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Studies on Chemotherapy of Parasitic Helminths (XXVIII). *In Vivo* Efficacy of Milbemycin D against Larval Stages of *Angiostrongylus cantonensis* and *A. costaricensis*

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Abstract

Anti-larval effects of milbemycin D were examined on *A. cantonensis* in rats and mice and on *A. costaricensis* in mice. 1) In rats inoculated with 20 infective larvae of *A. cantonensis*: Compared with non-treated control group, significant reductions in the first stage larval counts in feces (LPG/female), number of worms recovered and host lung-body weight ratio were seen in the group receiving 10 successive daily doses of 5.0 mg/kg. In groups receiving a single dose of 5.0 or 25.0 mg/kg, no noticeable change was seen in any parameters. 2) In mice inoculated with 50 infective larvae of *A. cantonensis*: In non-treated control group, severe loss in body weight and death of host animals were caused by the worms. Ten successive doses of 5.0 mg/kg remarkably inhibited these changes and only slight effects were seen by 10 consecutive doses of 1.0 mg/kg. 3) In mice with 20-larval infection of *A. costaricensis*: In non-treated control group, host animals were also affected severely by the worms. These changes were inhibited effectively in groups receiving 5 or 10 successive doses of 5.0 mg/kg. From these results, it was suggested that milbemycin D had conspicuous *in vivo* effects against larval stages of *A. costaricensis* as well as *A. cantonensis*.

Key words: Milbemycin D, anti-larval effects, *in vivo*, *Angiostrongylus cantonensis*, *A. costaricensis*

Introduction

Milbemycin D is one of 13 chemically-related antibiotics which have been recently isolated by Takiguchi *et al.* (1980, 1983) from submerged cultures of *Streptomyces hygroscopicus* subsp. *aureolacrimosus*. As its chemical structure is closely related to that of avermectins (Campbell, 1985), it is presumed that milbemycin D also may have insecticidal, acaricidal and nematocidal activities. Indeed, insecticidal and acaricidal activities were detected in all milbemycins (Takiguchi *et al.*, 1980). In addition, antinematode effects of milbemycin D were also reported in intestinal nematodes including *Ancylostoma caninum* (Shiramizu and Abu, 1985) and in filarial worms such as *Dirofilaria immitis* (Sakamoto *et al.*, 1985) and *Litomosoides carinii* (Nakamura *et al.*, 1985).

In our serial studies on the effects of many antinematode drugs, the remarkable *in vitro* and *in vivo* effects of milbemycin D on adult worms of *Angiostrongylus cantonensis* were detected, while little effect was seen on *A. costaricensis* (Sano *et al.*, 1986; Dharejo *et al.*, 1986). It was further suggested that the paralyzing effect of milbemycin D on adult *A. cantonensis* was probably caused through the gabergic mechanism like avermectins (Terada *et al.*, 1984; Sano *et al.*, 1986). However, anthelmintics with anti-larval activities are imperatively wanted for the clinical chemotherapy of human angiostrongylosis cantonensis because this nematode hardly develops to adult

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stage in abnormal hosts like man and the mouse (Alicata and Jindrak, 1970). Thus, larvicidal effects of milbemycin D in *A. cantonensis* and *A. costaricensis* were examined in the present study.

Materials and Methods

Compound tested: Milbemycin D was kindly supplied by Sankyo Co. Ltd. as an 1% powder and was suspended in a 30% glycerol solution for administration to experimental animals.

Animal treatment: Five-week-old male ddY mice (25–27 g in body weight) and 4-week-old male Wistar rats (80–90 g in body weight) were used as host animals.

The isolation of infective third stage larvae of *A. cantonensis* and *A. costaricensis* from intermediate snails, *Biomphalaria glabrata* and infection of host animals with each of these infective larvae were carried out as described by Ishii *et al.* (1983).

In the experiment I, effects of milbemycin D against larval stages of *A. cantonensis* in rats were examined. Three groups of 5 rats infected with 20 infective larvae of *A. cantonensis* each were treated orally with a single dose of 5.0 or 25.0 mg/kg on 6 days post infection (pi) and with 10 successive daily doses of 5.0 mg/kg on 6 to 15 days pi. Additional non-treated control group of 5 rats received equal volumes of vehicle only. The first stage larval counts in rat feces were determined at 7th and 9th week pi, being represented as larvae per gram of feces per female worm recovered (LPG/female). All rats of treated and control groups were sacrificed 15 weeks pi and the number of worms in the heart and lungs was examined. The lung-body weight ratio was also determined on each rat by such calculation as 100 x lung weight/body weight, as this ratio was suggested to be one of good indicators of drug effects in the parasitic disease (Ishii, 1986).

In the experiment II, anti-larval effects of milbemycin D on *A. cantonensis* in mice were examined. According to Hayashi *et al.* (1984), three groups of 10 mice were inoculated with 50 infective larvae of *A. cantonensis*, and two of them were treated orally with 10 successive daily doses of 1.0 or 5.0 mg/kg on 6 to 15 days pi. The remaining group served as non-treated control. Body weight, clinical signs and number of surviving host animals in all groups were monitored until 30 days pi.

In the experiment III, effects of milbemycin D against larval stages of *A. costaricensis* in mice were examined. Four groups of 10 mice with 20-larval infection of *A. costaricensis* were treated orally with a single dose of 5.0 mg/kg on 6 days pi and with 5 or 10 consecutive daily doses of 1.0 or 5.0 mg/kg on 6–10 or 6–15 days pi. Additional one group of 10 mice served as non-treated control. Body weight, clinical signs and number of surviving host animals in all groups were monitored until 30 days pi.

To study effects on larval stages of worms in these experiments, the time of drug administration was decided as described above referring to Alicata and Jindrak (1970) for *A. cantonensis* and to Monge *et al.* (1978) for *A. costaricensis.*

Results

Experiment I: Effects of milbemycin D against larval stages of *A. cantonensis* in rats – Compared with non-treated control group, significant reductions were seen in such parameters as LPG/female at 7th and 9th week pi, and number of worms recovered and host lung-body weight ratio at sacrifice in the group treated with 10 successive doses of 5.0 mg/kg. In groups receiving a single dose of 5.0 or 25.0 mg/kg, no noticeable change was seen in any parameters (Table 1).

Experiment II: Anti-larval effects of milbemycin D on *A. cantonensis* in mice – As shown in Fig. 1, host body weight of non-treated control group rapidly decreased from around 12 days pi, but later the surviving mice regained. Various clinical signs as depression and bristled hair were seen in all mice at 15 days pi, and some of them showed wryneck and claudication. Some mice died after these signs became severe, but others were restored later. Between

Table 1 Effects of milbemycin D against larval stages of *Angiostrongylus cantonensis* in rats (Experiment I)

Oral dose (mg/kg)	LPG/female ($\times 10^3$)† 7th week	9th week	Rat body weight at sacrifice‡	Rat lung-body weight ratio at sacrifice	No. of worms recovered
0.0	8.81 ± 1.48	11.98 ± 2.28	308.5 ± 11.5	1.40 ± 0.07	16.8 ± 0.8
5.0 x 1	7.52 ± 0.79	9.38 ± 0.85	324.3 ± 4.5	1.34 ± 0.10	15.0 ± 1.3
25.0 x 1	7.34 ± 0.57	13.25 ± 0.44	324.7 ± 5.3	1.26 ± 0.16	12.2 ± 1.9
5.0 x 10	0.00 ± 0.00* :	0.00 ± 0.00*	328.3 ± 3.2	0.73 ± 0.02*	2.2 ± 0.7*

Results are shown as mean ± SE of five animals each. *: Significant difference by Student's t-test at $P < 0.01$, †: Larvae per gram of rat feces per female worm recovered, ‡: All animals were sacrificed 15 weeks post infection (pi). A single dose of 5.0 or 25.0 mg/kg was administered on 6 days pi and 10 successive daily doses of 5.0 mg/kg were on 6 to 15 days pi.

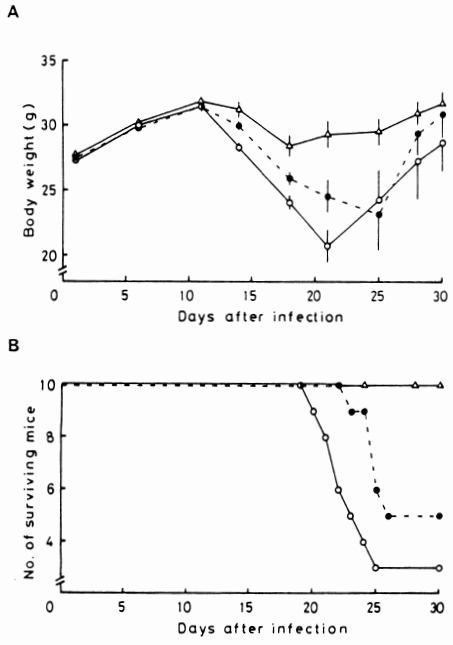

Fig. 1 Effects of milbemycin D on body weight (A) and surviving number (B) of mice infected with *A. cantonensis* (Experiment II). All mice were inoculated with 50 infective larvae and each group had 10 mice. Ten successive daily doses of 1.0 mg/kg (●- -●) or 5.0 mg/kg (△—△) were administered on 6 to 15 days post infection. Control mice (○—○) received vehicle (a 30% glycerol solution) only.

19–25 days pi 7 out of 10 mice died. No death and only a slight reduction in body weight were observed in animals treated with 10 successive doses of 5.0 mg/kg, and less effects were also seen in animals receiving 10 consecutive doses of 1.0 mg/kg.

Experiment III: Effects of milbemycin D against larval stages of *A. costaricensis* in mice – Though there were some reports describing that cotton rats were useful as a definitive host of the nematode (Morera *et al.*, 1977; Monge *et al.*, 1978), there was few information on its experimental model with mice for chemotherapeutic studies. Thus, a preliminary examination on abdominal angiostrongylosis in mice was made to know influence of worm burden on parameters like changes in body weight, clinical signs and death of host animals, and number of worms recovered. When 3 groups of 10 mice were inoculated with 10 infective larvae, host body weight kept increasing slightly throughout the experimental period and 50% or more died until 43.0 ± 2.3 (mean ± SE) days pi. Four to 5 mice in each group had survived until sacrifice (50 to 55 days pi), and 5.6 ± 0.3 (mean ± SE) adult worms were recovered from the mesenteric arteries of the surviving 14 mice. When 4 groups of 10 mice were inoculated with 20 third stage larvae, these changes were more conspicuous. Host body weight started to decrease from around 16 days pi and 50% or more died until 25.5 ± 1.2 days pi without remarkable clinical signs except bristled hair and/or hemafecia. One to 2 out of 10 mice in each group had survived until 50 to 55 days pi when they were sacrificed, and 10.3 ± 0.9 adult worms were recovered from the mesenteric

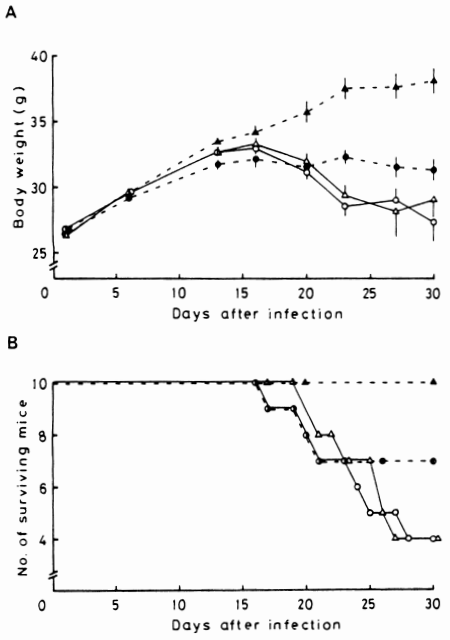

Fig. 2 Effects of milbemycin D on body weight (A) and surviving number (B) of mice infected with *A. costaricensis* (Experiment III). All mice were inoculated with 20 infective larvae and each group had 10 animals. A single dose of 5.0 mg/kg (△—△) was administered on 6 days post infection (pi) and 10 consecutive daily doses of 1.0 mg/kg (●- -●) or 5.0 mg/kg (▲- -▲) were on 6 to 15 days pi. Control mice (○—○) received vehicle only.

arteries of these surviving 6 mice. Thus, the mice with 20-larval infection were used for examining chemotherapeutic effects of milbemycin D in *A. costaricensis.*

As compared to these non-treated control animals, remarkable effects were observed in animals receiving 5 or 10 successive doses of 5.0 mg/kg; no decrease in body weight and no death of host animals were seen (Fig. 2). Though results on the 5-dosed group are not contained in Fig. 2 because of little difference from those of the 10-dosed group regarding these parameters, a noticeable difference was seen in their number of adult worms recovered. No worm was recovered 50 days pi from all of 10 animals of the 10-dosed group, but only 6 adult worms were from the mesenteric arteries of 3 animals of the 5-dosed group (1 female and 1 male from a mouse and 2 females from each of 2 mice). In animals treated with 10 doses of 1.0 mg/kg, effects were rather slight (Fig. 2); 50% or more of host mice died on 36 days pi, and at sacrifice 50 days pi 6.8 ± 1.1 worms were recovered from the surviving 4 mice.

Discussion

In our previous studies on *in vitro* and *in vivo* effects of milbemycin D against adult worms of *A. cantonensis* and *A. costaricensis,* it was shown that this anthelmintic was remarkably effective only in *A. cantonensis.* The efficacy was demonstrated not only from the *in vitro* motility (Sano *et al.,* 1986) but also from various parameters *in vivo* such as host lung-body weight ratio, the first stage larval count in rat feces (LPG/female) and number of adult worms recovered (Dharejo *et al.,* 1986). In addition, the *in vivo* efficacy was conspicuous when 5 or 10 successive daily doses of 5.0 mg/kg were given to animals. As it was reported in intestinal nematodes like *Ancylostoma caninum* that almost 100% worms were expelled by milbemycin D at lower doses of 0.05 to 0.1 mg/kg (Shiramizu and Abu, 1985), poor absorption of the drug from the intestinal tracts may explain the difference in the effective doses between intestinal and blood nematodes.

On the other hand, in the present study milbemycin D had conspicuous effects on larval stages of *A. costaricensis* in mice as well as of *A. cantonensis* in rats and mice. The efficacy against *A. cantonensis* in rats was seen from such parameters as LPG/female, host lung-body weight ratio and number of worms recovered. Anti-larval effects against *A. cantonensis* and *A. costaricensis* in mice were observed from clinical signs, changes in body weight and death of host animals. The anti-larval effects were also remarkable in host animals receiving 5 or 10 consecutive daily doses of 5.0 mg/kg. From these results, it was suggested that milbemycin D had at least two effects obviously differentiated: the one on the motility and/or reproductive system of adult worms of *A. cantonensis* and the other on development and

growth of larval stages of both species of nematodes. Therefore, it seems probable that milbemycin D acts primarily on some sites or functions of worms and that the actions inhibit secondarily the changes or death of host animals caused by the worms.

As described in introduction, anti-larval effects seem essential against human angiostrongylosis cantonensis, and such effects have been reported in animal experiments on drugs like thiabendazole (Cuckler *et al.*, 1965; Nishimura, 1965/66), *l*-tetramisole (Jindrak and Alicata, 1969), mebendazole (Lämmler and Weidner, 1975; Hayashi *et al.*, 1982), flubendazole (Maki and Yanagisawa, 1983), avermectin Bıa (Ishii *et al.*, 1983) and ivermectin (Ishii *et al.*, 1985). Among these, some such as avermectins were also effective against later stages of the worm including adult *A. cantonensis* and milbemycin D was included in the drugs. Moreover, compared to drugs such as thiabendazole and *l*-tetramisole, the effective doses of milbemycin D seem rather lower. Therefore, its *in vivo* efficacy against larval stages of *A. cantonensis* may be worthy of further investigation.

On the other hand, some anthelmintics like diethylcarbamazine and thiabendazole have been used from urgent necessity for the treatment of human abdominal angiostrongylosis, but whether the treatment was actually effective or not has never been confirmed yet (Loria-Cortes and Lobo-Sanahuja, 1980). As *A. costaricensis* develops to adult stage in humans, anti-adult effects of anthelmintics on this nematode must be desirable. It was reported that some anthelmintics including levamisole and diethylcarbamazine were effective on the *in vitro* motility of the adult nematode (Sano *et al.*, 1985). Though anti-larval effects of milbemycin D were observed in the present study, unfortunately this drug had little effect on adult worms of *A. costaricensis in vivo* (Dharejo *et al.*, 1986).

Recently, we could immunize mice against *A. costaricensis* by treating the animals with milbemycin D during larval stages of worms after infection and promising results on inhibition of reinfection by the nematode were obtained (Terada *et al.*, 1986). We think other approaches like this may be necessary and useful for these parasites which are extremely difficult to treat with chemotherapeutic drugs.

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