dose could not be determined owing to the wide range of measured concentrations of monensin in the feed.

In four parallel subchronic toxicity studies conducted according to GLP, rats were fed diets containing crystalline monensin sodium or three different preparations of mycelial monensin sodium at 0, 50, 200 or 400 mg/kg (equivalent to 0, 2.5, 10 and 20 mg/kg bw per day) for 3 months. Mortality occurred in four females and one male exposed to the highest dose of mycelial monensin and in one female in the middle dose group. The cause of death could not be determined; however, a relationship with the treatment could not be ruled out. Decreased body weight gain was observed for all forms of monensin starting at 200 mg/kg in the diet and in female rats exposed to the mycelial forms of monensin at 50 mg/kg in the diet. Doses of 200 and 400 mg mycelial monensin/kg in the diet reduced feed consumption compared with crystalline monensin and led to decreased body weight gain in male rats. Slight quantitative differences in body weight gain and feed consumption between the crystalline and mycelial forms were not considered biologically significant. Focal degeneration and interstitial myositis of the diaphragm and skeletal muscle occurred in higher incidence in treated female rats than in controls; however, overall incidence and severity were low. Owing to the decreased body weight gain at the lowest dose, a NOAEL could not be determined.

In a non-GLP-compliant study, rats of both sexes were exposed in feed to crystalline or mycelial monensin sodium at 0, 50, 200 or 400 mg/kg (equivalent to 0, 2.5, 10 and 20 mg/kg bw per day) for 3 months. The toxicological responses to both forms of monensin did not differ. Three high-dose females (one in the crystalline group, two in the mycelial group) died during the study. A severe reduction in body weight gain was observed for both formulations starting at 200 mg/kg in the diet. A slight, transient decrease in body weight gain was observed for females in the 50 mg/kg diet group for the first 2 weeks of the study. Increased total bilirubin and alkaline phosphatase levels and decreased mean serum glucose and creatinine levels were seen in all animals receiving either form at 400 mg/kg in the diet and in females receiving 200 mg/kg in the diet. Female rats in all treatment groups also had decreased alanine aminotransferase levels. Initial histopathological examination revealed a non-dose-dependent incidence of scattered foci of a few myocardial fibres, with degeneration, necrosis and infiltration of mononuclear cells, particularly in males, in all three dose groups for both forms of monensin. A second independent pathology evaluation concluded that the scattered myocardial lesions were not adverse and that the incidence was similar to the control incidence. The Committee concluded that a NOAEL could not be identified because of a slight, transient reduction in body weight gain in females in the lowest dose group, which became severe and non-transient in the next dose group.

In a non-GLP-compliant study, two dogs per sex per group were administered monensin sodium orally in capsules at daily doses of 0, 2.5, 5, 11 or 25 mg/kg bw for 90 days. Deaths attributed to treatment with monensin occurred in two males of the highest dose group and one female of the second highest dose group. Females of the highest dose group developed ataxia, tremors, loss of muscular control and slight relaxation of the nictitating membrane; therefore, treatment was stopped after 5 days. There were no signs of toxicity in surviving male and female dogs receiving 11 and 5 mg/kg bw per day or less, respectively. Haematology, clinical chemistry, urinalysis, organ weights and gross pathology of

all animals were normal, with the exception of transiently elevated serum alanine aminotransferase levels in the second highest dose group. The NOAEL was 5 mg/kg bw per day.

In a second non-GLP-compliant study, dogs were orally administered monensin sodium at doses of 0, 5, 15 or 50 mg/kg bw per day for 91 days in gel capsules. Two males in the highest dose group and one male in the middle dose group did not survive the study and exhibited myopathy of the heart with degeneration of the muscle fibre, macrophage infiltration and visceral congestion. Dogs dosed with 15 and 50 mg monensin sodium/kg bw per day vomited more frequently, lost body weight, had transient increases in lactate dehydrogenase and aspartate aminotransferase and developed muscular weakness, ataxia, arrhythmias, convulsions and mydriasis. Degenerative changes in striated muscle were observed starting at 15 mg/kg bw per day. A slight loss of body weight was observed in dogs in all dose groups. Since toxic effects were seen at the lowest dose, a NOAEL could not be identified.

In a 1-year study, mycelial monensin sodium was orally administered as an equally divided dose in gel capsules twice daily to dogs at doses of 0, 1.25, 2.5, 5 or 7.5 mg/kg bw. No data on feed intake were reported. Dogs receiving 5 and 7.5 mg/kg bw per day showed transient signs of toxicity, which included hypoactivity, muscle weakness (particularly legs and neck), stilted gait, difficulty standing and anorexia. Increased alanine aminotransferase and creatine phosphokinase levels were observed during the first 2 weeks of monensin administration in dogs receiving 5 and 7.5 mg/kg bw per day, and several dogs in these groups also had periodic increases in alanine aminotransferase and creatine phosphokinase levels throughout the entire study period. A decrease in body weight gain was seen in male dogs receiving 2.5, 5 or 7.5 mg/kg bw per day, which exceeded 10% for the highest dose. No histopathological changes were seen that were related to monensin exposure. Since body weight gain was decreased at the next higher dose, the NOAEL was 1.25 mg/kg bw per day.

Long-term (1- and 2-year) studies were carried out in mice and rats orally administered mycelial or crystalline monensin sodium. All long-term toxicity studies were conducted according to GLP guidelines.

Mice were given mycelial monensin sodium in the diet at concentrations of 0, 10, 25, 75 or 150 mg/kg (equal to 0, 1.2, 3.1, 10.2 and 22.6 mg/kg bw per day for males and 0, 1.4, 3.5, 11.7 and 25.6 mg/kg bw per day for females) for 2 years. Significant decreases in body weight and body weight gain occurred in mice receiving 25 mg/kg in the diet or greater, and a statistically significant, dose-dependent decrease in leukocyte counts was observed in males receiving monensin at concentrations of 25, 75 or 150 mg/kg in the diet. Minimal increases in urea nitrogen, creatinine, bilirubin, aspartate aminotransferase and creatine phosphokinase occurred with the highest dose. No substance-related deaths, physical signs or behavioural changes were reported. There was no evidence of carcinogenicity that could be attributed to monensin. A NOAEL of 10 mg/kg in the diet (equal to 1.2 mg/kg bw per day) was identified.

Rats were given crystalline monensin sodium in the diet at 0, 25, 56 or 125 mg/kg (equal to 0, 1.14, 2.57 and 5.91 mg/kg bw per day in males and 0, 1.46, 3.43 and 8.68 mg/kg bw per day in females) for 2 years. Body weight and body weight gain were significantly decreased in animals receiving 125 mg/kg in the diet, and a decrease in these parameters was observed during the first 4 months in rats receiving 56 mg/kg in the diet. Feed conversion efficiency was decreased in the animals receiving 56 or 125 mg/kg in the diet, and mean feed consumption was decreased in animals in the highest dose group during the first 5 weeks of the trial. Crystalline monensin sodium did not produce carcinogenicity. The NOAEL was 25 mg/kg in the diet (equal to 1.14 mg/kg bw per day).

In a second study, rats that were exposed to monensin in utero were further exposed to 0, 33, 50 or 80 mg mycelial monensin sodium/kg in the diet for 2 years (equal to 0, 1.40, 2.18 and 3.60 mg/kg bw per day in males and 0, 1.72, 2.86 and 5.02 mg/kg bw per day in females). Survival in both sexes increased in a dose-dependent manner. A transient decrease in body weight was observed at the beginning of the study in all animals receiving 80 mg/kg in the diet and in females receiving 50 mg/kg in the diet. Body weight gain was also significantly decreased during the 1st week in males receiving monensin at 33 and 80 mg/kg in the diet and during the first 2 weeks in females receiving the highest dose. There was a statistically significant increase in feed intake in females in the highest dose group. In utero exposure followed by 2 years of exposure to mycelial monensin sodium at doses up to 80 mg/kg in the diet did not lead to carcinogenicity. As the observed decrease in body weight gain was transient and restricted to the first few weeks of the 2-year study, the effect was not considered to be adverse. The NOAEL was 80 mg/kg in the diet (equal to 3.60 mg/kg bw per day).

Monensin produced negative results in an adequate range of in vitro and in vivo genotoxicity studies. The Committee concluded that monensin had no genotoxic potential.

In a GLP-compliant multigeneration study, mycelial monensin was administered to three generations of rats and their offspring at doses of 0, 33, 50 or 80 mg/kg in the diet (equivalent to 0, 1.6, 2.5 and 4 mg/kg bw per day). Reduced body weight gain was seen in animals of both sexes in every generation and at all doses. There were no significant differences in fertility, litter size, gestation length, parent and progeny survival or sex distribution, and no evidence of teratogenicity was observed. A NOAEL for parental and offspring toxicity could not be determined owing to the reduction in body weight gain in both sexes in every generation and at all doses. The NOAEL for reproductive toxicity was 80 mg/kg in the diet (equivalent to 4 mg/kg bw per day), the highest dose tested.

In a one-generation, non-GLP-compliant reproduction study, female rats received diets containing a non-specified preparation of monensin at concentrations of 0, 100 or 300 mg/kg (equivalent to 0, 5 or 15 mg/kg bw per day) until premating weights achieved 185 g and during pregnancy and lactation. Female body weight was significantly decreased in the highest dose group after 8 days of treatment and remained lower throughout the study. No significant differences in female fertility were observed. All females became pregnant, with the exception of two in the high

dose group, which did not show vaginal opening and could not be mated. Weight gain during pregnancy was not affected by treatment. Gestation length, litter size and number of stillborn pups were also unaltered by monensin treatment. The body weights of male and female pups in the highest dose group were reduced from postnatal day 10 until postnatal day 21. Male offspring in the low dose group showed body weight reduction only on postnatal day 21. No external signs of malformation were detected in the pups. Females perinatally exposed to 100 mg monensin/kg in the diet showed a delay of incisor eruption; this effect was not seen in the high dose group. No other treatment-related effects were observed. Owing to effects on body weight in males in the low dose group on postnatal day 21, a NOAEL for developmental toxicity could not be identified.

In a non-GLP-compliant teratogenicity study, pregnant rabbits received monensin sodium at doses of 0, 0.076, 0.38 or 0.76 mg/kg bw per day from gestation days 6 to 18. Decreased maternal feed consumption was observed in the high dose group during treatment only. Doses up to 0.76 mg/kg bw per day did not affect litter size, corpora lutea number, implantation occurrence, fetal viability or resorption occurrence. In addition, sex distribution, progeny survival and mean fetal weights did not differ between groups. Fetal abnormalities occurred in low incidence and were unrelated to monensin treatment. The NOAEL for maternal toxicity was 0.76 mg/kg bw per day, the highest dose tested. The NOAEL for teratogenicity was 0.76 mg/kg bw per day, the highest dose tested.

In dogs that received monensin sodium by gavage at a single dose of 0, 0.138, 0.345, 0.690 or 1.38 mg/kg, coronary artery flow was significantly increased at 0.690 and 1.38 mg/kg bw, whereas heart rate and blood pressure remained unchanged. The elevation in coronary blood flow was maximal 13–17 min after dosing and returned to normal by 30 min. The threshold for pharmacological effects on the heart was 0.345 mg/kg bw. The observed transient increase in coronary blood flow in dogs given a single oral dose of monensin was considered treatment related but not adverse, owing to the absence of effects on blood pressure or heart rate.

Monensin is not used in human medicine. An evaluation of the medical records of employees involved in the manufacture of monensin from 1968 to 2001 provided no evidence of chronic diseases that could be related to monensin exposure. Several employees developed IgE-mediated allergic responses, including transient urticaria, swelling of the face or tongue, pruritis, chest congestion and chest tightness, which resolved upon their removal from the monensin manufacturing area.

Two case-reports are available in the literature regarding accidental exposure of humans to monensin. In the first case, a 17-year-old boy ingested an unknown amount of monensin sodium; in the second, a 16-year-old boy consumed approximately 500 mg of monensin. In both cases, a similar pattern of toxicity was observed as has been seen previously during overdosing in domestic animal species. Monensin caused rhabdomyolysis, which led to acute kidney failure in both patients and heart failure in the 16-year-old boy. Death occurred in both patients

within 11 days of consumption. The primary targets of monensin overdose in humans appear to be skeletal and heart muscles.

3.3 Microbiological data

A JECFA decision tree approach that was adopted at the sixty-sixth meeting of the Committee (Annex 1, reference *181*) and complies with VICH GL36 (VICH, 2004) was used by the Committee to determine the impact of monensin residues on the intestinal microbiota.

The Committee evaluated MIC susceptibility, faecal binding interaction and biological activity of monensin metabolites in the assessment of monensin.

Monensin is microbiologically active against some bacterial genera and species representative of the human intestinal flora.

In tests using the higher bacterial inoculum density of 10^9 cfu/ml, monensin exerted little or no antibacterial activity (MIC_{50} values >128 μg/ml) against *Bacteroides fragilis*, other *Bacteroides* species and *Escherichia coli*. This is consistent with the known spectrum of activity for this compound, which has low activity against Gram-negative bacteria. Monensin also exerted very weak activity against *Bifidobacterium* species. Monensin activity was clearly demonstrable against the other six bacterial groups tested at the higher inoculum density. *Peptostreptococcus* was the most susceptible group (MIC_{50} of 0.5 μg/ml), whereas *Fusobacterium* was the least susceptible.

To determine the effect of faecal binding on the antibacterial activity of monensin, selected monensin concentrations of 0, 1, 2, 5, 10, 20, 50 and 100 μg/ml were incubated with increasing concentrations of sterilized human faeces (0, 10, 20 and 50% w/v in Mueller Hinton Broth), collected from three individual donors for time periods between 0 and 12 h. All three faecal samples had maximal binding of monensin (>90% binding) at 50% concentration. The results demonstrated the rapid and extensive binding of monensin to human faeces. An additional faecal interaction study was conducted, incorporating the microbiological and chemical assays. The mean proportions of monensin that became unavailable after 12 h interaction with faeces, as determined by the growth inhibition assay and chemical assay, were 96.8% and 94.3–98.6%, respectively. This confirmed the conclusions from the earlier study that the antibiotic activity of monensin in the colon would be reduced by >90% by contact with faecal material.

In terms of resistance development, monensin does not appear to select for true acquired resistance in bacteria and is not a drug used in human medicine. Results from the microbiological studies suggest that the development of resistance to monensin and cross-resistance to a number of commonly used antimicrobials in veterinary and human medicine is unlikely. Thus, the only potential adverse effect on human intestinal microbiota would be disruption of the colonization barrier as the end-point of concern for determining the microbiological ADI. However, since the majority of monensin residues in the colon are bound to faeces and are biologically inactive, the bioavailable concentration is below the lowest MIC_{50} of any of the representative human intestinal bacteria. Therefore, monensin residues are

unlikely to disrupt the colonization barrier of the human intestine. Consequently, there is no need to determine a microbiological ADI for monensin residues.

4. EVALUATION

Oral exposure to monensin results in skeletal and cardiac muscle damage and a decrease in leukocyte count and body weight gain. The effects on leukocytes and body weight gain occur at similar doses, which are lower than those that induce the effects in muscle. The effect on body weight gain was consistent, at similar doses, across studies in mice, rats and dogs; based on feed intake data in a rat study, it was considered to be a true treatment-related effect. The observed transient increase in coronary blood flow in dogs given a single oral dose of monensin was considered treatment related but not adverse, owing to the absence of effects on blood pressure or heart rate.

The Committee considered the effects of high doses of monensin on muscle tissue to be important adverse effects. The Committee also considered the consistent decrease in body weight gain at lower doses as a conservative indicator of monensin toxicity, even though the exact mechanism of this effect is not known. On the basis of the toxicological findings, the Committee selected the lowest relevant NOAEL of 1.14 mg/kg bw per day in the 2-year oral rat study, based on a decrease in body weight gain at the next higher dose, as the basis for derivation of the ADI. The Committee noted that this NOAEL was supported by similar NOAELs for this effect in other species. An ADI of 0–10 µg monensin/kg bw was established by applying a safety factor of 100 to this NOAEL and rounding to one significant figure.

5. REFERENCES

Adams, E.R. (1981) *A multi-generation reproductive study with monensin sodium (Compound 63080) in the Wistar rat*. Unpublished GLP studies Nos R-78, R-958 and R-29 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Anadón, A. & Reeve-Johnson, L. (1999) Macrolide antibiotics, drug interactions and microsomal enzymes: Implications for veterinary medicine. *Res. Vet. Sci.*, **66**(3), 197–203.

Anderson, W.I., Reed, W.M. & McDougald, L.R. (1976) Efficacy of monensin against turkey coccidiosis in laboratory and floor pen experiments. *Avian Dis.*, **20**, 387–394.

Arthur, B.H. (1968) *Monensin guinea pig dermal toxicity and sensitization study G-D-9-68.* Unpublished study No. G-D-9-68 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Arthur, B.H. (1980) *Acute dermal toxicity of Coban 45 in rabbits*. Unpublished report from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Arthur, B.H. & Downs, O.S. (1979) *Acute oral, dermal, and ocular toxicity testing of Coban®, lot A-39287, a pre-mix containing 9.9% of compound 47039 (monensin)*. Unpublished studies Nos R-0-528-79, B-D-113, B-D-205-79, B-E-100 and B-E-112-79 from the

Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Arthur, B.H., Gossett, F.O., Gibson, W.R. & Morton, D.M. (1976) *Acute oral toxicity of crystalline monensin sodium in rabbits*. Unpublished study from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Boder, G.B., Donoho, A.L., Holland, D.R., Jeffers, T.K., Rockhold, F.W. and Wong, D.T. (1979) *Comparative activity of monensin and* O-*desmethylmonensin in a variety of biological systems*. Unpublished study from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Broddle, W.D. & Worth, H.M. (1976) *Acute oral toxicity of mycelial monensin sodium, QA-182D, given to rodents*. Unpublished study from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Caldeira, C., Neves, W.S., Cury, P.M., Serrano, P., Baptista, M.A. & Burdmann, E.A. (2001) Rhabdomyolysis, acute renal failure, and death after monensin ingestion. *Am. J. Kidney Dis.*, **38**, 1108–1112.

Calhoun, M.C. (1986) *Target animal safety study*. Unpublished study from the Freedom of Information Master File No. 5055 (Ir4), Food and Drug Administration, United States Department of Health and Human Services, Rockville, MD, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Callaway, T.R., Edrington, T.S., Rychijk, J.L., Genovese, K.J., Poole, P.L., Jung, Y.S., Bischoff, K.M., Anderson, R.C. & Nisbet, D.J. (2003) Ionophores: Their use as ruminant growth promotants and impact on food safety. *Curr. Issues Intest. Microbiol.*, **4**, 43–51.

Ceppa, L., Dacasto, M., Carletti, M., Montesissa, C. & Nebbia, C. (1997) In vitro interactions of monensin with hepatic xenobiotic metabolizing enzymes. *Pharmacol. Res.*, **36**, 249–254.

Codex Alimentarius Commission (2007) *Report of the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods, Breckenridge, CO, USA, 3–7 September 2007*. Rome, Italy, Food and Agriculture Organization of the United Nations (ALINORM 08/31/31; http://www.codexalimentarius.net/web/archives.jsp?year=08).

CLSI (2004) *Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard*, 6th ed. Wayne, PA, USA, Clinical and Laboratory Standards Institute (CLSI Document M11-A6).

Davison, K.L. (1984) Monensin absorption and metabolism in calves and chickens. *J. Agric. Food Chem.*, **32**, 1273–1277.

de Souza Spinosa, H., Nicolau, A.A., Maruo, V.M. & Bernardi, M.M. (1999) Effects of monensin feeding during development on female rats and their offspring. *Neurotoxicol. Teratol.*, **21**, 467–470.

Donoho, A.L. (1984) Biochemical studies on the fate of monensin in animals and the environment. *J. Anim. Sci.*, **58**(6), 1528–1539.

Donoho, A.L. (1985) *[^{14}C] monensin metabolism in rats*. Unpublished GLP study No. ABC-0053 from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Donoho, A.L. & Herberg, R.J. (1977) *^{14}C monensin swine balance-excretion study in a barrow*. Unpublished study No. 702-Q63-118 from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to FAO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Donoho, A., Manthey, J., Occolowitz, J. & Zornes, L. (1978) Metabolism of monensin in the steer and rat. *J. Agric. Food Chem.*, **26**(5), 1090–1095.

Donoho, A.L., Galloway, R.B., Lee, D.E., Jeffers, T.K., Boder, G.B., Wong, D.T. & Holland, D.R. (1979) *Comparative activity of monensin and* O*-desmethylmonensin in a variety of biological assays.* Unpublished report from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Donoho, A.L., Herberg, R.J. & Van Duyn, R.L. (1980) *[^{14}C] monensin tissue residue study in chickens*. Unpublished GLP study No. ABC-0043 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Donoho, A.L., Herberg, R.J. & Ruggles, D.E. (1982a) *[^{14}C] monensin in turkeys.* Unpublished GLP study No. ABC-0151 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Donoho, A.L., Herberg, R.J., Zornes, L.L. & Van Duyn, R.L. (1982b) Excretion and tissue distribution of [^{14}C] monensin in chickens. *J. Agric. Food Chem.*, **30**, 909–913.

Dorato, M.A. & Howard, L.C. (1983) *Subchronic inhalation toxicity of mycelial monensin sodium (compound 63080) in Beagle dogs.* Unpublished study No. D3730 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Dutta, G.N., Devriese, L.A. & Van Assche, P.F. (1983) Susceptibility of clostridia from farm animals to 21 antimicrobial agents including some used for growth promotion. *J. Antimicrob. Chemother.*, **12**, 347–356.

Elanco Animal Health (1998) *Appendix 1. Rate and route of excretion in a lamb of orally administered ^{14}C monensin*. Unpublished experiment No. B48-3242 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to FAO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Garriott, M.L. & Gilbert, J.L. (2002) *The effects of monensin sodium on the in vitro induction of chromosome aberrations in Chinese hamster ovary cells*. Unpublished studies Nos 010926CAB0692, 011003CAB0692 and 011024CAB0692 from the Toxicology Research Laboratories, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Garriott, M.L. & Schwier, L.S. (2001) *The effect of monensin sodium on the induction of reverse mutations in* Salmonella typhimurium *and* Escherichia coli *using the Ames test*. Unpublished GLP studies Nos 010919AMT0692 and 011017AMS0692 from the Toxicology Research Laboratories, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Gibson, W.R., Koenig, G.R., Marroquin, F. & Young, S.S. (1974) *Safety studies with monensin premix in the dog*. Unpublished study No. D4753 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Giera, D.D., Herberg, R.J., Thomson, T.D. & Handy, P.R. (1984a) *^{14}C monensin tissue residue study in sheep*. Unpublished GLP study No. ABC-0270 from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to FAO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Giera, D.D., Herberg, R.J., Thomson, T.D. & Handy, P.R. (1984b) *^{14}C monensin tissue residue study in swine*. Unpublished GLP study No. ABC-0279 from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to FAO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Goodrich, R.D., Garrett, J.E., Gast, D.R., Kirick, M.A., Larson, D.A. & Meiske, J.C. (1984) Influence of monensin on the performance of cattle. *J. Anim. Sci*., **58**(6), 1484–1498.

Gossett, F.O., Markham, J.K., Adams, E.R., Owen, N.V., Young, S.S. & Kiplinger, G.F. (1974) *A teratology study on monensin sodium in the rabbit*. Unpublished study No. B-7293 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Gossett, F.O., Arthur, B.H., Marroquin, F., Worth, H.M., Gibson, W.R. & Morton, D.M. (1976) *Subacute inhalation toxicity studies in rats of monensin sodium in a mycelia mass and as a crystalline product.* Unpublished study from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Gossett, F.O., Emmerson, J.L., Gibson, W.R., Worth, H.M. & Morton, D.M. (1977a) Acute effects of a single oral dose of mycelial sodium in rhesus monkeys (*Macaca mulatta*). Unpublished studies Nos M-6017 and M-6067 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Gossett, F.O., Gibson, W.R., Hoffman, D.G., Marroquin, F., Emerson, J.L., Worth, H.M., Young, S.S. & Morton, D.M. (1977b) *The effects of feeding crystalline or mycelial monensin sodium to rats in a subchronic study for three months.* Unpublished study No. R-296 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Griffon, B. (2002a) *Evaluation of skin sensitization potential in mice using the local lymph node assay (LLNA).* Unpublished GLP study No. 22847 TSS (T1FMFR0202) from the Centre International de Toxicologie, Evreux, France. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Griffon, B. (2002b) *Evaluation of skin sensitization potential in mice using the local lymph node assay (LLNA).* Unpublished GLP study No. 22848 TSS (T1FMFR0201) from the Centre International de Toxicologie, Evreux, France. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Grundy, J.S., Bewley, G.W., Buck, J.M., Kiehl, D.E., Krabel, B.J. & Moran, J.W. (1998) *[^{14}C] monensin residue decline and metabolism in broiler chickens.* Unpublished study No. T1F759701 from Elanco Animal Health, Animal Science Chemistry & Biopharmaceutical Research, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Halstead, B., Holmstrom, S.D., Jolly, R., Liang, Z. & Riggs, K.L. (2007) *Pilot laboratory study: Comparative antimicrobial and ionophoretic activity of monensin and monensin metabolites*. Unpublished study No. T1F920615 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Haney, M.E., Jr & Hoehn, M.M. (1967) Monensin, a new biologically active compound. 1. Discovery and isolation. *Antimicrob. Agents Chemother*., **7**(7), 349–352.

Herberg, R.J. (1967) *Radioactivity balance experiment with chickens given feed containing H^3 monensin.* Unpublished study from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Herberg, R.J. (1973a) *Rate and route of excretion in a chicken of orally administered [^{14}C] monensin*. Unpublished study No. VPR-231-766 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Herberg, R.J. (1973b) *Excretion of radioactivity from a steer orally dosed with [^{14}C] monensin.* Unpublished study from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Herberg, R.J. (1973c) *Rate and route of excretion in a rat of orally administered [^{14}C] monensin*. Unpublished study No. 268-122-201 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Herberg, R.J. (1974a) *Excretion of [^{14}C] monensin in steers*. Unpublished study from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Herberg, R.J. (1974b) *Excretion of radioactivity from two steers orally dosed with [^{14}C] monensin*. Unpublished study from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Herberg, R.J. (1975) *[^{14}C] monensin three-chicken balance-excretion experiments*. Unpublished study No. VPR-245-766 from Elanco Animal Health, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Herberg, R.J. & Donoho, A.L. (1977) *^{14}C monensin swine balance-excretion experiment.* Unpublished study No. 702-Q63-155 from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Herberg, R.J., Donoho, A.L. & Zornes, L.L. (1978) *Radioactivity in cattle tissues following oral dosing with [^{14}C]monensin.* Unpublished study No. ABC-0001 from Elanco Animal Health, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Herrera, M.J., Holmstrom, S.D. & Riggs, K.L. (2005) *Pilot laboratory study: Comparative metabolism of monensin in liver microsomes from dogs, horses, and humans*. Unpublished study No. T1F920505 from Elanco Animal Health, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Holland, D.R. (1978) *Cardiovascular and respiratory effects of sodium monensin, i.v., in dogs and pigs.* Unpublished report from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Holland, D.R., Turk, J.A. & Johnson, S.R. (1981) *Cardiovascular effects of sodium monensin, i.v. and p.o. in conscious dogs*. Unpublished GLP report from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Holmstrom, S.D. (2007) *Comparative metabolism of monensin in liver microsomes from dogs, horses, and humans*. Unpublished study No. T1F920505, Report Amendment 1, from Elanco Animal Health, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C. (1980a) *A toxicity study in Wistar rats maintained for three months on diets containing mycelial monensin sodium (Compound 63080).* Unpublished study No. R-306 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C. (1980b) *A one year chronic toxicity study of mycelial monensin sodium (63080) administered orally to Beagle dogs.* Unpublished GLP study No. D-3018 from the

Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C. (1981a) *A subchronic toxicity study in B6C3F1 mice maintained for three months on diets containing mycelial monensin sodium (EL-980, compound 63030).* Unpublished GLP study No. M00879 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C. (1981b) *A chronic toxicity–oncogenicity study in Wistar rats maintained for two years on diets containing mycelial monensin sodium (EL-980, 63080).* Unpublished GLP studies Nos R06378 and R06478 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C. & Lobb, K.L. (1979) *Radiocarbon excretion from Wistar rats after administration of a single oral dose of [^{14}C] monensin; excretion of radioactivity into bile from Wistar rats administered single oral doses of [^{14}C] monensin; distribution of radioactivity into tissues and organs from Wistar rats administered single oral doses of [^{14}C] monensin*. Unpublished GLP studies Nos R-409, R04779, R08879, R09179, R09579, R05279, R05579, R05879 and R06079 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C. & Lobb, K.L. (1981a) *Excretion of radioactivity into bile from Wistar rats administered single oral doses of [^{14}C]monensin.* Unpublished studies Nos R08879, R09179 and R09579 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C. & Lobb, K.L. (1981b) *Distribution of radioactivity into tissues and organs from Wistar rats administered single oral doses of [^{14}C]monensin.* Unpublished studies Nos R05279, R05579, R05879 and R06079 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C. & Usher, R.W. (1984) *A chronic toxicity–oncogenicity study in B6C3F1 mice maintained for two years on diets containing mycelial monensin sodium.* Unpublished GLP studies Nos M00281 and M00381 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C. & Young, S.S. (1981) *Toxicity in rats maintained for three months on diets containing monensin sodium: Discussion of comparative effects of crystalline and double-drum-dried, azeotrope or flash-dried processed mycelial forms of monensin sodium.* Unpublished GLP studies Nos R04879, 04979 and 05179 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Howard, L.C., Gries, C.L. & Novilla, M.N. (1981) *A chronic toxicity–oncogenicity study in Wistar rats maintained for two years on diets containing crystalline monensin sodium (EL-980, 63080).* Unpublished GLP studies Nos R11478 and R11578 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Kouyoumdjian, J.A., Morita, M.D., Sato, A.K. & Pissolatti, A.F. (2001) Fatal rhabdomyolysis after acute sodium monensin (Rumensin) toxicity: Case report. *Arq. Neuropsiquiatr.*, **59**, 596–598.

Nebbia, C., Ceppa, L., Dascasto, M., Carletti, M. & Nachtmann, C. (1999) Oxidative metabolism of monensin in rat liver microsomes and interactions with tiamulin and other

chemotherapeutic agents: Evidence for the involvement of cytochrome P-450 3A subfamily. *Drug Metab. Dispos.*, **27**(9), 1039–1044.

Nebbia, C., Ceppa, L., Dascasto, M., Nachtmann, C. & Carletti, M. (2001) Oxidative monensin metabolism and cytochrome P450 3A content and functions in liver microsomes from horses, pigs, broiler chicks, cattle and rats. *J. Vet. Pharmacol. Ther.*, **24**(6), 399–403.

Phelps, J.B. & Murphy, G.P. (2002) *The effect of monensin sodium (Compound 063080) given orally by gavage for 2 consecutive days on the induction of micronuclei in bone marrow of ICR mice*. Unpublished study No. 020123MNT0692 from Toxicology Research Laboratories, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pierson, C.L. (1981) *Hazard evaluation of mycelial monensin sodium*. Unpublished report for studies Nos R-0-389-79, R-0-393-79, B-D-43-79, B-E-55-79 and R-H-11-79 from Toxicology Research Laboratories, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pressman, B.C. & Fahim, M. (1982) Pharmacology and toxicology of the monovalent carboxylic ionophores. *Annu. Rev. Pharmacol. Toxicol.*, **22**, 465–490.

Pridmore, A. (2004a) *Activity of monensin against bacterial strains representing the normal human intestinal microbiota: Determination of minimum inhibitory concentration (MIC).* Unpublished GLP report No. AA9CUK0401 from Don Whitley Scientific Limited, Shipley, West Yorkshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pridmore, A. (2004b) *Effect of faecal binding on antibacterial activity of monensin*. Unpublished GLP report No. AA9CUK0402 from Elanco Animal Health, Eli Lilly and Company, Basingstoke, Hampshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pridmore, A. (2007a) *Effect of interaction of monensin with faeces on the human intestinal bioavailability of monensin*. Unpublished GLP report No. T1FCUK0602 from Don Whitley Scientific Limited, Shipley, West Yorkshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pridmore, A. (2007b) *Optimization of an assay system to estimate the human intestinal bioavailability of monensin*. Unpublished GLP report No. T1FCUK0601 from Don Whitley Scientific Limited, Shipley, West Yorkshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pridmore, A. (2007c) *Antimicrobial activity of monensin and monensin metabolites against selected bacterial strains: Determination of minimum inhibitory concentration (MIC).* Unpublished GLP report No. T1FCUK0604 from Don Whitley Scientific Limited, Shipley, West Yorkshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Russell, J.B. (1987) A proposed mechanism of monensin action in inhibiting ruminal bacterial growth: Effects on ion flux and protonmotive force. *J. Anim. Sci.*, **64**, 1519–1525.

Russell, J.B. & Strobel, H.J. (1989) Effect of ionophores on ruminal fermentation. *Appl. Environ. Microbiol.*, **55**(1), 1–6.

Scott, R., Andrews, C. & Smith, M.D. (1999) *Susceptibility of selected strains of human enteric bacteria to monensin in vitro*. Unpublished report from Elanco Animal Health, Eli Lilly and Company, Basingstoke, Hants, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Shumard, R.F. & Callender, M.E. (1967) Monensin, a new biologically active compound. VI. Antimicrobial activity. *Antimicrob. Agents Chemother.*, **7**, 369–377.

Twenty, J.D. (2001) *Monensin: The safety to workers*. Unpublished report from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

USFDA (1986) *Use of monensin premix for the prevention of coccidiosis in goats.* Freedom of Information Master File No. 5055 (Ir4) from the Food and Drug Administration, United States Department of Health and Human Services, Rockville, MD, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Van Nevel, C.J. & Demeyer, D.I. (1977) Effect of monensin on rumen metabolism in vitro. *Appl. Environ. Microbiol.*, **34**, 251–257.

VICH (2004) *Studies to evaluate the safety of residues of veterinary drugs in human food: General approach to establish a microbiological ADI.* Brussels, Belgium, International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH Guideline 36; http://www.vichsec.org/pdf/05_2004/Gl36_st7_F_rev.pdf).

WHO (2007) *Critically important antimicrobials for human medicine: Categorization for the development of risk management strategies to contain antimicrobial resistance due to non-human antimicrobial use. Report of the Second WHO Expert Meeting, Copenhagen, 29–31 May 2007*. Geneva, World Health Organization, Department of Food Safety, Zoonoses and Foodborne Diseases (http://www.who.int/foodborne_disease/resistance/antimicrobials_human.pdf).

Worth, H.M., Gibson, W.R., Harris, P.N., Owen, N.V., Todd, G.C. & Pierce, E.C. (1967) *Subacute toxicity study with Monensin Dog Study D-148-66, supplementary study*. Unpublished study from Lilly Toxicology Laboratories, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

NARASIN

First draft prepared by

Dr Pamela L. Chamberlain,[1] Dr Carl Cerniglia[2] and Dr J.G. (Jock) McLean[3]

[1] Covance Laboratories, Vienna, VA, United States of America (USA)
[2] National Center for Toxicological Research, Food and Drug Administration, Department of Health and Human Services, Jefferson, AR, USA
[3] Camberwell, Victoria, Australia

Explanation 133
Biological data 135
- Biochemical aspects 135
  - Absorption, distribution and excretion 135
  - Biotransformation 138
  - Effects on enzymes and other biochemical parameters 140
- Toxicological studies 141
  - Acute toxicity 141
  - Short-term studies of toxicity in laboratory animals 143
  - Non-laboratory animal safety/toxicology studies 151
  - Long-term studies of toxicity and carcinogenicity 157
  - Genotoxicity 159
  - Reproductive and developmental toxicity 159
  - Special studies 163
- Observations in humans 168

Comments 169
- Biochemical data 169
- Toxicological data 169
- Microbiological data 175

Evaluation 175
References 176

1. EXPLANATION

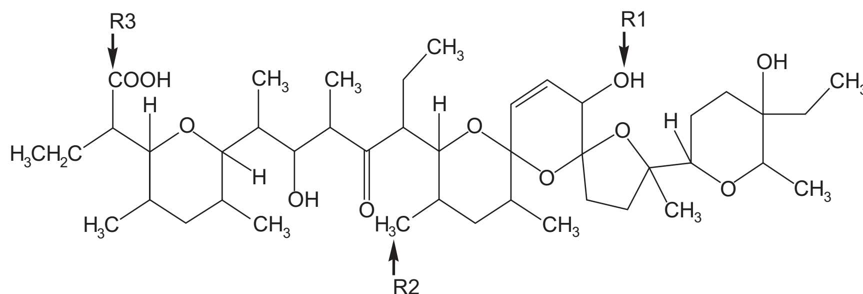
Narasin belongs to the polyether monocarboxylic acid class of ionophores produced by *Streptomyces aureofaciens* strain NRRL 8092. Narasin is composed of 96% narasin A, 1% narasin B, 2% narasin D and 1% narasin I. Narasin A has the major activity (85%). The biological activity of narasin is based on its ability to form lipid-soluble and dynamically reversible complexes with cations. Narasin functions as a carrier of these ions, mediating an electrically neutral exchange–diffusion type of ion transport across membranes. The resultant changes in transmembrane ion gradients and electrical potentials produce critical effects on cellular function and metabolism of coccidia. Narasin is effective against sporozoites and early and late asexual stages of coccidia infections in broiler chickens, caused by *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. mivati*, *E. necatrix* and *E. tenella.* Narasin is also used for the prevention of necrotic enteritis in broiler chickens. In cattle, narasin

is used as a growth promoter. Narasin's growth-promoting effect is attributed to its effect on modulation of rumen fermentation, resulting in enhancement of propionate production with some rumen deaminase inhibition. In addition to increasing the ratio of propionic acid to acetic acid production, enhancement of propionate production and deaminase inhibition results in decreased methane production and decreased protein degradation to ammonia, effects generally attributable to a shift in the ruminal bacterial population (Berg & Hamill, 1978; Jeffers, 1981).

The antimicrobial spectrum of activity of narasin is limited mainly to Gram-positive bacteria, including *Enterococcus* spp., *Staphylococcus* spp. and *Clostridium perfringens*. Narasin is not used in human medicine and was therefore not classified as a critically important antibiotic for humans by the 2007 World Health Organization (WHO) expert meeting on categorization of critically important antimicrobials for human medicine for the development of risk management strategies to contain antimicrobial resistance due to non-human antimicrobial use (WHO, 2007).

The structure of narasin and descriptions of narasin variants are shown in Figure 1.

Figure 1. Structural variants of narasin



Structural variants of narasin	R1	R2	R3
A	OH	CH_3	COOH
B	=O	CH_3	COOH
D	OH	C_2H_5	COOH
I	OH	CH_3	$COOCH_3$

Narasin has not been previously reviewed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Narasin was evaluated on priority request by the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods (Codex Alimentarius Commission, 2007). The Committee was requested to establish an acceptable daily intake (ADI) and recommend maximum residue limits (MRLs) for narasin residues in chicken, pigs and cattle.

The Committee considered the results of studies on pharmacokinetics and metabolism, acute, short-term and long-term toxicity, carcinogenicity, genotoxicity, reproductive and developmental toxicity, general pharmacology, microbiological safety and exposures in humans. Studies were conducted with the dried biomass form of narasin referred to as mycelial narasin or the purified crystalline form of narasin. Mycelial narasin is used in the manufacture of narasin products used in food-producing animals.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Absorption

In a rodent study, bile-cannulated rats were given a single oral dose of [^{14}C]-narasin in acacia suspension. Thirty-five per cent of the dose was recovered in the bile, and 6% in the urine. These results suggest that at least 40% of the dose is absorbed in rats (Manthey et al., 1983).

Although no bioavailability data are available to determine the extent of absorption of narasin in chickens, studies are available that show narasin and its related metabolites in the edible tissues and excreta of broiler chickens dosed orally with narasin. In a comparative metabolism study in which broilers were dosed with [^{14}C]narasin at a level equivalent to 100 mg/kg in feed, three metabolites (NM-1, NM-2 and NM-3) accounted for 14% of the label in excreta, and there were numerous radiolabelled metabolites in the liver. The most abundant compound present in liver was unchanged narasin, which represented approximately 8.8% of the total radioactivity. While these data do not provide a quantitative measure of the extent of absorption, they do provide evidence that narasin is absorbed to some extent in the chicken (Manthey, 1977a; Readnour, 2000).

Three groups of cattle (two steers and one heifer per group) were dosed orally by gelatine capsules with [^{14}C]narasin at 20 g/t ration, for 3, 5 or 7 consecutive days per group, and sacrificed at a practical zero-time withdrawal of approximately 12 h. Liver contained the highest levels of radiochemical residues (calculated as narasin equivalents). After 7 days of dosing, the amount of parent narasin present in liver was 6.5–12% of the total radiochemical residue. The results of this study show that narasin is absorbed to some extent in cattle, with extensive hepatic metabolism (Manthey et al., 1982).

(b) Distribution

The distribution of narasin at zero-time withdrawal has been studied in several species of target animals, including chickens, cattle and pigs. In all species, liver contains the highest levels of narasin residue at zero withdrawal times.

A study was carried out to determine the dosage period necessary to establish steady-state equilibrium of residues in chickens dosed orally with narasin

and to determine the levels of residue at a practical zero-time withdrawal. Male and female broiler chickens (three per sex) were fed a ration containing 100 mg [^{14}C]-narasin/kg for 4 or 6 days. Three males and three females were sacrificed at each time interval after a zero-time withdrawal of 6 h off treatment. Kidney, liver, skin, fat and muscle samples from each chicken were assayed for total radioactivity by combustion analysis and liquid scintillation counting. The narasin level in fat was determined by microbiological analysis. A steady-state equilibrium of tissue residues was achieved after 4 days of dosing. The combined net levels of radioactivity calculated as narasin were, in decreasing order: liver, 0.50 mg/kg; fat, 0.27 mg/kg; skin, 0.16 mg/kg; kidney, 0.13 mg/kg; and muscle, 0.01 mg/kg. There were no apparent differences in residue levels between males and females. Approximately one half of the total radioactivity in fat was narasin (Manthey et al., 1981).

A study compliant with Good Laboratory Practice (GLP) was conducted to demonstrate the dosing interval required to achieve a steady-state equilibrium of residues in the major edible tissues of Hereford cattle following oral dosing with narasin and to determine the levels of radiochemical residues in those tissues at a practical zero withdrawal time of 12 h post-dosing. Nine naive Hereford feedlot cattle (three groups of two steers and one heifer) were administered a total daily dose of approximately 20 g [^{14}C]narasin/t, or 1.5 times the highest recommended narasin use level, by gelatine capsule, twice daily (10 g [^{14}C]narasin/t per dose) for 3, 5 or 7 days. Animals were sacrificed 12 h post-dosing, and muscle, kidney, liver and back fat were assayed for radioactivity. The narasin levels in the livers of all animals dosed for 7 days were also determined by thin-layer chromatographic (TLC) bioautography. The data demonstrated that steady-state levels of radiochemical residues were established in the cattle tissues within 3 days of dosing. Liver contained the greatest amount of total radiochemical residue (calculated as narasin equivalents), corresponding to 0.918, 0.739 and 0.839 mg narasin/kg from cattle dosed for 3, 5 and 7 days, respectively. Parent narasin represented approximately 6.5–12% of the total radiochemical residue in the liver. Little more than trace levels of residue were found in the other tissues. The mean residue levels for tissues after 3, 5 or 7 days of dosing were not statistically different by one-way analysis of variance of the means. The metabolism of narasin was found to be qualitatively similar for steers and heifers for all dosing intervals (Manthey et al., 1982).

A companion, GLP-compliant, [^{14}C]narasin tissue decline study in Hereford cattle (six steers, three heifers) compared withdrawal intervals of 0, 1 or 3 days following oral dosing for 5 days with a total daily dose of [^{14}C]narasin equivalent to 13 g/t medicated ration. The dose was administered by gelatine capsule, twice daily (6.6 g [^{14}C]narasin/t per dose). Liver contained the highest concentrations of radioactivity, with 0.492, 0.233 and 0.050 mg narasin equivalents/kg at withdrawal times of 0, 1 and 3 days, respectively (three steers and one heifer per interval). Less than 5% of the total liver radioactivity was parent narasin. At 1-day withdrawal, only one of three livers had detectable levels of narasin. At the 3-day withdrawal, there was no detectable narasin residue in any of the three livers. Muscle, fat and kidney all contained less than 0.02 mg/kg at zero withdrawal (Manthey et al., 1984a).

In a GLP-compliant study, three groups of four cross-bred pigs (two per sex per group) were fed [^{14}C]narasin rations for 7 days at either 30 μg [^{14}C]narasin/g

followed by 0- or 3-day withdrawal or 45 μg [^{14}C]narasin/g with a 0-day withdrawal. The edible tissues (liver, kidney, muscle, fat and skin) were assayed for total radioactivity. Liver contained the greatest amount of residue for all treatment groups. Pigs fed 45 μg/g had a mean liver residue of 1.48 μg/g at zero withdrawal. Pigs fed 30 μg/g had a mean concentration of 0.75 μg/g at zero withdrawal, which declined to 0.17 μg/g after a 3-day withdrawal. The amount of radioactivity extractable from liver by methanol extraction averaged 56% (n = 2) and 59% (n = 2) for 0-day withdrawal at the 30 μg/g and 45 μg/g dose levels, respectively. These results show no concentration-dependent difference in the percentage of extractable residue at zero withdrawal. At the 30 μg/g dose level, unextractable liver radioactivity after a 3-day withdrawal was 22% of 0-day withdrawal values (Sweeney et al., 1995).

(c) Excretion

A number of studies in several different species have demonstrated that narasin and/or its metabolites are primarily excreted via the faeces, with only a very small proportion eliminated in the urine.

Studies have been completed in rats and chickens to determine the rate of faecal excretion of [^{14}C]narasin. A single mature male rat of unspecified strain was given a single oral gavage dose of 2.3 mg [^{14}C]narasin (study B79-3394-256). Within 52 h post-dosing, 75% of the total radioactivity was recovered in the urine and faeces of the rats. Only 1.1% of the total excreted radioactivity was found in the urine, and the remainder (98.9%) was in the faeces. Three young rats of an unspecified strain with biliary cannulae were given a single oral gavage dose of 2.3 mg [^{14}C]narasin (study B79-3391-241). Up to 16% of the total radioactivity was excreted in the bile within 24 h, indicating that a substantial portion of the [^{14}C]-narasin dose was absorbed and processed through the hepatic system. In a rat respiration study (study Q61-3415-44), less than 0.2% of a dose of approximately 3.1 mg [^{14}C]narasin was recovered from two young Wistar rats as $^{14}CO_2$ (Manthey, 1977b).

Four 8-week-old broiler chickens preconditioned on feed containing 80 mg narasin/kg were given an oral dose of [^{14}C]narasin. The total dose (doses given twice daily) corresponded to the daily average intake of narasin from 80 mg/kg feed (studies Q61-3414-42 and Q61-3422-68). More than 85% of the recovered radioactivity was excreted within 2 days. In three of the four chickens, the total recovery of the dose ranged from 90% to 114%, with a mean of 99%. Low recovery from the fourth chicken (66%) was attributed to incomplete recovery of faeces (Manthey, 1977b).

When the metabolite pattern following administration of [^{14}C]narasin was compared in faeces from four broiler chickens (dose equivalent to 80 mg/kg feed) and four Wistar rats (approximate oral dose of 10 mg/kg body weight [bw]), the metabolite pattern was qualitatively similar for the two species, but there were some quantitative differences. The most abundant metabolites were a group of three, which were designated NM-1, NM-2 and NM-3. In rat faeces, these represented approximately 4, 19 and 10% of the total faecal radioactivity, respectively. In chicken excreta, they represented approximately 7, 4 and 3%, respectively. Mass

spectrometric analysis of NM-3 isolated from chicken excreta tentatively identified this metabolite as the sodium salt of dihydroxynarasin. No other metabolite from either chicken or rat excreta appeared to have a relative abundance greater than 3%. Numerous radiolabelled metabolites were present in the liver of the four [^{14}C]-narasin-fed chickens, with no single metabolite accounting for more than 5% of the total liver radioactivity. The most abundant compound was unchanged [^{14}C]narasin, which represented approximately 8.8% of the total radioactivity. The metabolite pattern in the liver was qualitatively similar to that in chicken and rat excreta. Metabolites NM-1, NM-2 and NM-3 were identified by TLC comparison with excreta metabolites (Manthey, 1977a).

In a GLP-compliant study to determine the rate, route and quantitativeness of excretion of radioactivity from cattle, two Hereford heifers in metabolic cages were each given a single bolus dose of [^{14}C]narasin at a dose corresponding to about one half of a day's intake of narasin for 240-kg cattle fed 13.2 mg/kg ration. The calculated intake of narasin would be 79.2 mg/day. The dose was administered in a gelatine capsule containing about 0.4 MBq of radioactivity. Basal ration and water were offered ad libitum throughout the study. At 24-h daily intervals, urine and faeces were quantitatively collected and assayed radiochemically for ^{14}C content. Within 4 days post-dosing, up to 98.0% of the total faecal radioactivity had been excreted in faeces. Less than 0.5% of the radioactivity of the dose was recovered in urine. Total recoveries of the radioactivity of the labelled narasin dose were 93.4% and 80.1% in each of the two heifers. These results demonstrate that in cattle, a dose of [^{14}C]narasin is rapidly excreted and eliminated nearly quantitatively in the faeces (Manthey et al., 1984b).

In a GLP-compliant study of ^{14}C tissue residue and metabolism in pigs, three groups of cross-bred pigs (two per sex per group) approximately 7 weeks of age and weighing approximately 14 kg were fed [^{14}C]narasin rations for 7 days at 30 μg/g with a 0-day withdrawal, 45 μg/g with a 3-day withdrawal and 45 μg/g with a 0-day withdrawal. The majority of the total radioactivity was recovered in faeces (95–97%), and a relatively small percentage (3–5%) was recovered from urine, indicating that the primary route of excretion in pigs is in the faeces (Sweeney et al., 1995).

2.1.2 Biotransformation

In metabolism studies, hydroxylated metabolites of narasin and narasin B have been identified in chicken excreta. The majority of these metabolites were either di- or trihydroxylated metabolites of narasin or narasin B. Up to 15 separate hydroxylated metabolites were identified in chicken excreta by liquid chromatography/mass spectrometry. In liver, the chromatographic distribution and relative magnitude of radioactivity were similar to those in excreta, suggesting that liver metabolites are the same as those found in excreta. Bound residues at zero withdrawal represented approximately one third of the residue in liver. In fat, radioactivity was found to be predominantly parent narasin, with bound residue constituting less than 12% of the total residues. The residues in skin/fat, muscle and kidney were in concentrations too low to allow for the identification of metabolites (Readnour, 2000).

In a GLP-compliant comparative metabolism study, cattle, a dog and rats were dosed orally for up to 7 days with [^{14}C]narasin. The radiochemical residues extracted from liver and faeces were subjected to fractionation by solvent partitioning, silica gel column chromatography and TLC. All three species produced multiple narasin metabolites. Faecal extracts contained more than 20 radioactive metabolites, and no single metabolite constituted a significant proportion of the total radioactivity. Comparison of column elution profiles and TLC autoradiograms indicated that metabolite patterns were qualitatively similar among cattle, rats and dogs; however, quantitative differences were noted. In addition to the eight dihydroxy- or trihydroxynarasin metabolites previously identified in rats and chickens, two additional metabolites were isolated in cattle faeces and liver in this study. These two, NM-12 and NM-13, were identified as monohydroxy- and dihydroxynarasin metabolites, respectively. Both were produced by the rat and dog as well, but were most prevalent in cattle. Quantitative fractionation of the radioactivity in cattle liver indicated that metabolite NM-12 was the most abundant (approximately 16%) and that metabolites NM-3 and NM-6, both dihydroxy derivatives, each accounted for approximately 4% of the total. The remainder of the liver radioactivity consisted of several metabolites with low relative abundance, approximately 10% non-extractable radioactivity and 10–15% intractable polar residues. Very little (<3%) of the liver residue was parent narasin. It can be concluded from this comparative study that the target animal (cattle) and the toxicological test animals (rats and dog) were exposed to the same metabolites following oral dosage with narasin (Manthey & Goebel, 1986).

In order to specifically determine narasin metabolism in a laboratory species, Fischer 344 rats (10 males and 10 females) were given daily oral doses of 5 mg mycelial narasin/kg bw for 5 days. Urine and faeces were collected daily and pooled by sex. Faecal samples from male and female rats after the 5th day of dosing were combined and extracted. Narasin metabolites in the extract were identified by high-performance liquid chromatography/ion spray mass spectrometry (HPLC/ISP-MS). At least four structural isomers of trihydroxynarasin were present in the faeces, as evidenced by the differences in the retention time between the peaks. Four peaks were identified as containing at least some dihydroxynarasin. Another four peaks were identified as containing trihydroxynarasin B, and four were identified as containing dihydroxynarasin B. Some other peaks contained monohydroxynarasin or monohydroxynarasin B. Using HPLC/ISP-MS, the exact position of hydroxylation could not be determined in this study; however, a previous study in chickens indicated that the hydroxylation of narasin occurs on the rings. These results indicate that the same pathways (oxidation/hydroxylation) for metabolism in the chicken are shared by the rat, and hydroxylated metabolites found in chickens were tested by autoexposure in rat toxicity studies with narasin (Sweeney & Kennington, 1994).

In a GLP-compliant tissue residue and metabolism study in pigs, three groups of four pigs each were fed [^{14}C]narasin rations for 7 days at 30 μg/g with zero withdrawal (treatment 01), 30 μg/g with a 3-day withdrawal (treatment 02) and 45 μg/g with zero withdrawal (treatment 03). Urine and faeces were collected daily throughout the study. At slaughter, liver, kidney, muscle, fat, skin and bile were

collected. The tissues were assayed for total radioactivity by solubilization and scintillation counting. The metabolites identified in liver, bile and faeces were oxidative and hydroxylated metabolites. The most abundant metabolite in liver, a dihydroxynarasin B metabolite (N-4), was also one of the most abundant metabolites in faeces for treatments 01 and 03. A trihydroxynarasin metabolite (N-1) was identified in faeces for treatment 03 and in liver for both treatments, where it co-eluted with trihydroxynarasin B. Another trihydroxynarasin metabolite (N-2) was identified in faeces but not in liver for both treatments. A trihydroxynarasin B metabolite (N-3) was identified in faeces and liver in both treatments but co-eluted with other peaks in the liver and faeces for treatment 03. A dihydroxynarasin B metabolite (N-5) was found in the liver and faeces for both treatments. A dihydroxynarasin metabolite (N-6) was identified in the faeces for both treatments but not in the liver. Another dihydroxynarasin metabolite (N-7) was identified in the liver and faeces for both treatments. Although bile contained the same metabolites as liver and faeces, an exact match was not possible because of the differences in retention times (Sweeney et al., 1995).

Hydroxylation of narasin leads to polar metabolites that are more readily excreted via the bile. Therefore, hydroxylation appears to be the major route for the inactivation and rapid elimination of administered narasin. The metabolites found in the rat were the same as those found in the chicken, indicating that these metabolites were tested by autoexposure in the rat toxicology studies.

2.1.2 Effects on enzymes and other biochemical parameters

The genome sequences of many human and animal pathogens encode primary membrane sodium ion (Na^+) pumps, Na^+-transporting dicarboxylate decarboxylases, Na^+-translocating reduced nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductases and a number of Na^+-dependent permeases. These provide potential antimicrobial targets for inhibitors of the Na^+ cycle, particularly in microbes having membrane bioenergetics that are solely dependent on Na^+ circulation. In general, ionophores can disrupt secondary transport processes and energy-conserving reactions by collapsing ion gradients. In addition to their antimicrobial effects, some polyether ionophores such as salinomycin have the potential to perturb liver mitochondrial functions by acting as a mobile carrier for alkali cations through membranes (Mitani et al., 1976).

Ionophores are highly lipophilic substances that are able to shield and delocalize the charge of ions and thus facilitate their movements across membranes composed of lipid bilayers by an electro-neutral exchange. Cations are carried across the lipid barrier of these membranes as lipid-soluble cation complexes by a passive diffusion process. Gram-negative bacteria are generally ionophore resistant owing to the presence of this outer membrane, but Gram-positive bacteria lack this outer membrane and are thus usually sensitive to ionophores (Russell & Strobel, 1989).

The effects of narasin metabolites on adenosine triphosphatase (ATPase) and oxygen uptake in rat liver mitochondria have been further characterized (Wong, 1978) following the earlier observation of Wong et al. (1977) that in isolated rat

liver mitochondria, the ATPase or adenosine triphosphate (ATP) hydrolysis induced by the addition of either valinomycin or monazomycin and alkali metal cation was reduced by narasin. In the more recent study, the four metabolites tested were metabolite F (a dihydroxynarasin), NM-3 (a dihydroxynarasin), NM-2 (a trihydroxynarasin) and a mixture of NM-6 (a dihydroxynarasin) and NM-3. These four were individually tested in terms of their effects on ATPase activity and oxygen uptake upon oxidation of malate and glutamate in rat liver mitochondria. The results indicated that the four narasin metabolites exhibited relatively weak effects on ATPase activity and oxygen uptake rates of rat liver mitochondria; that is, the four metabolite preparations were only 1/215th or less as effective as narasin in producing ionophorous effects in rat liver mitochondria (Wong, 1978).

2.2 Toxicological studies

Mycelial and purified crystalline forms of narasin were tested for toxicity. Doses of both forms of narasin in the toxicological studies were reported on the basis of the microbiological activity of narasin relative to that of the crystalline form.

2.2.1 Acute toxicity

The results of acute toxicity studies with narasin are summarized in Table 1. The clinical signs of toxicity following an oral or intravenous dose of mycelial or crystalline narasin were similar in mice, rats and chickens. Clinical signs of toxicity were characterized by hypoactivity, leg weakness and ataxia. In rabbits, following an oral dose of mycelial narasin equivalent to 10 mg mycelial narasin activity/kg bw, no clinical signs of toxicity were present. In dogs given a similar dose, only vomiting was observed 1 h after dosing.

Horses, known to be highly sensitive to the toxic effects of polyether ionophorous antibiotics, had the lowest oral median lethal dose (LD_{50}) of all species tested (0.8 mg mycelial narasin activity/kg bw). Clinical signs of narasin toxicosis in horses were anorexia, tachycardia, evidence of pain, incoordination and intermittent profuse sweating. Microscopic examination of kidney, liver, lung, spleen, stomach, heart and skeletal muscle revealed early degenerative and necrotic changes in the heart and skeletal muscle.

No clinical signs of toxicity were observed in rats exposed for 30 min to an atmosphere containing particulate mycelial narasin at an active concentration of 9.72 mg/m^3 of air.

The topical application of 250 mg mycelial narasin activity/kg bw to rabbits held under occlusion for 24 h caused no obvious toxicity or dermal irritation.

Eyes of rabbits exposed to 1.7 mg mycelial narasin activity (40 mg total dose of mycelial narasin) developed severe corneal, iridal and conjunctival irritation. Two of six treated eyes developed pannus, indicative of permanent eye damage. Eyes rinsed 2 min after exposure developed slight irritation, which healed within 48 h (Broddle et al., 1978).

Table 1. Acute toxicity studies with narasin

Species (strain)	Sex	Route	LD_{50} ± SD (mg/kg bw)	Compound ID (lot number)	References
Mouse (ICR)	M	Oral	22.8 ± 2.9	A28086 (C16-D48-200)	Worth & Gibson (1974)
	F	Oral	36.7 ± 4.3		
Mouse (ICR)	M	Oral	33 (30–37)[a]	Mycelial 79891 (X-40533)	Bridge (1984a)[b]
	F	Oral	34 (30–39)[a]		
Mouse (ICR)	M	Oral	15.8 ± 2.6	Mycelial 79891 (X-22719)	Broddle et al. (1978)
	F	Oral	16.7 ± 2.1		
Rat (Wistar)	M	Oral	40.8 ± 4.0	A28086 (C16-D48-200)	Worth & Gibson (1974)
	F	Oral	33.8 ± 6.0		
Rat (Fischer 344)	M	Oral	22 (19–26)[a]	Mycelial 79891 (X-40533)	Bridge (1984b)[b]
	F	Oral	24 (21–27)[a]		
Rat (Fischer 344)	M	Oral	31.6 ± 3.06	Mycelial 79891 (not provided)	Levitt et al. (1979)[b]
	F	Oral	44.3 ± 5.78		
Rat (Wistar)[c]	F	Intravenous	1.96 ± 0.14	Mycelial 79891 (not provided)	Broddle et al. (1978)
Rabbit	M & F	Oral	15.5 ± 3.9	A28086 (C16-D48-257-7)	Worth & Gibson (1974)
Chicken[d,e]	M	Oral	87.7 ± 16.4 89.1 ± 24.1 80.1 ± 14.3	See footnotes	Van Duyn & Russell (1978a)
Chicken[d,f]	M	Oral	54 ± 19.7 40.2 ± 22.6 75.5 ± 8.5	See footnotes	Van Duyn & Russell (1978b)
Chicken[g,h]	M	Oral	24.9 ± 5.7 53.9 ± 11.6 42.9 ± 8.5 43.3 ± 5.0	See footnotes	Van Duyn & Russell (1978c)
Chicken[c]	M & F	Oral	51.6 ± 27.02	Mycelial 79891 (X-22719)	Broddle et al. (1978)

Table 1 (contd)

Species (strain)	Sex	Route	$LD_{50} \pm SD$ (mg/kg bw)	Compound ID (lot number)	References
Bobwhite quail	M	Oral	73.96 ± 9.15	Mycelial 79891 (X-24458)	Karnak et al. (1979b)
	F	Oral	70–100		
	M & F	Oral	102.9 (46.6–227.5)[a]	Mycelial 79891 (X-40533)	Cochrane (1985b)[b]
Pigs	Gilts[i]	Oral	6.9 ± 1.88	Mycelial 79891 (X-40533)	Nelson & Novilla (1987)
Horses	M & F	Oral	0.8	Mycelial 79891 (X-40533)	Novilla & Van Duyn (1987)[b]

F, female; LD_{50}, median lethal dose; M, male; SD, standard deviation.
[a] LD_{50} with 95% confidence limits.
[b] GLP-compliant study.
[c] Expressed as narasin activity/kg.
[d] Expressed as narasin activity/kg for three different lots (361-R113-152-1 [1000 mg activity/g], 361-R113-102-1 [943.4 mg activity/g] and 381-781AD-263-2 [82.6 mg activity/g], respectively).
[e] Dose levels were 63, 100, 160 and 250 mg/kg bw.
[f] Dose levels were 40, 63, 100 and 160 mg/kg bw.
[g] Expressed as narasin activity/kg for four different lots (317-W79-257-2 [93.3 mg activity/g], 873-W79-76-1, 2, 3 [110 mg activity/g], F14-W37-156-1 [86.4 mg activity/g] and F14-W37-130-1 [82.5 mg activity/g], respectively.
[h] Dose levels were 25, 40, 63 and 100 mg/kg bw.
[i] LD_{50} for barrows fed narasin alone could not be calculated. Doses were 5, 10 and 15 mg/kg bw.

2.2.2 Short-term studies of toxicity in laboratory animals

(a) Mice

In a non-GLP-compliant 3-month toxicity study, ICR mice approximately 30 days of age were randomly separated into groups of 15 per sex per dose and fed mycelial narasin (ID 79891, lot X-22719, 4.3% narasin activity) at levels of 0, 10, 20 and 40 mg by weight (w/w) narasin activity/kg in the diet, equivalent to 0, 1.5, 3 and 6 mg narasin activity/kg bw per day. Food and water were provided ad libitum. The mice were inspected daily, and body weight and food consumption were determined weekly. Eye examinations were performed at the beginning and end of the study. At the termination of the study, individual blood samples were obtained, and six haematology and six clinical chemistry parameters were evaluated. The mice were killed and the weights of five major organs recorded. Sections of those organs and 17 other tissues or organs were prepared for histological examination.

All animals survived for the duration of the study. No clinical signs associated with narasin were present. The scheduled eye inspections failed to reveal any abnormalities. No growth impairment due to drug treatment was observed (no body weight data were provided, only figures). The organ weights for the experimental and control groups were similar. The haematology values in all the dosed groups were within the normal range. The clinical chemistry data indicated that creatinine values were decreased in males and females of the high dose group. Values for total bilirubin were decreased in the middle- and high-dose males, but were significantly higher statistically in the middle- and high-dose females than in controls. These findings were considered to be of no toxicological significance, because other serum chemistry parameters were normal and no compound-related liver lesions were found. The pathological entities observed in this study were those commonly found in this strain of mouse and not due to narasin administration.

The administration of mycelial narasin at levels of 10, 20 and 40 mg narasin activity/kg in the diet for 3 months resulted in no clinical, haematological, clinical chemistry or pathological changes in mice (Marroquin et al., 1978a). The no-observed-adverse-effect level (NOAEL) for males and females was 40 mg/kg in the diet, equivalent to 6 mg/kg bw per day, the highest dose tested.

In a second non-GLP-compliant 3-month toxicity study, ICR-SPF mice approximately 27 days of age were randomly separated into groups of 15 per sex per dose and fed narasin (ID 79891, lot X-23213, 10.16% narasin activity) at levels of 0, 60, 80 and 100 mg (w/w) narasin activity/kg in the diet, equivalent to 0, 9, 12 and 15 mg/kg bw per day. Food and water were provided ad libitum. The mice were inspected daily, and body weight and food consumption were determined weekly. Eye examinations were performed at the beginning and end of the study. At the termination of the study, individual blood samples were obtained, and six haematology and six clinical chemistry parameters were evaluated. The mice were killed and the weights of five major organs recorded. Sections of those organs and 16 other tissues or organs were prepared for histological examination.

All but one animal survived to study termination. Male 304 in the 100 mg/kg dietary group died on day 57. Balanoposthitis and distended urinary bladder were abnormal findings in this animal. Based on this single incident and absence of similar findings in animals that survived to scheduled sacrifice, the death of this animal is considered incidental and unrelated to treatment with narasin. No clinical signs associated with narasin were present. The final eye inspections failed to reveal any abnormalities. At termination, mean body weights of males in the 80 and 100 mg/kg dietary groups were decreased by 26% and 27% of controls, respectively. The mean body weight of 100 mg/kg females was decreased by 14% relative to control. On the basis of the magnitude of the differences from control, these decreases were considered to be treatment related and adverse. The decreased mean body weight in these groups relative to control was initially observed on treatment day 35 and persisted throughout the study. Mice consuming narasin at 100 mg/kg of the diet had increased relative testes and uterus weights. Narasin-treated mice had decreased organ weights, except for the testes in males and spleen and uterus in females. These changes were of no toxicological consequence, because they were not accompanied by any pathological findings. The

haematology data suggested that haemoconcentration occurred in a few narasin-treated males at all dose levels and in a few narasin-treated females at some dose levels. Therefore, it is likely that haemoconcentration reflects the degree of hydration in these animals and was not directly related to narasin. The alanine aminotransferase (ALT) values were elevated in a few males of the 60 mg/kg dietary group. These changes were unlikely to be due to narasin treatment because they were absent at the higher dose level. The pathological entities observed in this study were those commonly found in this strain of mouse and not due to narasin administration.

The only change of toxicological significance in this 3-month study was the decrease in mean body weight relative to control in males at the 80 and 100 mg/kg dietary levels and in females at the 100 mg/kg dietary level (Owen, 1978). Therefore, the NOAEL in this study was 60 mg/kg in the diet, equivalent to 9 mg narasin activity/kg bw per day.

(b) Rats

In a 3-month, non-GLP-compliant study to determine the subacute effects of narasin in rats, Wistar-derived rats, 28–35 days of age, were separated into groups of 15 per sex per dose and fed mycelial narasin (ID 79891, lot X-22719, 4.3% narasin activity) at levels of 0, 15, 30 and 60 mg (w/w) narasin activity/kg in the diet, equal to 0, 1.1, 2.2 and 4.7 mg narasin activity/kg bw per day for males and 0, 1.1, 2.6 and 5.7 mg narasin activity/kg bw per day for females. Food and water were available ad libitum. The rats were examined daily, and body weight and food consumption were determined weekly. Eye examinations were performed at the beginning and end of the study. At the termination of the study, individual blood samples were obtained from each rat, and six standard haematological parameters were measured. Also, prothrombin time was determined on half of the samples, and six routine clinical chemistry parameters were assayed on the remaining half of the samples. At the end of the study, the rats were killed, and the tissues and organs were examined. Ten major organs, including the prostate and testes or uterus and ovaries, were weighed. Sections of these organs as well as sections from 18 additional tissues or organs were prepared for histological examination.

All animals survived to the end of the study. No clinical signs associated with narasin administration were observed. At the end of the study, feed conversion ratios were comparable or slightly increased compared with control in males and females of the 15 mg/kg dietary group and decreased by 21% relative to control in males of the 60 mg/kg dietary group and by 16% and 46% relative to control in females of the 30 and 60 mg/kg dietary groups, respectively. Decreases in mean body weight gain relative to control were seen in males and females of the 60 mg/kg dietary group. In males, the decreases were 25% of control values; in females, the differences were 40% of controls. Mean body weight was decreased in males (18%) and females (21%) relative to control at 60 mg/kg diet. The difference in females was statistically significant ($P \leq 0.05$). Based on the magnitude of the differences, the aforementioned effects on food conversion, mean body weight and mean body weight gain are considered to be treatment related and adverse. Statistically significant ($P \leq 0.05$) increases in red blood cell counts, haematocrit and

haemoglobin values were found in males of the treated groups. Elevated white blood cell counts also occurred in a few control and narasin-treated males at all dose levels. The changes in the aforementioned red blood cell parameters are consistent with haemoconcentration and likely related to the hydration status in the affected males and not directly related to narasin treatment, as only males were affected. However, an effect of narasin cannot be ruled out completely based on the dose–response increase in these parameters and absence of other data (e.g. bone marrow evaluation or serum albumin concentrations) to support a more definitive conclusion. Clinical chemistry findings included an elevated ALT value in one female in the 15 mg/kg dietary group, significantly ($P \leq 0.05$) higher blood glucose levels in the treated females and also in the middle-dose males and blood urea nitrogen levels that were decreased for both sexes in the treated groups. These findings were not likely related to narasin treatment because they were not dose related, they were within the normal ranges for this laboratory and no treatment-related pathological findings were noted. The terminal body and organ weight data indicated a treatment-related decrease in mean body weight relative to controls for males and females in the 30 and 60 mg/kg dietary groups. For 60 mg/kg dietary females, the difference was statistically significant ($P < 0.05$). Rats of the 60 mg/kg dietary group had increased relative testes and ovarian weights. Organ weights for the kidneys, heart, spleen, thyroid and adrenals of females given 60 mg narasin/kg in the diet were significantly ($P < 0.05$) higher than control values. Because no treatment-related lesions were found, these changes were of no toxicological significance.

On the basis of adverse effects on food conversion, mean body weight and mean body weight gain in the 30 and 60 mg/kg dietary groups, the NOAEL was 15 mg/kg of the diet, equal to 1.1 mg/kg bw per day (Marroquin et al., 1978b).

A 1-year non-GLP-compliant toxicity study in Wistar rats (15 per sex per group) was initiated in which the offspring derived from a multigenerational toxicity study were fed mycelial narasin (ID 79891, lot X-23542, 8% narasin activity) at 0, 7.5, 15 or 30 mg narasin activity/kg in the diet for 1 year. These levels were equal to 0, 0.49, 1.0 and 1.9 mg narasin activity/kg bw per day for males and 0, 0.57, 1.2 and 3.2 mg narasin activity/kg bw per day for females.

There were eight unscheduled deaths during the study (0/15, 2/15, 2/15 and 0/15 males and 0/15, 0/15, 3/15 and 1/15 females in the 0, 7.5, 15 and 30 mg/kg dietary groups, respectively). None of the deaths were attributable to treatment with narasin. At the end of the study, a treatment-related decrease (13%) in mean body weight relative to control was noted for 30 mg/kg dietary females. Mean body weights of males were comparable to control at all dose levels. Mean food consumption was unaffected in most groups. Significantly increased values for weekly mean food consumption were observed in 30 mg/kg dietary females, with the exception of week 1, in which mean food consumption was significantly decreased. The decreased consumption during week 1 was attributed to the unpalatability of the diet. The increased mean food consumption values were attributable to spillage. This was evidenced by a significant decrease in mean food consumption and mean body weight loss in females in the 30 mg/kg dietary group in week 1, followed by significantly increased weekly mean food consumption in most weeks thereafter. Non-statistical increases in weekly food consumption, likely related to unpalatability

and spillage, were also noted for 15 mg/kg dietary females, but there was no associated effect on mean body weight. The cumulative feed conversion ratios for 15 and 30 mg/kg dietary females were decreased by 20% and 29% of control, respectively. The effects on mean body weight and food conversion in 30 mg/kg dietary females and effects on food conversion in 15 mg/kg females were assessed as indirect effects of treatment due to the unpalatability of the diet and were not toxicologically relevant. Statistically significant changes in some haematology parameters were observed in male and female rats at the 15 and 30 mg/kg dietary levels, but all of these changes were slight, and the values were within acceptable normal ranges. Males at the 7.5 mg/kg dietary level and females at the 15 mg/kg dietary level had significant increases in mean glucose values relative to controls, which appeared to be unrelated to narasin administration on the basis of sporadic occurrence and absence of a dose–response. Decreased blood urea nitrogen and ALT concentrations were observed in females in the middle and high dose groups, respectively, but were of no biological or toxicological significance. Some mild and non-dose-related changes in female spleen and thyroid weights were noted but were not considered to be important biological effects of narasin, since no microscopic changes were seen. Cardiac and skeletal muscle lesions in control and treated rats were observed with variable incidence, but they showed no dose–response and were considered to be related to ageing. Neoplasms of the pituitary, mammary gland, lymph nodes, thyroid, adrenal, peritoneum and subcutis were seen sporadically across treatment groups, and the incidences did not indicate any carcinogenic propensity by narasin.

In conclusion, narasin fed to male and female rats at 7.5, 15 and 30 mg/kg in the diet for 1 year was tolerated with no toxicity or serious adverse effects. Effects on mean body weight and food conversion were indirect effects of the test article resulting from the unpalatability of the diet and were not toxicologically relevant. The NOAEL was 30 mg narasin activity/kg in the diet, equal to 1.9 mg/kg bw per day, which was the highest dose tested (Novilla & Young, 1979).

(c) Dogs

In a 3-month, non-GLP-compliant study, male and female Beagle dogs (6.0–10.2 kg, 13–16 months old) were placed into one of four groups with four dogs per sex in each group. Narasin (ID 79891, lot X-22719, 4.3% activity) was administered orally by gelatine capsules at doses of 0, 0.5, 1.0 and 2.0 mg narasin activity/kg bw per day. Each dog received 300 g dog food per day and water ad libitum. The toxicity parameters studied included physical appearance, behaviour, eye examinations at the initiation and termination of the study, weekly body weight, haematology, bone marrow, blood clinical chemistry and urinalysis. Haematology (10 parameters) and clinical chemistry tests (8 parameters) were completed prior to the study and then periodically at weeks 1, 2, 4, 8 and 12. Urine was collected at the same intervals and analysed for five parameters. Unscheduled electrocardiograms (ECGs) were performed once on high-dose animals only. Organ weights were determined, and histopathological examinations of 29 tissue or organ samples from each animal on study were completed postmortem.

All animals survived for the duration of the study. No changes related to narasin administration were recorded for body weights, haematology or clinical chemistry parameters, bone marrow cell counts, organ weights, pathology or urinalysis end-points. Clinical signs of toxicity included leg weakness and incoordination during the first 3 weeks in four dogs and 3 months in one animal at the 2.0 mg/kg bw per day level. Bradycardia (decreased heart rate) was apparent in six of the eight dogs given 2.0 mg/kg bw per day, and normal to markedly abnormal sino-arrhythmias were present. In three of these dogs, signs of sino-arrest (some T–R intervals >1 s) were noted. In most of the dogs dosed with 2.0 mg/kg bw per day, the QRS interval was short and the S-wave was deep. One animal had normal heart rate, but both P- and T-waves were very erratic (amplitude 0 to greater than 1 mV) and abnormal in shape. The significance of the ECGs was difficult to evaluate because there were no pre-study ECGs available for comparison, no histological lesions were found in the hearts of these same animals and the blood chemistry values were not greatly altered.

In conclusion, the daily oral administration of narasin to dogs by gelatine capsule was well tolerated at dose levels of 0.5 or 1.0 mg/kg bw per day for 3 months, as evidenced by the absence of treatment-related effects on clinical signs, body weights, haematology, clinical chemistry and histopathology. On the basis of clinical signs of leg weakness, incoordination and abnormal ECG findings seen at 2.0 mg/kg bw per day, the NOAEL was 1.0 mg narasin activity/kg bw per day (Marroquin et al., 1978c).

In an effort to further define the signs of toxicity and to evaluate the effects of narasin on ECGs in dogs, a 6-month, non-GLP-compliant study was conducted in male and female Beagle dogs ranging from 12 to 16 months of age. The dogs were separated into four groups (four per sex per group) and administered narasin (ID 78981, lot X-23542, 8% activity) orally by gelatine capsule at dose levels of 0, 0.5, 1.0 and 1.5 mg narasin/kg bw per day before the morning feeding. Animals received water ad libitum and 300 g of dog food daily. Physical appearance, behaviour and gait were observed daily. Body weights were recorded weekly. Ophthalmoscopic examinations were completed at the study start and termination. A battery of 10 haematological and 7 clinical chemistry tests were performed on samples of venous blood from each animal at a pretreatment time and then again at intervals of 1 and 2 weeks and 1, 2, 3, 4, 5 and 6 months on study. Urine was collected at the same intervals for the determination of five parameters. Bone marrows were evaluated at the termination of the study. ECGs were conducted on all dogs prior to treatment and then again 2 h after dosing at 1 week and at 1, 3 and 6 months. At 6 months, the dogs were euthanized, and the weights of seven organs, including either the testes or the ovaries, were recorded after dissection and gross examination. Samples of those organs as well as samples of 22 other tissues and organs, including the uterus and eyes, were collected, fixed and processed further for eventual histopathological examination.

No animals died during the course of the study. There were no changes in body weights attributable to narasin treatment. There were no physical signs of toxicity considered to be directly attributable to narasin treatment. The ophthalmoscopic examinations did not reveal any eye abnormalities. The

haematological values for haematocrit, haemoglobin and red blood cell, white blood cell, prothrombin and platelet counts were within the normal range recorded in this laboratory. The mean values obtained for blood glucose, blood urea nitrogen, creatinine, total bilirubin, alkaline phosphatase, ALT and creatine phosphokinase were within the normal range. There were no changes in the urinalysis data or in the bone marrow examinations associated with narasin administration. Only one dog, a female in the high dose group, had an aberrant ECG (bradycardia, low-amplitude R-waves, elevated T-waves and a marked suppression of the ST segment), which occurred 1 month after initiation of treatment. These findings were deemed treatment related, because treatment-related effects on ECGs were seen in a separate 3-month oral toxicity study of narasin in dogs (Marroquin et al., 1978c). However, ECG changes in this animal were absent at the 3- and 6-month ECG examinations, providing evidence of reversibility, and there was no evidence of microscopic changes in the heart. No compound-related changes in the absolute or relative organ weights were noted. The gross pathological or microscopic lesions observed were of a sporadic occurrence and were typical of naturally occurring lesions observed in this laboratory (Novilla et al., 1978).

In conclusion, on the basis of the abnormal ECG findings reported for dogs in the Marroquin et al. (1978c) study, the abnormal ECG findings in the single high-dose female in this study are identified as a treatment-related effect. Therefore, the NOAEL in this study is 1.0 mg/kg bw per day.

In a GLP-compliant study, 5-month-old Beagle dogs (four per sex per group) were given daily oral doses of mycelial narasin (ID 79891, lot X-40533, 10.6% activity) by gelatine capsule at 0, 0.5, 1.0 or 2.0 mg narasin activity/kg bw per day for 1 year. An additional group (four per sex) was given crystalline narasin (lot X-24458) orally by capsule at a dose level of 2.0 mg/kg bw per day for a side-by-side comparison of potential toxicity induced by mycelial and crystalline narasin in young dogs. The dogs were observed for signs of toxicity throughout the period. Pretreatment, interim and/or terminal evaluations included physical examination, neurological evaluation, ophthalmoscopy, haematology, clinical chemistry, electrocardiography, bone marrow examination, urinalysis, body weight and food consumption. All animals were necropsied, and the tissues were collected and examined microscopically. The weights of specified individual organs were recorded, and the ratios of organ weight to body weight and organ weight to brain weight were calculated as well.

One dog did not survive until the scheduled termination of the study. A male from the 2.0 mg/kg bw per day mycelial group was found moribund and killed on study day 13. Prior to death, this animal had clinical signs of anorexia, excessive salivation, laboured respiration and recumbency. Clinical signs and microscopic lesions observed in the heart indicated that the moribund condition of this animal was treatment related. Effects related to treatment with mycelial narasin at 2.0 mg/kg bw per day in all surviving animals were slight (barely discernible) to severe (unable to stand; two males, one female) leg weakness and excessive salivation. Bilateral absence or inhibition of the patellar reflex was observed in all dogs at the end of the study. Less frequent effects were convulsions or opisthotonos (two males, one female; observed during the 5th, 8th or 9th month), bilateral

dropped carpus (one male, one female), transient occurrences of tremors, decreased food consumption, hypoactivity and laboured respiration. Loss of muscle tone of the shoulder and thigh muscles was observed in all dogs at study termination. Clinical signs in the 2.0 mg/kg bw per day crystalline narasin group were of lower incidence or severity and included leg weakness, ataxia, excessive salivation, depressed appetite and laboured respiration. None of the crystalline narasin–treated animals lost the ability to stand, although one animal on a single day during the 9th month was reluctant to move but could walk and had bilateral absence of the patellar reflex. At study termination, bilateral absence or inhibition of the patellar reflex was observed in all crystalline narasin–treated dogs, and three dogs had decreased muscle tone. In the 1.0 mg/kg bw per day mycelial narasin group, transient leg weakness was seen in one male, occasional salivation was seen in two females and slight atrophy of the posterior thigh muscles was seen in one male. Decreases in body weight gain were assessed as slight (three animals) to marked (two animals) in the 2.0 mg/kg bw per day mycelial narasin group and slight (five animals) in the crystalline narasin group. Mean body weight was decreased relative to control for males (11% and 15%) and females (29% and 11%) in the 2.0 mg/kg bw per day mycelial and crystalline groups, respectively. These decreases were associated with decreases of 10% or greater in monthly mean food consumption relative to control at multiple intervals during the study. Dogs from the 0.5 mg/kg bw per day mycelial narasin group tolerated daily oral doses for 1 year without signs of toxicity. Ophthalmoscopic and ECG examinations revealed no treatment-related abnormalities. No toxicologically important changes occurred in haematology, urinalysis or bone marrow parameters of dogs in any treatment groups. Slight transient increases in creatine phosphokinase and aspartate aminotransferase (AST) occurred in some dogs from both 2.0 mg/kg bw per day groups during the first 4 weeks of treatment, an effect probably related to active muscle damage. The values returned to the normal range with continued narasin administration. No other important clinical chemistry changes were found. Pathological evaluation demonstrated the occurrence of dose-related alterations in heart, skeletal muscles and sciatic, tibial and other unspecified peripheral nerves. Heart muscle lesions were found only in the 2.0 mg/kg bw per day mycelial narasin dog that was killed in extremis. Focal degeneration of skeletal muscles, including the diaphragm, occurred in three males and one female from each of the 2.0 mg/kg bw per day mycelial and crystalline narasin groups, but the changes appeared to be more severe in the mycelial group. All dogs in the 2.0 mg/kg bw per day crystalline narasin group had less severe peripheral neuropathy, with no involvement of the spinal cord. Three dogs from the 1.0 mg/kg bw per day mycelial narasin group had minimal to slight peripheral nerve changes. The three also had minimal focal skeletal muscle degeneration. There were no treatment-related lesions in the dogs from the 0.5 mg/kg bw per day mycelial narasin group.

In conclusion, daily oral capsule doses of 0, 0.5, 1.0 or 2.0 mg mycelial narasin or 2.0 mg crystalline narasin activity/kg bw per day for 1 year produced dose-related adverse effects. Compound-related effects were physical signs of toxicity, including excessive salivation, leg weakness, decreased appetite, laboured respiration, hypoactivity and recumbency, reduction in body weight, transient increases in creatine phosphokinase and AST values, and degenerative and/or

regenerative changes in cardiac and skeletal muscles and peripheral nerves. Mycelial narasin was less well tolerated than crystalline narasin. A higher proportion of dogs showed clinical signs and had lesions in the 2.0 mg/kg bw per day mycelial narasin group than in the 1.0 mg/kg bw per day mycelial narasin group. On the basis of mortality, clinical signs, effects on body weight, clinical chemistry and microscopic findings in skeletal and cardiac muscle and peripheral nerves, the NOAEL was 0.5 mg narasin activity/kg bw per day (Novilla & Bernhard, 1986).

2.2.3 Non-laboratory animal safety/toxicology studies

(a) Aquatic species

Bluegills (*Lepomis macrochirus*) were exposed to nominal concentrations of 0.25, 0.5, 0.7, 1.0 or 2.0 mg crystalline narasin/l (ID 79891, lot X-24458, 100% activity) for 96 h. No compound-related effects were observed at concentrations of ≤0.7 mg/l. Fish exposed to concentrations of ≥1.0 mg/l showed a dose–response pattern of hypoactivity, loss of equilibrium and death. The 96-h median lethal concentration (LC_{50}) was between 1.0 and 1.4 mg/l. The no-observed-effect concentration (NOEC) was 0.7 mg/l (Karnak et al., 1978).

In a second GLP-compliant study, juvenile bluegills were exposed to nominal crystalline narasin (ID 79891, lot X-24458, activity 101.9%) concentrations of either 0 mg narasin/l (control) or 1 of 10 concentrations ranging from 0.82 to 3.92 mg narasin/l. A total of 10 fish were tested at each treatment level. The toxicity assessments (i.e. NOEC and LC_{50}) were based on signs of sublethal toxicity and frequencies of mortality observed in the exposed populations. Based on the responses, the 96-h LC_{50} with 95% confidence limits was 3.27 mg/l (3.04–3.55 mg/l). Because physical signs of toxicity ranging from hypoactivity to death were observed at all treatment levels, a NOEC could not be determined in this study (Grothe & Francis, 1983).

In a similar GLP-compliant bluegill study using mycelial narasin (lot X-40533, 10.6% activity), juvenile bluegill were exposed to concentrations of either 0 mg/ml (control) or one of nine concentrations ranging from 0.88 to 9.55 mg/ml. Behavioural signs of toxicity ranged from hypoactivity to prostration at narasin concentrations of ≥2.8 mg/l. No mortality or behavioural signs of toxicity were found for fish exposed to narasin concentrations of ≤1.66 mg/ml. The NOEC was 1.66 mg/l, and the 96-h LC_{50} with 95% confidence limits was 5.02 mg/l (4.88–5.15 mg/l) (Hamelink, 1985a).

Rainbow trout (*Salmo gairdneri*) were exposed to one of eight nominal concentrations of crystalline narasin (ID 79891, lot X-24458, activity 100%) ranging from 0.5 to 5.0 mg/l for 96 h. No compound-related effects were observed at the 0.5 mg/l level. Fish exposed to narasin concentrations above 0.7 mg/l showed a concentration–response pattern of hypoactivity, loss of equilibrium, irregular swimming behaviour, prostration and death. The 96-h LC_{50} was between 1.4 and 2.0 mg/l. The NOEC was 0.5 mg/l (Karnak et al., 1979a).

In a later GLP-compliant study, rainbow trout were exposed to mycelial narasin (ID 79891, lot X-40553, 10.6% activity) at concentrations of either 0.0 mg/l (control) or one of eight concentrations ranging from 0.103 to 5.26 mg/l. The toxicity

assessments (i.e. NOEC and LC_{50}) were based on signs of sublethal toxicant stress and frequencies of mortality observed in the exposed populations. Behavioural signs of toxicity ranged from hypoactivity to laboured respiration at narasin concentrations of ≥3.16 mg/ml. No mortalities or behavioural signs of toxicity were found for fish exposed to narasin at concentrations of ≤0.190 mg/ml. Based on the responses, the acute NOEC of mycelial narasin was 0.190 mg/l. The 96-h LC_{50} with 95% confidence limits was 2.21 mg/l (1.79–2.73 mg/l) (Hamelink, 1985b).

The acute immobilization effect of narasin (ID 79891, lot X-24458, activity 100%) was determined on first-instar *Daphnia magna* ≤20 h of age using five nominal test concentrations of 1.1–16.0 mg/l and a 0 mg/l control. These studies resulted in a 24-h median effective concentration (EC_{50}) that was above 16.0 mg/l and a 48-h EC_{50} that was 7.72 ± 0.48 mg/l. The NOECs at 24 and 48 h were 4.0 and 2.25 mg/l (Karnak et al., 1979c). A second similar GLP-compliant study with mycelial narasin (ID 79891, lot X-40533, 10.6% activity) using concentrations of 4.69–42.18 mg narasin/l resulted in an acute NOEC of <5.0 mg/l and a 48-h EC_{50} with 95% confidence limits of 20.56 mg/l (9.19–68.1 mg/l) (Grothe & Mohr, 1985).

(b) Birds

In a GLP-compliant study, groups of 10 10-day-old mallards (*Anas platyrhynchos*) were fed diets containing mycelial narasin (ID 79891, lot X-40533, 10.6% activity) at 0 (control), 157, 313, 625, 1250, 2500 or 5000 mg/kg for 5 days, followed by 3 days on basal diet. Three of 10 birds died in the 5000 mg/kg treatment group. The mortalities occurred on days 4 and 5. Lethargy was observed in birds at the 2500 and 5000 mg/kg dose levels. During the 5-day treatment phase, a significant ($P < 0.05$) reduction in mean body weight gain corresponding with a treatment-related reduction in food consumption was observed at narasin levels of ≥625 mg/kg. No signs of toxicity were observed at ≤313 mg/kg. During the 3-day basal diet phase, food consumption in treated and control groups was similar. Based on the mortality that occurred in this study, the LC_{50} concentration is >5000 mg narasin/kg, the highest dietary level tested. The no-observed-adverse-effect concentration (NOAEC) in this study was 313 mg/kg, equal to 190 mg/kg bw per day. A repeat of this study (study A01983) resulted in mortality for 4 of 10 and 6 of 10 birds in the 2500 and 5000 mg/kg dietary levels, respectively. An 8-day LC_{50} with its 95% confidence limits for narasin activity in the diet of mallards was 3800 mg/kg (2590–5570 mg/kg). The nominal dietary concentration of 313 mg/kg was the highest level of narasin activity tested that had no mortalities, signs of toxicity or treatment-related changes in food consumption and body weight gain (Cochrane, 1985a).

In two GLP-compliant studies, adult bobwhite (*Colinus virginianus*) were administered mycelial narasin (ID 79891, lot X-40533, 10.6% activity) in the diet at 0 (control), 50, 100, 200, 400, 800 or 1600 mg/kg for 5 days, followed by 3 days on basal diet. Treatment-related mortalities occurred at the 400, 800 and 1600 mg/kg dose levels (2/10, 4/10 and 9/10 birds in the first study and 3/10, 4/10 and 10/10 in the second study, respectively). Significant ($P \leq 0.05$) body weight loss or reductions in mean body weight gain values occurred at all treatment levels during the treatment period and corresponded with treatment-related decreased food

consumption at all dose levels relative to controls during the 5-day treatment period. Treatment levels of ≥200 mg/kg produced ataxia and lethargy. The LC_{50} values with 95% confidence intervals were 630 mg/kg (460–870 mg/kg) in the first study and 800 mg/kg (580–1090 mg/kg) in the second study. A NOAEL was not established in these studies (Cochrane, 1985c,d).

(c) Chickens

In an 8-week GLP-compliant safety study under simulated use conditions, 1696 day-old Hubbard × White Mountain cross broiler chickens (848 cockerels and 848 pullets) were allotted to 1 of 16 floor pens. Four pens of pullets and four pens of cockerels were randomly assigned to each of four treatments, and the chickens were fed narasin in a pelleted complete ration at narasin concentrations of 0, 80, 240 and 400 mg/kg ad libitum for 8 weeks. Narasin dried fermentation product (lot X-30764) was used to prepare the premix (lot X-32430). The effects of narasin were determined by clinical signs, necropsy and microscopic findings for unscheduled deaths, bird mortality, prothrombin times, weight gain, feed consumption, litter condition and litter feather scores.

Daily observations during the course of the 8-week study revealed a dose-associated decrease in size. Chickens on the 240 mg/kg and especially the 400 mg/kg treatments were hypoactive. Feathering was also poor at these two doses, particularly at the higher one. Feeding narasin to broiler chickens for 8 weeks at these dose levels resulted in a dose-associated increase in mortality. Mortality for 80 mg/kg males (5%) was increased relative to control males and females (2.8% and 2.4%, respectively), whereas mortality for 80 mg/kg females (1.4%) was decreased relative to controls. Mortality was 10.4% and 6.1% for 240 mg/kg males and females and 31.1% and 18.9% for 400 mg/kg males and females, respectively. Prothrombin values from birds on treatment for 4 days and 8 weeks showed no changes that were regarded as being indicative of toxicity.

There was a significant ($P < 0.05$) dose-related decrease in body weight after 4 days on test for the 240 and 400 mg/kg treatments for both male and female broilers sacrificed at 4 days. Body weights for the male and female broilers were significantly ($P < 0.05$) decreased for the 240 and 400 mg/kg treatments after 3 weeks on study. After 8 weeks on treatment, there was a significant ($P < 0.05$) dose-related decrease in body weights for the 80, 240 and 400 mg/kg treatments for both males and females when compared with controls. Feed consumption was significantly ($P < 0.05$) decreased at 80, 240 and 400 mg/kg for the female broilers, but only at 240 and 400 mg/kg for the males, during the first 3 weeks of treatment. This same trend in feed consumption continued for weeks 4–8. Gross necropsy observations of birds that died or were killed in extremis revealed that the birds were small, dehydrated and cachectic. Microscopically, birds treated with 240 or 400 mg narasin/kg had focal degeneration of skeletal muscles and congestive heart failure. Birds on 80 mg/kg treatment had no lesions regarded as treatment related. Based on significant and dose-related decreases in body weights after 8 weeks of treatment in males and females at all doses, a NOAEL could not be established in this study (Van Duyn & Novilla, 1982).

A subsequent GLP-compliant safety study was completed with narasin (ID 79891, lot X-40533, activity 10.6%) administered to 1590 day-old Hubbard × White Mountain cross broiler chickens under simulated use conditions. Treatment groups were 0, 70, 80, 120 and 210 mg narasin activity/kg, and all diets were fed ad libitum. There were three pens per treatment, each with 53 cockerels and 53 pullets. The study duration was 49 days on medicated feed followed by a 3-day withdrawal from medicated feed. The effects of narasin were determined by clinical signs, weight gain, feed consumption, feed/gain ratios, litter moisture, feather scores, necropsy of dead birds and prothrombin times (day 4). At the end of the treatment period, 39 randomly preselected birds per sex from each treatment group were killed for necropsy. Heart, kidney and liver were weighed, and organ to body weight ratios were calculated. Major organs were collected and examined microscopically. Blood was collected from 5 of the 13 birds for haematology and clinical chemistry determinations.

There was no significant difference in bird mortality between control and treatment groups. The number of birds that did not survive until study termination was 16, 19, 13, 14 and 6, respectively, from the 0, 70, 80, 120 and 210 mg narasin/kg treatment groups. Clinical signs of toxicity were seen after the 3rd week, when birds fed 210 mg narasin/kg were consistently smaller in size than both the control birds and birds at lower dose levels. Significant adverse effects on growth performance, evidenced by significantly reduced weight gain resulting from reduced feed intake, occurred at the 120 and 210 mg narasin/kg treatment groups. There were no toxicologically important changes in haematology or organ weights of birds treated with narasin. There was a treatment-related increase in AST values in males at 210 mg/kg. Organ weight effects included significant decreases, relative to controls, in absolute weights for liver, kidney and heart in males and for kidney in females at 210 mg/kg. Because there were no pathological correlates, these differences were attributed to body weight changes. An increased incidence of slight focal regeneration of skeletal muscle in birds fed 120 and 210 mg narasin/kg indicated repair of a previous mild injury. A dose-related increased incidence of congestive heart failure seen at 80 (one male), 120 (one male, one female) and 210 mg narasin/kg (two males, one female) was considered to be unrelated to narasin administration on the basis of low incidence and lack of correlation with increased mortality and because similar changes in broilers have previously been attributed to a variety of causes. However, because congestive heart failure was not seen in controls and has been seen in birds in previous studies, congestive heart failure seen in animals at 80, 120 and 210 mg/kg might be treatment related.

In conclusion, compound-related adverse effects were restricted to the top two levels of 120 and 210 mg narasin/kg. These included growth depression, reduced feed efficiency, an increase in mild skeletal muscle alterations and mildly increased AST values (Novilla & Van Duyn, 1985). Based on the possibility that congestive heart failure was a treatment-related effect at 80, 120 and 210 mg/kg, the NOAEL in this study is 70 mg/kg.

In a third safety study conducted in accordance with Japanese Ministry of Agriculture, Forestry and Fisheries GLP regulations, 120-day-old Chankey (Ross) broiler chicks (20 per sex per group) were fed narasin in feed at concentrations of

0, 80 (the designated maximum dose) and 240 mg/kg (3 times the maximum dose) for 6 weeks and then non-medicated control feed during a 1-week withdrawal period. The effects of narasin were determined by clinical observations, body weight and body weight gain, feed intake and feed efficiency. On withdrawal days 0 and 7, clinical pathology and anatomic pathology (necropsy, organ weights and histopathology) examinations were performed on five animals per sex per group.

Mortalities in males occurred at 80 mg/kg (two animals) and 240 mg/kg (one animal). The causes of death at 80 mg/kg were cardiovascular failure and ascites. The cause of death at 240 mg/kg was cardiovascular failure. There was one female death in each group. The cause of death was ascites at 0 and 240 mg/kg and undetermined at 80 mg/kg. One female at 240 mg/kg had depression, anorexia and tachypnoea 3 days before death. Leg weakness was observed in five birds; the incidence was 2, 1 and 0 for males and 1, 0 and 1 for females in the control, 80 and 240 mg/kg treatment groups, respectively. Given the incidence in controls and the absence of a clear dose–response, leg weakness was not related to treatment with narasin in this study. During the medication period, body weight gain for the 240 mg/kg pullet group was lower than controls and attributable to decreased feed intake. No changes in haematology parameters related to narasin were observed for either cockerels or pullets in the 80 and 240 mg/kg groups. Total cholesterol and urate for pullets in the 240 mg/kg group were significantly higher than controls at withdrawal day 0. These changes were mild and reversible, recovering quickly by withdrawal day 7, and no other changes in lipid or sugar metabolism were observed (Hashizume, 2000).

On the basis of the early death attributable to cardiovascular failure in one 80 mg/kg male, a NOAEL was not established in this study.

(d) Pigs

Narasin has been evaluated for safety following dietary administration to fattening pigs. In a GLP-compliant study, narasin was fed to male and female pigs (three per sex per group) for 63–65 days at nominal concentrations of 0, 30, 45 and 60 mg/kg, which represent 0, 1, 1.5 and 2 times the maximum proposed dose level, respectively, in the diet. At study initiation, the average body weights were 34 kg and 36 kg for males and females, respectively.

There were two unscheduled deaths during the study in the 30 mg/kg dose group. Neither was related to treatment with narasin. One male was killed for complications resulting from a submandibular abscess and lameness. One female was killed with clinical signs of weight loss, impaired mobility/prone posture, pyrexia and tachycardia. There were no unusual necropsy findings, and these clinical signs were not seen in other animals at this dose level or higher. Therefore, this animal's condition was not likely related to treatment with narasin. There were no test article–related findings in body weight, food consumption, clinical observation or necropsy data.

In conclusion, administration of 30, 45 or 60 mg/kg in the diet was without deleterious or stimulatory effect on food intake, overall body weight or the rate of body weight gain throughout the duration of the study. Clinical signs observed in

animals receiving 45 and 60 mg/kg were of an isolated nature, and their occurrence was not considered to be treatment related. There was no indication of any differences in any parameter related to sex and narasin treatment. The treatment of male and female pigs with narasin in the diet at nominal rates of 30, 45 or 60 mg/kg was without adverse signs. The NOAEL for this study is 60 mg/kg, the highest dose tested (Ferguson, 1995).

A second safety evaluation study of narasin involved 168 pigs separated into four groups of 21 barrows and 21 gilts each and fed one of four levels of narasin (ID and lot number not provided) (0, 25, 75 and 125 mg/kg) for 69–82 days. Animals were observed daily for clinical signs of toxicity, and two pigs per sex per group were evaluated at study termination for haematology, serum chemistry and anatomic pathology effects. Performance was evaluated by weighing the pigs twice weekly and measuring feed intake by pen.

Clinical signs consistent with narasin toxicity were observed in pigs fed 75 and 125 mg/kg 8–14 days after trial administration. Observations included anorexia, dyspnoea, depression, lethargy, unwillingness to rise or walk, leg weakness, knuckling, tremors, ataxia, stiff gait, recumbency and rare episodes of convulsions. There was no evidence of treatment-related effects in the clinical or anatomic pathology data at any treatment level (Nelson & Novilla, 1992). The NOAEL in this study was 25 mg/kg.

(e) Cattle

To determine the safety of feeding narasin or a combination of narasin and tylosin to beef-type cattle in a complete ration under simulated use conditions, 40 Hereford and Hereford-cross cattle (20 heifers and 20 steers weighing 220–320 kg each) were allotted to four treatment groups (five per sex per group). The cattle were treated for 154 days with narasin at 0, 16.5 and 50 g/t of complete ration or the combination of 50 and 33 g/t of narasin and tylosin, respectively. Treatment effects were evaluated by clinical laboratory tests and growth performance. At the end of the study, all cattle were euthanized and necropsied, and tissues were collected for histological examination. Terminal urinalysis was performed, and organ weights were recorded.

All but one of the cattle survived the treatment period. One steer in the 50 g/t group died on the 23rd day of treatment from congestive heart failure, possibly attributable to narasin treatment, in view of the significant degenerative lesions found in sections of the heart, pectoral muscle and diaphragm, severe diffuse oedema in the lungs and centrilobular congestion in the liver. Pathological findings from surviving cattle revealed no treatment-associated lesions. Compared with control values, significantly ($P < 0.05$) different values for alkaline phosphatase, urea nitrogen and uric acid were noted, but there was no dose association, and they were random with respect to time and therefore were not considered as being treatment related. Cholesterol values were significantly elevated over control values for the narasin–tylosin combination treatment at days 56 and 112 and all treatments at study termination. These latter treatment-related changes were not regarded as signs of toxicity, but instead may reflect the improved performance of the treated

cattle. There were no changes in haematology, urinalysis or organ weights that would be indicative of toxicity. Average weight gain was higher for the treated groups than for the controls, and feed utilization was also more efficient in the treated animals than in controls (Van Duyn & Novilla, 1983). Because the death of one animal in the 50 g/t group was possibly related to treatment with narasin, the NOAEL in this study was 16.5 g/t.

2.2.4 Long-term studies of toxicity and carcinogenicity

(a) Mice

The chronic toxicity and oncogenic potential of narasin (mycelial: lot X-40533, 10.6% narasin activity; crystalline: lot X-24458, 99.7% narasin activity) were assessed in a GLP-compliant study in B6C3F1 mice randomly distributed to two replicate studies and maintained for 2 years on diets containing one of five narasin concentrations. For each replicate study, five treatment groups of 30 males and 30 females were fed mycelial narasin at 0, 5, 15 or 50 mg narasin activity/kg of the diet, equal to 0, 0.59, 1.91 and 7.16 mg narasin activity/kg bw per day for males and 0, 0.71, 2.29 and 8.72 mg narasin activity/kg bw per day for females. An additional group of 30 mice per sex were fed crystalline narasin at 50 mg/kg in the diet, equal to 8.24 and 9.49 mg/kg bw per day for males and females, respectively. The summary and conclusions are based on the interpretation of the combined data from both replicates (60 per sex per group). Lot numbers for the mycelial and crystalline forms were X-40533 (10.5% activity) and X-24458 (99.7% activity), respectively. The mice were examined daily for general physical condition and behaviour. Animals found dead or in extremis were submitted for necropsy. Body weights were determined weekly for the first 13 weeks in each replicate study and every other week during the remainder of the study. At study termination, each animal was necropsied, and organ weight, haematology and clinical chemistry determinations were made. Histopathological evaluations were performed on tissues and organs collected at necropsy.

Feeding narasin to mice for 2 years had no adverse effect on survival. Overall survival rates for both sexes for the combined replicates were 73, 82, 87, 78 and 84% for mice in the 0, 5, 15 and 50 mg/kg dietary mycelial narasin groups and the 50 mg/kg dietary crystalline narasin group, respectively. Antemortem observations, except for thinness, were unrelated to narasin administration. Narasin treatment was associated with consistent decreases in mean body weight and body weight gain in the 15 and 50 mg/kg dietary groups. Body weight decreases in the 50 mg/kg dietary crystalline narasin group were greater than those in the 50 mg/kg dietary mycelial narasin group. Decreases in mean body weight and body weight gain in males and females in the 5 mg/kg dietary group were inconsistent and not significant and therefore were not treatment related. Narasin caused no toxicologically important changes in haematological and clinical chemistry parameters, organ weights or pathology. There were no treatment-related lesions or neoplasms. Benign and malignant tumours occurred sporadically across treatment groups, and the frequency and occurrence did not indicate a carcinogenic effect.

In summary, narasin fed to mice at concentrations up to 50 mg/kg in the diet continuously for 2 years had no effect on survival. Mice tolerated this level of narasin treatment with no evidence of toxicity or carcinogenicity (Novilla & Probst, 1986). Based on treatment-related decreases in mean body weight and body weight gain in males and females, the NOAEL was 5 mg narasin activity/kg in the diet, equal to 0.59 mg narasin activity/kg bw per day.

(b) Rats

The chronic toxicity and oncogenic potential of narasin (lot X-23542, 8% activity) were determined by administering narasin in the diet to Wistar-derived rats in replicate studies. Both studies had 60 rats per sex in the control and 40 rats per sex in the narasin-treated groups, except the second study (R-776) had 39 males and 41 females in the high dose group. The rats used in these chronic studies were offspring derived from a multigeneration teratology study of narasin. These rats were fed the same respective dietary concentrations of 0, 7.5, 15 and 30 mg narasin activity/kg in the diet as the parental rats for 2 years. The highest dose had been shown to be the maximum concentration that could be given to the F_0 parents and still result in viable offspring, although body weight gain was significantly reduced at this dietary level in those parental animals. The two lower doses represented one half and one quarter the concentration of the highest dose, one of which was anticipated to constitute a NOAEL. This study was conducted in 1978 prior to the effective date of GLP regulations (20 June 1979). In 1980, the final report was audited by the sponsor's quality assurance unit, which found that the report accurately reflected the data. Intake of narasin was similar for males and females at each dose level in the two studies. In study R-766, the 24-month average narasin intakes were 0, 0.31, 0.59 and 1.15 mg narasin activity/kg bw per day for males and 0, 0.38, 0.76 and 2.0 mg narasin activity/kg bw per day for females. In study R-776, the 24-month average intakes were 0, 0.29, 0.62 and 1.26 mg narasin activity/kg bw per day for males and 0, 0.37, 0.88 and 2.34 mg narasin activity/kg bw per day for females.

Reduced body weight gain in female rats in the 30 mg/kg dietary narasin group was the only finding that was clearly related to narasin treatment. The test compound did not adversely affect the survival of treated rats. The survival rate of treated rats, which was increased in the middle and high dose groups, was correlated with a decrease in the severity of progressive glomerulonephritis, a prevalent disease of ageing rats. Food consumption values of high-dose females were higher than in control rats, but actual feed intake was probably reduced due to feed spillage. Narasin produced no adverse effects on haematology, clinical chemistry or organ weights. Moreover, narasin did not induce cardiac, skeletal muscle or other non-neoplastic lesions or neoplasms. Benign and malignant tumours occurred sporadically across treatment groups, and the frequency of occurrence did not indicate a carcinogenic effect of the compound.

Signs of toxicity or oncogenicity were not evident in rats fed mycelial narasin at 7.5, 15 or 30 mg narasin activity/kg in the diet. A significant decrease in body weight gain was observed in female rats in the 30 mg/kg dietary group. On the basis of this body weight effect, the NOAEL was 15 mg narasin activity/kg diet, equal

to 0.76 mg narasin activity/kg bw per day in the first study and 0.88 mg narasin activity/kg bw per day in the second study (Novilla, 1980).

2.2.5 Genotoxicity

Crystalline narasin was tested for the potential to induce microbial gene mutations in eight Ames *Salmonella typhimurium* tester strains and in the *Escherichia coli* strains WP2 and WP2uvrA. Narasin was also tested in the L5178Y $TK^{+/-}$ mouse lymphoma cell gene and chromosomal mutation assay. The in vitro microbial and mammalian cell gene mutation studies were completed in the presence and absence of metabolic activation using S9 fraction prepared from the livers of Aroclor 1254–induced rats to compare the direct genotoxic potential and the genotoxic potential of any major narasin metabolites. Additional in vitro testing included the testing of narasin in rat primary hepatocyte cultures for the induction of unscheduled deoxyribonucleic acid (DNA) repair (UDS). In vivo testing included screening for the induction of sister chromatid exchange (SCE) in Chinese hamster bone marrow.

The results of these genotoxicity assays are shown in Table 2.

2.2.6 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity in rats

A multigeneration study was conducted in Wistar rats to determine if continuous ingestion of narasin (ID 79891, lot X-23542, activity 8%) by successive generations of rats had any effect on the reproductive process. A total of four generations, three parental generations and their offspring, were exposed to narasin. This multigeneration study was also used as the source of rats for the 1- and 2-year chronic toxicity trials conducted for narasin (see sections 2.2.2 and 2.2.4). The study was initiated on 18 March 1976 and terminated on 17 June 1977, which was prior to the effective date of GLP regulations (20 June 1979). In 1980, the final report was audited by the sponsor's quality assurance unit, which found that the report accurately reflected the data. Dose levels of 0, 15, 30 and 60 mg narasin activity/kg in the diet were initially selected for this study based on a previous 90-day rat study that produced body weight gain impairment at 30 and 60 mg/kg in a dose-related manner, with no other adverse effects observed. During the first breeding trial, however, progeny weights were significantly depressed in the 60 mg/kg dietary group. Consequently, the remainder of the study was conducted using dietary levels of 7.5, 15 and 30 mg narasin activity/kg in the diet. These dietary concentrations resulted in the following milligram per kilogram body weight equivalents of narasin activity during the growth phases: 7.5 mg narasin activity/kg in the diet, 0.8 mg/kg bw per day; 15 mg narasin activity/kg in the diet, 1.8 mg/kg bw per day; 30 mg narasin activity/kg in the diet, 3.8 mg/kg bw per day; and 60 mg narasin activity/kg in the diet, 5.9 mg/kg bw per day.

Table 2. Results of genotoxicity assays with narasin

Test system	Test object	Narasin concentration	Results	References
In vitro				
Bacterial mutagen screen[a]	*S. typhimurium* C3076, D3052, G46, TA98, TA100, TA1535, TA1537 and TA1538 and *E. coli* WP2 and WP2uvrA tester strains	0.1–1000 µg/plate	Not mutagenic	Thompson & McMahon (1978)
Ames reverse mutation assay[a,b]	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 tester strains	125–1000 µg/plate	Not mutagenic	Rexroat (1984)
Mammalian cell gene and chromosomal mutation assay[a,b,c]	L5178Y $TK^{+/-}$ mouse lymphoma cells	Eight concentrations from 0.1 to 10 µg/ml	Not mutagenic	Oberly (1984)
UDS[b,d]	Adult rat primary hepatocyte cultures	Eight concentrations from 0.5 to 1000 nmol/ml for 20 h	Negative; no induction of UDS	Hill (1981)
		Eight concentrations from 0.0005 to 1 µg/ml for 20 h	Negative; no induction of UDS	Hill (1983)
In vivo				
SCE induction[b,e]	Chinese hamster bone marrow	1, 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 mg/kg bw for 19 h	Negative; no induction of SCE	Neal (1984)

[a] With and without Aroclor 1254–induced rat liver S9 fraction.
[b] This testing was GLP compliant, except that the concentration of the dose preparations and stability of test articles in vehicle were not determined.
[c] Ethylmethanesulfonate and 3-methylcholanthrene were used as positive controls.
[d] *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine and 2-acetylaminofluorene were used as positive controls.
[e] 50 mg/kg cyclophosphamide was used in the positive control groups.

In the 15 mg/kg dietary group, mean body weights for the female parents were generally lower than the respective control body weights. Also at this dose, mean progeny weights were significantly depressed in the F_0 and F_2 breeding trials. Parental and progeny body weights at the 7.5 mg/kg and 15 mg/kg narasin levels were similar to the control findings. There was no evidence of an effect of the narasin treatment on the parameters of parental survival and condition, reproductive performance or any other progeny data.

In summary, narasin was well tolerated, with no evidence of reproductive impairment or effect on the progeny when fed at levels of 0, 7.5 and 15 mg/kg of the diet. At the 30 mg/kg dietary level, parental female body weight was generally lower than control, and mean progeny body weights were significantly decreased. However, the reproductive capacity of the rats was normal. The NOAEL based on effects on body weight in parents and offspring was 15 mg narasin activity/kg of the diet, equal to 1.8 mg narasin activity/kg bw per day. The NOAEL for reproductive effects was 30 mg narasin activity/kg of the diet, equal to 3.8 mg narasin activity/kg bw per day, which was the highest dose tested (Markham & Blubaugh, 1980a).

(b) A teratology study in the rat

In conjunction with the previously cited multigeneration rat study (Elanco studies R-916, R-1056 and R-127; Markham & Blubaugh, 1980a), a teratology study was performed during each of the three parental generations to specifically determine the effects of narasin on the rat fetus. Mycelial narasin was fed at 0, 7.5, 15 and 30 mg narasin activity/kg in the diet throughout the teratology segments. These dietary concentrations resulted in the following approximate milligram per kilogram body weight equivalents of narasin activity at the time of mating: 7.5 mg/kg dietary group, 0.5 mg/kg bw per day; 15 mg/kg dietary group, 1.3 mg/kg bw per day; and 30 mg/kg dietary group, 3.5 mg/kg bw per day. The females were sacrificed on gestation day 20 and examined for relevant reproduction and fetal data. The final termination date for this study was 16 August 1977, which was prior to the effective date of GLP regulations (20 June 1979). In 1980, the final report was audited by the sponsor's quality assurance unit, which found that the report accurately reflected the data.

In the 30 mg/kg dietary group, maternal body weights were significantly lower than the control values. Necropsy examinations of the females revealed no treatment-related findings. The reproductive performance of the animals, as determined by the findings of fertility, litter size, resorption occurrence, implantation number and corpora lutea number, was not impaired by the narasin treatment. The narasin treatment was without effect on the developing fetus. Hydronephrosis, a common finding in the fetal rat population of this laboratory, occurred more frequently in the control progeny than in the offspring from the narasin treatment groups. Other defects occurred in low incidence with no relationship to the narasin treatment.

On the basis of significantly decreased maternal mean body weight in the 30 mg/kg dietary group, the NOAEL for maternal toxicity was 15 mg narasin activity/kg in the diet, equal to 1.3 mg narasin activity/kg bw per day. The NOAEL

for teratogenicity was 30 mg narasin activity/kg in the diet, equal to 3.5 mg narasin activity/kg bw per day, the highest dose tested (Markham & Blubaugh, 1980b).

(c) A teratology study in the rabbit

In a pilot study, narasin (ID 79891, lot X-23542, activity 8%) was administered to pregnant Dutch Belted rabbits (15 per dose) in daily oral gavage doses of 0, 0.6, 1.2, 1.8 or 2.4 mg narasin activity/kg bw on gestation days 6 through 18 to determine the maternal toxicity of the chemical and its effects on reproductive performance. The highest dose administered in this pilot study was considered to be the maximum multiple dose that might be tolerated in the pregnant rabbit based on the acute toxicity of narasin in the non-pregnant rabbit (LD_{50} = 15.5 ± 3.9 mg/kg bw). The termination date for this study was 9 March 1976, which was prior to the effective date of GLP regulations (20 June 1979). In 1980, the final report was audited by the sponsor's quality assurance unit, which found that the report accurately reflected the data

Two rabbits in the 2.4 mg/kg bw per day dose group died. Additional signs of toxicity included depressed values of food consumption, depressed water intake, body weight losses and abortions. Although these effects occurred at all dose levels, they were most pronounced at the highest dose level of 2.4 mg/kg bw per day. One fetus at the lowest dose had a shortened tail; however, no morphological defects were seen in the other fetuses. Although one rabbit each aborted at both the 0.6 and the 1.2 mg/kg bw per day treatment levels, the reproductive performance of the rabbits at the 1.8 mg/kg bw per day dosage level was not impaired. The results of this pilot study indicated that the maximum multiple dose of narasin activity tolerated by the pregnant rabbit is approximately 1.8 mg/kg bw per day (Markham, 1980a).

Subsequently, in the definitive study, pregnant Dutch Belted rabbits were given daily oral doses of 0, 0.6, 1.2 or 1.8 mg narasin activity/kg bw on gestation days 6 through 18 to determine the teratogenic potential of this chemical. On gestation day 28, the females were killed and evaluated for reproductive performance, and the fetuses were examined for any abnormalities. The final termination date for this study was 21 October 1976, which was prior to the effective date of GLP regulations (20 June 1979). In 1980, the final report was audited by the sponsor's quality assurance unit, which found that the report accurately reflected the data.

Four rabbits, two from the middle dose and two from the high dose, aborted. Two of the rabbits of the high dose group were found moribund and were killed. Leg weakness and incoordination were observed in three of these rabbits (one at the middle dose; two at the high dose). Mean values for food consumption and body weight were slightly depressed in the middle and high dose groups; however, these findings were not statistically significant. Mean live litter size was slightly decreased and resorption occurrence was slightly increased at the high dose. There was no evidence of an effect of the narasin treatment on the other reproductive parameters or on the fetal values of viability, sex or weight. External defects were confined to open eyelids and/or cleft palate in one fetus at the low dose, five littermates at the middle dose and one fetus at the high dose. Visceral defects consisted of internal

hydrocephalus and aplastic kidney (one low-dose fetus) and an enlarged heart (one middle-dose fetus). The corresponding incidences of abnormality observed in the cumulative control data for 3646 animals were 3 and 6 for these external defects and 13, 1 and 1 for these visceral defects, respectively. The developmental deviations of 13 ribs, incomplete development of the dorsal cranial bones and sternebral irregularities occurred as frequently in the control progeny as in the progeny from the narasin treatment groups.

It was concluded from this study that the oral administration of narasin to pregnant rabbits on gestation days 6 through 18 resulted in maternal toxicity and abortions at doses of 1.2 or 1.8 mg/kg bw per day and an increase in the occurrence of resorptions at 1.8 mg/kg bw per day. On the basis of maternal toxicity and effects on body weight observed at 1.2 and 1.8 mg narasin activity/kg bw per day and non-significant decreases in mean live litter size and a slight increase in resorptions relative to control at 1.8 mg/kg bw per day, the NOAEL for maternal toxicity was 0.6 mg narasin activity/kg bw per day. The NOAEL for teratogenicity was 1.8 mg narasin activity/kg bw per day, which was the highest dose tested (Markham, 1980b).

2.2.7 Special studies

(a) General pharmacology studies

The general pharmacology of narasin was investigated in mice, rats and rabbits for 1) effects on the central nervous system, 2) effects on autonomic nerves, 3) effects on somatic nerves and 4) effects on kidney function. For these studies, narasin was suspended in 5% gum arabic solution for oral administration or was dissolved in ethanol and then emulsified with 5% dextrose for intravenous administration. The six individual studies were completed as follows.

(i) General behavioural and central nervous system signs in mice

Narasin was administered orally to three male and three female mice at doses of 0 (control), 1, 3, 10, 30 and 100 mg/kg bw. No effects were observed in either males or females from the 1 and 3 mg/kg bw dose groups. Decreased motor activity, awareness, mood and reflexes, abnormal position, ptosis, piloerection and decreased respiration in males and females and staggering gait in females were observed at doses of ≥10 mg/kg bw. In addition to these behavioural signs, decreased body tone and abdominal tone were observed at 100 mg/kg bw. One 30 mg/kg bw female and all 100 mg/kg bw animals died from 1 to 24 h after administration. No effects on general behaviour were observed in controls.

(ii) General behaviour and central nervous system signs in rabbits

Narasin was administered orally to three male rabbits at doses of 0 (control), 10, 30 and 100 mg/kg bw. No effects were observed at 10 mg/kg bw. In animals given 30 mg/kg bw or higher doses, decreased locomotor activity, weakness in extremities and ataxia upon walking were observed 3 h after administration. In addition to these behavioural signs, relaxation of abdominal muscle, prone position, ptosis, decreased respiration and unusual breathing were present. Decreased

locomotor activity, relaxation of abdominal muscle, prone position, ptosis and unusual breathing were still observable 6 h following narasin administration, but the signs of weakness in extremities and ataxia were not seen at the 6-h time point.

(iii) Charcoal meal transit in mice

Narasin was administered orally to six male mice at doses of 0 (control), 0.1, 0.3, 1, 3 and 10 mg/kg bw. No effects were observed at doses of ≤3 mg/kg bw. Charcoal transit was significantly decreased at 10 mg/kg bw; hence, intestinal transit was suppressed at the highest dose.

(iv) In situ uterine motility in the rabbit

Narasin was administered intravenously to three female multiparous rabbits at escalating doses of 0 (solvent), 0.03, 0.1, 0.3 and 1 mg/kg bw. No effects were observed from the administration of solvent vehicle or the 0.03 mg/kg bw dose. At the 0.1 mg/kg bw or higher doses, dose-related decreases in amplitude and frequency of uterine contractions were observed from 3 to 15 min after administration, with apparent recovery of motility after about 30 min.

(v) In situ contractions of anterior tibial muscle in the rabbit

Narasin was administered intravenously to four male rabbits at escalating doses of 0 (solvent), 0.03, 0.1, 0.3 and 1 mg/kg bw. No effects were observed from the administration of the solvent vehicle or the doses of 0.03 and 0.1 mg/kg bw. At doses of ≥0.3 mg/kg bw, a dose-related, slight increase in amplitude of contractions by direct stimuli and a dose-related slight or mild increase in amplitude of contractions by indirect stimuli were observed. Findings were similar in the animal that had intact ischiatic nerves.

(vi) Urine excretion in rats

Narasin was administered orally to six male rats per group at doses of 0 (control), 0.3, 1, 3, 10 and 30 mg/kg bw. Two animals from the 30 mg/kg bw group died. No changes were observed for qualitative tests, and no significant differences in urine volume, osmotic pressure or concentration of sodium, potassium or chloride were observed between narasin groups and control.

(vii) Summary

To summarize, the major effects of narasin on the central nervous system in mice were systemic suppression, including signs of decreased motor activities, awareness, reflexes and muscle tones, abnormal position, ptosis, piloerection and decreased respiration. In rabbits, the signs were decreased locomotor activity, weakness in the extremities, ataxia, ptosis and suppression of respiration. For the peripheral nervous system, the effects included suppression of intestinal transit in mice, suppression of uterine motility in rabbits and an increase in the contractions of the anterior tibial muscle in rabbits. No effects on kidney function were observed in rats based on urine qualitative and quantitative tests (Yamamoto, 1999).

(b) Sensitization

The sensitization potential of narasin was studied in a group of 10 female albino guinea-pigs 2–3 months of age. A series of 10 intracutaneous injections of 0.025% narasin (ID 79891, lot C16-D48-245-7, isolated crystalline) in safflower oil were given to each animal over a 3-week period. A group of eight guinea-pigs served as controls and were injected with the safflower oil vehicle only. Two weeks after the 10th injection, a retest (challenge) injection was made. Twenty-four hours after each retest injection, the irritation wheal diameters were measured. The reaction following retest was compared with the average readings taken after each of the original 10 injections to determine sensitization potential. No indication of delayed hypersensitization was found after the intracutaneous challenge injection of narasin. Decreased body weight was attributable to narasin injections (Arthur et al., 1978).

(c) Microbiological effects

A JECFA decision tree approach that was adopted at the sixty-sixth meeting of the Committee (Annex 1, reference *181*) and complies with Guideline 36 of the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH GL36) (VICH, 2004) was used by the Committee to determine the need to establish a microbiological ADI for narasin. The decision tree approach initially seeks to determine if there may be microbiologically active narasin residues entering the human colon. If the answer is “no” to any of the first three steps, then no microbiological ADI is necessary. However, should such residues be present, then two end-points of public health concern are to be considered: 1) disruption of the colonization barrier and 2) increase of the population(s) of resistant bacteria. At Step 4 of the decision tree process, it is possible to provide scientific justification to eliminate testing (i.e. the need for a microbiological ADI) for either one or both end-points. Step 5 is where a microbiological ADI would be determined. Should a microbiological ADI not be necessary, then the toxicological or pharmacological ADI would be used. The Committee evaluated minimum inhibitory concentration (MIC) susceptibility, faecal binding interaction and biological activity of narasin metabolites and used the decision tree to answer the following questions in the assessment of narasin.

Step 1: Are residues of the drug, and (or) its metabolites, microbiologically active against representatives of the human intestinal flora?

Yes. Narasin is microbiologically active against some bacterial genera and species representative of the human intestinal flora.

In a GLP-compliant study, the MIC of narasin was determined against 100 bacterial strains, comprising 10 isolates from each of 10 groups of genera representing the normal human intestinal microbiota (Pridmore, 2006a). All strains were sourced from the faecal microbiota of healthy unmedicated human volunteers. The test system was standardized agar dilution MIC methodology using quality control strains as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2004). MICs were determined using inoculum levels recommended by CLSI guidelines, which ranged from 10^7 to 10^8 colony-forming units (cfu)/ml for each strain. Narasin activity against each bacterial group is summarized in Table 3. MIC_{50}, MIC_{90} and MIC range were calculated for each bacterial group. The

degree of narasin activity varied considerably between each bacterial taxonomic group. Narasin activity was clearly demonstrable against *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Lactobacillus*, *Fusobacterium* and *Peptostreptococcus*. *Peptostreptococcus* was the most susceptible group, with a MIC range of 0.062–0.5 µg/ml and a MIC_{50} of 0.125 µg/ml. Narasin exerted very weak activity against *Bacteroides fragilis* and other *Bacteroides* spp., with a MIC_{50} of 32 µg/ml. Narasin exerted no measurable antibacterial activity against *Escherichia coli* (MIC >128 µg/ml).

Table 3. Susceptibility of representative human intestinal bacteria to narasin[a]

Bacterial group (10 strains/group)	Summary of MIC parameters (µg/ml)		
	MIC range	MIC_{50}	MIC_{90}
Bacteroides fragilis	8–32	32	32
Bacteroides (other species)	16–32	32	32
Bifidobacterium	0.125–4	0.5	1
Clostridium	0.125–1	0.25	5
Enterococcus	All 0.5	0.5	0.5
Escherichia coli	All >128	>128	>128
Eubacterium	0.25–0.5	0.25	0.5
Fusobacterium	0.125–32	8	16
Lactobacillus	0.5–4	1	2
Peptostreptococcus	0.062–0.5	0.125	0.5
All strains (*n* = 100)	0.062–>128	0.5	32

[a] From Pridmore (2006a).

Step 2: Do residues enter the human colon?

Yes. A number of residue studies using ^{14}C radiolabelling to detect total residues and analytical and microbiological assay methods to detect parent narasin have been conducted in chickens, pigs and cattle. Muscle contains little or no narasin-derived residue regardless of the period between withdrawal of medication and slaughter. However, residues may be present at low levels in offal, fat and skin. Narasin accounted for approximately 5% of total residues in pig and rat faeces following oral administration (Manthey, 1977a). Therefore, narasin-related residues could enter the colon of a person ingesting tissues from treated animals.

Step 3: Do the residues entering the human colon remain microbiologically active?

No. Narasin residue is extensively transformed to hydroxylated metabolites with very reduced activity prior to entering the colon of the consumer; within the colon, it will become substantially bound (>99%) to faecal material.

To determine the effect of faecal binding on the antibacterial activity of narasin, narasin at 12 concentrations of 0, 0.5, 1, 2, 5, 10, 20, 50, 100, 120, 140 and 160 μg/ml was incubated for up to 12 h with increasing concentrations of sterilized human faeces (0, 25 and 50% weight by volume [w/v] in Mueller Hinton Broth), collected from three individual donors who had not had diarrhoea within the previous 4 weeks or received antibiotic therapy during the previous 3 months (Pridmore, 2006b). Narasin activity was determined using *Enterococcus faecalis* as the indicator organism, as it is susceptible to narasin. The antibacterial activity of the supernatant obtained from each incubation time was assessed for the presence or absence of bacterial growth before and after incubation with faeces. All three faecal samples had maximal (99.4%) binding of narasin at both 25% and 50% concentrations (Table 4). Narasin binding (>99%) to faeces occurred immediately after mixing. The 50% faecal concentration provided the closest representation of the in vivo situation. The results demonstrated the rapid and extensive binding of narasin to human faeces. Based on this in vitro study, it can be estimated that the binding of narasin residues to undiluted faecal material is rapid and would likely exceed 99%.

Table 4. Determination of narasin binding to human faeces: Definitive experiment with faecal sample 042/05/002[a]

Incubation time (h)	Broth only (no faeces)	25% w/v faeces		50% w/v faeces	
	Initial drug concentration (μg/ml) required to inhibit growth (a)	Initial drug concentration (μg/ml) required to inhibit growth (c)	% of drug bound to faeces [(c – a) / c] × 100	Initial drug concentration (μg/ml) required to inhibit growth (d)	% of drug bound to faeces [(d – a) / d] × 100
0	1	>160	>99.4	>160	>99.4
1	1	>160	>99.4	>160	>99.4
2	1	100	99.0	>160	>99.4
6	1	100	99.0	>160	>99.4
8	1	100	99.0	>160	>99.4
12	1	100	99.0	>160	>99.4

[a] From Pridmore (2006b). Concentrations of narasin used in this experiment were 0, 0.5, 1, 2, 5, 10, 20, 50, 100, 120, 140 and 160 μg/ml.

Narasin is extensively metabolized and converted to numerous metabolites in pigs and rats. Hydroxylation appears to be the major metabolic pathway. Hydroxylated metabolites of narasin A and B were identified in the liver, bile and faeces (Manthey, 1977a; Sweeney et al., 1995). Antimicrobial activity of six hydroxylated metabolites of narasin was determined by bioautography against *Bacillus subtilis*. In this bioassay, all of the metabolites were at least 20 times less

active than narasin (Manthey & Goebel, 1982). The ionophoric potency of di- and trihydroxylated narasin metabolites isolated from chicken and cattle excreta was further investigated by Wong (1978). Di- and trihydroxylated narasin metabolites had 200 times less activity than narasin.

Step 4: Is there any scientific justification to eliminate testing for either one or both end-points of concern, i.e. disruption of the colonization barrier or resistance development?

Yes. The only potential adverse effect on human intestinal microbiota would be disruption of the colonization barrier as the end-point of concern for determining the microbiological ADI. However, since the majority of narasin residues in the colon are bound to faeces (99.4%) and biologically inactive, the bioavailable concentration is below the MIC_{50} of 0.125 µg/ml for *Peptostreptococcus*, which is the most sensitive genus of the representative human intestinal bacteria listed in Table 3. In addition, to study the ability of narasin to affect faecal excretion of *Salmonella* in poultry, broiler chickens were fed narasin at 100 mg/kg of the diet and the next day dosed with *Salmonella* for periods up to 8 weeks. Narasin treatment had no statistically significant effect on the faecal excretion of *Salmonella* or on the proportion of faecal coliforms resistant to narasin (Bennett & Elliott, 1976). Therefore, narasin did not affect the normal intestinal microbiota. Based on MIC susceptibility, faecal binding interaction and in vivo colonization barrier studies, narasin residues were not shown to disrupt the colonization barrier of the human gastrointestinal tract. Narasin does not appear to select for acquired resistance in bacteria, since there is no genetically encoded resistance gene for ionophores. Consequently, there is no need to determine a microbiological ADI for narasin residues.

2.4 Observations in humans

No controlled studies have been conducted in which humans were intentionally exposed to any formulation of narasin. It is recognized, however, that unintentional occupational exposure may occur in the workplace during the manufacture of premixes or complete feeds. Narasin and narasin products have been manufactured at the Clinton Laboratories of Eli Lilly and Company for more than 20 years, with a Lilly exposure guideline of 11 µg/m^3 for 12 h. In February 2000, an extensive evaluation of the medical records of 163 employees who had been involved with the manufacturing process during that time was completed. This included results from the annual health surveillance exams, employee records of visits to the Employee Health Services and incident reports resulting from industrial injuries or potential exposures to narasin. Particular emphasis was placed on any reports of skin rashes, allergic manifestations, neuromuscular disorders and cardiac symptoms that may have resulted from known exposure to narasin.

The health surveillance screening process included haematology (haemoglobin, haematocrit, red blood cell indices, white blood cell and platelet counts), blood chemistry (glucose, blood urea nitrogen, creatinine, cholesterol, total bilirubin, AST, alkaline phosphatase, lactate dehydrogenase, uric acid, total protein, electrolytes and albumin), pulmonary functions (forced vital capacity [FVC], forced

expiratory volume [FEV], FEV/FVC) and completion of a health questionnaire. A review of the results for these 163 employees revealed no significant values that could be related to narasin exposure. Specifically, there was no evidence of blood dyscrasias, hepatic or renal dysfunction, pulmonary function abnormalities, neoplasm, chronic skin disease, or neuromuscular, cardiac or other abnormalities.

A review of the medical records and incident/injury reports for these same 163 employees indicated two cases of immunoglobulin E (IgE)–mediated allergic response to narasin, which was manifested by transient facial uticaria, pruritis, nasal congestion and chest tightness. In addition, three other cases were found that were suspicious for allergic symptoms but not confirmed by intradermal testing. These cases may have represented local respiratory irritation rather than an allergic response. The symptoms in all five cases were transient and resolved completely after removal of the employees from the narasin manufacturing area, with no long-term consequences (Twenty, 2000).

3. COMMENTS

3.1 Biochemical data

Owing to the principal effect of narasin on the microflora of the gastrointestinal tract (including coccidia), few conventional pharmacokinetic studies have been performed. Studies in both target and laboratory animals indicate that narasin depletes quickly in plasma. Narasin is metabolized by the liver by oxidation/hydroxylation pathways, resulting in polar metabolites that are readily excreted in bile. The primary route of excretion of narasin is in the faeces, with total elimination occurring within 3–4 days.

The metabolism and excretion of narasin were evaluated in a series of studies in rats. Following a single oral dose of 2.3 mg of [^{14}C]narasin, 75% of the total radioactivity was recovered in the urine and faeces of rats by 52 h post-dosing. Only 1.1% of the total excreted radioactivity was found in the urine, and the remainder (98.9%) was in the faeces. In a second study, more than 16 structural isomer metabolites of narasin and narasin B were identified in the faeces using HPLC/ISP-MS. In another study, it was concluded that [^{14}C]narasin was metabolized by rats to more than 20 metabolites, and the pattern in faeces and liver was qualitatively similar.

3.2 Toxicological data

Mycelial and crystalline forms of narasin were tested for toxicity. Doses of both forms of narasin in the toxicological studies were reported on the basis of the microbiological activity of narasin relative to that of the crystalline form.

The acute oral toxicity of narasin varies between species, being extremely toxic (defined as an LD_{50} of 1 mg/kg bw or less) to horses, highly toxic (defined as an LD_{50} of 1–50 mg/kg bw) to pigs, mice, rats and rabbits, and moderately toxic (defined as an LD_{50} of 50–500 mg/kg bw) to chickens and quail. The primary clinical

signs of acute toxicity following an oral dose of mycelial or crystalline narasin were hypoactivity, leg weakness and ataxia.

In a non-GLP-compliant 3-month oral toxicity study, mice (15 per sex per group) were fed mycelial narasin at 0, 10, 20 or 40 mg narasin activity/kg in the diet, equivalent to 0, 1.5, 3 and 6 mg narasin activity/kg bw per day. There were no toxicologically relevant changes in survival, clinical signs, body weight, or haematological, clinical chemistry or anatomic pathology. The NOAEL was 40 mg/kg in the diet, equivalent to 6 mg narasin activity/kg bw per day, which was the highest dose tested.

In a second 3-month non-GLP-compliant oral toxicity study, mice (15 per sex per group) were fed mycelial narasin at levels of 0, 60, 80 or 100 mg narasin activity/kg in the diet, equivalent to 0, 9, 12 and 15 mg narasin activity/kg bw per day. At termination, mean body weights of males in the 80 and 100 mg/kg dietary groups were decreased by 26% and 27%, respectively. The mean body weight of females in the 100 mg/kg dietary group was decreased by 14%. On the basis of the magnitude of the body weight changes at 80 and 100 mg/kg in the diet, the NOAEL was 60 mg/kg in the diet, equivalent to 9 mg narasin activity/kg bw per day.

In a 3-month non-GLP-compliant oral toxicity study, rats (15 per sex per group) were fed mycelial narasin at levels of 0, 15, 30 and 60 mg narasin activity/kg in the diet, equal to 0, 1.1, 2.2 and 4.7 narasin activity/kg bw per day for males and 0, 1.1, 2.6 and 5.7 mg narasin activity/kg bw per day for females. Decreases in feed conversion ratios, mean body weight and mean body weight gain were adverse (exceeding 10% of control values) narasin-related effects seen in males and females at the 30 and 60 mg/kg dietary levels. The decreased mean body weight in females in the 60 mg/kg dietary group was statistically significant. On the basis of decreased feed conversion ratios, mean body weight and mean body weight gain, the NOAEL was 15 mg/kg in the diet, which was equal to 1.1 mg narasin activity/kg bw per day.

In a 1-year non-GLP-compliant oral toxicity study, rats (15 per sex per group) were fed mycelial narasin at levels of 0, 7.5, 15 or 30 mg narasin activity/kg in the diet, equal to 0, 0.49, 1.0 or 1.9 mg narasin activity/kg bw per day for males and 0, 0.57, 1.2 or 3.2 mg narasin activity/kg bw per day for females. Decreases in mean body weight and feed conversion ratios relative to control in females in the 30 mg/kg dietary group and decreased feed conversion ratios in females in the 15 mg/kg dietary group were attributed to unpalatability and spillage of the diet. This was evidenced by a significant decrease in mean food consumption and mean body weight loss in females in the 30 mg/kg dietary group in week 1, followed by significantly increased weekly mean food consumption in most weeks thereafter. Non-significant increases in weekly mean food consumption by females in the 15 mg/kg dietary group were also observed. Therefore, the effects on mean body weight and feed conversion ratio are not toxicologically relevant findings. There were no treatment-related effects in males or females at any dose level. In the absence of evidence of toxicity, the NOAEL was 30 mg narasin activity/kg in the diet, equal to 1.9 mg narasin activity/kg bw per day, which was the highest dose tested.

In a 3-month non-GLP-compliant oral toxicity study, dogs (four per sex per group) were given mycelial narasin in gelatine capsules at dose levels of 0, 0.5, 1.0 or 2.0 mg narasin activity/kg bw per day. Clinical signs of leg weakness and incoordination at 2.0 mg/kg bw per day were attributed to treatment with narasin. The ECGs of six of the eight 2.0 mg/kg bw per day animals showed decreased heart rate, with normal to markedly abnormal sino-arrhythmias present. Three of these dogs had signs of sino-arrest. No histological lesions were found in the hearts of these same animals, and there were no corresponding changes in blood chemistry values. On the basis of clinical signs of leg weakness, incoordination and abnormal ECG findings seen at 2.0 mg/kg bw per day, the NOAEL was 1.0 mg narasin activity/kg bw per day.

To further define the signs of toxicity and evaluate the effects of narasin on ECGs in dogs, a non-GLP-compliant 6-month study was conducted in dogs (four per sex per group) given mycelial narasin orally by gelatine capsule at dose levels of 0, 0.5, 1.0 or 1.5 mg narasin activity/kg bw per day before the morning feeding. Abnormal ECG findings consisting of bradycardia, low-amplitude R-waves, elevated T-waves and marked suppression of the ST segment were seen in a single 1.5 mg/kg bw per day female at the 1-month interval. These findings were deemed treatment related, because treatment-related effects on ECGs were seen in a separate 3-month oral toxicity study of narasin in dogs. However, ECG changes in this animal were absent at the 3- and 6-month ECG examinations, providing evidence of reversibility, and there was no evidence of microscopic changes in the heart. On the basis of the reversible abnormal ECG findings, the NOAEL was 1.0 mg narasin activity/kg bw per day.

In a GLP-compliant study, male and female dogs (four per sex per group) were given daily oral doses of mycelial narasin by gelatine capsule at 0, 0.5, 1.0 or 2.0 mg narasin activity/kg bw per day for 1 year. An additional group (four per sex) was given crystalline narasin orally by capsule at a dose level of 2.0 mg/kg bw per day. One male in the 2.0 mg/kg bw per day mycelial group was found moribund, with clinical signs of anorexia, excessive salivation, laboured respiration and recumbency, and was killed on day 13. Clinical signs and microscopic lesions observed in the heart indicated that the moribund condition of this animal was treatment related. Effects related to treatment with mycelial narasin at 2.0 mg/kg bw per day in all surviving animals were slight to severe leg weakness, excessive salivation, loss of muscle tone and patellar reflex deficits. Less frequently observed effects were convulsions or opisthotonos, bilateral dropped carpus, transient occurrences of tremors, decreased food consumption, hypoactivity and laboured respiration. Effects related to treatment with crystalline narasin at 2.0 mg/kg bw per day were of lower incidence or severity and included leg weakness, ataxia, excessive salivation, depressed appetite, laboured respiration, decreased muscle tone and patellar reflex deficits. Effects related to treatment in the 1.0 mg narasin activity/kg bw per day mycelial narasin group included transient leg weakness and slight muscle atrophy observed clinically, but not histologically, in one male and occasional salivation in two females. Decreases in body weight gain were observed in the 2.0 mg/kg bw per day mycelial and crystalline narasin groups, but were of greater magnitude in the mycelial narasin group. Mean body weight was decreased

in males (11% and 15%) and females (29% and 11%) in the 2.0 mg/kg bw per day mycelial and crystalline narasin groups, respectively. These decreases were associated with decreases of 10% or greater in monthly mean food consumption at multiple intervals during the study. There were no treatment-related ECG findings. Transient increases in creatine phosphokinase and AST values and degenerative and/or regenerative changes in cardiac and skeletal muscles and sciatic, tibial and other unspecified peripheral nerves were narasin-related effects seen in animals given mycelial or crystalline narasin at 2.0 mg/kg bw per day. The histopathological changes were more severe in the mycelial narasin group. Heart muscle lesions were observed only in the 2.0 mg/kg bw per day dog that was killed in extremis. On the basis of mortality, clinical signs, effects on body weight, clinical chemistry and microscopic findings in skeletal and cardiac muscles and peripheral nerve, the NOAEL was 0.5 mg narasin activity/kg bw per day.

In replicate 2-year, GLP-compliant combined toxicity/carcinogenicity studies, mice (30 per sex per group per replicate) were fed mycelial narasin at 0, 5, 15 or 50 mg/kg of the diet, equal to 0, 0.59, 1.91 and 7.16 mg narasin activity/kg bw per day for males and 0, 0.71, 2.29 and 8.72 mg narasin activity/kg bw per day for females. An additional group of 30 mice per sex was fed crystalline narasin at 50 mg/kg in the diet, equal to 8.24 and 9.49 mg/kg bw per day for males and females, respectively. Mice tolerated these levels of exposure with no treatment-related effects on survival and no evidence of carcinogenicity. Decreases in mean body weight and body weight gain were observed for males and females at mycelial and crystalline narasin dietary levels of 15 and 50 mg/kg. Body weight decreases in the 50 mg/kg dietary crystalline narasin group were greater than those in the 50 mg/kg dietary mycelial narasin group. In both replicates, on the basis of effects on body weight, the NOAEL was 5 mg narasin activity/kg in the diet, equal to 0.59 narasin activity/kg bw per day.

In rats, two non-GLP-compliant 2-year combined chronic toxicity/carcinogenicity studies were conducted. The first study had 60, 40, 40 and 40 rats per sex per group, and the second study had 60, 40, 40 and 39 males:41 females per sex per group, for the control, low dose, middle dose and high dose groups, respectively. In both studies, rats were fed mycelial narasin for 2 years at 0, 7.5, 15 or 30 mg narasin activity/kg of the diet, equal to 0, 0.31, 0.59 and 1.15 mg narasin activity/kg bw per day for males and 0, 0.38, 0.76 and 2.0 mg narasin activity/kg bw per day for females in the first study and 0, 0.29, 0.62 and 1.26 mg narasin activity/kg bw per day for males and 0, 0.37, 0.88 and 2.34 mg narasin activity/kg bw per day for females in the second study. In both studies, the rats tolerated these levels of exposure with no treatment-related effects on survival and no evidence of carcinogenicity. The only clinical finding clearly related to treatment with narasin was decreased body weight gain in females at the 30 mg/kg dietary narasin level. On the basis of this body weight effect, the NOAEL was 15 mg narasin activity/kg diet, equal to 0.76 mg narasin activity/kg bw per day in the first study and 0.88 mg narasin activity/kg bw per day in the second study.

Crystalline narasin produced negative results in an adequate range of in vitro and in vivo genotoxicity studies. The Committee concluded that narasin had no genotoxic potential.

In a non-GLP-compliant multigeneration reproductive toxicity study, rats (45 per sex per group for the parental generation and 25 per sex per group in subsequent generations) were fed mycelial narasin at levels of 0, 7.5, 15 and 30 mg narasin activity/kg in the diet, equal to 0, 0.8, 1.8 and 3.8 mg narasin activity/kg bw per day during the growth phases. A total of four generations, three parental generations and their offspring, were exposed to narasin. Narasin was well tolerated, with no evidence of reproductive impairment or effect on the progeny when fed at levels of 0, 7.5 and 15 mg/kg of the diet. At the 30 mg/kg dietary level, parental female body weight was generally lower than control, and mean progeny body weights were significantly decreased. However, the reproductive capacity of the rats was normal. The NOAEL based on effects on body weight in parents and offspring was 15 mg narasin activity/kg of the diet, equal to 1.8 mg narasin activity/kg bw per day. The NOAEL for reproductive effects was 30 mg narasin activity/kg of the diet, equal to 3.8 mg narasin activity/kg bw per day, which was the highest dose tested.

The teratogenicity of mycelial narasin in rats was evaluated as a segment in each of the three generations from the aforementioned multigeneration reproductive toxicity study. Rats (22–25 per sex per group) were fed narasin at levels of 0, 7.5, 15 or 30 mg narasin activity/kg in the diet, equivalent to 0, 0.5, 1.3 or 3.5 mg narasin activity/kg bw per day. Pregnant females were killed on gestation day 20. Maternal mean body weight at the 30 mg/kg dietary level was significantly decreased compared with control. The NOAEL for maternal toxicity was 15 mg narasin activity/kg in the diet, equal to 1.3 mg narasin activity/kg bw per day. The NOAEL for teratogenicity was 30 mg narasin activity/kg in the diet, equal to 3.5 mg narasin activity/kg bw per day, the highest dose tested.

Pregnant rabbits (15 per dose) were dosed by oral gavage with mycelial narasin at dose levels of 0, 0.6, 1.2 or 1.8 mg narasin activity/kg bw per day. Maternal toxicity occurred at 1.2 and 1.8 mg narasin activity/kg bw per day and included mortality, abortions, leg weakness and incoordination. Non-significant decreases in mean body weight and food consumption relative to control were also noted at these dose levels. Non-significant decreases in mean live litter size and a slight increase in resorptions relative to control were noted at 1.8 mg/kg bw per day. There was no evidence of teratogenicity. The NOAEL for maternal toxicity was 0.6 mg narasin activity/kg bw per day. The NOAEL for teratogenicity was 1.8 mg narasin activity/kg bw per day, which was the highest dose tested.

A review of the medical records and incident/injury reports for 163 employees occupationally exposed to narasin indicated two cases of confirmed IgE-mediated allergic responses to narasin, which were manifested by transient facial uticaria, pruritis, nasal congestion and chest tightness. Three additional cases with similar respiratory symptoms were not confirmed by intradermal testing and might have represented local respiratory irritation rather than an allergic response. After relocation away from the narasin manufacturing area, the symptoms in all five cases resolved completely, with no long-term consequences.

The studies considered for the determination of the toxicological ADI are summarized in Table 5.

Table 5. Summary of lowest NOAELs in toxicology studies for narasin

Study type	Species and study no.	Type of narasin	Doses (mg narasin activity/ kg diet)	Doses (mg/kg bw per day)	NOAEL (mg/kg bw per day)
90-day oral	Mouse	Mycelial	0, 10, 20, 40	0, 1.5, 3, 6	6
90-day oral	Mouse M-9046	Mycelial	0, 60, 80, 100	0, 9, 12, 15	9
90-day oral	Rat R-885	Mycelial	0, 15, 30, 60	0, 1.1, 2.2, 4.7 (M); 0, 1.1, 2.6, 5.7 (F)	1.1
Multigeneration reproductive toxicity	Rat	Mycelial	0, 7.5, 15, 30	0, 0.8, 1.8, 3.8	1.8
Teratology	Rat	Mycelial	0, 7.5, 15, 30	0, 0.5, 1.3, 3.5	1.3
Teratology	Rabbit B-7396	Mycelial	N/A	0, 0.6, 1.2, 1.8	0.6
90-day oral capsule	Dog D-3815	Mycelial	N/A	0, 0.5, 1.0, 2.0	1.0
6-month oral capsule	Dog	Mycelial	N/A	0, 0.5, 1.0, 1.5	1.0
1-year oral capsule	Dog D04183	Mycelial	N/A	0, 0.5, 1.0, 2.0	0.5
		Crystalline	N/A	2.0	
1-year oral	Rat R-1046	Mycelial	0, 7.5, 15, 30	0, 0.49, 1.0, 1.9 (M); 0, 0.57, 1.2, 3.2 (F)	1.9
2-year oral oncogenicity (mycelial and crystalline)	Mice M01783 and M01883	Mycelial	0, 5, 15, 50	0, 0.59, 1.81, 7.16 (M); 0, 0.71, 2.29, 8.72 (F)	0.59
		Crystalline	50	8.24 (M); 9.49 (F)	
2-year oral oncogenicity	Rat R766		0, 7.5, 15, 30	0, 0.31, 0.59, 1.15 (M); 0, 0.38, 0.76, 2.0 (F)	0.76

Table 5 (contd)

Study type	Species and study no.	Type of narasin	Doses (mg narasin activity/kg diet)	Doses (mg/kg bw per day)	NOAEL (mg/kg bw per day)
	R776,			0,0.29, 0.62, 1.26 (M); 0, 0.37, 0.88, 2.34 (F)	

F, female; M, male; N/A, not applicable.

3.3 Microbiological data

The JECFA decision tree approach that was adopted by the sixty-sixth Committee (Annex 1, reference *181*) and complies with VICH GL36 (VICH, 2004) was used to evaluate the impact of narasin residues on the intestinal microflora.

Narasin activity was clearly demonstrable against *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Lactobacillus*, *Fusobacterium* and *Peptostreptococcus*. *Peptostreptococcus* was the most susceptible group, with a MIC range of 0.062–0.5 µg/ml and a MIC_{50} of 0.125 µg/ml. Narasin exerted very weak activity against *Bacteroides fragilis* and other *Bacteroides* spp., with a MIC_{50} of 32 µg/ml. Narasin exerted no measurable antibacterial activity against *Escherichia coli* (MIC >128 µg/ml). Therefore, the only potential adverse effect on human intestinal microbiota would be disruption of the colonization barrier as the end-point of concern for determining the microbiological ADI. However, since the majority of narasin residues in the colon are bound to faeces (99.4%) and biologically inactive, the bioavailable concentration is below the MIC_{50} of 0.125 µg/ml for *Peptostreptococcus*, which is the most sensitive genus of the representative human intestinal bacteria. In addition, to study the ability of narasin to affect faecal excretion of *Salmonella* in poultry, broiler chickens were fed narasin at 100 mg/kg of the diet and the next day dosed with *Salmonella* for periods up to 8 weeks. Narasin treatment had no statistically significant effect on the faecal excretion of *Salmonella* or on the proportion of faecal coliforms resistant to narasin. Therefore, narasin did not affect the normal intestinal microbiota. Based on MIC susceptibility, faecal binding interaction and in vivo colonization barrier studies, narasin residues were considered unlikely to disrupt the colonization barrier of the human gastrointestinal tract. Narasin does not appear to select for acquired resistance in bacteria, as there is no genetically encoded resistance gene for ionophores. The Committee concluded that it was not necessary to determine a microbiological ADI for narasin.

4. EVALUATION

The lowest NOAEL was established in the GLP-compliant, 1-year oral toxicity study in the dog. The NOAEL in this study was 0.5 mg/kg bw per day, based on clinical and pathological signs of toxicity at the higher doses. A safety factor of

10 for interspecies differences and 10 for interindividual variability in the population was applied, for a total safety factor of 100. This safety factor was considered appropriate in view of the extensive database of adequately conducted studies for a compound that has a long history of use. Therefore, an ADI of 0–5 μg/kg bw was established on the basis of the toxicological data.

5. REFERENCES

Arthur, B.H., Gibson, W.R. & Morton, D.M. (1978) *An intracutaneous sensitization study of narasin (Lilly compound 79891) in guinea pigs*. Unpublished study No. G-D-5-74 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Bennett, T.H. & Elliott, R.A. (1976) *The effect of passage of seven microorganisms in subinhibitory levels of narasin on their resistance to 14 antibiotics.* Unpublished study from the Animal Science Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Berg, D.H. & Hamill, R.L. (1978) The isolation and characterization of narasin, a new polyether antibiotic. *J. Antibiot. (Tokyo)*, **31**(1), 1–6.

Bridge, T.L. (1984a) *The acute oral toxicity of narasin (compound 79891) in the ICR mouse*. Unpublished GLP studies Nos M-0-129-83 and M-0-128-83 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Bridge, T.L. (1984b) *The acute oral toxicity of narasin (compound 79891) in the Fischer 344 rat*. Unpublished GLP studies Nos R-0-81-83 and R01883 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Broddle, W.D., Arthur, B.H., Griffing, W.J., Gibson, W.R. & Morton, D.M. (1978) *Acute toxicity studies of narasin (Lilly compound 79891) in various species*. Unpublished studies Nos R-V-8-76, M-0-76-75, M-0-77-75, R-0-121-75, R-0-122-75, B-0-12-75, D-3455, H-8505, R-H-38-75, B-D-69-75, B-D-85-75, B-E-52-75 and B-E-68-75 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

CLSI (2004) *Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard*, 6th ed. Wayne, PA, USA, Clinical and Laboratory Standards Institute (CLSI Document M11-A6).

Cochrane, R.L. (1985a) *The toxicity of narasin (compound 79891) to mallards in a five-day dietary study*. Unpublished GLP studies Nos A01283 and A01983 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Cochrane, R.L. (1985b) *The toxicity of narasin (compound 79891) to bobwhite in a fourteen-day acute oral study*. Unpublished GLP study No. A00983 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Cochrane, R.L. (1985c) *The toxicity of narasin (compound 79891) to bobwhite in a five-day dietary study*. Unpublished GLP study No. A01083 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Cochrane, R.L. (1985d) *The toxicity of narasin (compound 79891) to bobwhite in a five-day dietary study*. Unpublished GLP study No. A02183 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Codex Alimentarius Commission (2007) *Report of the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods, Breckenridge, CO, USA, 3–7 September 2007*. Rome, Italy, Food and Agriculture Organization of the United Nations (ALINORM 08/31/31; http://www.codexalimentarius.net/web/archives.jsp?year=08).

Ferguson, E. (1995) *Narasin: Safety evaluation following dietary administration to fattening swine as Monteban 100 premix.* Unpublished study No. 1388/1-1011 from Corning Hazelton, Harrogate, North Yorkshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Grothe, D.W. & Francis, P.C. (1983) *The acute toxicity of crystalline narasin (compound 79891) to bluegill (*Lepomis macrochirus*) in a static test system.* Unpublished GLP study No. F08182 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Grothe, D.W. & Mohr, R.R. (1985) *The acute toxicity of narasin (compound 79891) to* Daphnia magna *in a static test system.* Unpublished GLP study No. C01883 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Hamelink, J.L. (1985a) *The acute toxicity to bluegill (*Lepomis macrochirus*) of narasin (compound 79891).* Unpublished GLP study No. F05183 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Hamelink, J.L. (1985b) *The acute toxicity to rainbow trout (*Salmo gairdneri*) of narasin (compound 79891).* Unpublished GLP study No. F05283 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Hashizume, M. (2000) *The safety of narasin in broiler chickens under simulated use conditions*. Unpublished GLP study No. T2NJA9836 from the Research Institute for Animal Science in Biochemistry and Toxicology, Kanagawa Prefecture, Japan. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Hill, L.E. (1981) *The effect of narasin (Lilly compound 79891) on the induction of DNA repair synthesis in primary cultures of adult rat hepatocytes.* Unpublished GLP study No. 801021-332 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Hill, L.E. (1983) *The effect of narasin (Lilly compound 79891, EL-991) on the induction of DNA repair synthesis in primary cultures of adult rat hepatocytes.* Unpublished GLP studies Nos 8030927UDS816 and 831005UDS816 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Jeffers, T.K. (1981) Resistance and cross-resistance studies with narasin, a new polyether antibiotic anticoccidial drug. *Avian Dis*., **25**(2), 395–403.

Karnak, R.E., Kehr, C.C. & Hamelink, J.L. (1978) *The toxicity of compound 79891 (narasin) to bluegills in a 96-hour static test*. Unpublished study No. 2116-78 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Karnak, R.E., Kehr, C.C. & Hamelink, J.L. (1979a) *The toxicity of compound 79891 (narasin) to rainbow trout in a 96-hour static study*. Unpublished study No. 2080-78 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Karnak, R.E., Kehr, C.C. & Hamelink, J.L. (1979b) *The toxicity of compound 79891 (narasin) to bobwhite quail in an acute oral study*. Unpublished study No. 703578 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Karnak, R.E., Kehr, C.C. & Hamelink, J.L. (1979c) *The toxicity of compound 79891 (narasin) to* Daphnia magna *in a 48-hour static study*. Unpublished study No. 5056-78 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Levitt, M.I., Arthur, B.H. & Jordan, W.H. (1979) *Single dose oral toxicity testing of narasin (Lilly compound 79891) in rats*. Unpublished GLP studies Nos R-0-202-79 and R-0-203-79 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Manthey, J.A. (1977a) *Comparative metabolism of [^{14}C] narasin in the chicken and the rat.* Unpublished report from Agricultural Chemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to FAO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Manthey, J.A. (1977b) *Excretion of [^{14}C] narasin by chickens and rats.* Unpublished studies Nos Q61-3414 and Q61-3422-68 from Agricultural Chemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Manthey, J.A. & Goebel, G.V. (1982) *Isolation and characterization of narasin metabolites derived from excreta of orally dosed chickens*. Unpublished study from Elanco Animal Health, Eli Lilly and Company, Greenfield, IN, USA. Submitted to FAO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Manthey, J.A. & Goebel, G.V. (1986) *Comparative metabolism of [^{14}C] narasin in orally dosed cattle, dog and rats.* Unpublished GLP studies Nos ABC-0126 and ABC-0127 from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Manthey, J.A., Handy, P.R., Van Duyn, R.L. & Herberg, R.J. (1981) *Determination of residue levels in tissues of chickens dosed orally with 100 ppm ^{14}C narasin ration for four or six days.* Unpublished study No. ABC-0059 from Agricultural Chemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Manthey, J.A., Herberg, R.J. & Van Duyn, R.L. (1982) *A ^{14}C narasin tissue residue and comparative metabolism study in cattle*. Unpublished study No. ABC-0137 from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Manthey, J.A., Herberg, R.J., Mattingly, C.L., Hanasono, G.K. & Donoho, A.L. (1983) *[^{14}C] narasin tissue residue bioavailability study*. Unpublished study No. ABC-0150 from Elanco Animal Health, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Manthey, J.A., Herberg, R.J. & Thomson, T.D. (1984a) *A study to determine the rate of decline of ^{14}C narasin residues from edible tissues of cattle dosed orally for five days with narasin.* Unpublished GLP study No. ABC-0264 from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Manthey, J.A., Herberg, R.J. & Van Duyn, R.L. (1984b) *^{14}C narasin balance-excretion study in cattle*. Unpublished GLP study No. ABC-0125 from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Markham, J.K. (1980a) *A pilot reproduction study with narasin (compound 79891) in the rabbit*. Unpublished GLP study No. B-7086 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Markham, J.K. (1980b) *A teratology study with narasin (compound 79891) in the rabbit.* Unpublished GLP study No. B-7396 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Markham, J.K. & Blubaugh, M.M. (1980a) *A multigeneration study with narasin (compound 79891) in the rat.* Unpublished GLP studies Nos R-236, R-916, R-1056 and R-127 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Markham, J.K. & Blubaugh, M.M. (1980b) *Teratology studies conducted during a multigeneration study with narasin (compound 79891) in the rat.* Unpublished GLP studies Nos R-916, R-1056 and R-127 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Marroquin, F., Gibson, W.R. & Morton, D.M. (1978a) *Subacute effects in mice of the oral administration of narasin (Lilly compound 79891) for 3 months*. Unpublished study No. M-9125 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Marroquin, F., Gibson, W.R. & Morton, D.M. (1978b) *Subacute effects in rats of the oral administration of mycelial narasin (Lilly compound 79891) for 3 months*. Unpublished study No. R-885 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Marroquin, F., Gibson, W.R. & Morton, D.M. (1978c) *The subacute toxicological evaluation of oral administration of narasin (Lilly compound 79891) in dogs for 3 months*. Unpublished study No. D-3815 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Mitani, M., Yamanishi, T., Miyazaki, Y. & Otake, N. (1976) Salinomycin effects on mitochondrial ion translocation and respiration. *Antimicrob. Agents Chemother.*, **9**(4), 655–660.

Neal, S.B. (1984) *The effect of crystalline narasin (compound 79891) on the in vivo induction of sister chromatid exchange in bone marrow of Chinese hamsters*. Unpublished GLP studies Nos 830913SCE816, 830922SCE816, 831011SCE816 and 840109SCE816 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Nelson, J.R. & Novilla, M.N. (1987) *An acute toxicity study of narasin alone or in combination with growth promotants in pigs*. Unpublished study No. T2N-CA-8601 from Elanco, Division of Eli Lilly Canada Inc., London, Ontario, Canada. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Nelson, R.J. & Novilla, M.M. (1992) *Safety evaluation of narasin in swine rations: Growth performance and margin of safety*. Unpublished studies Nos T6K-CA-8701 and T6K-CA-8702 from Elanco, Division of Elli Lilly Canada Inc., London, Ontario, Canada. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Novilla, M.N. (1980) *Two-year chronic toxicity studies with narasin (compound 79891) in Wistar rats derived from parents fed narasin*. Unpublished GLP studies Nos R-766 and R-776 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Novilla, M.N. & Bernhard, N.R. (1986) *A chronic toxicity study of narasin (79891) administered to Beagle dogs for one year*. Unpublished GLP study No. D04183 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Novilla, M.N. & Probst, K.S. (1986) *A chronic toxicity/oncogenicitiy study in B6C3F1 mice maintained for two years on diets containing narasin (79891).* Unpublished GLP studies Nos M01783 and M01883 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Novilla, M.N. & Van Duyn, R.L. (1985) *A safety evaluation of narasin fed to broiler chickens under simulated use conditions*. Unpublished GLP study No. T2NVX8402 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Novilla, M.N. & Van Duyn, R.L. (1987) *An acute oral toxicity study of narasin (compound 79891) in horses*. Unpublished GLP study No. T2NVX8409 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Novilla, M.N. & Young, S.S. (1979) *The toxicological evaluation of narasin (Lilly compound 79891) fed to rats for one year*. Unpublished study No. R-1046 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Novilla, M.N., Carter, J.L., Gibson, W.R., Todd, G.C. & Morton, D.M. (1978) *The chronic toxicological evaluation of narasin (Lilly compound 79891) given orally to dogs for six months*. Unpublished report No. D-3236 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Oberly, T.J. (1984) *The effect of crystalline narasin (EL-991; Compound 79891) on the induction of forward mutation at the thymidine kinase locus of L5178Y mouse lymphoma cells.* Unpublished GLP study No. 830927MLA0816 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Owen, N.V. (1978) *Subacute effects in mice of the oral administration of narasin (Lilly compound 79891) for three months.* Unpublished study No. M-9046 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA.

Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pridmore, A. (2006a) *Non-clinical laboratory study: Activity of narasin against bacterial strains representing the normal human intestinal microbiota: Determination of minimum inhibitory concentration (MIC).* Unpublished GLP study No. DWS/041/05 from Don Whitley Scientific Limited, Shipley, West Yorkshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pridmore, A. (2006b) *Non-clinical laboratory study: Effect of fecal binding on the antibacterial activity of narasin.* Unpublished GLP study No. DWS/042/05 from Don Whitley Scientific Limited, Shipley, West Yorkshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Readnour, R.S. (2000) *Monteban. Expert report on tissue residue documentation for poultry*. Unpublished report from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Rexroat, M.A. (1984) *The effect of crystalline narasin (compound 79891) on the induction of reverse mutations in* Salmonella typhimurium *using the Ames test.* Unpublished GLP study No. 831128AMS816 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Russell, J.B. & Strobel, H.J. (1989) Effect of ionophores on ruminal fermentation. *Appl. Environ. Microbiol*., **55**(1), 1–6.

Sweeney, D.J. & Kennington, A.S. (1994) *Narasin metabolite study with rat feces*. Unpublished study No. T4H969401 from Elanco Animal Health, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Sweeney, D.J., Kennington, A.S., Buck, J.M., Ehrenfried, K.M. & Kiehl, D.E. (1995) *[^{14}C] narasin tissue residue and metabolism study in swine*. Unpublished GLP study No. T6M969501 from Animal Science Product Development, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Thompson, C.Z. & McMahon, R.E. (1978) *The effect of Lilly compound 79891, narasin, on bacterial systems known to detect mutagenic events.* Unpublished study from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Twenty, J.D. (2000) *Narasin: Safety to workers*. Unpublished report, Employee Health Services, Tippecanoe Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Van Duyn, R.L. & Novilla, M.N. (1982) *The safety of narasin in broiler chickens maintained under simulated use conditions for eight weeks.* Unpublished GLP study No. T2N758001 from the Animal Science Division, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Van Duyn, R.L. & Novilla, M.N. (1983) *The safety of narasin alone or in combination with tylosin when administered orally in a complete feed*. Unpublished study No. T3C757702 from Animal Science Research, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA

Van Duyn, R.L. & Russell, E.L. (1978a) *The acute toxicity for three preparations of narasin following a single oral administration of each to four-week-old broiler cockerels.* Unpublished study No. VPR-326-766 from the Animal Science Division, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Van Duyn, R.L. & Russell, E.L. (1978b) *The acute toxicity for three preparations of narasin following a single oral administration of each to four-week-old broiler cockerels.* Unpublished study No. VPR-331-766 from the Animal Science Division, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Van Duyn, R.L. & Russell, E.L. (1978c) *The LD_{50} values for narasin following a single oral administration to four-week-old broiler cockerels.* Unpublished study No. 766-G125-93 from the Animal Science Division, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

VICH (2004) *Studies to evaluate the safety of residues of veterinary drugs in human food: General approach to establish a microbiological ADI.* Brussels, Belgium, International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH Guideline 36; http://www.vichsec.org/pdf/05_2004/Gl36_st7_F_rev.pdf).

WHO (2007) *Critically important antimicrobials for human medicine: Categorization for the development of risk management strategies to contain antimicrobial resistance due to non-human antimicrobial use. Report of the Second WHO Expert Meeting, Copenhagen, 29–31 May 2007*. Geneva, World Health Organization, Department of Food Safety, Zoonoses and Foodborne Diseases (http://www.who.int/foodborne_disease/resistance/antimicrobials_hum an.pdf).

Wong, D. (1978) *Effects of narasin metabolites on ATPase and oxygen uptake in rat liver mitochondria.* Unpublished study No. DTW-82-01 from Elanco Animal Health, Eli Lilly and Company, Greenfield, IN, USA. Submitted to FAO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Wong, D.T., Berg, D.H., Hamill, R.H. & Wilkinson, J.R. (1977) Ionophorous properties of narasin, a new polyether monocarboxylic acid antibiotic, in rat liver mitochondria. *Biochem. Pharmacol.*, **26**, 1373–1376.

Worth, H.M. & Gibson, W.R. (1974) *Acute toxicity studies of A28086 in various species*. Unpublished studies Nos M-0-315-73, M-0-316-73, R-0-188-73, R-0-189-73 and B-0-5-73 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Yamamoto, Y. (1999) *Narasin general pharmacology*. Unpublished study No. T2NJA9833 from the Research Institute for Animal Science in Biochemistry and Toxicology, Kanagawa Prefecture, Japan. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

TYLOSIN

First draft prepared by

Dr Gladwin Roberts,[1] Dr Carl Cerniglia[2] and Dr João Palermo Neto[3]

[1] Consultant, Preston, Victoria, Australia
[2] National Center for Toxicological Research, Food and Drug Administration, Department of Health and Human Services, Jefferson, AR, United States of America (USA)
[3] Department of Pathology, Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil

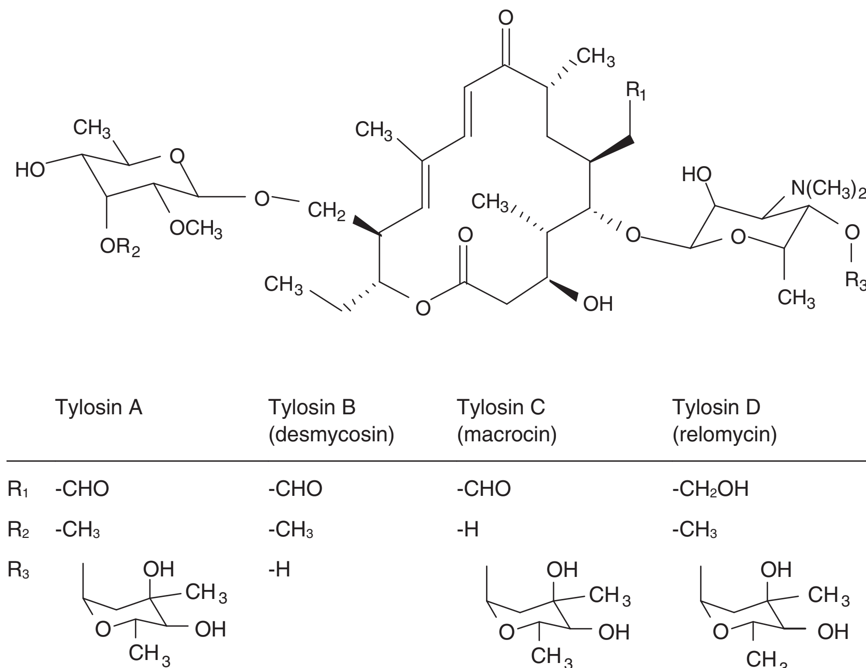
Explanation 183
Biological data 185
 Biochemical aspects 185
  Absorption, distribution, biotransformation and excretion 185
 Toxicological studies 189
  Acute toxicity 189
  Short-term studies of toxicity 191
  Long-term studies of toxicity and carcinogenicity 195
  Genotoxicity 196
  Reproductive and developmental toxicity 197
  Special studies 199
 Observations in humans 207
Comments 207
 Biochemical data 207
 Toxicological data 208
 Microbiological data 209
Evaluation 211
References 211

1. EXPLANATION

Tylosin is a macrolide antibiotic produced by fermentation from a strain of the soil microorganism, *Streptomyces fradiae.* The compound is active against Gram-positive bacteria, mycoplasma and certain Gram-negative bacteria. Like other macrolide antibiotics, tylosin inhibits protein synthesis by inhibiting aminoacyl-transfer ribonucleic acid (tRNA) and peptidyl-tRNA binding to the ribosomes.

Tylosin consists of one major factor, tylosin A. Three minor factors—desmycosin (tylosin B), macrocin (tylosin C) and relomycin (tylosin D)—may also be present in varying amounts, depending upon the manufacturing source (Figure 1). Most of the microbiological activity resides with tylosin A. Tylosin B, C and D and dihydrodesmycosin (a metabolite) have approximately 83%, 75%, 35% and 31% of the activity of tylosin A, respectively.

Figure 1. Structures of tylosin factors A, B (desmycosin), C (macrocin) and D (relomycin)



	Tylosin A	Tylosin B (desmycosin)	Tylosin C (macrocin)	Tylosin D (relomycin)
R_1	-CHO	-CHO	-CHO	$-CH_2OH$
R_2	$-CH_3$	$-CH_3$	-H	$-CH_3$
R_3		-H		

Tylosin and its phosphate and tartrate salts are used in pigs, cattle and poultry for the treatment of infections caused by organisms sensitive to tylosin. They may be administered to calves, orally in the milk replacer, at a dose of 40 mg/kg body weight (bw) and to cattle, by intramuscular injection, at a dose of 4–10 mg/kg bw. In pigs, tylosin is used for the prevention and control of diseases such as swine dysentery and enzootic pneumonia. It may be administered in the drinking-water at a dose of 25 mg/kg bw, in the feed at a dose of 3–7 mg/kg bw or by intramuscular injection at a dose of 2–10 mg/kg bw. Tylosin is administered to poultry in the drinking-water at a dose equivalent to 75 mg/kg bw. It is used primarily in the treatment of chronic respiratory disease complex in chickens and infectious sinusitis in turkeys caused by *Mycoplasma gallisepticum*. Tylosin has also been approved for emergency use in the control of American foulbrood of honey bees.

Tylosin was evaluated at the twelfth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1968 (Annex 1, reference *17*). It was concluded that tylosin used in animal feed or in veterinary medicine should not give rise to detectable residues in edible products of animal origin. No acceptable daily intake (ADI) was established.

The drug was subsequently evaluated at the thirty-eighth meeting of the Committee in 1991 (Annex 1, reference *97*). Because of deficiencies in the toxicological and microbiological data, the Committee was not able to establish an

ADI. Before reviewing the compound again, the Committee wished to have the following information:

1. Detailed information from the reported reproduction and teratogenicity studies.
2. Studies designed to explain the positive result that was obtained in the mouse lymphoma genotoxicity assay in the absence of metabolic activation.
3. Studies designed to test the hypothesis that the increased incidence of pituitary adenomas in male rats after the administration of tylosin is a consequence of the greater rate of body weight gain in these rats.
4. Studies from which a no-observed-effect level (NOEL) for microbiological effects in humans can be determined.
5. Additional studies of residues in eggs using more sensitive analytical methods.
6. Additional information on microbiologically active metabolites of tylosin.
7. Studies on the contribution of the major metabolites of tylosin to the total residues in edible tissues of cattle and pigs.

At the sixty-sixth meeting of the Committee in 2006 (Annex 1, reference *181*), none of the requested information was provided. A review of the available toxicological data in the published scientific literature indicated that publicly available data were insufficient to allow a toxicological and residue evaluation of tylosin to be performed. Therefore, tylosin could not be evaluated at the sixty-sixth meeting.

Tylosin was included on the agenda and call for data for the current meeting of the Committee as a result of a request from the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods (Codex Alimentarius Commission, 2007).

New data on microbiological effects and detailed information on the reproductive and developmental toxicity studies reported at the thirty-eighth meeting were made available for the present meeting. This report consolidates the available information for tylosin relevant to the establishment of an ADI and maximum residue limits (MRLs).

The Committee considered the results of studies on pharmacokinetics, pharmacology, acute and short-term toxicity, genotoxicity, fertility and developmental toxicity, microbiological safety and studies in humans. The genotoxicity and microbiological studies and some acute toxicity studies were carried out according to appropriate standards. The majority of the other studies were performed prior to the establishment of standards for study protocol and conduct. Several were available in summary form only and thus could not be independently assessed.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution, biotransformation and excretion

Unless otherwise mentioned, tylosin activity was measured using a microbiological assay against *Sarcina lutea*.

Pharmacokinetic data for tylosin in plasma of rats, rabbits, dogs and cats are summarized in Table 1.

Table 1. Summary of plasma kinetics in rats, rabbits, dogs and cats

Species	Test substance, dose and route	C_{max} (µg/ml)	T_{max} (h)	References
Rats, intact	Tylosin base; 50 mg/kg bw; oral	<LOQ–1.0	1	Anderson (1960); Anderson et al. (1966a)
	Tylosin tartrate; 50 mg/kg bw; oral	0.11–1.35	2	
Rabbits, intact	Tylosin hydrochloride; 10 mg/kg bw; intramuscular	0.26–0.88	1.5	Anderson (1960); Anderson et al. (1966a)
	Tylosin tartrate; 10 mg/kg bw; intramuscular	0.96–1.4	1	
	Tylosin tartrate; 25 mg/kg bw; intramuscular	4.7–7.2	1	
Dogs, intact	Tylosin base; 25 mg/kg bw; oral	1.4–2.7	2	Anderson (1960); Anderson et al. (1966a)
	Tylosin base; 100 mg/kg bw; oral	2.7–4.6	4	
Dogs, duodenal fistula	Tylosin base; 25 mg/kg bw; intraduodenal	1.98	0.5	Anderson (1960); Anderson et al. (1966a)
Dogs, intact	Tylosin hydrochloride; 10 mg/kg bw; intravenous	6.8	0.25	Anderson (1960); Anderson et al. (1966a)
Dogs, intact	Tylosin; 11 mg/kg bw; intramuscular	2.5	1	Van Duyn et al. (undated)
Dogs, intact	Tylosin; 11 mg/kg bw; intramuscular	1.8	2	Van Duyn & Kline (undated)
Cats, intact	Tylosin; 11 mg/kg bw; intramuscular	3.0	2	

C_{max}, maximum concentration in plasma; LOQ, limit of quantification; T_{max}, time to reach the C_{max}.

(a) Rats

Rats were given oral doses of 50 mg tylosin/kg bw as the base or tartrate. After 15 and 30 min and 1, 2, 4, 5, 7 and 24 h, groups of five rats were killed, and

the serum was assayed for tylosin activity. A small amount of activity was present 1–2 h after treatment (≤1.35 μg/ml), but no clear pattern was established because of high individual variability. Serum levels decreased to below the limit of quantification (LOQ = 0.1 μg/ml) within 5 h (Anderson, 1960; Anderson et al., 1966a).

Four anaesthetized rats were given an intraperitoneal injection of 100 mg tylosin/kg bw, and bile was collected for 2 h. The bile:serum ratio for tylosin activity ranged from 143 to 266 (Anderson, 1960; Anderson et al., 1966a).

Four rats received oral doses of 10 mg unlabelled tylosin/kg bw per day for 3 days, followed by similar doses of [^{14}C]tylosin for 5 days. The animals were killed 4 h after the last dose. Tissue levels of radioactivity were 0.23 mg equivalents/kg in liver, 0.18 mg equivalents/kg in kidney and 0.08 mg equivalents/kg in fat. Approximately 99% of the radiolabel was excreted in faeces, and 1% in urine. The proportion of radioactivity in faeces that was extractable was 93%. The major components of the extractable residue in rat faeces were relomycin (10%), tylosin A (6%) and dihydrodesmycosin (4%). The remaining more polar metabolites were not identified. The metabolic fate of tylosin was shown to be qualitatively similar in rats and pigs (Sieck et al., 1978).

Fischer 344 rats were given oral doses of 10 mg [^{14}C]tylosin/kg bw per day for 4 days. Urine and faeces were collected daily. Approximately 95% of the excreted radioactivity was found in faeces. The rats were euthanized 4 h after the last dose, when the liver had a mean radioactivity concentration of 0.09 mg equivalents/kg. Fractionation of radioactivity indicated the presence of multiple metabolites in liver, including tylosin A, relomycin, dihydrodesmycosin and cysteinyl-tylosin A, although evidence for the latter was not conclusive. The faeces contained relomycin (24%) and dihydrodesmycosin (11%) as the major components. Minor components in the faeces included tylosin A, macrocin, the seco acid of tylosin A, the seco acid of relomycin and desmethyl dihydrodesmycosin. The seco acids are products of hydrolysis of the lactone in the macrolide ring (Kennington & Donoho, 1994).

(b) Rabbits

Three rabbits were given an intramuscular dose of 10 mg tylosin/kg bw as the hydrochloride. Blood was collected at 1.5, 4, 7 and 24 h after treatment and assayed for tylosin activity. The highest level of activity (≤0.88 μg/ml) was found in the samples taken at 1.5 h. A further four and two rabbits were given an intramuscular injection of 10 or 25 mg tylosin/kg bw as the tartrate, respectively. Blood was taken at 1, 2, 4, 6 and 24 h after treatment and assayed for tylosin activity. Peak serum levels of activity were found at 1 h after treatment (≤1.4 and ≤7.2 μg/ml). In all animals, activity had declined to less than the limit of detection (LOD = 0.5 μg/ml) by 6–7 h (Anderson, 1960; Anderson et al., 1966a).

(c) Dogs

Tylosin activity was determined in the serum of two dogs given daily doses of 25 or 100 mg tylosin base/kg bw by capsule for 1, 15 or 29 days. Blood samples

were taken at 0, 1, 2, 3, 4, 5, 6 and 7 h after administration. Peak serum activity occurred at 2–4 h (≤2.7 and ≤4.6 μg/ml) and was less than proportional to dose (Anderson, 1960; Anderson et al., 1966a).

In a group of four dogs with a duodenal fistula, intraduodenal application of 25 mg tylosin base/kg bw gave rise to higher serum levels of activity than after oral dosing. Urinary recovery amounted to 7.2% of the dose in 5 h after intraduodenal application, but only 2% of the oral dose (Anderson et al., 1966a).

In a 2-year toxicity study in dogs, tylosin activity in blood was determined in groups of eight animals after 8, 148, 622 and 723 doses of 1, 10 or 100 mg tylosin base/kg bw per day given in capsules. After 8 days, activity was detected in the serum at all dose levels, but was less than dose proportional (≤2.15, ≤2.15 and ≤9.5 μg/ml, respectively). At later times, none of the dogs given the lowest dose contained tylosin in blood above the LOD of 0.15 μg/ml. Dogs given 10 mg/kg bw per day showed the presence of tylosin activity in the blood at levels of <LOD–1.9 μg/ml. At the 100 mg/kg bw per day dose, blood levels at 2 h were <LOD–35 μg/ml. In an extension of the study, dogs given 200 or 400 mg/kg bw per day had serum tylosin activity of 8–29 μg/ml 2 h after dosing. Activity levels were no higher and in fact appeared to be lower as the study progressed, indicating that there was no accumulation (Anderson, 1960; Anderson et al., 1966a).

Four dogs received an intravenous injection of 10 mg tylosin/kg bw as the hydrochloride. Blood was collected at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 h after treatment and assayed for tylosin activity. The highest activity in serum (6.8 μg/ml) was detected at 0.25 h and decreased with a half-life of 48 min. Urinary recovery amounted to 15.7% in the first 2 h and 18.8% over a 6-h period (Anderson, 1960; Anderson et al., 1966a).

A single intramuscular injection of Tylocine Injection at a dose of 11 mg tylosin/kg bw was given to five Beagle dogs. Blood samples were collected at 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 48 h after injection, and the serum was assayed for tylosin activity. The mean tylosin activity in the blood reached a peak of 2.5 μg/ml at 1 h; by 10 h after the injection, the concentration of tylosin in the blood had declined to the LOD of 0.1 μg/ml (Van Duyn et al., undated).

Six Beagle dogs (three male and three female) were given two intramuscular injections of Tylocine Injection at a dose of 11 mg tylosin/kg bw 12 h apart. Samples of blood were collected and assayed for tylosin activity at 2-h intervals up to 24 h after the first injection. Tylosin reached its maximum concentration (1.9 and 1.7 μg/ml) in the blood at approximately 2 h after each injection and had disappeared again after approximately 12 h (Van Duyn & Kline, undated).

One anaesthetized dog was given an intravenous injection of 10 mg tylosin/kg bw as the hydrochloride. The bile:serum ratio for tylosin activity ranged from 1233 to 3778. Approximately 13.7% of the dose was recovered in the bile in 5 h, and 25.2% was recovered in the urine (Anderson, 1960; Anderson et al., 1966a).

(d) Cats

Six cats (three males and three females) were given two intramuscular injections of Tylocine Injection at a dose of 11 mg tylosin/kg bw 12 h apart. Samples of blood were collected and assayed for tylosin activity at 2-h intervals up to 24 h after the first injection. Tylosin reached its maximum concentration (3.0 μg/ml) in the blood at approximately 2 h after each injection and had disappeared again after approximately 12 h (Van Duyn & Kline, undated).

2.2 Toxicological studies

2.2.1 Acute toxicity

In the majority of the studies, the sex of the rodent used was not specified, and the tests employed an observation period of 7 days following dose administration. This observation period is less than the 14 days currently required by regulatory guidelines. Most of these results were provided in summary form only. They are summarized in Table 2.

Table 2. Summary of acute toxicity studies with tylosin

Tylosin form	Species	No. of animals per dose (M & F)	Route of administration	Dose range (mg/kg bw)	LD_{50} (mg/kg bw)	References
Phosphate	Mouse	10	Oral	4000–6200	>6200	Anderson & Worth (1961)
Phosphate	Mouse	10	Intraperitoneal	400–800	492	Anderson & Worth (1961)
Phosphate	Mouse	10	Subcutaneous	620–1250	784	Anderson & Worth (1961)
Phosphate	Mouse	10	Intravenous	275–500	386	Anderson & Worth (1961)
Phosphate	Rat	10	Oral	4000–6200	>6200	Anderson & Worth (1961)
Phosphate	Rat	10	Intraperitoneal	700–3650	1001	Anderson & Worth (1961)
Phosphate	Rat	10	Subcutaneous	2750–5000	4083	Anderson & Worth (1961)
Base	Mouse	5–10	Oral	2500–3650	>3650	Anderson et al. (1966a)
Base	Mouse	10	Subcutaneous	1250–2500	>2500	Anderson et al. (1966a)

Table 2 (contd)

Tylosin form	Species	No. of animals per dose (M & F)	Route of administration	Dose range (mg/kg bw)	LD_{50} (mg/kg bw)	References
Base	Mouse	10	Intraperitoneal	400–800	594	Anderson et al. (1966a)
Base	Rat	10	Oral	5000	>5000	Anderson et al. (1966a)
Base	Dog	2	Oral	10–800	>800	Anderson et al. (1966a)
Tylosin hydrochloride	Mouse	5	Intravenous	400–800	582	Anderson et al. (1966a)
Lactate	Mouse	5	Intravenous	400–620	589	Anderson et al. (1966a)
Tartrate	Mouse	10	Oral	4000–6200	>6200	Anderson et al. (1966a)
Tartrate	Mouse	5–10	Oral	2500–5000	>5000	Morton (1988)
Tartrate	Mouse	5	Oral	4500–5600	>5600	Morton (1988)
Tartrate	Mouse	10	Subcutaneous	1000–2000	1354	Anderson et al. (1966a)
Tartrate	Mouse	5–10	Subcutaneous	1000–2000	1439	Morton (1988)
Tartrate	Mouse	10	Intravenous	450–700	589	Anderson et al. (1966a)
Tartrate	Mouse	5	Intravenous	400–800	668	Morton (1988)
Tartrate	Mouse	10	Intravenous	400–500	435	Morton (1988)
Tartrate	Mouse	10	Intravenous	400–620	436	Morton (1988)
Tartrate	Mouse	10	Intravenous	225–500	321	Quarles (1983)
Tartrate	Rat	10	Oral	4000–6200	>6200	Anderson et al. (1966a)
Tartrate	Rat	10	Subcutaneous	1000–3000	>3000	Anderson et al. (1966a)
Tartrate	Rat	10	Intravenous	450–900	695	Anderson et al. (1966a)
Tartrate	Rat	10	Intraperitoneal	620–2500	>2500	Anderson et al. (1966a)

F, female; LD_{50}, median lethal dose; M, male.

Intraperitoneal treatment of mice with tylosin base caused vocalizing and fighting, suggesting irritation or pain. Death of mice was preceded by depression and prostration. High doses of tylosin base resulted in salivation, vomiting and defecation in dogs (Anderson et al., 1966a).

The intravenous toxicities of tylosin tartrate, desmycosin (tylosin B) and macrocin (tylosin C) were compared in the female ICR mouse. This study utilized 10 females per dose, and the animals were observed for 14 days following administration. Signs of toxicity included tremors, clonic convulsions and leg weakness with each compound. In addition, poor grooming was seen with tylosin, and hypoactivity and ataxia occurred with desmycosin. Body weights were unaffected. The median lethal doses (LD_{50}s) were 321, 193 and 189 mg/kg bw for tylosin, desmycosin and macrocin, respectively (Quarles, 1983).

The acute toxicity of desmycosin was determined for the mouse. The LD_{50}s for the oral, subcutaneous and intraperitoneal routes of administration of desmycosin were >5000, 1593 and 483 mg/kg bw, respectively. Values for the oral, subcutaneous and intravenous routes of administration of the tartrate salt of desmycosin were >5000, 1706 and 323 mg/kg bw, respectively (Morton, 1988).

Smitherman cross cockerels, 10 per group, were administered single doses of tylosin phosphate either orally or by subcutaneous injection. The LD_{50} was 3765 mg/kg bw orally and 501 mg/kg bw subcutaneously (Richards & Berkman, undated).

Bobwhite quail (*Colinus virginianus*), five per sex per group, were given a single oral dose of 0, 1000 or 2000 mg tylosin base/kg bw. There were no deaths, but transient diarrhoea occurred in the treated birds (Kehr et al., 1978a).

2.2.2 Short-term studies of toxicity

(a) Rats

Groups of five female Harlan rats were treated subcutaneously with tylosin base suspended in 5% acacia at 10, 25, 50 or 100 mg/kg bw per day for 28 days. Food intake and body weight gains were similar between groups, and terminal blood cell counts were within normal limits. Gross and microscopic examination of the heart, lungs, kidney, liver, spleen, ovary, uterus, stomach and intestines and salivary, thymus, pancreas, adrenal and thyroid glands revealed no abnormalities. This study had no control group, and the report was provided in summary form only (Anderson et al., 1966a,b).

Tylosin tartrate was administered by the subcutaneous route at doses of 0, 100, 250, 500 or 1000 mg/kg bw per day to groups of six male and six female Harlan rats for 28 days. Diarrhoea occurred during the 1st week of treatment in the groups receiving 250, 500 and 1000 mg/kg bw per day. Occasional soft faeces were noted at all treatment levels. There were no significant differences in body weight gain, relative organ weights or haematological parameters and no treatment-related gross or microscopic abnormalities in heart, lungs, liver, spleen, kidney, stomach, intestines, skeletal muscle, gonads, salivary gland, pancreas, adrenal, thymus, thyroid or mesenteric lymph nodes. This study was provided in summary form only (Anderson et al., 1966a,b).

Groups of six male (29 days old) and six female (28 days old) Wistar rats were given gavage doses of 0, 0.005, 0.2, 10 or 200 mg tylosin base/kg bw per day for 6 weeks. Diarrhoea was noted in most rats receiving 200 mg/kg bw per day. Body weight gain and feed intake, the day of vaginal opening and preputial separation were unaffected. At study termination, slight changes included increased platelet volume, decreased white blood cells and decreased monocytes at all doses. In females, serum levels of alanine aminotransferase (ALT) and total bilirubin were increased at the highest dose, whereas lactate dehydrogenase, follicle stimulating hormone and prolactin were decreased at doses of 0.2–200 mg/kg bw per day. In males, ALT was decreased and testosterone increased at 10 and 200 mg/kg bw per day, and prolactin and luteinizing hormone were decreased at doses of 0.2–200 mg/kg bw per day. Thyroid hormones were unaltered. Immunoglobulins G and M were decreased at 0.2 mg/kg bw per day and above. Enlarged caeca were observed at doses of 0.2–200 mg/kg bw per day, but organ weights were not meaningfully altered. Infiltration of basophilic materials or eosinophilic fluids in renal tubular epithelial cells and reduced sperm counts were found in tylosin-treated groups, but were not dose related. Sperm motility was increased at 200 mg/kg bw per day. Ribonucleic acid (RNA) isolated from pituitary glands was used to analyse gene expression. There was a dose-related increased induction of genes related to cell proliferation and adhesion in females and to metabolism, regulation of cell cycle and neuronal development in males (Jeong et al., 2007).

Groups of 15 male and 15 female Wistar rats were administered tylosin base in the diet for 1 year at levels of 0, 1000, 5000 and 10 000 mg/kg in the feed. These rats were the F_1 offspring from the fertility study (see section 2.2.5) in which parents were fed test diets for approximately 10 weeks prior to mating and thereafter. The highest level was chosen because it had been tolerated without any effect in the previous three-generation rat study. Based on food intake, the doses were equal to 0, 68–76, 345–391 and 684–842 mg/kg bw per day for weeks 1–13 and 0, 39–64, 192–283 and 391–586 mg/kg bw per day for weeks 14–52. Treated rats appeared moderately hyperirritable and hyperactive from 7 to 12 months on test, but there was no mortality attributable to treatment. Toxicologically significant changes were not observed for food consumption, body weight gain, blood chemistry parameters or organ weights. Significantly increased numbers of lymphocytes, significantly decreased numbers of neutrophils and significantly increased urine pH were observed in females given 5000 and 10 000 mg/kg diet. Microscopic examination revealed a slight increase in pituitary tumours in females of all treated groups: 1, 3, 4 and 3 adenomas and 0, 0, 1 and 0 carcinomas at 0, 1000, 5000 and 10 000 mg/kg diet, respectively. Since the tumour incidences were not dose related, the no-observed-adverse-effect level (NOAEL) was considered to be 1000 mg/kg diet, equal to 39 mg/kg bw per day (Broddle et al., 1978a).

(b) Dogs

Two female dogs were given oral doses of 25 or 100 mg tylosin base/kg bw per day in capsules for 30 days. Haematological parameters were within normal ranges. Bone marrow was normal, and ratios of myeloid to erythroid precursors

in bone marrow (M/E ratios) were within expected ranges. Haematuria and albuminuria occurred in both dogs. Gross and microscopic examinations of heart, lung, liver, pancreas, spleen, gastrointestinal tract, kidney, bladder, adrenal, thyroid and bone marrow were normal, except for the bladder, which showed mild cystitis in both animals. This study had no control group, and the report was provided in summary form only (Anderson et al., 1966a,b).

One male and one female dog were given oral doses of 25 mg tylosin base/kg bw per day in capsules twice daily for 25 days. Haematological parameters and bone marrow were normal, and the M/E ratios were within expected limits. Although a slight amount of albumin was found in the urine of the male dog, urine from the female was albumin-free. All tissues appeared normal on gross and microscopic examination. This study had no control group, and the report was provided in summary form only (Anderson et al., 1966a,b).

In a 2-year study, Beagle dogs and mixed-breed dogs were divided into groups of eight animals (both sexes) and given oral doses of 0, 1, 10 or 100 mg tylosin base/kg bw per day by capsule. After this study had progressed for 153 days, it was expanded by adding further groups of two male and two female mongrel dogs given doses of 200 or 400 mg tylosin base/kg bw per day for the remainder of the study period. Occasional diarrhoea and vomiting occurred in dogs given 10–400 mg/kg bw per day. Transient elevated bromosulfophthalein retention times were recorded in two dogs at 100 mg/kg bw per day and one dog at 400 mg/kg bw per day. Haematology, urinalysis, bone marrow sections, body weights and organ weights were unaffected, and no changes were noted in the faecal microbiological flora. At necropsy, mild pyelonephritis was found in one dog given 200 mg/kg bw per day, and bilateral nephrosis, mild chronic pyelonephritis and mild chronic cystitis were seen in one dog given 400 mg/kg bw per day. Since diarrhoea and vomiting are common observations in untreated dogs, the NOAEL was 100 mg/kg bw per day (Anderson et al., 1966a).

(c) Quail

Bobwhite quail chicks (5–10 per group) were fed diets containing 0, 1250, 2500 or 5000 mg tylosin base/kg for 5 days, followed by 3 days of basal diet. There were no treatment-related deaths or overt signs of toxicity. Food consumption and body weight gains were unaffected (Kehr et al., 1978b).

(d) Ducks

Groups of 10 mallard ducklings (*Anas platyrhynchos*) were fed diets containing 0, 1250, 2500 or 5000 mg tylosin base/kg for 5 days, followed by 3 days of basal diet. There were no overt signs of toxicity and no deaths. Food consumption and weight gains were depressed during the treatment period in all tylosin-fed ducklings, presumably due to rejection of food. All birds showed normal or accelerated weight gains during the withdrawal period (Kehr et al., 1978c).

(e) Chickens

Replicate groups of 10 1-day-old unsexed White Rock chickens were fed tylosin tartrate at levels of 0, 220, 550, 1100 or 3300 g equivalent base activity per tonne of feed. The dietary mixtures were prepared in Lilly Broiler ration for the first 9 weeks of treatment and subsequently in Lilly Pullet Grower ration. The chickens were on treatment for a total period of 18 weeks. At week 8, five chickens from each replicate of each treatment group were sacrificed, and the remaining birds were killed terminally. Body weight gain and feed conversion, haematology, organ weights and histopathology were similar between groups (Berkman & Van Duyn, undated).

Seventy-five male and 75 female 1-week-old Cobb broiler chickens were allocated into three equal groups that received 0, 0.5 or 1.5 g tylosin tartrate/l in drinking-water for 8 days. All birds remained in good health throughout the study, and no treatment-related effects on body weight gains or feed and water consumption were noted (Cameron, 1990a).

(f) Turkeys

Seventy-five male and 75 female 11-day-old “Big 6” type turkey poults were allocated into three equal groups that received 0, 0.5 or 1.5 g tylosin tartrate/l in drinking-water for 5 days. All birds remained in good health throughout the study, and no treatment-related effects on body weight gains or feed consumption were noted. There was a slight dose-related reduction in water consumption, but all group values remained within normal limits (Cameron, 1990b).

(g) Pigs

Nine male and nine female weaned pigs, approximately 8 weeks of age, were allocated into three equal treatment groups that received 0, 0.25 or 0.75 g tylosin tartrate/l in drinking-water for 10 days. All animals remained in good health throughout the study, and no treatment-related effects on body weight gains or food and water consumption were noted (Cameron, 1990c).

(h) Cattle

Nine male and nine female calves were allocated into three equal treatment groups that received 0, 1 or 3 g tylosin tartrate per calf in milk replacer feeds for 14 days. All treated animals remained in good health throughout the study. Hard, dry faeces were noted during the 2nd week of the test period in both groups treated with tylosin, but no other treatment-related clinical abnormalities were observed. There were no treatment-related effects on body weight gain or milk feed consumption (Cameron, 1990d).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Rats

Harlan rats (three animals per sex) were given diets containing 0, 1000, 3000 or 10 000 mg tylosin base/kg for 17 months. There were no effects on body weight, and haematological parameters were within normal limits. Ovary size and weights were depressed, and uterus thickening and weights were increased in 1/3, 3/3, 2/3 and 2/3 rats in the control, 1000, 3000 and 10 000 mg/kg diet groups, respectively. Squamous metaplasia in the uterus was observed in two females given 10 000 mg/kg diet. These effects were considered to be age related rather than treatment related. This study was provided in summary form only (Anderson et al., 1966a,b).

Groups of about 25 Harlan rats per sex per dose were given 0, 10, 100 or 1000 mg tylosin base/kg in the diet for 2 years. Survival at 2 years was 30%, 41%, 70% and 51% with increasing dose. There were no effects on body weight, and haematological parameters were within normal limits. There were no differences in organ weights, nor were any gross or histopathological findings attributable to tylosin treatment. This study was provided in summary form only (Anderson et al., 1966a,b).

Groups of 10 male and 10 female rats were fed diets containing 0, 20 000, 50 000, 100 000 or 200 000 mg tylosin base/kg diet for 2 years. Body weight gain and food intake were reduced at the two highest doses. All high-dose rats died within 12 months and exhibited high incidences of malnutrition and atrophy/necrosis of lymphoid organs. This study was provided in summary form only (Anderson et al., 1966a,b).

Harlan albino rats were given 0, 100 or 10 000 mg tylosin base/kg admixed in the diet for 2 years. Each group consisted of approximately 30 animals per sex. After the 16th week of feeding, 10 females and 5 males from the control and high dose groups were mated within groups. Pregnant females were allowed to litter and complete lactation and, following a 1-week rest period, were remated with a different male from the same group. This process was continued for a minimum of six pregnancies. The effects on reproductive indices are reported in section 2.2.5. Survival was enhanced in males and females of the 10 000 mg/kg diet group (57% versus 29%). Food intake and body weight gain were similar between groups, and haematology, urinalysis and organ weights were unaffected. Slightly increased fatty change in the liver was observed in both sexes given 10 000 mg/kg diet. The NOAEL was 100 mg/kg diet, equivalent to 5 mg/kg bw per day (Harris, 1961).

Weanling Wistar rats, the F_1 offspring from the fertility study (see section 2.2.5), were used in replicate 2-year chronic studies. Each study consisted of a control group of 60 rats per sex and treated groups of 40 per sex per dose given 1000, 5000 and 10 000 mg tylosin base/kg in the diet. Based on food intake, the mean doses were equal to 106, 517 and 1080 mg/kg bw per day during the 1st week of study and 39, 192 and 402 mg/kg bw per day during the last week of study. The findings were similar in both replicates. Physical signs of toxicity, haematology, blood chemistry, urinalysis and organ weights were not affected. Survival during the

final 3–6 months was slightly higher (5–10%) in tylosin-treated male rats, but was not dose related. Food consumption was increased in males and females given 5000 and 10 000 mg/kg diet, and body weight gains were generally increased in males given the highest dose. The incidence of bacterial pneumonia was decreased in a dose-related manner. The incidences of benign pituitary adenomas in male rats are outlined in Table 3. The incidences in one middle dose group and one high dose group were above the historical control range for this laboratory (1.7–23.3%). Tylosin had no effect on the incidence of malignant tumours in males or females (Gries, 1980a).

Table 3. Incidences of benign pituitary adenomas in male rats (Gries, 1980a)

	Incidence of pituitary adenomas at following dietary doses			
	0 mg/kg	1000 mg/kg	5000 mg/kg	10 000 mg/kg
Replicate 1	1/60	3/40	10/40[a]	8/40
Replicate 2	5/60	6/40	8/40	12/40[a]

[a] Incidence was higher than historical control range.

Further information was provided that purported to demonstrate a high correlation between benign pituitary tumours in male rats and food consumption/ body weight. These studies documented comparative findings from experiments in which four strains of rat were placed on restricted diets or were fed ad libitum and from another 10 separate chronic studies in Wistar rats conducted in the above laboratory. The increase in pituitary adenomas was considered not to be due to tylosin per se but secondary to the increased food consumption and survival associated with tylosin ingestion (Ross et al., 1970; Gries, 1980b; Gries & Young, 1982). Pituitary tumours are commonly observed in ageing rats, and the apparent increase in some treated groups was considered to be associated with the increased survival in treated males. The NOAEL was 10 000 mg/kg diet, equal to 402 mg/kg bw per day, the highest dose tested.

2.2.4 Genotoxicity

Tylosin was evaluated for potential genotoxicity in a battery of in vitro and in vivo tests. The results are summarized in Table 4. The tests included 1) an in vitro assay for the induction of gene mutations in L5178Y mouse lymphoma cells, 2) an in vitro assay for the induction of gene mutations in $HGPRT^+$ Chinese hamster ovary cells, 3) an in vitro chromosomal damage assay in Chinese hamster ovary cells and 4) an in vivo assay for cytogenetic damage in mouse bone marrow.

A total of three experiments were conducted with mouse lymphoma cells in the absence of metabolic activation. The mutation frequency was increased by only 2.7- to 3.8-fold at 1000 µg/ml and by 2.7- to 2.8-fold at 850 µg/ml. These concentrations were cytotoxic, and the mean per cent survival was 13% and 25% at 1000 and 850 µg/ml, respectively. Owing to decreased survival of cells, the positive mutagenicity in the mouse lymphoma assay was considered to represent

Table 4. Results of tests for genotoxicity with tylosin

End-point	Test object	Concentration/ dose[a]; tylosin form	Result	References
Forward mutation in vitro[b]	L5178Y mouse lymphoma cells	10–1000 μg/ml −S9, 10–750 μg/ml +S9[c]; tylosin (salt unknown)	Positive[d]	Michaelis (1990)
Forward mutation in vitro[b]	Chinese hamster ovary cells	100–1500 μg/ml ±S9[c]; tylosin (salt unknown)	Negative	Garriott et al. (1991a)
Chromosomal damage in vitro[e]	Chinese hamster ovary cells	500–1000 μg/ml −S9, 250–750 μg/ml +S9[c]; tylosin (salt unknown)	Negative	Kindig (1990)
Chromosomal damage in vivo[f]	Micronucleus formation in bone marrow from ICR mice	Two daily oral doses of 1250, 2500 or 5000 mg/kg bw; tylosin base	Negative	Gries et al. (1990)

[a] Concentrations/doses are expressed as tylosin base.
[b] Positive controls were ethylmethane sulfonate in the absence of S9 and 3-methylcholanthrene in the presence of S9.
[c] 9000 × *g* supernatant of rat liver used for metabolic activation.
[d] The mutation frequency was increased at 850 and 1000 μg/ml −S9.
[e] Positive controls were mitomycin C in the absence of S9 and cyclophosphamide in the presence of S9.
[f] Positive control was cyclophosphamide.

an unreliable result. The sponsor advised that the majority of mutant colonies associated with tylosin treatment were small in nature (Garriott et al., 1991b), suggesting that tylosin produces a clastogenic effect rather than a mutagenic effect (Clive et al., 1979; Moore et al., 1985). However, no satisfactory data to support this contention were provided. Owing to the markedly decreased survival of cells, the positive mutagenicity in the mouse lymphoma assay was considered to represent an unreliable result. It is concluded that tylosin presents a low potential for genetic damage.

2.2.5 Reproductive and developmental toxicity

(a) Fertility studies

(i) Mice

Groups of 7–8 male and 14–17 female ICR mice were paired and given diets containing 0, 1000 or 10 000 mg tylosin base/kg continuously for two litters in each of two generations. The commencement of treatment was variable, but in most cases it began prior to conception in the F_0 mice. Females were allowed to deliver naturally and rear their young for 4 weeks in each generation. There were no

significant effects on litter size, growth of pups, number of young weaned or fertility. The NOAEL was 10 000 mg/kg diet, equivalent to 1500 mg/kg bw per day, the highest dose tested (Tsubura et al., undated).

(ii) Rats

A rat reproduction study was conducted as part of a 2-year study in Harlan albino rats (see section 2.2.3). Groups of approximately 30 animals per sex were given 0 or 10 000 mg tylosin base/kg admixed in the diet. After the 16th week of feeding, 10 females and 5 males were mated within groups. Pregnant females were allowed to litter and complete lactation and, following a 1-week rest period, were remated with a different male from the same group. This process was continued for a minimum of six pregnancies. First litters were discarded, and 5 males and 10 females per group were selected from second litters for the subsequent F_1, F_2 and F_3 generations. Growth curves and survival of offspring and fertility and reproductive indices were similar for the control and treated groups in each succeeding generation. The NOAEL was 10 000 mg/kg diet, equivalent to 500 mg/kg bw per day, the only dose tested (Tsubura et al., undated; Harris, 1961).

Wistar rats (35 control, 25 treated per sex per dose) were fed diets containing 0, 1000, 5000 or 10 000 mg tylosin base/kg for 10 weeks prior to mating and until about 6 weeks after their offspring were distributed to 1-year chronic toxicity studies (see section 2.2.2) and 2-year carcinogenicity studies (see section 2.2.3), a total period of about 5 months. Based on food intake, the doses were equal to 0, 61–70, 311–379 and 635–795 mg/kg bw per day. There were no treatment-related clinical signs, and food intake and weight gain were similar in all groups. White blood cell counts were statistically significantly decreased in males given 10 000 mg/kg diet, but the value was within the normal range. Blood chemistry, fertility and reproductive performance of the parental generation and growth and survival of the F_1 progeny were not affected. Sera collected from parental rats after approximately 150 days on experimental diets did not contain detectable levels (LOD = 0.1 µg/ml) of tylosin. The NOAEL was 10 000 mg/kg diet, equal to 635 mg/kg bw per day, the highest dose tested (Broddle et al., 1978b; Hoyt & Higdon, 1991).

(b) Developmental studies

(i) Mice

Tylosin base was given by gavage to A/Jax or CBA strain mice (10 females per group) at doses of 0, 100, 500 or 1000 mg/kg bw per day on gestation days 7–12. Another two females (A/Jax × CBA hybrid) per group were given 0 or 1000 mg/kg bw per day on the same schedule. Four A/Jax mice per group given 0 or 500 mg/kg bw per day were allowed to deliver and rear their young for 4 weeks. The remainder were killed on gestation day 18. There were no treatment-related differences in maternal body weight gains. There were no effects on survival or external, visceral or skeletal development of fetuses. Delivered offspring were maintained until they were 9 weeks old. No adverse effects were noted in growth, survival, vaginal patency or testicular descent in the pups. Activity was normal when measured at 7 and 9 weeks, and no changes were found in sensory measurements. There were no treatment-related findings in the visceral or skeletal examinations

performed at 9 weeks. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Tsuchikawa & Akabori, undated; Kelich & Byrd, 1991).

(ii) Rats

Groups of female Wistar rats were given diets containing 0, 1000, 10 000 or 100 000 mg tylosin base/kg. Based on food intake, the doses were equal to 0, 60.5, 725 and 4800 mg/kg bw per day. Ten control and 15 animals from each treated group were dosed on gestation days 0–20 and killed on gestation day 20. There were no treatment-related effects on survival during gestation and no treatment-related fetal abnormalities from external, visceral or skeletal examination. At 100 000 mg/kg diet, body weight was depressed in dams and fetuses, and ossification was retarded. Fifteen females from the control and the two highest dose groups were treated from gestation day 0 to postnatal day 21 and allowed to deliver and rear their litters until postnatal day 21. The body weight gain of offspring was lower at 100 000 mg/kg diet. There were no remarkable findings from external, visceral or skeletal examination of the weanlings. The NOAEL was 10 000 mg/kg diet, equal to 725 mg/kg bw per day (Terashima & Maeda, undated; Kelich & Byrd, 1991).

2.2.6 Special studies

(a) Pharmacology

The general pharmacological properties of tylosin have been evaluated in dogs (Anderson, 1960; Anderson et al., 1966a). The effects of tylosin on blood pressure, cardiac activity, intestinal mobility and respiration were determined in six anaesthetized dogs. Tylosin, as the hydrochloride, was administered intravenously in doses of 10–40 mg/kg bw.

Injection at all dose levels was followed by a decrease in mean arterial pressure. This varied from a 13–18% fall following 10 mg/kg bw to 20–40% following 40 mg/kg bw. In some instances, the depressor action was transient; in other cases, it persisted for 1 h or more. This depressor action of tylosin was similar to that reported for erythromycin. In three dogs, there was a slight increase in respiration rate. There were no changes in heart rate, as indicated by the electrocardiogram (ECG). However, in one dog, the ECG pattern showed an increase in height of the T- and S-waves following the dose of 40 mg/kg bw. Duodenal motility generally tended to increase for 10–25 min. In two dogs, there was no change following 10 mg/kg bw. In one dog, there was a relaxation of the duodenum following 20 mg/kg bw and a relaxation followed by stimulation after 40 mg/kg bw.

(b) Neurotoxicity

One female and three male cats were treated subcutaneously with 200 mg tylosin tartrate/kg bw per day for 90 days. Three cats served as untreated controls. There was a slight reduction (25–35%) in the post-rotatory nystagmus response, but hearing appeared unimpaired. When the cats were allowed to fall from a height of approximately 1 m, each landed on all four feet. In addition, there was no ataxia.

It was concluded that there was no significant indication of neurotoxicity. This study was provided in summary form only (Anderson et al., 1966a).

(c) Intracellular accumulation

The uptake and intracellular accumulation of tylosin were studied in primary swine and bovine alveolar macrophage cultures using ^{14}C-labelled material. Tylosin reached a concentration of 197 μg/ml in swine macrophages and 122 μg/ml in bovine macrophages after a 24-h incubation time in culture medium containing 20 μg/ml of antibiotic. The ratio of intracellular to extracellular concentrations for tylosin was 9.8 and 6.1 for swine and bovine alveolar macrophage cultures, respectively (Chamberland, 1993).

(d) Interaction with metabolic enzymes

Sprague-Dawley rats were given intraperitoneal injections of 500 mg tylosin tartrate/kg bw per day for 3 days and killed 24 h after the last injection. Cytochrome P450 (CYP) content in liver microsomes was similar to that in untreated animals, and formation of a tylosin metabolite–CYP complex was not detected. By comparison, a number of macrolide antibiotics, most notably troleandomycin and erythromycin, induced CYP content and formed a CYP–iron–nitrosoalkane metabolite complex, leading to inhibition of enzymatic activity. Structural factors were thought to be responsible for the differing responses (Delaforge et al., 1983).

The relative CYP3A inhibiting potency and CYP–iron–nitrosoalkane metabolite complex formation of a series of macrolide antibiotics were studied in liver microsomal fractions of goat and cattle and in a cell line expressing bovine CYP3A. Tylosin tartrate showed typical complex formation as measured by spectral analysis, but was a weak inhibitor (≤10%) of the CYP3A-catalysed hydroxylation of testosterone in microsomes and was not an inhibitor in the V79 bovine CYP3A cell line. Higher complex formation and greater inhibition were exhibited by triacetyl oleandomycin and erythromycin (Zweers-Zeilmaker et al., 1999).

(e) Skin and eye irritation

Eight dogs and eight cats received two intramuscular injections of Tylocine Injection, once in each leg, at a dose of 11 mg tylosin/kg bw. The animals were killed 7 and 14 days post-injection, when internal and external examination of the injection sites showed no evidence of irritation (Van Duyn & Kline, undated).

Tylosin was applied topically to the skin of New Zealand White rabbits at a dose of 2.0 ml Tylan 200 Injection/kg bw or 2000 mg Tylosin Concentrate or Tylan Soluble/kg bw. After 24-h exposure, the treatment sites were rinsed with warm water, and the rabbits were observed for the subsequent 14 days. No treatment-related deaths or signs of systemic toxicity were observed. Very slight dermal irritation occurred, which cleared within 48 h after treatment with Tylan 200 Injection. No dermal irritation occurred after exposure to Tylosin Concentrate. Very slight irritation, which cleared within 8 days, and slight desquamation in two animals were attributed to the treatment with Tylan Soluble (Downs & Negilski, 1985a,b; Negilski & Downs, 1985).

Tylosin was placed in one eye of New Zealand White rabbits in an amount of 0.1 ml, 52 mg or 58 mg of Tylan 200 Injection, Tylosin Concentrate or Tylan Soluble, respectively. Tylan 200 Injection caused very slight conjunctival hyperaemia, which cleared within 48 h. Tylosin Concentrate caused corneal dullness, slight corneal opacity, slight to moderate iritis and moderate conjunctivitis within 1 h post-exposure. However, all irritation cleared within 14 days. Tylan Soluble caused slight to moderate corneal opacity, marked iritis and moderate conjunctivitis within 1 h. In this study, all irritation cleared within 7 days post-exposure (Downs & Negilski, 1985a,b; Negilski & Downs, 1985).

(f) Sensitization

Ten guinea-pigs were given an intraperitoneal injection of 10 mg tylosin base. One week later, the seven surviving animals were given a second 10 mg injection of tylosin. All animals died during the following 3 weeks prior to administration of the challenge dose (Anderson et al., 1966a).

Groups of three guinea-pigs were given a single intraperitoneal injection of 2, 4 or 7 mg tylosin hydrochloride/kg bw, and eight guinea-pigs were given a single intraperitoneal injection of 10 mg/kg bw. The three, two, one and two animals (respectively) surviving after 5 weeks were challenged with an intravenous dose of 5 mg tylosin/kg bw. None exhibited overt signs, indicating the absence of a sensitization response. This study was provided in summary form only (Anderson et al., 1966a).

These studies were conducted prior to the development of more advanced methods of determining sensitization potential and were also compromised by poor survival due to general systemic toxicity unrelated to sensitization.

(g) Antigenicity

Attempts were made to produce antibodies in eight rabbits injected intradermally with a combination of 100 mg tylosin lactate, human serum albumin and Freund’s complete adjuvant. Blood was collected 3 days after the sensitization phase, and the resulting sera were tested for the production of passive cutaneous anaphylaxis in guinea-pigs. There were no reactions following intravenous challenge with the antigens (Horiuchi, undated).

(h) Stimulation of hormones in vitro

Using transformed HeLa cells expressing human thyroid hormone responsive elements, tylosin tartrate, at concentrations up to 100 µmol/l, failed to exhibit any direct interaction with the receptors. However, concentrations of 1 pmol/l to 100 µmol/l inhibited triiodothyronine stimulation of receptors, with a flat dose–response (Jeong et al., 2007).

Tylosin tartrate at concentrations up to 100 µmol/l did not affect the synthesis of growth hormone in cultured rat pituitary epithelial tumour cells (ATCC CCL-82.1). The release in growth hormone stimulated by triiodothyronine was inhibited by

tylosin at concentrations from 1 pmol/l to 100 μmol/l, with a flat dose–response (Jeong et al., 2007).

(i) Microbiology

A JECFA decision tree approach that was adopted at the sixty-sixth meeting of the Committee (Annex 1, reference *181*) and complies with Guideline 36 of the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH GL36) (VICH, 2004) was used by the Committee to determine the need to establish a microbiological ADI for tylosin. The decision tree approach initially seeks to determine if there may be microbiologically active tylosin residues entering the human colon. If the answer is "no" to any of the first three steps, then no microbiological ADI is necessary. However, should such residues be present, then two end-points of public health concern are to be considered: 1) disruption of the colonization barrier and 2) increase of the population(s) of resistant bacteria. At Step 4 of the decision tree process, it is possible to provide scientific justification to eliminate testing (i.e. the need for a microbiological ADI) for either one or both end-points. Step 5 is where a microbiological ADI would be determined. Should a microbiological ADI not be necessary, then the toxicological or pharmacological ADI would be used. The Committee evaluated minimum inhibitory concentration (MIC) susceptibility, faecal binding interaction and biological activity of tylosin metabolites and used the decision tree to answer the following questions in the assessment of tylosin.

Step 1: Are residues of the drug, and (or) its metabolites, microbiologically active against representatives of the human intestinal flora?

Yes. Tylosin is microbiologically active against the majority of the bacterial genera and species representative of the human intestinal flora. Most of the intestinal bacteria tested were susceptible to tylosin, with the Gram-positive anaerobes being more susceptible than the Gram-negative strains. *Bifidobacterium* spp. and *Clostridium* spp. had the lowest MIC_{50} values (0.062 μg/ml). Tylosin did not show activity against any of the strains of *Escherichia coli* (MIC_{50} >128 μg/ml).

MICs for tylosin were determined in 100 bacterial strains representative of the normal human intestinal microflora. The organisms were isolated from faeces of healthy volunteers who had not received antibiotic therapy during the 3 months prior to faecal collection and who had experienced no symptoms of diarrhoea within 4 weeks of sample collection. Ten predominant human faecal flora species were isolated, and 10 strains of each bacterial species were grown for use in the MIC tests. For each bacterial strain used in the test, the standardized inoculum was enumerated to demonstrate compliance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2004). The MIC range, MIC_{50} and MIC_{90} results are presented in Table 5. Tylosin activity varied considerably between bacterial groups and also within the majority of groups. A consistent absence of antibacterial activity was apparent against any of the strains of *Escherichia coli*, which had MIC values >128 μg/ml. Antibacterial activity was demonstrated among the strains from other species; the most susceptible were the Gram-positive anaerobes, *Bifidobacterium*, *Clostridium*, *Eubacterium* and *Peptostreptococcus*. The MIC_{50} of *Bifidobacterium* spp. and *Clostridium* spp. was 0.062 μg/ml (Pridmore, 2006a).

Table 5. MIC values of tylosin on human microflora

Microflora[a]	Inoculum density ($\times 10^8$ cfu/ml)	Tylosin MIC values (µg/ml)			
		MIC range	MIC_{50}	MIC_{90}	Geometric mean MIC
Bacteroides fragilis	1.5–5.8	0.5–128	1	32	2.6
Bacteroides (other species)	1.8–12	0.25–32	0.5	32	1.3
Bifidobacterium spp.	0.34–6.5	0.031–2	0.062	1	0.12
Clostridium spp.	0.21–13	0.031–0.5	0.062	0.25	0.094
Enterococcus spp.	1.3–5.6	1–4	1	4	2.0
Eubacterium spp.	0.46–2.4	0.125–1	0.25	0.5	0.31
Fusobacterium spp.	0.46–3.4	0.062–64	1	4	1.5
Lactobacillus spp.	0.23–8	0.5–8	2	8	2.0
Peptostreptococcus	0.33–5.5	0.125–0.5	0.5	0.5	0.41
Escherichia coli	2.3–59	>128	>128	>128	>128

cfu, colony-forming unit.
[a] Ten isolates per organism (total 100 isolates) were used.

Step 2: Do residues enter the human colon?

Yes. Residue studies using ^{14}C radiolabelling to detect total residues have been conducted or analytical methods to detect parent tylosin have been determined in poultry, pigs, cattle, eggs and milk. Under the most conservative assumptions, tylosin residues may be present at low levels in meat products consumed by humans. Therefore, tylosin-related residues could enter the colon of a person ingesting edible tissues or milk from tylosin-treated animals.

Step 3: Do the residues entering the human colon remain microbiologically active?

Yes. Tylosin residue will be extensively transformed to metabolites with reduced activity prior to entering the colon of the consumer; within the colon, it will become diluted and substantially bound (~36%) to faecal material. Therefore, approximately 64% of tylosin residues in the human colon are free (unbound). Since tylosin metabolites reaching the colon would have 35% of the activity of tylosin A (Teeter & Meyerhoff, 2003), then approximately 22.4% of the fraction of oral dose is available and potentially microbiologically active.

Faecal binding experiments were conducted using 12 tylosin concentrations between 0 and 3.3 µg/ml in linear arithmetic increments of 0.3 µg/ml. The reference bacterial strain was *Enterococcus faecalis*, which is susceptible to tylosin. Each drug concentration was mixed with three separate sterile faecal samples at concentrations of 0 (Mueller Hinton Broth only), 25% and 50% faeces and incubated for 0, 1, 2, 6, 8 or 12 h. The antibacterial activity of the supernatant obtained from each of the incubation times was assessed for the presence or absence of bacterial

growth before and after incubation with faeces. The results are summarized in Table 6. Binding of tylosin to faeces was not influenced by the concentration of faeces present, but it was time dependent. Within 1 h, binding was of the order of 20–28%. Maximal binding of tylosin to each concentration of faeces occurred between 1 and 8 h, and the extent of the binding varied between 28.6% and 42.9%. Practical limitations made it impossible to perform in vitro faecal binding assays using faecal concentrations that exceeded 50% weight by volume (w/v). Therefore, the 50% concentration provided the closest possible in vitro representation of the in vivo situation with regard to the binding of ingested tylosin residues to intestinal contents. On this basis, it was estimated that maximal tylosin binding of undiluted faecal material would occur within 1–8 h and would probably exceed 30% (Pridmore, 2006b).

Table 6. Determination of tylosin binding to human faeces[a]

Incubation time (h)	Broth only (no faeces)	25% w/v faeces		50% w/v faeces	
	Initial drug concentration (µg/ml) required to inhibit growth (a)	Initial drug concentration (µg/ml) required to inhibit growth (c)	% of drug bound to faeces [(c − a)/c] × 100	Initial drug concentration (µg/ml) required to inhibit growth (d)	% of drug bound to faeces [(d − a)/d] × 100
0	1.2	1.2	0.0	1.5	20.0
1	1.2	1.5	20.0	1.5	20.0
2	1.2	1.2	0.0	1.5	20.0
6	1.2	1.8	33.3	1.8	33.3
8	1.2	2.1	42.9	2.1	42.9
12	1.2	2.1	42.9	2.1	42.9

[a] Concentrations of tylosin used in this experiment were 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4, 2.7, 3.0 and 3.3 µg/ml.

Tylosin is extensively metabolized in pigs and rats, demonstrated by in vivo and in vitro radiolabelled tylosin studies. The metabolic pathway in humans is unknown. The metabolism of tylosin in rats is similar to that in pigs; however, the metabolic profiles in the two species are different. Major metabolites found were tylosin D, dihydrodesmycosin and seco acid of tylosin D (Kennington & Donoho, 1994). Tylosin D and dihydrodesmycosin have about 35% of the biological activity of tylosin A. Seco acids of tylosin D are biologically inactive (Teeter & Meyerhoff, 2003).

Step 4: Is there any scientific justification to eliminate testing for either one or both end-points of concern, i.e. disruption of the colonization barrier or resistance development?

Yes. There is potential for a disruption of the colonization barrier of the human gastrointestinal tract, as tylosin is most active against both *Bifidobacterium* spp. and *Clostridium* spp. Even though the majority of tylosin residues in the colon are bound to faeces and biologically inactive and the bioavailable concentration is close to the lowest MIC_{50} (0.062 µg/ml) for both *Bifidobacterium* spp. and *Clostridium* spp., as listed in Table 4, there is a need to determine a microbiological ADI, as there were no definitive analytical chemical assay data submitted to validate the faecal binding inactivation bioassay results. Tylosin residues are unlikely, but they may disrupt the colonization barrier of the human gastrointestinal tract. In terms of resistance development, all of the bacterial species representative of the human gastrointestinal tract had MIC values above the incurred active tylosin concentrations present in the human gastrointestinal tract. Thus, the small amount of active tylosin present in the human gastrointestinal tract would exert negligible pressure in selecting for resistance. Consequently, there is no need to consider resistance development as an end-point of concern.

Step 5: 1) Determine the no-observed-adverse-effect concentrations/levels (NOAECs/NOAELs) for the end-point(s) of concern as established in Step 4; and 2) The most appropriate NOAEC/NOAEL should be used to determine the ADI_{mic}*.*

The formula for calculating the upper bound of the microbiological ADI for the end-point of concern (disruption of the colonization barrier) is as follows:

$$\text{Upper bound of the ADI } (\mu g/kg \text{ bw}) = \frac{MIC_{calc} \times \text{Mass of colon content}}{\text{Fraction of oral dose available to microorganisms} \times \text{Body weight}}$$

The equation terms are derived as described below.

MIC_{calc}: In accordance with Appendix C of VICH GL36, the estimated NOAEC (MIC_{calc}) for colonization barrier disruption uses MIC values from the lower 90% confidence limit (CL) of the mean MIC_{50} for the most relevant and sensitive human colonic bacterial genera for which the drug is active.

MIC_{calc} is derived as follows:

$$\text{Lower 90\% CL} = \text{Mean } MIC_{50} - \frac{\text{Std Dev}}{\sqrt{n} \times t_{0.10,df}}$$

where:

- Mean MIC_{50} is the mean of the log-transformed MIC_{50} values;
- Std Dev is the standard deviation of the log-transformed MIC_{50} values;
- n is the number of MIC_{50} values used in the calculations; and
- $t_{0.10,df}$ is the 90th percentile from a central t-distribution with degrees of freedom (df) = $n - 1$.

The tylosin ADI was derived from in vitro MIC data as described in VICH GL36 (VICH, 2004). The nine strains needed to determine the MIC_{calc} were chosen according to these guidelines, which state that an intrinsically resistant bacterial genus should not be included, so *Escherichia coli* was excluded. Additionally,

although VICH GL36 requires the use of genera with a MIC_{50}, the Committee used the same genera but used the MIC_{90} to determine the MIC_{calc}. The justification is based on CLSI guidelines (CLSI, 2004) and the observation that some of the strains within each of the genera tested exhibited high MICs, presumably due to resistance. Given that the strains with the high MICs are more likely to fill niches vacated by susceptible strains (i.e. those with low MICs), the argument is advanced that these strains represent a more realistic scenario of what might occur in the human colon.

The MIC_{90} values for *Bacteroides fragilis*, other *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium* spp., *Enterococcus* spp., *Eubacterium* spp., *Fusobacterium* spp., *Lactobacillus* spp. and *Peptostreptococcus* (Table 4) were used to obtain the following values:

- MIC_{90} = 5.44
- t = 1.397
- Lower 90% CL = 3.76
- MIC_{calc} = 1.698

Mass of colon content: A value of 220 g is based on the colon content measured from humans.

Fraction of oral dose available to microorganisms: It is recommended that the fraction of an oral dose available for colonic microorganisms be based on in vivo measurements for the drug administered orally. Alternatively, if sufficient data are available, the fraction of the dose available for colonic microorganisms can be calculated as 1 minus the fraction (of an oral dose) excreted in urine. Human data are encouraged, but, in their absence, non-ruminant animal data are recommended. In the absence of data to the contrary, it should be assumed that metabolites have antimicrobial activity equal to the parent compound. The fraction may be lowered if the applicant provides quantitative in vitro or in vivo data to show that the drug is inactivated during transit through the intestine.

From the discussion above, the mix of metabolites reaching the colon would have 35% of the activity of tylosin A based on results in pigs as a surrogate model for the human. The faecal inactivation study indicates that at least 36% of tylosin A is bound to faeces, so 64% is available to microorganisms. Multiplying these two "availability" factors together (i.e. 0.35 × 0.64) gives 0.224. This is the fraction of an oral dose available to microorganisms.

Body weight: The body weight of an adult is assumed to be 60 kg.

The upper bound of the microbiological ADI can therefore be calculated as follows:

$$\text{Upper bound of ADI} = \frac{1.698 \times 200\ \text{g}}{0.224 \times 60\ \text{kg}}$$

$$= 25.3\ \mu\text{g/kg bw}$$

2.3 Observations in humans

Groups of 11 or 12 human volunteers were given daily oral doses of 20 mg tylosin or a placebo control for 6 months. There was no significant increase or decrease in the total number of staphylococci or lactobacilli in weekly faecal samples. The total number of streptococci was significantly increased at the end of the 6-month period. The occurrence of tylosin-resistant organisms followed a random pattern and showed no difference between groups. In addition, no overgrowth of coliform or yeast forms occurred. Of the 336 staphylococci isolates obtained from a hospital where other antibiotics were in use but tylosin was not, only 2 were resistant to tylosin lactate at 5 μg/ml. In both studies, no regular pattern was observed in cross-resistance to related and other antibiotics (Malin & Silliker, 1966).

Healthy adults (two per group) were given tylosin at oral doses of 0, 2 or 5 mg/day for 3 months. Faeces were inspected for *Escherichia coli*, enterococci and staphylococci at intervals of 1–2 weeks from 2 months prior to administration to 3 months after the start of administration. Bacterial counts were highly variable, but were not influenced by treatment with tylosin. At no time was any change in susceptibility or resistance pattern observed (Kuwabara, undated).

Of 3812 human cultures of *Staphylococcus aureus*, *Streptococcus pyogenes* and *Campylobacter* spp. isolated between May 1985 and April 1987, only 1% was found to be resistant to tylosin. There was no evidence for a significant animal source of these resistant cultures (Lacey, 1987).

Cases of occupational dermatitis following exposure to tylosin have been reported (Veien et al., 1980; Verbov, 1983). These reports suggest that, in humans, tylosin may cause irritation and/or allergic dermatitis.

3. COMMENTS

3.1 Biochemical data

Administration by the oral route in rats and dogs gave rise to peak serum concentrations within 2 h, followed by a rapid decline. Based on the findings in dogs, absorption appeared to take place in the intestine rather than in the stomach. In dogs given increasing doses, absorption was less than proportional to dose. Limited tissue distribution studies in rats with radiolabelled drug revealed higher levels in liver and kidney than in fat. The urinary recovery of oral doses in rats and dogs accounted for 1–2% of the dose, with the remainder in the faeces. Biliary excretion was not quantified, but tylosin concentrations were considerably higher in bile than in serum in both rats and dogs.

The biotransformation of tylosin was extensive in rats. Major compounds found in the liver were tylosin A, relomycin and dihydrodesmycosin. In the faeces, major compounds were relomycin and dihydrodesmycosin, and minor compounds included tylosin A, macrocin and a range of metabolites arising from hydrolysis of the lactone ring.

3.2 Toxicological data

Tylosin was relatively non-toxic in mice, rats and dogs after single oral dosing with the base and a variety of salts. The oral LD_{50} was generally >5000 mg/kg bw in rodents and >800 mg/kg bw in dogs.

Several short-term toxicity studies were performed in rats and dogs that received oral doses. Studies of up to 1 month in duration were provided in summary form only and were unsuitable for evaluation. In a 1-year rat study, there were increases in the numbers of lymphocytes and urinary pH was elevated at doses of 5000 mg/kg in the feed and above. The NOAEL in rats was 1000 mg/kg in the feed, equal to 39 mg/kg bw per day.

In a 2-year dog study, tylosin was given by capsule at doses of 0, 1, 10, 100, 200 or 400 mg/kg bw per day. Pyelonephritis was seen at 200 mg/kg bw per day, and pyelonephritis, nephrosis and cystitis were seen at 400 mg/kg bw per day. The NOAEL in dogs was 100 mg/kg bw per day.

Five long-term studies in orally dosed rats were available. Three studies were provided in summary form only and were unsuitable for evaluation. In the other studies, survival was enhanced by exposure to tylosin. In one study, fatty change in the liver was increased at a dose of 10 000 mg/kg in the feed, equivalent to 500 mg/kg bw per day. The other study revealed increased food intake at 5000 and 10 000 mg/kg in the feed and body weight gain at 10 000 mg/kg in the feed. At these same doses, the incidences of pituitary adenomas were increased in males. This type of tumour is commonly observed in ageing rats, and the apparent increase in some treated groups was considered to be associated with the increased survival in treated males. The overall NOAEL in rats was 5000 mg/kg in the feed, equal to 192 mg/kg bw per day.

Tylosin was evaluated for potential genotoxicity in an in vitro assay for the induction of gene mutations in L5178Y mouse lymphoma cells, in an in vitro assay for the induction of gene mutations in $HGPRT^{+}$ Chinese hamster ovary cells, in an in vitro chromosomal damage assay in Chinese hamster ovary cells and in an in vivo assay for cytogenetic damage in mouse bone marrow. Negative findings were obtained in Chinese hamster ovary cells investigated for gene mutations and chromosomal damage and in mouse bone marrow investigated for cytogenetic damage. An increase in gene mutations was detected in mouse lymphoma cells, in the absence of metabolic activation only. Owing to the markedly decreased survival of cells, the positive mutagenicity in the mouse lymphoma assay was considered to represent an unreliable result. It is concluded that tylosin presents a low potential for genetic damage.

Multigeneration reproduction studies were conducted in mice and rats. No adverse effects were found on reproductive indices or on growth and survival of offspring. The NOAEL was 10 000 mg/kg in the feed in all studies, equivalent to 1500 mg/kg bw per day in mice and 500 mg/kg bw per day in rats.

In developmental studies, fetal growth and development were unaffected at the highest administered doses of 1000 mg/kg bw per day in mice (given by gavage) and 725 mg/kg bw per day in rats (given as 10 000 mg/kg in the feed).

The most relevant study for determining a toxicological NOAEL is the 2-year study in dogs. The NOAEL was 100 mg/kg bw per day, and a safety factor of 100 was considered appropriate. Therefore, an ADI of 0–1 mg/kg bw could be established on the basis of the toxicological data.

3.3 Microbiological data

A JECFA decision tree approach that was adopted by the sixty-sixth meeting of the Committee (Annex 1, reference *181*) and complies with VICH GL36 (VICH, 2004) was used by the Committee to determine the need for, and to establish, if necessary, a microbiological ADI for tylosin. Studies of microbiological activity against bacterial strains representative of the human colonic flora were presented. A consistent absence of antibacterial activity against any of the strains of *Escherichia coli*, which had MIC values >128 µg/ml, was apparent. Antibacterial activity was demonstrated among strains from other species. The most susceptible groups were the Gram-positive anaerobes—*Bifidobacterium*, *Clostridium*, *Eubacterium* and *Peptostreptococcus*. The MIC_{50} of both *Bifidobacterium* spp. and *Clostridium* spp. was 0.062 µg/ml.

Faecal binding experiments were conducted using tylosin concentrations between 0 and 3.3 µg/ml. The reference bacterial strain was *Enterococcus faecalis*, which is susceptible to tylosin. The binding of tylosin to faeces was not influenced by the concentration of faeces present (up to 50%), but it was time dependent. Maximal binding of tylosin to faeces at each concentration occurred between 1 and 8 h, and the extent of the binding varied between 28.6% and 42.9%. Practical limitations made it impossible to perform in vitro faecal binding assays using faecal concentrations that exceeded 50% w/v. Therefore, the 50% concentration provided the closest possible in vitro representation of the in vivo situation with regard to the binding of ingested tylosin residues to intestinal contents. On this basis, it was estimated that maximal tylosin binding of undiluted faecal material would occur within 1–8 h and would probably exceed 30%.

In studies in human volunteers given up to 20 mg tylosin orally for 6 months, there was no evidence for an increase in resistant microorganisms in the faeces or for the emergence of cross-resistance to other antibiotics.

Tylosin residues may disrupt the colonization barrier of the human gastrointestinal tract, as tylosin is most active against *Bifidobacterium* spp. and *Clostridium* spp. In terms of the development of resistance, all of the bacterial species representative of the human gastrointestinal tract had MIC values above the incurred active tylosin concentrations present in the human gastrointestinal tract. Thus, the small amount of active tylosin present in the human gastrointestinal tract would exert negligible pressure in selecting for resistance. Consequently, there is no need to consider the development of resistance as an end-point of concern.

The formula for calculating the upper bound of the microbiological ADI is as follows:

$$\text{Upper bound of ADI } (\mu g/kg \text{ bw}) = \frac{MIC_{calc} \times \text{Mass of colon content}}{\text{Fraction of oral dose available to microorganisms} \times \text{Body weight}}$$

The equation terms are derived as described below.

MIC_{calc}: In accordance with Appendix C of VICH GL36, calculation of the estimated NOAEC (MIC_{calc}) for colonization barrier disruption uses MIC values from the lower 90% confidence limit (CL) of the mean MIC_{50} for the most relevant and sensitive human colonic bacterial genera.

MIC_{calc} is derived as follows:

$$\text{Lower 90\% CL} = \text{Mean } MIC_{50} - \frac{\text{Std Dev}}{\sqrt{n} \times t_{0.10,df}}$$

where:

- Mean MIC_{50} is the mean of the log-transformed MIC_{50} values;
- Std Dev is the standard deviation of the log-transformed MIC_{50} values;
- n is the number of MIC_{50} values used in the calculations; and
- $t_{0.10,df}$ is the 90th percentile from a central t-distribution with degrees of freedom (df) = $n - 1$.

The tylosin ADI was derived from in vitro MIC data as described in VICH GL36 (VICH, 2004). The strains needed to determine the MIC_{calc} were chosen according to these guidelines, which state that an intrinsically resistant bacterial genus should not be included; thus, *Escherichia coli* was excluded. Additionally, although VICH GL36 requires the use of genera with a MIC_{50}, the Committee used the same genera, but used the MIC_{90} to determine the MIC_{calc}. The justification is based on CLSI guidelines (CLSI, 2004) and the observation that some of the strains within each of the genera tested exhibited high MICs, presumably due to resistance. Given that the strains with the high MICs are more likely to fill niches vacated by susceptible strains (i.e. those with low MICs), the argument is advanced that these strains represent a more realistic scenario of what might occur in the human colon.

The MIC_{90} values for *Bacteroides fragilis*, other *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium* spp., *Enterococcus* spp., *Eubacterium* spp., *Fusobacterium* spp., *Lactobacillus* spp. and *Peptostreptococcus* were used to obtain the following values:

- $MIC_{90} = 5.44$
- $t = 1.397$
- Lower 90% CL = 3.76
- $MIC_{calc} = 1.698$

Mass of colon content: A value of 220 g is based on the colon content measured from humans.

Fraction of oral dose available to microorganisms: It is recommended that the fraction of an oral dose available for colonic microorganisms be based on in vivo

measurements for the drug administered orally. Alternatively, if sufficient data are available, the fraction of the dose available for colonic microorganisms can be calculated as 1 minus the fraction (of an oral dose) excreted in urine. Human data are encouraged, but, in their absence, non-ruminant animal data are recommended. In the absence of data to the contrary, it should be assumed that metabolites have antimicrobial activity equal to that of the parent compound. The fraction may be lowered if the applicant provides quantitative in vitro or in vivo data to show that the drug is inactivated during transit through the intestine.

The mix of metabolites reaching the colon would have 35% of the activity of tylosin A based on results in pigs as a surrogate model for the human. The faecal inactivation study indicates that at least 36% of tylosin A is bound to faeces, so 64% is available to microorganisms. Multiplying these two "availability" factors together (i.e. 0.35 × 0.64) gives 0.224. This is the fraction of an oral dose available to microorganisms.

Body weight: The body weight of an adult is assumed to be 60 kg.

Therefore, the upper bound of the ADI is calculated as follows:

$$\text{Upper bound of ADI} = \frac{1.698 \times 200\ \text{g}}{0.224 \times 60\ \text{kg}}$$

$$= 25.3\ \mu\text{g/kg bw}$$

Therefore, a microbiological ADI of 0–30 μg/kg bw (rounded to one significant figure) could be derived from in vitro MIC susceptibility testing and faecal binding data.

4. EVALUATION

The sponsor addressed the concerns expressed by the Committee at its thirty-eighth meeting, which enabled a reconsideration of all the toxicological and related information and a review of new studies on microbiological effects. The Committee considered that microbiological effects were more relevant than toxicological effects for the establishment of an ADI for tylosin. Therefore, the Committee established an ADI of 0–30 μg/kg bw on the basis of the MIC data. This ADI is significantly lower than it would be if it were based on a toxicological end-point and is 6400-fold lower than the lowest dose associated with increased pituitary tumours in rats.

5. REFERENCES

Anderson, R.C. (1960) *Tylosin absorption and excretion studies*. Unpublished report No. 893/FAANIM/AM from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Anderson, R.C. & Worth, H.M. (1961) *The acute toxicity of tylosin phosphate*. Unpublished study No. 893/TACUTE/AM from Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Anderson, R.C., Harris, P.N., Lee, C.C., Maze, N., Small, R.M. & Worth, H.M. (1966a) *The toxicology and pharmacology of tylosin, an antibiotic, and some salts of tylosin*. Unpublished report No. VAR.100/c/9 from Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Anderson, R.C., Worth, H.M., Small, R.M. & Harris, P.N. (1966b) Toxicological studies on tylosin: Its safety as a food additive. *Food Cosmet. Toxicol*., **4**, 1–15.

Berkman, R.N. & Van Duyn, R.L. (undated) *Tylosin toxicity study in poultry*. Unpublished study No. VPR-53-418 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Broddle, W.D., Gossett, F.O., Adams, E.R., Hoffman, D.G., Gries, C.L., Gibson, W.R. & Morton, D.M. (1978a) *Chronic toxicity of tylosin fed to rats for one year.* Unpublished study No. R-307 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Broddle, W.D., Gossett, F.O., Adams, E.R., Hoffman, D.G., Kitchen, D.N., Gibson, W.R. & Morton, D.M. (1978b) *A study of a parental population of rats bred to produce offspring assigned to one- and two year dietary studies of tylosin.* Unpublished study No. R-1176 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Cameron, D.M. (1990a) *Tylosin (Tylan Soluble) dose tolerance study in broiler chickens*. Unpublished study No. LLY 24/90799 from the Department of Animal Science, Huntington Research Centre, Huntington, Cambridgeshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Cameron, D.M. (1990b) *Tylosin (Tylan Soluble) dose tolerance study in turkeys*. Unpublished study No. LLY 25/90800 from the Department of Animal Science, Huntington Research Centre, Huntington, Cambridgeshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Cameron, D.M. (1990c) *Tylosin (Tylan Soluble) dose tolerance study in pigs*. Unpublished study No. LLY 22/90797 from the Department of Animal Science, Huntington Research Centre, Huntington, Cambridgeshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Cameron, D.M. (1990d) *Tylosin (Tylan Soluble) dose tolerance study in calves*. Unpublished study No. LLY 23/90798 from the Department of Animal Science, Huntington Research Centre, Huntington, Cambridgeshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Chamberland, S. (1993) *Intracellular accumulation of tylosin in primary swine and bovine alveolar macrophages*. Unpublished study from the Département de Microbiologie, Centre de Recherche du CHUL, Université Laval, Ste-Foy, Québec, Canada. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Clive, D., Johnson, K.O., Spector, J.F.S., Batson, A.G. & Brown, M.M.M. (1979) Validation and characterization of the L5178Y/TK$^{+/-}$ mouse lymphoma mutagen assay system. *Mutat. Res*., **59**, 61–108.

CLSI (2004) *Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard*, 6th ed. Wayne, PA, USA, Clinical and Laboratory Standards Institute (CLSI Document M11-A6).

Codex Alimentarius Commission (2007) *Report of the 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods, Breckenridge, CO, USA, 3–7 September 2007*. Rome, Italy, Food and Agriculture Organization of the United Nations (ALINORM 08/31/31; http://www.codexalimentarius.net/web/archives.jsp?year=08).

Delaforge, M., Jaouen, M. & Mansuy, D. (1983) Dual effects of macrolide antibiotics on rat liver cytochrome P-450. Induction and formation of metabolite-complexes: A structure–activity relationship. *Biochem. Pharmacol.*, **32**(15), 2309–2318.

Downs, O.S. & Negilski, D.S. (1985a) *The acute oral, dermal, and ocular toxicity of granulated tylosin concentrate*. Unpublished studies Nos R-0-365-79, R-0-366-79, B-D-109-79 and B-E-94-79 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Downs, O.S. & Negilski, D.S. (1985b) *The acute oral, dermal, ocular and inhalation toxicity of Tylan 200 Injection*. Unpublished studies Nos R-0-344-79, R-0-343-79, B-D-103-79, B-E-87-79 and R-H-39-79 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Garriott, M.L., Michaelis, K.C. & Gries, C.L. (1991a) *The effect of tylosin on the induction of forward mutation at the HGPRT+ locus of Chinese hamster ovary cells*. Unpublished study No. 910612CHO3279 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Garriott, M.L., Oberly, T.J. & Gries, C.L. (1991b) *A revision to the summary of genetic toxicology studies with tylosin.* Unpublished studies Nos 891109CTX3279, 891129CAB3279, 891220CAB3279, 891212MNT3279, 891011MLT3279, 891017MLA3279, 891114MLA3279, 910501CHT3279, 910605CHT3279 and 910612CHO3279 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Gries, C.L. (1980a) *The toxicological evaluation of tylosin (compound 27892) given to Wistar rats in the diet for two years*. Unpublished studies Nos R-287 and R-297 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Gries, C.L. (1980b) *Compound 27892 (tylosin), studies R-287 and R-297*. Unpublished memorandum to Dr M.E. Amundson from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Gries, C.L. & Young, S.S. (1982) Positive correlation of body weight with pituitary tumor incidence in rats. *Fundam. Appl. Toxicol.*, **2**(3), 145–148.

Gries, C.L., Parton, J.W. & Garriott, M.L. (1990) *The effect of tylosin (compound 027892) on the in vivo induction of micronuclei in bone marrow of ICR mice.* Unpublished study No. 891212MNT3279 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Harris, P.N. (1961) *Chronic toxicity study R3-59*. Unpublished study No. R3-59 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Horiuchi, Y. (undated) *Antigenicity of tylosin lactate*. Unpublished study from Faculty of Medicine, Hokkaido University, Sapporo, Japan. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Hoyt, J.A. & Higdon, G.L. (1991) *A reproduction study of rats fed diets containing tylosin: Data tables for reproduction and progeny indices.* Unpublished report No. R-1176 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Jeong, S.-H., Kang, J.-W., Cho, J.-H., Choi, B.-K., Kim, E.-J., Jean, Y.-H., Woo, G.-H. & Chung, G.-S. (2007) *Toxicological study of tylosin tartrate in rats and mammalian cells.* Unpublished report from Toxicology and Chemistry Division, National Veterinary Research and Quarantine Service, Anyang, Republic of Korea. Submitted to WHO by Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

Kehr, C.C., Hamelink, J.L., Brannon, D.R. & Amundson, M.E. (1978a) *The toxicity of compound 27892 (tylosin) to bobwhite quail in an acute oral study.* Unpublished study No. 7017-78 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Kehr, C.C., Hamelink, J.L., Brannon, D.R. & Amundson, M.E. (1978b) *The toxicity of compound 27892 (tylosin) to bobwhite quail in an eight day dietary study.* Unpublished study No. 7025-78 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Kehr, C.C., Hamelink, J.L., Brannon, D.R. & Amundson, M.E. (1978c) *The toxicity of compound 27892 (tylosin) to mallard ducks in an eight-day dietary study*. Unpublished study No. 7022-78 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Kelich, S.L. & Byrd, R.A. (1991) *Teratology studies of tylosin in mice and rats: Summary tables and figures.* Unpublished report from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Kennington, A.S. & Donoho, A.L. (1994) *^{14}C-tylosin rat metabolism study*. Unpublished study No. T1X759102 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Kindig, D.E.F. (1990) *The effect of tylosin on the in vitro induction of chromosome aberrations in Chinese hamster ovary cells.* Unpublished studies Nos 891109CTX3279, 891129CAB3279 and 891220CAB3279 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Kuwabara, S. (undated) *A study on the effect of a continuous and minute amount of tylosin on human intestinal flora*. Unpublished study from the Medical Department, Toho University, Tokyo, Japan. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Lacey, R.W. (1987) *Rarity of tylosin resistance in human pathogenic bacteria.* Unpublished report from the Department of Microbiology, University of Leeds, Leeds, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Malin, B. & Silliker, J.H. (1966) *Low level tylosin and the emergence of antibiotic-resistant bacteria in humans*. Abstract, American Society of Microbiology 6th Interscience Conference on Antimicrobial Agents and Chemotherapy, Philadelphia, PA, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Michaelis, K.C. (1990) *The effect of tylosin on the induction of forward mutation at the thymidine kinase locus of L5178Y mouse lymphoma cells.* Unpublished studies Nos 891011MLT3279, 891017MLA3279 and 891114MLA3279 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Moore, M.M., Clive, D., Howard, B.E., Batson, A.G. & Turner, N.T. (1985) In situ analysis of trifluorothymidine-resistant (TFT^r) mutants of L5178Y/TK$^{+/-}$ mouse lymphoma cells. *Mutat. Res.*, 1**51,** 147–159.

Morton, D.M. (1988) *Tylan products. Expert report on toxicological documentation.* Unpublished report from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Negilski, D.S. & Downs, O.S. (1985) *The acute oral, dermal, ocular and inhalation toxicity of Tylan Soluble.* Unpublished studies Nos R-0-367-79, R-0-368-79, B-D-94-79, B-E-90-79 and R-H-40-79 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pridmore, A. (2006a) *Non-clinical laboratory study: Activity of tylosin against bacterial strains representing the normal human intestinal microbiota: Determination of minimum inhibitory concentration (MIC).* Unpublished study No. 039/05 from Don Whitley Scientific Limited, Shipley, West Yorkshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Pridmore, A. (2006b) *Non-clinical laboratory study: Effect of fecal binding on the antibacterial activity of tylosin.* Unpublished study No. 040/05 from Don Whitley Scientific Limited, Shipley, West Yorkshire, England. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Quarles, J.P. (1983) *Acute comparative intravenous toxicity testing of tylosin, desmycosin, and macrocin in the ICR mouse.* Unpublished studies Nos M-V-46-83, M-V-45-83 and M-V-44-83 from the Toxicology Division, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Richards, F.A. & Berkman, R.N. (undated) *LD_{50} determination for tylosin phosphate in chickens*. Unpublished study No. VPR-116-418 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Ross, M.H., Bras, G. & Ragbeer, M.S. (1970) Influence of protein and caloric intake upon spontaneous tumor incidence of the anterior pituitary gland of the rat. *J. Nutr.*, **100**, 177–189.

Sieck, R.F., Graper, L.K., Giera, D.D., Herberg, R.J. & Hamill, R.L. (1978) *Metabolism of tylosin in swine and rat.* Unpublished study from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Teeter, J.S. & Meyerhoff, R.D. (2003) Aerobic degradation of tylosin in cattle, chicken and swine excreta. *Environ. Res.*, **93**, 45–51.

Terashima, H. & Maeda, J. (undated) *The effect of tylosin on a fetus and a suckling-young of Wistar strain rat.* Department of Pathology, Osaka City University Medical School, Osaka City, Japan. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Tsubura, Y., Toyoshima, K., Sano, S., Nishii, Y. & Tani, M. (undated) *Effect of tylosin on mouse breeding.* Unpublished study from Second Department of Pathology, Nara Medical College, Kashihara, Japan. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Tsuchikawa, K. & Akabori, A. (undated) *On the teratogenicity of tylosin.* Unpublished study from National Institute of Genetics, Shizuoka, Japan. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Van Duyn, R.L. & Kline, R.M. (undated) *Additional studies on the pharmacology and toxicology of Tylocine® Injection in dogs and cats.* Unpublished study from Lilly Research

Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Van Duyn, R.L., Kline, R.M. & Russell, E. (undated) *Tylocine® intramuscular canine blood levels.* Unpublished study No. VPR-139-766 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Submitted to WHO by Elanco Animal Health, Division of Eli Lilly and Company, Indianapolis, IN, USA.

Veien, N.K., Hattel, T., Justesen, O. & Norholm, A. (1980) Occupational contact dermatitis due to spiramycin and/or tylosin among farmers. *Contact Dermatitis*, **6**, 410–413.

Verbov, J. (1983) Tylosin dermatitis. *Contact Dermatitis*, **9**, 325–326.

VICH (2004) *Studies to evaluate the safety of residues of veterinary drugs in human food: General approach to establish a microbiological ADI.* Brussels, Belgium, International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH Guideline 36; http://www.vichsec.org/pdf/05_2004/Gl36_st7_F_rev.pdf).

Zweers-Zeilmaker, W.M., Van Miert, A.S.J.P.A.M., Horbach, G.J. & Witkamp, R.F. (1999) In vitro complex formation and inhibition of hepatic cytochrome P450 activity by different macrolides and tiamulin in goats and cattle. *Res. Vet. Sci.*, **66**(1), 51–55.

ANNEXES

ANNEX 1

REPORTS AND OTHER DOCUMENTS RESULTING FROM PREVIOUS MEETINGS OF THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

1. *General principles governing the use of food additives* (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. *Procedures for the testing of intentional food additives to establish their safety for use* (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. *Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants)* (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. I. *Antimicrobial preservatives and antioxidants*, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. *Specifications for identity and purity of food additives (food colours)* (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. II. *Food colours*, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. *Evaluation of the carcinogenic hazards of food additives* (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. *Evaluation of the toxicity of a number of antimicrobials and antioxidants* (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. *Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents* (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. *Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants* (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. *Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants*. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).

10. *Specifications for identity and purity and toxicological evaluation of food colours*. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.
11. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases* (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).
12. *Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases.* FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.
13. *Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances* (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.
14. *Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non nutritive sweetening agents* (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.
15. *Toxicological evaluation of some flavouring substances and non nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.
16. *Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.
17. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics* (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.
18. *Specifications for the identity and purity of some antibiotics.* FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.
19. *Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances* (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.
20. *Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances.* FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.
21. *Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives.* FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.
22. *Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some*

antimicrobial agents. (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

23. *Toxicological evaluation of some extraction solvents and certain other substances.* FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.
24. *Specifications for the identity and purity of some extraction solvents and certain other substances.* FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.
25. *A review of the technological efficacy of some antimicrobial agents.* FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.
26. *Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants* (Fifteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.
27. *Toxicological evaluation of some enzymes, modified starches, and certain other substances.* FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.
28. *Specifications for the identity and purity of some enzymes and certain other substances.* FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.
29. *A review of the technological efficacy of some antioxidants and synergists.* FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.
30. *Evaluation of certain food additives and the contaminants mercury, lead, and cadmium* (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.
31. *Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate.* FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.
32. *Toxicological evaluation of certain food additives with a review of general principles and of specifications* (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).
33. *Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents.* FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.
34. *Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers.* FAO Food and Nutrition Paper, No. 4, 1978.
35. *Evaluation of certain food additives* (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series,

No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.
36. *Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives.* FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.
37. *Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives.* FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.
38. *Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances.* (Nineteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 55, 1975; WHO Technical Report Series, No. 576, 1975.
39. *Toxicological evaluation of some food colours, thickening agents, and certain other substances.* FAO Nutrition Meetings Report Series, No. 55A, 1975; WHO Food Additives Series, No. 8, 1975.
40. *Specifications for the identity and purity of certain food additives.* FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.
41. *Evaluation of certain food additives* (Twentieth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Meetings Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.
42. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 10, 1976.
43. *Specifications for the identity and purity of some food additives.* FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.
44. *Evaluation of certain food additives* (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.
45. *Summary of toxicological data of certain food additives.* WHO Food Additives Series, No. 12, 1977.
46. *Specifications for identity and purity of some food additives, including antioxidant, food colours, thickeners, and others.* FAO Nutrition Meetings Report Series, No. 57, 1977.
47. *Evaluation of certain food additives and contaminants* (Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 631, 1978.
48. *Summary of toxicological data of certain food additives and contaminants.* WHO Food Additives Series, No. 13, 1978.
49. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 7, 1978.
50. *Evaluation of certain food additives* (Twenty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 648, 1980, and corrigenda.
51. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 14, 1980.

52. *Specifications for identity and purity of food colours, flavouring agents, and other food additives.* FAO Food and Nutrition Paper, No. 12, 1979.
53. *Evaluation of certain food additives* (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.
54. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 15, 1980.
55. *Specifications for identity and purity of food additives (sweetening agents, emulsifying agents, and other food additives).* FAO Food and Nutrition Paper, No. 17, 1980.
56. *Evaluation of certain food additives* (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.
57. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 16, 1981.
58. *Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives).* FAO Food and Nutrition Paper, No. 19, 1981.
59. *Evaluation of certain food additives and contaminants* (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.
60. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 17, 1982.
61. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 25, 1982.
62. *Evaluation of certain food additives and contaminants* (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.
63. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 18, 1983.
64. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 28, 1983.
65. *Guide to specifications General notices, general methods, identification tests, test solutions, and other reference materials.* FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.
66. *Evaluation of certain food additives and contaminants* (Twenty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 710, 1984, and corrigendum.
67. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 19, 1984.
68. *Specifications for the identity and purity of food colours.* FAO Food and Nutrition Paper, No. 31/1, 1984.
69. *Specifications for the identity and purity of food additives.* FAO Food and Nutrition Paper, No. 31/2, 1984.

70. *Evaluation of certain food additives and contaminants* (Twenty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 733, 1986, and corrigendum.
71. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 34, 1986.
72. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 20. Cambridge University Press, 1987.
73. *Evaluation of certain food additives and contaminants* (Thirtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 751, 1987.
74. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 21. Cambridge University Press, 1987.
75. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 37, 1986.
76. *Principles for the safety assessment of food additives and contaminants in food.* WHO Environmental Health Criteria, No. 70. Geneva, World Health Organization, 1987 (out of print). The full text is available electronically at www.who.int/pcs.
77. *Evaluation of certain food additives and contaminants* (Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 759, 1987 and corrigendum.
78. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 22. Cambridge University Press, 1988.
79. *Specifications for the identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 38, 1988.
80. *Evaluation of certain veterinary drug residues in food* (Thirty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 763, 1988.
81. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 23. Cambridge University Press, 1988.
82. *Residues of some veterinary drugs in animals and foods*. FAO Food and Nutrition Paper, No. 41, 1988.
83. *Evaluation of certain food additives and contaminants* (Thirty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 776, 1989.
84. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 24. Cambridge University Press, 1989.
85. *Evaluation of certain veterinary drug residues in food* (Thirty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 788, 1989.
86. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 25, 1990.
87. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/2, 1990.
88. *Evaluation of certain food additives and contaminants* (Thirty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 789, 1990, and corrigenda.

89. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 26, 1990.
90. *Specifications for identity and purity of certain food additives.* FAO Food and Nutrition Paper, No. 49, 1990.
91. *Evaluation of certain veterinary drug residues in food* (Thirty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 799, 1990.
92. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 27, 1991.
93. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/3, 1991.
94. *Evaluation of certain food additives and contaminants* (Thirty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 806, 1991, and corrigenda.
95. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 28, 1991.
96. *Compendium of food additive specifications (Joint FAO/WHO Expert Committee on Food Additives (JECFA)). Combined specifications from 1st through the 37th meetings, 1956–1990*. Rome, Food and Agriculture Organization of the United Nations, 1992 (2 volumes).
97. *Evaluation of certain veterinary drug residues in food* (Thirty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 815, 1991.
98. *Toxicological evaluation of certain veterinary residues in food.* WHO Food Additives Series, No. 29, 1991.
99. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/4, 1991.
100. *Guide to specifications—General notices, general analytical techniques, identification tests, test solutions, and other reference materials.* FAO Food and Nutrition Paper, No. 5, Ref. 2, 1991.
101. *Evaluation of certain food additives and naturally occurring toxicants* (Thirty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 828, 1992.
102. *Toxicological evaluation of certain food additives and naturally occurring toxicants.* WHO Food Additive Series, No. 30, 1993.
103. *Compendium of food additive specifications: addendum 1.* FAO Food and Nutrition Paper, No. 52, 1992.
104. *Evaluation of certain veterinary drug residues in food* (Fortieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 832, 1993.
105. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 31, 1993.
106. *Residues of some veterinary drugs in animals and food.* FAO Food and Nutrition Paper, No. 41/5, 1993.
107. *Evaluation of certain food additives and contaminants* (Forty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 837, 1993.

108. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 32, 1993.
109. *Compendium of food additive specifications: addendum 2.* FAO Food and Nutrition Paper, No. 52, Add. 2, 1993.
110. *Evaluation of certain veterinary drug residues in food* (Forty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 851, 1995.
111. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 33, 1994.
112. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/6, 1994.
113. *Evaluation of certain veterinary drug residues in food* (Forty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 855, 1995, and corrigendum.
114. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 34, 1995.
115. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/7, 1995.
116. *Evaluation of certain food additives and contaminants* (Forty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 859, 1995.
117. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 35, 1996.
118. *Compendium of food additive specifications: addendum 3.* FAO Food and Nutrition Paper, No. 52, Add. 3, 1995.
119. *Evaluation of certain veterinary drug residues in food* (Forty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 864, 1996.
120. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 36, 1996.
121. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/8, 1996.
122. *Evaluation of certain food additives and contaminants* (Forty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 868, 1997.
123. *Toxicological evaluation of certain food additives.* WHO Food Additives Series, No. 37, 1996.
124. *Compendium of food additive specifications, addendum 4.* FAO Food and Nutrition Paper, No. 52, Add. 4, 1996.
125. *Evaluation of certain veterinary drug residues in food* (Forty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 876, 1998.
126. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 38, 1996.
127. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/9, 1997.

128. *Evaluation of certain veterinary drug residues in food* (Forty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 879, 1998.
129. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 39, 1997.
130. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/10, 1998.
131. *Evaluation of certain food additives and contaminants* (Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 884, 1999.
132. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 40, 1998.
133. *Compendium of food additive specifications: addendum 5.* FAO Food and Nutrition Paper, No. 52, Add. 5, 1997.
134. *Evaluation of certain veterinary drug residues in food* (Fiftieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 888, 1999.
135. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 41, 1998.
136. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/11, 1999.
137. *Evaluation of certain food additives* (Fifty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 891, 2000.
138. *Safety evaluation of certain food additives.* WHO Food Additives Series, No. 42, 1999.
139. *Compendium of food additive specifications, addendum 6.* FAO Food and Nutrition Paper, No. 52, Add. 6, 1998.
140. *Evaluation of certain veterinary drug residues in food* (Fifty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 893, 2000.
141. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 43, 2000.
142. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/12, 2000.
143. *Evaluation of certain food additives and contaminants* (Fifty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 896, 2000.
144. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 44, 2000.
145. *Compendium of food additive specifications, addendum 7.* FAO Food and Nutrition Paper, No. 52, Add. 7, 1999.
146. *Evaluation of certain veterinary drug residues in food* (Fifty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 900, 2001.
147. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 45, 2000.

148. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/13, 2000.
149. *Evaluation of certain food additives and contaminants* (Fifty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 901, 2001.
150. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 46, 2001.
151. *Compendium of food additive specifications: addendum 8.* FAO Food and Nutrition Paper, No. 52, Add. 8, 2000.
152. *Evaluation of certain mycotoxins in food* (Fifty-sixth report of the Joint FAO/ WHO Expert Committee on Food Additives). WHO Technical Report Series No. 906, 2002.
153. *Safety evaluation of certain mycotoxins in food.* WHO Food Additives Series, No. 47/FAO Food and Nutrition Paper 74, 2001.
154. *Evaluation of certain food additives and contaminants* (Fifty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 909, 2002.
155. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 48, 2002.
156. *Compendium of food additive specifications: addendum 9.* FAO Food and Nutrition Paper, No. 52, Add. 9, 2001.
157. *Evaluation of certain veterinary drug residues in food* (Fifty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 911, 2002.
158. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 49, 2002.
159. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/14, 2002.
160. *Evaluation of certain food additives and contaminants* (Fifty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 913, 2002.
161. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 50, 2003.
162. *Compendium of food additive specifications: addendum 10*. FAO Food and Nutrition Paper No. 52, Add. 10, 2002.
163. *Evaluation of certain veterinary drug residues in food* (Sixtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 918, 2003.
164. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 51, 2003.
165. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/15, 2003.
166. *Evaluation of certain food additives and contaminants* (Sixty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 922, 2004.
167. *Safety evaluation of certain food additives and contaminants.* WHO Food Additives Series, No. 52, 2004.

168. *Compendium of food additive specifications: addendum 11.* FAO Food and Nutrition Paper, No. 52, Add. 11, 2003.
169. *Evaluation of certain veterinary drug residues in food* (Sixty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 925, 2004.
170. *Residues of some veterinary drugs in animals and foods.* FAO Food and Nutrition Paper, No. 41/16, 2004.
171. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 53, 2005.
172. *Compendium of food additive specifications: addendum 12.* FAO Food and Nutrition Paper, No. 52, Add. 12, 2004.
173. *Evaluation of certain food additives* (Sixty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 928, 2005.
174. *Safety evaluation of certain food additives*. WHO Food Additives Series, No. 54, 2005.
175. *Compendium of food additive specifications: addendum 13.* FAO Food and Nutrition Paper, No. 52, Add. 13 (with Errata), 2005.
176. *Evaluation of certain food contaminants* (Sixty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 930, 2005.
177. *Safety evaluation of certain contaminants in food*. WHO Food Additives Series, No. 55/FAO Food and Nutrition Paper, No. 82, 2006.
178. *Evaluation of certain food additives* (Sixty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 934, 2006.
179. *Safety evaluation of certain food additives.* WHO Food Additives Series, No. 56, 2006.
180. *Combined Compendium of food additive specifications.* FAO JECFA Monographs 1, 2005.
181. *Evaluation of certain veterinary drug residues in food* (Sixty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 939, 2006.
182. *Residue evaluation of certain veterinary drugs.* FAO JECFA Monographs 2, 2006.
183. *Toxicological evaluation of certain veterinary drug residues in food*. WHO Food Additives Series, No. 57, 2006.
184. *Evaluation of certain food additives and contaminants* (Sixty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 940, 2007.
185. *Compendium of Food Additive Specifications.* FAO JECFA Monographs 3, 2006.
186. *Safety evaluation of certain food additives and contaminants*. WHO Food Additives Series, No. 58, 2007.
187. *Evaluation of certain food additives and contaminants* (Sixty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 947, 2007.

188. *Safety evaluation of certain food additives and contaminants*. WHO Food Additives Series, No. 59, 2008.
189. *Compendium of Food Additive Specifications*, FAO JECFA Monographs 4, 2007.
190. *Evaluation of certain food additives* (Sixty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 952, 2009.
191. *Safety evaluation of certain food additives*. WHO Food Additives Series, No. 60, 2009.
192. *Compendium of Food Additive Specifications*, FAO JECFA Monographs 5, 2009.
193. *Evaluation of certain veterinary drug residues in food* (Seventieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 954, 2009.
194. *Toxicological evaluation of certain veterinary drug residues in food.* WHO Food Additives Series, No. 61, 2009.
195. *Compendium of Food Additive Specifications*, FAO JECFA Monographs 6, 2009 (in press).

ANNEX 2

ABBREVIATIONS USED IN THE MONOGRAPHS

A	adenine
ADI	acceptable daily intake
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BMD	benchmark dose
BMD_{10}	benchmark dose (BMD) calculated for a benchmark response of 10% incidence above the modelled background incidence
BMDL	lower limit of a one-sided 95% confidence interval on the benchmark dose (BMD)
$BMDL_{10}$	lower limit of a one-sided 95% confidence interval on the benchmark dose (BMD) calculated for a benchmark response of 10% incidence above the modelled background incidence
BMDS	benchmark dose software (United States Environmental Protection Agency)
bw	body weight
CCRVDF	Codex Committee on Residues of Veterinary Drugs in Foods
cfu	colony-forming unit
CHO	Chinese hamster ovary
CLSI	Clinical and Laboratory Standards Institute
C_{max}	maximum concentration in plasma
CYP	cytochrome P450
DEN	*N*-nitrosodiethylamine
DIA	dichloroisoeverninic acid
DNA	deoxyribonucleic acid
ECG	electrocardiography; electrocardiogram
ER	estrogen receptor
ESI-MS	electrospray ionization mass spectrometry
F	female
F344	Fischer 344
FAO	Food and Agriculture Organization of the United Nations
FEV	forced expiratory volume
FVC	forced vital capacity
G	guanine
GC	gas chromatography
GD	gestation day
GGT	gamma-glutamyl transferase
GL36	Guideline 36
GLP	Good Laboratory Practice

HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
IC_{50}	50% inhibitory concentration
IgE	immunoglobulin E
IGF-1	insulin-like growth factor-1
ISP-MS	ion spray mass spectrometry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	liquid chromatography
LC_{50}	median lethal concentration
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LH	luteinizing hormone
LMG	leucomalachite green
LOD	limit of detection
LOQ	limit of quantification
M	male
MA	megestrol acetate
MG	malachite green
MGA	melengestrol acetate
MIC	minimum inhibitory concentration
MIC_{50}	minimum inhibitory concentration required to inhibit the growth of 50% of organisms
MOE	margin of exposure
MPA	medroxyprogesterone acetate
MRL	maximum residue limit
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NOEC	no-observed-effect concentration
NOEL	no-observed-effect level
NRU	neutral red uptake
OECD	Organisation for Economic Co-operation and Development
PB	phenobarbitone
PR	progesterone receptor
RT-PCR	real-time polymerase chain reaction
S9	9000 × *g* rat liver supernatant
SCGE	single-cell gel electrophoresis
SHE	Syrian hamster embryo

T	thymine
T_3	triiodothyronine
T_4	thyroxine
T_{max}	time to reach the maximum concentration in plasma
TDLU	terminal ductal lobular unit
TLC	thin-layer chromatography
TPO	thyroid peroxidase
tRNA	transfer ribonucleic acid
TSH	thyroid stimulating hormone
UDS	unscheduled DNA synthesis
USA	United States of America
v/v	by volume
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
w/v	weight by volume
w/w	by weight
WHO	World Health Organization

ANNEX 3

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

Geneva, 21–29 October 2008

MEMBERS

Professor Arturo Anadón, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, Madrid, Spain

Dr Dieter Arnold, Consultant, Berlin, Germany

Professor Alan R. Boobis, Faculty of Medicine, Imperial College London, London, England

Dr Richard Ellis, Consultant, Myrtle Beach, South Carolina, USA *(Joint Rapporteur)*

Dra Adriana Fernández Suárez, Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, Argentina

Dr Lynn G. Friedlander, Food and Drug Administration, Department of Health and Human Services, Rockville, MD, USA

Dr Kevin Greenlees, Food and Drug Administration, Department of Health and Human Services, Rockville, MD, USA *(Joint Rapporteur)*

Dr John C. Larsen, National Food Institute, Technical University of Denmark, Søborg, Denmark

Dr J.G. (Jock) McLean, Professor Emeritus, Camberwell, Victoria, Australia *(Chairman)*

Professor Joao Palermo-Neto, Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil

Dr Philip Reeves, Australian Pesticides and Veterinary Medicines Authority, Kingston, ACT, Australia

Dr Pascal Sanders, Laboratoire d'Etudes et de Recherches sur les Médicaments Vétérinaires et les Désinfectants, Agence Française de Sécurité Sanitaire des Aliments, Fougères, France

Professor G.E. Swan, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa *(Vice-Chairman)*

SECRETARIAT

Dr Susan Barlow, Consultant, Brighton, East Sussex, England *(WHO Temporary Adviser)*

Ir Astrid S. Bulder, RIKILT Institute of Food Safety – Wageningen UR, Wageningen, Netherlands *(WHO Temporary Adviser)*

Dr Carl Cerniglia, Food and Drug Administration, Department of Health and Human Services, Jefferson, AR, USA *(WHO Temporary Adviser)*

Dr Pamela Chamberlain, Covance Laboratories, Vienna, VA, USA *(WHO Temporary Adviser)*

Dr Myoengsin Choi, Department of Food Safety, Zoonoses and Foodborne Diseases, World Health Organization, Geneva, Switzerland *(WHO Staff Member)*

Dr Bernadette Dunham, Chair, Codex Committee on Residues of Veterinary Drugs in Foods, Director, Center for Veterinary Medicine, Food and Drug Administration, Department of Health and Human Services, Rockville, MD, USA *(WHO Temporary Adviser; unable to attend)*

Dr Donald Grant, Consultant, Ottawa, Ontario, Canada *(WHO Temporary Adviser)*

Dr Toshio Imai, National Institute of Health Sciences, Tokyo, Japan *(WHO Temporary Adviser)*

Dr Sang-Hee Jeong, Ministry for Food, Agriculture, Forestry and Fisheries, Anyang City, Republic of Korea *(WHO Temporary Adviser)*

Professor Bruno Le Bizec, Laboratoire d’Etude des Résidus et contaminants dans les aliments, Ecole Nationale Vétérinaire de Nantes, Nantes, France *(FAO Expert)*

Dr Jacek Lewicki, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, Warsaw, Poland *(FAO Expert)*

Dra Betty San Martín Nuñez, Faculdad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, La Pintana, Chile *(FAO Expert)*

Professor Len Ritter, Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada *(WHO Temporary Adviser)*

Dr Gladwin Roberts, Consultant, Preston, Victoria, Australia *(WHO Temporary Adviser)*

Ms Marla Sheffer, Orleans, Ontario, Canada *(WHO Editor)*

Dr Angelika Tritscher, Department of Food Safety, Zoonoses and Foodborne Diseases, World Health Organization, Geneva, Switzerland *(WHO Joint Secretary)*

Dr Annika Wennberg, Nutrition and Consumer Protection Division, Food and Agriculture Organization of the United Nations, Rome, Italy *(FAO Joint Secretary)*

Professor Shi-Xin Xu, Center for Veterinary Drug Evaluation, China Institute of Veterinary Drugs Control, Beijing, China *(FAO Expert)*

ANNEX 4

RECOMMENDATIONS ON COMPOUNDS ON THE AGENDA AND FURTHER INFORMATION REQUIRED

Avilamycin (antimicrobial agent)

Acceptable daily intake: The Committee established an ADI of 0–2 mg/kg bw on the basis of a NOAEL of 150 mg avilamycin activity/kg bw per day and a safety factor of 100 and rounding to one significant figure.

Residue definition: Dichloroisoeverninic acid (DIA)

Recommended maximum residue limits (MRLs)

Species	Skin/fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Pigs	200	200	300	200
Chickens	200	200	300	200
Turkeys	200	200	300	200
Rabbits	200	200	300	200

Dexamethasone (glucocorticosteroid)

Acceptable daily intake: The Committee established an ADI of 0–0.015 µg/kg bw at the 42nd meeting of the Committee (WHO TRS No. 851, 1995).

Residue definition: Dexamethasone

Recommended maximum residue limits (MRLs)

Species	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)	Milk (µg/l)
Cattle	1.0	2.0	1.0	0.3
Pigs	1.0	2.0	1.0	
Horses	1.0	2.0	1.0	

Malachite green (antimicrobial agent and contaminant)

Acceptable daily intake: The Committee considered it inappropriate to establish an ADI for malachite green and did not support the use of malachite green for food-producing animals.

Residues: The Committee did not recommend MRLs for malachite green and leucomalachite green, as it did not support the use of malachite green for food-producing animals.

Melengestrol acetate (production aid)

Acceptable daily intake: The Committee established an ADI of 0–0.03 μg/kg bw at its 54th meeting (WHO TRS No. 900, 2001). It did not consider it necessary to reconsider the ADI at the current meeting on the basis of new data provided.

Residues: The MRLs that were recommended by the 66th meeting of the Committee (WHO TRS No. 939, 2006) were not reconsidered and were maintained.

Monensin (antimicrobial agent and production aid)

Acceptable daily intake: The Committee established an ADI of 0–10 μg/kg bw on the basis of a NOAEL of 1.14 mg/kg bw per day and a safety factor of 100 and rounding to one significant figure.

Residue definition: Monensin

Recommended maximum residue limits (MRLs)

Species	Fat (μg/kg)	Kidney (μg/kg)	Liver (μg/kg)	Muscle (μg/kg)	Milk (μg/kg)
Cattle	100	10	10	10	2
Sheep	100	10	10	10	
Goats	100	10	10	10	
Chickens	100	10	10	10	
Turkeys	100	10	10	10	
Quail	100	10	10	10	

Narasin (antimicrobial agent and production aid)

Acceptable daily intake: The Committee established an ADI of 0–5 μg/kg bw on the basis of a NOAEL of 0.5 mg/kg bw per day and a safety factor of 100.

Residues: Narasin A

Recommended maximum residue limits (MRLs)

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Cattle	50[a]	15[a]	50[a]	15[a]
Chickens	50	15	50	15
Pigs	50	15	50	15

[a] The MRL is temporary. Before re-evaluation of narasin with the aim of recommending MRLs in tissues of cattle, the Committee would require a detailed description of a regulatory method, including its performance characteristics and validation data. This information is required by the end of 2010.

Tilmicosin (antimicrobial agent)

Acceptable daily intake:	The Committee established an ADI of 0–40 µg/kg bw at its 47th meeting (WHO TRS No. 876, 1998).
Residue definition:	Tilmicosin

Recommended maximum residue limits (MRLs)

Species	Skin/fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Chickens	250	600	2400	150
Turkeys	250	1200	1400	100

The Committee was not able to recommend a MRL for sheep milk.

Before a re-evaluation of tilmicosin with the aim of recommending MRLs in tissues of rabbits, the Committee would require adequately designed residue studies with doses and routes of administration under authorized conditions of use and using a validated method suitable for the purpose.

Triclabendazole (anthelminthic)

Acceptable daily intake:	The Committee established an ADI of 0–3 µg/kg bw at its 40th meeting (WHO TRS No. 832, 1993).
Residue definition:	Ketotriclabendazole

Recommended maximum residue limits (MRLs)

Species	Fat (μg/kg)	Kidney (μg/kg)	Liver (μg/kg)	Muscle (μg/kg)
Cattle	100	400	850	250
Sheep	100	200	300	200

Tylosin (antimicrobial agent)

Acceptable daily intake: The Committee established an ADI of 0–30 μg/kg bw based on a microbiological end-point derived from in vitro MIC susceptibility testing and faecal binding data (MIC_{calc} = 1.698).

Residue definition: Tylosin A

Recommended maximum residue limits (MRLs)

Species	Fat (μg/kg)	Kidney (μg/kg)	Liver (μg/kg)	Muscle (μg/kg)	Skin/fat (μg/kg)	Milk (μg/kg)	Eggs (μg/kg)
Cattle	100	100	100	100		100	
Pigs	100	100	100	100			
Chickens		100	100	100	100		300

This volume contains monographs prepared at the seventieth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 21 to 29 October 2008.

The toxicological monographs in this volume summarize data on the veterinary drug residues that were evaluated toxicologically by the Committee: the antimicrobial agents avilamycin and tylosin, the antimicrobial agent and contaminant malachite green, the production aid melengestrol acetate, and the antimicrobial agents and production aids monensin and narasin.

This volume and others in the WHO Food Additives Series contain information that is useful to those who produce and use food additives and veterinary drugs and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

ISBN 978 92 4 166061 7