Milbemycin: Discovery and Development

Junya IDE*#, Takao OKAZAKI[a], Michihisa ONO[b], Akio SAITO, Keiko NAKAGAWA[c], Satoru NAITO, Kazuo SATO[d], Keiji TANAKA[e], Hiroji YOSHIKAWA[c], Mitsuru ANDO[d], Shigeru KATSUMI[e], Kiyoshi MATSUMOTO[f], Toshimitsu TOYAMA, Mitsugi SHIBANO[g] and Masanori ABE[f]

Medicinal Chemistry Research Laboratories, SANKYO CO., LTD., 2-58, Hiromachi 1-chome, Shinagawa-ku, Tokyo 140, JAPAN

a) Tsukuba Research Laboratories
b) Research Planning Department
c) Fermentation Research Laboratories
d) Agricultural Chemicals Research Laboratories
e) Agrochemicals Development and Technical Services Department
f) Special Merchandise Development Department
g) Process Development Laboratories

三共研究所年報　Vol. 45　平成5年（1993）　別冊

Annu. Rep. Sankyo Res. Lab. **45**, 1-98 (1993)
(Sankyo Kenkyusho Nenpo)

Review

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f) Special Merchandise Development Department
g) Process Development Laboratories

(Received September 22, 1993)

ミルベマイシンの発見とその研究および開発

井手純也*#, 岡崎尚夫[a], 小野道久[b], 斉藤彰夫, 中川恵子[c], 内藤 覚, 佐藤一雄[d], 田中啓司[e]
吉川博治[c], 安藤 満[d], 勝見 茂[e], 松本 清[f], 遠山俊光, 芝野弥次[g], 阿部正則[f]
三共株式会社化学研究所
a) 筑波研究所, *b*) 研究企画部, *c*) 醗酵研究所, *d*) 農薬研究所, *e*) 農薬開発普及部, *f*) 特品開発部, *g*) 第二生産技術研究所
#Present address: Research Institute.
*The author to whom inquiries concerning this paper should be addressed.

I. Introduction

(Junya Ide)

The discovery of milbemycins dates back more than two decades, to 1967. Mr. A. Aoki[1], at Hokkai Sankyo, found that, during the screening of fermentation broths of various microorganisms, the metabolite B-41, from an actinomycete, showed dramatic acaricidal activity against adult plant mites and plant mite eggs. However, the productivity of B-41, a mixture of many structurally similar compounds, was very low, because of the physical fragility of the producing strain. We at Sankyo, in cooperation with Hokkai Sankyo, made efforts to improve productivity, and succeeded significantly by obtaining B-41 in gram scale. In 1972, the structural elucidation of one of the metabolites of B-41 was done by Dr. H. Mishima[2] at Sankyo, using X-ray crystallographic analysis of the *p*-bromophenylurethane derivative, mass spectrometry, and ^1H and ^{13}C NMR. The structure of this metabolite of B-41 was basically a sixteen-membered lactone with a spiroketal ring system consisting of two six-membered rings and cyclohexenediol or phenol. B-41 was named Milbemycin from the German for mite and the conventional suffix for streptomycete antibiotics, although milbemycins have no antimicrobial activity. As acaricides, milbemycins exhibited remarkable activity against two-spotted spidermite and citrus red mite, and insects such as rice leaf beetle and tent caterpillar. The milbemycin-producing strains of the genus *Streptomyces* were of a new sub-species, belonging to *S. hygroscopicus*, designated *S. hygroscopicus* subsp. *aureolacrimosus*.[3] Milbemycins have a large diversity of functionality, and were originally given a somewhat complicated system of nomenclature. They fall into two main sections, however, those which possess a fused tetrahydrofuranyl ring, which is extensively associated with biological activity, and those in which the ring is missing.

Subsequently, in 1977, avermectins (**2**),[4] isolated from culture broth of *Streptomyces avermitilis* by a group from Merck, were discovered to have struc-

Table 11. Summary of acute toxicity studies on milbemycin D in mice and rats.

Animal		Mice		Rats	
Strain		RFVL		F344	
Age		5-week		7-week	
Number of Animal		10/dose		10/dose	
	Route	Male	Female	Male	Female
LD_{50} (mg/kg)	P.O.	1,847.1	1,609.5	2,467.0	2,998.5
	S.C.	>5,000	>5,000	>2,000	>2,000
	I.P.	667.9	772.3	2,000	2,000

5. Toxicology

(Masanori Abe)

1) Milbemycin D

i) Acute toxicity in mice and rats

Milbemycin D was administered orally, subcutaneously and intraperitoneally to RFVL mice and F344 rats. No severe toxicity was observed regardless of animal species or sex. LD_{50} values for each administration route and animal are summarized in Table 11.

ii) Subacute toxicity in rats

Milbemycin D was administered to F344 rats at doses of 0, 2, 10, 50 and 250 mg/kg/day for 4 weeks. Each dosage group consisted of 15 males and 15 females. After the administration period, recovery was observed for 4 weeks in 5 males and 5 females per dosage group.

No fatalities were observed, and no abnormalities were found in the 10 mg/kg group.

Although decreased locomotive activity and respiratory failure were observed in the 250 mg/kg group 3 to 5 hours after administration, no other symptoms were observed in clinical tests.

Food intake in males and females of the 250 mg/kg group temporarily decreased during the early period after administration compared with control-group values. However, they became comparable within 2 weeks.

No significant difference in body weight was observed between the treatment and control groups.

At the end of the 4-week administration period, males and females treated with 50 mg/kg or more showed an increase in RBC, a decrease in MCH and MCV, and an increase in relative organ weight of the liver. In addition to these, males and females in the 250 mg/kg group showed a decrease in total cholesterol content, an increase in ALP, and slightly hypertrophic liver cells.

These phenomena, however, tended to return to normal during the 4-week recovery period. From the above results, it is concluded that the no-adverse-effect level of milbemycin D in F344 rats is 10 mg/kg/day.

iii) Chronic toxicity in rats

Four groups of rats, each consisting of

10 males and 10 females, were given oral milbemycin D administration at doses of either 0, 2, 10 or 50 mg/kg/day for 13 weeks respectively.

No abnormalities in body-weight changes and food intake were found in the treated groups.

At the end of the 13-week administration period, males and females in the 50 mg/kg group showed an increase in RBC and liver weight, and a decrease in MCH and MCV. Males in this group also showed an increase in ALP and a decrease in total cholesterol. Pathological observation, however, found no changes in any organs. Moreover, no toxicity was found in the 2 or 10 mg/kg groups.

iv) Reproductive toxicity in rats: Segment I

Milbemycin D was given orally to male rats for 9 weeks from 6 weeks prior to mating age and to female rats for 2 weeks from 9 weeks prior to mating age at doses of 0, 10, 50 and 250 mg/kg/day. Administration to female rats was then continued until day 7 of pregnancy. Each dosage group consisted of 24 males and 24 females. The effects on reproductivity and the early stages of embryogenesis were examined.

Although suppression of locomotive activities and body weight changes in parents of the 250 mg/kg group were observed, neither fatalities of parents nor adverse effects on parental reproductivity were confirmed. Neither were lethal effects on embryos, retardation of fetal development or teratogenic effects noted.

v) Reproductive toxicity in rats: Segment II

Pregnant rats received milbemycin D orally from day 7 to day 17 of pregnancy at doses of 0, 10, 50 or 250 mg/kg/day. The effects on dams, embryos and newborns were examined.

The dams of the 250 mg/kg group showed transient suppression of body weight and food and water intakes. However, no embryo mortality, retardation of fetal development or teratogenic effects were noted in any treatment group. In newborns, no abnormalities were noted either.

vi) Mutagenicity

Possible mutagenicity of milbemycin D was investigated by a bacterial reverse mutagenicity test (Ames' Test). Four *Salmonella typhimurium* strains and one *Escherichia coli* strain were used. Revertant colonies were not induced by milbemycin D regardless of the presence of metabolic activator (S-9).

vii) Safety in dogs

Since the clinical usage of milbemycin D is for dogs, several studies were performed to see the possible adverse effects of milbemycin D in dogs. Particular attention was paid to dogs with microfilariae, larvae of heartworm (*Dirofilaria immitis*), or heartworm infection, and collie breeds, because they are said to be sensitive to milbemycin-like molecules.[90,120]

a) 10-Day oral toxicity study in beagles

Milbemycin D, as a 1% powder formulation, was given to beagle dogs at a daily

dose of 5 mg/kg, 5 times the recommended clinical use rate, for 10 consecutive days. The treatment group consisted of 5 males and 5 females.

No significant changes in the observed parameters including clinical observation, body-weight, water intake, urine volume, urinalysis, or hematological, biochemical or postmortem examination were noted, and, therefore, 5 mg/kg was estimated to be a no-adverse-effect level in this study.

b) 10-Month safety study in dogs

Since the clinical usage of milbemycin D for the prevention of heartworm infection is once-a-month oral administration during the whole mosquito season, the safety of the drug when administered for long periods was investigated.

Milbemycin D, as a 1% powder formulation, was given orally to 2 mongrel dog groups, each consisting of 13 animals, at doses of 0 and 5 mg/kg, 5 times the recommended clinical dose, every 15 days for 10 months.

No significant changes in the observed parameters including clinical observation, body-weight, water intake, urine volume, urinalysis, or hematological, biochemical or postmortem examination were noted.

c) Safety study in heartworm-infected dogs[90,91,93,118,119]

Mongrel dogs which were confirmed positive for the presence of microfilaria prior to the study received milbemycin D, as a 1% powder, orally at doses of 1- and 5 times the recommended use rate (corresponding to 1 and 5 mg/kg). The total number of dogs was 99 in the 1 mg/kg group and 65 in the 5 mg/kg group.

Dosing with milbemycin D resulted in several clinical symptoms, though no deaths were observed in any dosage group. Some of the treated dogs revealed severe adverse reactions such as Vena Cava Syndrome and shock. The results indicated that dogs with microfilaremia should be treated to remove adult heartworms and blood-circulating microfilariae prior to initiating milbemycin D administration.

d) Safety in collie dogs[94,113]

Milbemycin D, as a 1% powder formulation, was given to 5 collie dogs at daily doses of 1-, 2.5- and 5 times the recommended clinical rate (corresponding to 0, 1, 2.5 and 5 mg/kg respectively), for 10 days, with a 2-week washing-out period prior to initiating each dosage treatment. Ten Japanese shiba-breed dogs were also treated in the same manner as controls. Observed parameters included clinical, hematological, biochemical and postmortem examination.

Control shiba-breed dogs showed no abnormalities except arrhythmia. Collie dogs exhibited arrhythmia and slight increase of lymphocyte number at a dose of 1 time (1 mg/kg), arrhythmia and slight increase of RBC at a dose of 2.5 times (2.5 mg/kg), and arrhythmia, slight ataxia, shock-like symptoms, decrease of blood pressure and increase of RBC at a dose of 5 times recommended use rate (5 mg/kg). These findings, except arrhythmia and shock-like symptom, were considered to be within normal range.

From these results, it is concluded that milbemycin D will not cause severe

Table 12. Summary of acute toxicity studies on milbemycin 5-oxime in mice and rats.

Animal		Mice		Rats	
Strain		Crj: CD-1(ICR)		Crj: CD(SD)	
Age		5-week		5-week	
Number of Animal		10/dose(♂5, ♀5)		10/dose(♂5, ♀5)	
	Route	Male	Female	Male	Female
LD_{50} (mg/kg)	P.O.	946	722	863	532
	S.C.	>3,000	>3,000	>3,000	>3,000
	I.P.	138	120	454	318

clinical problems in collie dogs when administered at the recommended dose of 1 mg/kg.

2) Milbemycin 5-oxime

i) Acute toxicity in rats and mice

Milbemycin 5-oxime was administered orally, subcutaneously and intraperitoneally to mice and rats. No severe toxicity was observed regardless of animal species or sex. LD_{50} values for each administration route and animal are summarized in Table 12.

ii) Subacute toxicity in rats

Milbemycin 5-oxime was administered to rats at doses of 0, 10, 30, 100 and 300 mg/kg/day for 4 weeks. Each dosage group consisted of 6 males and 6 females. Observation parameters were general conditions, body weight, food intake, organ weight, urinalysis, and ophthalmological, hematological, biochemical and postmortem examinations.

In the 10 mg/kg group, no abnormalities were noted in any parameter examined.

In the 30 mg/kg group, an increase in RBC and decrease of extramedullary erythropoiesis in spleens were observed.

In the 100 mg/kg group, slight changes were noted in hematological and biochemical examinations such as decreases in MCH and MCV and an increase in total cholesterol, in addition to the changes observed in the 30 mg/kg group. Liver-weight increase accompanied by a relatively mild swelling of hepatocytes was also observed.

In the 300 mg/kg group, abnormalities in general conditions, decrease of body weight and fatty changes in hepatocytes were observed in addition to the findings in the 30 and 100 mg/kg groups.

From these results, the no-adverse-effect level of milbemycin 5-oxime was estimated to be 10 mg/kg/day.

iii) Chronic toxicity in rats

Milbemycin 5-oxime was administered orally to rats at doses of 0, 3, 15 and 100 mg/kg/day for 3 months. Each dosage group consisted of 10 males and 10 females. Observed parameters included general conditions, body weight changes, organ weight, urinalysis, and ophthal-

mological, hematological, biochemical and postmortem examinations.

In the 3 mg/kg group, no treatment-related changes were noted in any parameters examined.

In the 15 mg/kg group, changes in hematological and biochemical conditions such as an increase in RBC and decreases in MCH and MCV were noted. A decrease in extramedullary erythropoiesis in spleens was also observed.

In the 100 mg/kg group, hematological and biochemical changes such as an increase in platelet number and decrease in Hb and Ht values were found, in addition to those findings in the 15 mg/kg group. An increase in liver weight, accompanied by swellings and fatty changes of hepatocytes, was also observed.

From the above results, the no adverse effect level of milbemycin 5-oxime was estimated to be 3 mg/kg/day.

iv) Reproductive toxicity in rats and rabbits

a) Segment II study in rats

Rats given milbemycin 5-oxime orally from day 7 to day 17 of pregnancy at doses of 0, 3, 30 or 300 mg/kg/day. The effects on dams and embryos were examined.

Some dams of the 300 mg/kg group exhibited several clinical symptoms including suppression of body weight increase, decrease of food intake and diarrhoea. Furthermore, 1 dam died at day 19 of pregnancy. Although embryo examination discovered a suppression of weight of survived fetus and placenta, examination on embryo appearance showed only slight delay of ossification and no other findings were observed.

No abnormalities in either dams or fetuses of the 3 mg/kg and 30 mg/kg group were noted.

From these results, milbemycin 5-oxime is thought to have no teratogenic toxicity in rats.

b) Segment II study in rabbits

Milbemycin 5-oxime was administered orally to pregnant rabbits at daily doses of 0, 5, 30 or 180 mg/kg for 18 days from day 6 of pregnancy.

Some dams in the 180 mg/kg group showed several clinical symptoms including decrease of food-intake and body weight, and diarrhoea. About half of the dams miscarriage suffered fetus death. However, only slight ossification delay was observed in embryo appearance examination and no other findings were noted.

No abnormalities in either dams or fetuses in the 5 mg/kg and 30 mg/kg groups were noted.

In conclusion, milbemycin 5-oxime was thought to have no teratogenic toxicity in rabbits.

v) Mutagenicity

Possible mutagenicity of milbemycin 5-oxime was investigated by a bacterial reverse mutagenicity test (Ames' Test). Four *Salmonella typhimurium* strains and one *Escherichia coli* strain were used. Revertant colonies were not induced by milbemycin 5-oxime regardless of the presence of metabolic activator (S-9).

A chromosomal aberration test using cultured Chinese hamster lung cells both

with and without metabolic activator (S-9) was also performed. No significant increases in the number of cells with chromosomal abnormalities were confirmed.

vi) Safety in dogs

Because milbemycin 5-oxime, like milbemycin D, is aimed at dogs, thorough safety studies were performed to see the possible adverse effects in dogs. Particular attention was paid to dogs with heartworm (*Dirofilaria immitis*) infection, and collie dogs, for the same reason as with milbemycin D.[90~93,113,117,118,120]

a) 10-Day oral toxicity study in beagle dogs

Milbemycin 5-oxime was administered orally by powder formulation to beagle dogs at daily doses of 0, 1-, 3-, and 5 times the maximum recommended clinical use rate (corresponding to 0, 0.5, 1.5 and 2.5 mg/kg respectively) for 10 consecutive days. Each dosage group consisted of 3 males and 3 females. Observations on general conditions, measurement of body weight, food and water consumption, urinalysis, and hematological, biochemical, reflex function and postmortem examinations were performed.

No treatment-related changes were observed in any parameter in any dosage group. Thus, the highest dose employed in this study, 2.5 times the clinical use rate, or 2.5 mg/kg, was concluded to be safe for dogs.

b) 10-Month oral toxicity in beagle dogs[121]

Since milbemycin 5-oxime, like milbemycin D, is to be given once a month by oral administration during a whole mosquito season for the prevention of heartworm infection, the safety of the drug when administered for long periods was investigated.

The drug was administered orally by tablet to beagle dogs at dosage levels of 0, 1-, 3- and 5 times the maximum use rate (0.5, 1.5 and 2.5 mg/kg) for the first 3 days of each month for 10 months. Each dosage group consisted of 8 males and 8 females of 8 weeks of age at dosing initiation. Examined parameters included general condition observations, bodyweight and food consumption measurement, ophthalmology, hematology, biochemistry, urinalysis and anatomic pathology.

In the 1 time use group (0.5 mg/kg), all criteria evaluated were considered to be comparable with findings in the control group.

In the 3 times use group (1.5 mg/kg), a few dogs exhibited slight to mild transient trembling and/or ataxia during the first 3 days of the study. Any other criteria evaluated were considered to be comparable with findings in the control group.

In the 5 times use group (2.5 mg/kg), transient trembling and/or ataxia was observed in most of the dogs during the first 3 days of the study. No signs were observed for the remainder of the study.

Ataxia and/or trembling in the 3 times and 5 times rate groups were considered a consequence of overdosing; that is, the puppies showing these signs were 8 weeks of age and weighed about 2 kg at the dosing period while the tablets ad-

ministered to these puppies were formulated for dogs weighing about 11 kg and, thus, the actual dosage levels of these groups at the period were about 17 times (8.6 mg/kg) and 28 times (14.3 mg/kg) the maximum recommended use rate. These findings, however, were not seen at any subsequent dosing period. All other criteria evaluated were considered similar to those in the control group.

c) Acute puppy study in young beagle dogs of 8 to 12 weeks of age[121]

The clinical effects of milbemycin 5-oxime in puppies of 8, 10 and 12 weeks of age were investigated to further evaluate those noted in the previous 10-month oral toxicity study.

The study used 30 male and 30 female beagle puppies distributed with 10 males and 10 females, in each of the 8, 10 and 12 week age groups. milbemycin 5-oxime was administered orally by tablet in doses of 0, 1-, 5-, 15- and 25 times the maximum recommended use rate (corresponding to 0, 0.5, 2.5, 7.5 and 12.5 mg/kg), for 3 consecutive days. Each dosage group in each age group consisted of 2 males and 2 females. Specific study parameters evaluated were clinical observations, body weight and mortality.

The only effects noted in the parameters measured were ataxia and trembling. Essentially no effects were seen in the 1 time rate in any age group. Six of the 12 animals at 5 times showed no trembling or ataxia.

These effects demonstrated a slight age-related occurrence. With 25 times the maximum rate, 12-week old males and females were less affected than 8-week or 10-week old males and females. At 15 times, 12-week old females were less affected than 8-week or 10-week old females.

These effects were also dose-related at all ages. The incidence, severity and length of occurrence were greatest at 25 times, less at 15 times and very slight at 5 times.

The lack of effects noted at 1 time and the minor effects noted at 5 times indicated a sufficient margin of safety of milbemycin 5-oxime for use in young dogs.

d) Reproduction study in beagle dogs[121]

The objective of the study was to evaluate the effects of milbemycin 5-oxime on reproduction in dogs. The study was divided into two phases, the reproductive phase and puppy safety evaluation phase, in order to evaluate any possible subtle effects on both parent animals and nursing pups that would receive the drug through the milk from lactating bitches.

In the reproductive phase, 20 studs were dosed daily for a minimum of 90 days through the end of mating, and 20 bitches were dosed daily through mating until 1 week prior to whelping. In the pup safety evaluation phase, untreated pregnant bitches were dosed once in separate groups to evaluate the effects of dosing singly just before, on the day of, or soon after whelping. This dosing regimen is believed to simulate a possible real-life dosing situation. In both phases, the dose was 3 times the maximum recommended dose rate (corresponding to 1.5 mg/kg).

Specific study parameters were sur-

vival, appearance and behavior, body weight, food consumption, stud sperm evaluation, stud and bitch fertility indices, mean gestational length, pup viability, pup growth, pup survival, and pup malformation.

No effects on any parameters were evaluated in either the reproductive phase or pup safety evaluation phase. In conclusion, milbemycin 5-oxime is considered to be safe to use without adverse effects on reproduction.

e) Safety study in heartworm-infected dogs[119,121]

As mentioned earlier, dogs infected with heartworm (*Dirofilaria immitis*) are said to be hypersensitive to milbemycin-like molecules. Studies were conducted to clarify the possible effects of milbemycin 5-oxime in heartworm-infected dogs.

Two dosing regimens were applied: single dosing and multiple monthly dosing for 3 months. The later dosing regimen was intended to simulate a real dosing situation.

In the single-dosing study, 70 mongrel dogs were dosed orally with milbemycin 5-oxime by powder or granules at the minimum recommended use rate (equivalent to 0.25 mg/kg) and 42 mongrel dogs at the maximum use rate (equivalent to 0.5 mg/kg). All these dogs were confirmed positive for the presence of microfilaria prior to study initiation. The parameters evaluated were clinical signs, hematology, chemistry and necropsy.

Most of the dogs at both dose rates exhibited several side-effects such as slightly pale mucous membranes, vomiting and activity decreases, although no mortalities were observed in either group. Hematological and biochemical test results were also noted.

In the multiple-dosing study, mongrel dogs were dosed with milbemycin 5-oxime tablets at monthly doses of 0 (15 males and 10 females), 1 time (0.5 mg/kg; 14 males and 11 females), 3 times (1.5 mg/kg; 8 males and 5 females) and 5 times (2.5 mg/kg; 6 males and 6 females) the maximum clinical use rate for 3 months. All these dogs were confirmed positive for presence of microfilaria prior to the study. The parameters evaluated were clinical signs, body weight, food consumption, hematology, biochemistry, urinalysis and necropsy.

Treatment of heartworm-infected dogs with milbemycin 5-oxime resulted in minor clinical signs after the initial dosing which were transient and of generally slight to moderate severity such as abdominal respiration, pale mucous membranes, and decreased activity. Clinical signs were not observed after the second and third treatment.

From the above results, it was indicated that dogs with microfilaremia should be treated to remove adult heartworms and blood-circulating microfilariae prior to initiating milbemycin 5-oxime administration. It was also noted that although several clinical symptoms were seen in the heartworm-infected dogs treated with milbemycin 5-oxime, no clinically severe signs, like Vena Cava Syndrome or shock, which were observed in dogs treated with milbemycin D, were exhibited.

f) Safety in collie dogs[105,113,121]

As described before, collie dogs were said to be hypersensitive to milbemycin-like molecules and, therefore, studies were conducted to see the possible adverse effects of milbemycin 5-oxime in collie dogs.

The following treatments were conducted:

Dosing I: Ten collie dogs (5 males and 5 females) were assigned to one each of the following groups: 0.5 mg/kg (maximum recommended dose) or 2.5 mg/kg (5 times). Each dog received treatment twice, one week apart.

Dosing II: Six collie dogs (3 males and 3 females) were dosed at rising doses of 0.25 (minimum recommended dose), 0.5 (maximum recommended dose), 1.0 (2 times) and 2.5 mg/kg (5 times) with a 2-week interval period between each dosing. Six weeks after the final dosing, 3 of these dogs received a dose of 12.5 mg/kg (25 times), and 9 weeks after this dosing, the same dogs were further treated with a dose of 25 mg/kg (50 times).

Dosing III: Fourteen collie dogs (8 males and 6 females) were treated at rising doses of 2.5 (5 times), 5.0 (10 times), 10.0 (20 times) and 12.5 mg/kg (25 times) with 2 to 4-week intervals between each dosing.

No apparent adverse reactions were observed in any dogs of either Dosing I or Dosing II. No toxic reactions were seen in any dogs of Dosing III except 1 dog at 25 times which exhibited severe signs such as ataxia and periodic recumbency. However, it is highly unlikely that a dog could receive such a magnitude of overdose sufficient to induce severe toxicosis. Also, dogs in Dosing II receiving the 50 times dose did not exhibit any apparent side-effects. Therefore, it is concluded that unlike milbemycin D, milbemycin 5-oxime can be used with collie dogs with a high safety margin.

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