

Toxicological evaluation of certain veterinary drug residues in food

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NARASIN

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1. EXPLANATION

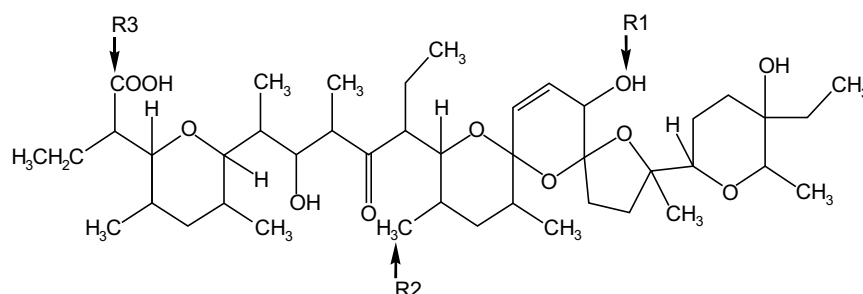
Narasin belongs to the polyether monocarboxylic acid class of ionophores produced by *Streptomyces aureofaciens* strain NRRL 8092. Narasin is composed of 96% narasin A, 1% narasin B, 2% narasin D and 1% narasin I. Narasin A has the major activity (85%). The biological activity of narasin is based on 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 ₂ H 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]-narsin/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 narsin 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 narsin 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 narsin (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 narsin 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]narsin/t, or 1.5 times the highest recommended narsin use level, by gelatine capsule, twice daily (10 g [^{14}C]narsin/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 narsin 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 narsin equivalents), corresponding to 0.918, 0.739 and 0.839 mg narsin/kg from cattle dosed for 3, 5 and 7 days, respectively. Parent narsin 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 narsin was found to be qualitatively similar for steers and heifers for all dosing intervals (Manthey et al., 1982).

A companion, GLP-compliant, [^{14}C]narsin 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]narsin equivalent to 13 g/t medicated ration. The dose was administered by gelatine capsule, twice daily (6.6 g [^{14}C]narsin/t per dose). Liver contained the highest concentrations of radioactivity, with 0.492, 0.233 and 0.050 mg narsin 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 narsin. At 1-day withdrawal, only one of three livers had detectable levels of narsin. At the 3-day withdrawal, there was no detectable narsin 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]narsin rations for 7 days at either 30 μg [^{14}C]narsin/g

followed by 0- or 3-day withdrawal or 45 µg [¹⁴C]narsin/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 narsin 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 [¹⁴C]narsin. A single mature male rat of unspecified strain was given a single oral gavage dose of 2.3 mg [¹⁴C]narsin (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 [¹⁴C]narsin (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 [¹⁴C]-narsin 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 [¹⁴C]narsin was recovered from two young Wistar rats as ¹⁴CO₂ (Manthey, 1977b).

Four 8-week-old broiler chickens preconditioned on feed containing 80 mg narsin/kg were given an oral dose of [¹⁴C]narsin. The total dose (doses given twice daily) corresponded to the daily average intake of narsin 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 [¹⁴C]narsin 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 quantitiveness 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 $\mu\text{g/g}$ with a 0-day withdrawal, 45 $\mu\text{g/g}$ with a 3-day withdrawal and 45 $\mu\text{g/g}$ 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]narsin. 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 narsin 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 trihydroxynarsin 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 dihydroxynarsin 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 narsin. 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 narsin (Manthey & Goebel, 1986).

In order to specifically determine narsin metabolism in a laboratory species, Fischer 344 rats (10 males and 10 females) were given daily oral doses of 5 mg mycelial narsin/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. Narsin metabolites in the extract were identified by high-performance liquid chromatography/ion spray mass spectrometry (HPLC/ISP-MS). At least four structural isomers of trihydroxynarsin 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 dihydroxynarsin. Another four peaks were identified as containing trihydroxynarsin B, and four were identified as containing dihydroxynarsin B. Some other peaks contained monohydroxynarsin or monohydroxynarsin 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 narsin 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 narsin (Sweeney & Kennington, 1994).

In a GLP-compliant tissue residue and metabolism study in pigs, three groups of four pigs each were fed [^{14}C]narsin rations for 7 days at 30 $\mu\text{g/g}$ with zero withdrawal (treatment 01), 30 $\mu\text{g/g}$ with a 3-day withdrawal (treatment 02) and 45 $\mu\text{g/g}$ 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/m³ 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 ± 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 ^c	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; LD₅₀, median lethal dose; M, male; SD, standard deviation.

^a LD₅₀ 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 LD₅₀ 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 LC₅₀) 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 LC₅₀ 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 (EC₅₀) that was above 16.0 mg/l and a 48-h EC₅₀ 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 EC₅₀ 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 LC₅₀ 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 LC₅₀ 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 $\times$ 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 F₀ 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	<i>S. typhimurium</i> C3076, D3052, G46, TA98, TA100, TA1535, TA1537 and TA1538 and <i>E. coli</i> WP2 and WP2uvrA tester strains	0.1–1000 µg/plate	Not mutagenic	Thompson & McMahon (1978)
Ames reverse mutation assay ^{a,b}	<i>S. typhimurium</i> 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 F₀ and F₂ 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 ($LD_{50} = 15.5 \pm 3.9$ 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. MIC₅₀, MIC₉₀ 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 MIC₅₀ of 0.125 µg/ml. Narasin exerted very weak activity against *Bacteroides fragilis* and other *Bacteroides* spp., with a MIC₅₀ 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
<i>Bacteroides fragilis</i>	8–32	32	32
<i>Bacteroides</i> (other species)	16–32	32	32
<i>Bifidobacterium</i>	0.125–4	0.5	1
<i>Clostridium</i>	0.125–1	0.25	5
<i>Enterococcus</i>	All 0.5	0.5	0.5
<i>Escherichia coli</i>	All >128	>128	>128
<i>Eubacterium</i>	0.25–0.5	0.25	0.5
<i>Fusobacterium</i>	0.125–32	8	16
<i>Lactobacillus</i>	0.5–4	1	2
<i>Peptostreptococcus</i>	0.062–0.5	0.125	0.5
All strains (<i>n</i> = 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 ¹⁴C 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 MIC₅₀ 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/m³ 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 urticaria, 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 [¹⁴C]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 [¹⁴C]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 LD₅₀ of 1 mg/kg bw or less) to horses, highly toxic (defined as an LD₅₀ of 1–50 mg/kg bw) to pigs, mice, rats and rabbits, and moderately toxic (defined as an LD₅₀ 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 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. 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 urticaria, 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 MIC₅₀ of 0.125 µg/ml. Narasin exerted very weak activity against *Bacteroides fragilis* and other *Bacteroides* spp., with a MIC₅₀ 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 MIC₅₀ 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.

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