

Toxicological evaluation of certain veterinary drug residues in food

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AVILAMYCIN

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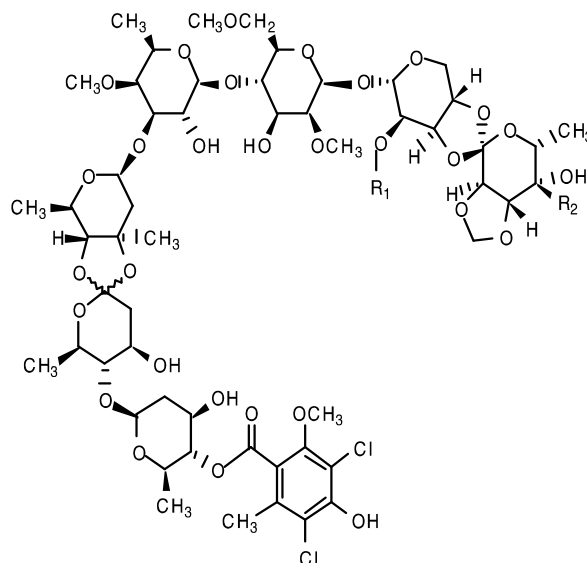
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Explanation	3
Biological data	5
Biochemical aspects	5
Absorption, distribution, excretion and metabolism	5
Biotransformation	8
Toxicological studies	8
Acute toxicity	8
Short-term studies of toxicity	8
Long-term studies of toxicity and carcinogenicity	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 dichloroisoevertinic 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 ($\geq 60\%$), 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 ₂ O ₃₂	1403	COCH(CH ₃) ₂	COCH ₃
B	C ₅₉ H ₈₄ Cl ₂ O 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 [¹⁴C]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 [¹⁴C]-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 eurenate 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 [¹⁴C]avilamycin (9.3 kBq/mg) incorporated into 450 g of diet. After the consumption of the diet containing [¹⁴C]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 ¹⁴C residues in both pigs were excreted in the first 4 days, with over 91% eliminated on days 2 and 3. The peak excretion of ¹⁴C 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 [¹⁴C]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 [¹⁴C]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 ¹⁴C 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 [¹⁴C]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 ($<0.1 \mu\text{g/g}$). The most abundant metabolite was flambic acid (metabolite B), which was present at concentrations up to $0.06 \mu\text{g/g}$. Parent [^{14}C]avilamycin concentrations in liver were less than $0.05 \mu\text{g/g}$. 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 $120 \mu\text{g/g}$ (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 $14.16 \text{ mg } [^{14}\text{C}]\text{avilamycin/kg}$ 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 $0.024 \mu\text{g/g}$, respectively, at all sampling times. The mean peak level of $0.039 \mu\text{g/g}$ 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 $0.024 \mu\text{g/g}$, 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 (LD₅₀) of the dried avilamycin products was generally higher than 5000 mg/kg bw in mice and rats. In terms of potency, the oral LD₅₀ was higher than 390 or 745 mg avilamycin activity/kg bw in both mice and rats. The LD₅₀ 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 LD₅₀ 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 LD₅₀ 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 (LC₅₀), 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 0.003–1000 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 (CrI: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	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538	1–25 µg/plate ^b	Negative	Williams & Rexroat (1983)
Ames test ^a	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537 <i>E. coli</i> WP2uvrA	0.003 33–10.0 µg/ml ^c (±S9 mix)	Negative	Mecchi (2004)
		0.1–100 µg/ml ^c (+S9 mix)	Negative	
		0.0333–33.3 µg/ml ^c (–S9 mix)	Negative	
Ames test ^f	<i>S. typhimurium</i> C3076, D3052, G46, TA98, TA100, TA1535, TA1537, TA1538	0.1–1000 µg/ml ^g (±S9 mix)	Negative	Williams & Thompson (1983a,b)
	<i>E. coli</i> 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 F₀ 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 F₂ 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 (CrI: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 μ l) 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. MIC₅₀, MIC₉₀, 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* (MIC₅₀ >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 (MIC₅₀ = 8 µg/ml), *Lactobacillus* (MIC₅₀ = 16 µg/ml) and *Bifidobacterium* spp. (MIC₅₀ = 16 µg/ml). *Peptostreptococcus* (MIC₅₀ = 0.25 µg/ml), *Eubacterium* (MIC₅₀ = 0.5 µg/ml) and *Clostridium* spp. (MIC₅₀ = 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
<i>Bacteroides fragilis</i>	4–128	8	>128	≥19.7	2–128	4	>128	≥9.8
Other <i>Bacteroides</i>	4–128	8	>128	≥16	2–128	8	>128	≥9.8
<i>Bifidobacterium</i>	2–128	16	>128	≥26	0.25–16	1	8	2.1
<i>Clostridium</i>	0.5–8	1	8	1.6	0.125–2	0.25	1	0.4
<i>Enterococcus</i>	2–4	2	4	2.5	1–2	1	2	1.1
<i>Escherichia coli</i>	All >128	>128	>128	>128	All >128	>128	>128	>128
<i>Eubacterium</i>	0.5–128	0.5	4	≥1.4	0.062–0.25	0.062	0.062	0.07
<i>Fusobacterium</i>	0.5–128	4	>128	≥8	0.5–128	1	32	3.5
<i>Lactobacillus</i>	8–128	16	>128	≥34	2–128	2	>128	≥12
<i>Peptostreptococcus</i>	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 $\mu\text{g/ml}$ 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 LD₅₀ 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 F₂ 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 F₂ 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* (MIC₅₀ >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* (MIC₅₀ = 0.25 µg/ml), *Eubacterium* (MIC₅₀ = 0.5 µg/ml) and *Clostridium* spp. (MIC₅₀ = 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.

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