

STRONGYLOIDES RATTI AND S. STERCORALIS: EFFECTS OF CAMBENDAZOLE, THIABENDAZOLE AND MEBENDAZOLE IN VITRO

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S U M M A R Y

The effects of *in vitro* incubation of three benzimidazole anthelmintics, thiabendazole, mebendazole and cambendazole on *Strongyloides* were compared. No drug affected hatching of *S. ratti* eggs or the viability of infective larvae or parasitic adult worms, but all three inhibited moulting of *S. ratti* larvae. In addition, cambendazole, but not thiabendazole or mebendazole, impaired the viability of *S. ratti* first- and second-stage larvae. The three drugs had no effect on isolated *S. stercoralis* free-living adult worms, but they all prevented development of *S. stercoralis* rhabditiform larvae. Thiabendazole and mebendazole had no effect on the infectivity of either *S. ratti* or *S. stercoralis* infective larvae, but infection with these worms was abrogated by prior incubation with cambendazole. These results indicate that cambendazole acts in a different manner to the other two drugs. Since it is active against larvae migrating through the tissues, it is potentially of much greater value than thiabendazole or mebendazole in the therapy of strongyloidiasis.

KEY WORDS: *Strongyloides ratti* — *Strongyloides stercoralis* — Action *in vitro* drugs — Cambendazole — Thiabendazole — Mebendazole.

I N T R O D U C T I O N

Strongyloidiasis is one of the major human intestinal nematode infections. It is resistant to most anthelmintics and thiabendazole has been the mainstay of therapy for many years. Unfortunately, this drug is not always effective, both in uncomplicated strongyloidiasis⁸ and in overwhelming infections in immunosuppressed patients^{14,21,24,25}. More recently, two related benzimidazole compounds, mebendazole^{4,17,20} and cambendazole^{1,3,13,19,23} have been used with variable success.

Initial studies of the modes of actions of benzimidazoles in strongyloidiasis simply assessed the effects of drugs on the excretions of parasites in the feces of humans and animals in-

fectured with various *Strongyloides* species. It was then shown in experimental studies of mice with *S. ratti* infections, that thiabendazole had no effect on migratory larvae and did not kill adult worms in the gastrointestinal tract, but did reduce the fecundity of these worms⁹. In contrast, mebendazole and cambendazole were active against both migratory larvae and adult worms in the gut. Furthermore, cambendazole but not mebendazole eradicated *S. stercoralis* larvae from the muscles of mice.

Since *Strongyloides* species complete part of their life cycle in the external environment, *in vitro* studies provide an alternative means for both screening of drugs for anti-*Strongyloidi-*

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des activity⁶ and for gaining insight into the anthelmintic mechanisms of those drugs. In this paper, the effects in vitro of three benzimidazole compounds, thiabendazole, mebendazole and cambendazole on *S. ratti* and *S. stercoralis* have been compared. A homogonic strain of *S. ratti* was used in which eggs hatched and rhabditiform larvae developed directly into infective larvae after two moults. *S. stercoralis*, however, was heterogonic with rhabditiform larvae moulting four times to produce free-living adult male or female worms which in turn produced rhabditiform larvae which moulted twice to become infective larvae.

METHODS

The source and maintenance of *S. ratti* by Sprague-Dawley rats have been described elsewhere⁵. *S. stercoralis* was obtained originally from infected humans⁷ and has since been maintained in dogs¹¹. The supply and preparation of thiabendazole, mebendazole and cambendazole have been detailed elsewhere⁹.

Feces were collected from infected rats or dogs, homogenized in water and the intensity of infection quantified. Aliquots were mixed with suspensions of thiabendazole or mebendazole prepared in 0.1M phosphate buffered saline, pH 7.4 (PBS) or a solution of cambendazole and placed on watch glasses at room temperature. In order to prepare infective larvae, moistened infected feces were placed on watch glasses in petri dishes containing a thin layer of water; larvae migrated into the water

and were recovered several days later for *S. ratti* and 10 days later for *S. stercoralis*.

The methods for infection of mice with infective larvae and quantification of eggs in the feces have been described earlier⁵. Female parasitic adult worms of *S. ratti* were recovered from rats by removal of the intestines seven days after infection. The intestines were slit longitudinally, cut in segments 2 cm long and incubated for one hour at 37°C in PBS in a petri dish; the adult worms were then picked out individually with a pipette. The technique for quantification of *S. stercoralis* larvae in the muscles of mice has been described in detail elsewhere⁹. Viability of worms was assessed on the basis of the morphology of intestinal structures and motility of the worms.

RESULTS

Effect on hatching of *S. ratti* eggs

Thirty-two small watch glasses were each set up with 100 µg aliquots of feces containing eggs. To each of these was added 400 µl PBS containing either no drug or 200 µg of thiabendazole, mebendazole or cambendazole giving a final concentration of 400 µg drug/ml. Eight dishes were in each group. The numbers of viable rhabditiform larvae were counted two hours after incubation. Each anthelmintic had no effect on hatching of eggs for there were no significant differences with any of the treatment groups when compared with the controls (Table I).

T A B L E I
Effects of anthelmintics on egg hatching, first stage larvae and the first moult of *S. ratti*

Hours of incubation with drug (**)	Type of Larva	Larvae per Sample (*)			
		Control mean ± S.D.	Thiabendazole mean ± S.D.	Mebendazole mean ± S.D.	Cambendazole mean ± S.D.
2	L1	17±11	14±6	14±5	18±12
7	L1	17.4±7.9	13±6.4	15±9.8	8.4±4.4
24	L1	2.3±1.0	7±4.3	6.1±5.1	1.5±2.3
	L2	23±5.3	0.25±0.5	0	0
31	L1	2.5±1.3	0.9±0.9	1.4±0.9	0.3±0.5
	L2	27±6.9	0.1±0.4	0.3±0.5	0

(*) Eight samples per group

(**) Drug was added at the time of moistening the faeces

L1 = first-stage (= rhabditiform) larva

L2 = second-stage larva

Effect on viability of first-stage *S. ratti* larvae

The same protocol as above was adopted except that the numbers of viable rhabditiform larvae were counted seven and 24 hours after incubation. Thiabendazole and mebendazole had no toxic effect on first-stage larvae but there was a significant ($P < 0.02$ "t" test) 52% reduction in the numbers of first-stage larvae in feces incubated with cambendazole for seven hours (Table I).

Effect on first moult of *S. ratti*

The same protocol as above was adopted except that the numbers of viable first — and second-stage larvae were counted 24 hours after incubation. At this time, 91% of larvae in the control feces had moulted. There was a highly significant reduction ($P < 0.001$, "t" test) in the numbers of second-stage larvae in feces treated with any of the anthelmintics, none being found in feces incubated with mebendazole or cambendazole and very few in that incubated with thiabendazole (Table I). At this time, there were large numbers of persistent, viable, rhabditiform larvae in feces incubated with thiabendazole or mebendazole. When the experiment was repeated after 31 hours of incubation similar results were obtained except

that very few viable first-stage larvae were now seen (Table I). These results suggest that each anthelmintic interferes with the first moult.

Effect on viability of *S. ratti* second-stage larvae.

A similar protocol was adopted except that drug was added 24 hours after incubation. The numbers of viable second-stage larvae in each group were counted 4 hours later. Thiabendazole and mebendazole had no effect on the viability of second-stage larvae but there was a significant ($P < 0.005$, "t" test) 89% reduction in the number of viable second-stage larvae in the feces incubated with cambendazole (Table II).

Effect on the second moult of *S. ratti*

The same protocol was adopted as in the preceding section except that the numbers of viable third-stage larvae were counted after 24 hours incubation with drug (48 hours after feces were moistened). No viable infective larvae were seen in feces treated with any of the anthelmintics (Table II); this difference was highly significant ($P < 0.001$, "t" test). These results suggest that each anthelmintic interferes with the second moult.

T A B L E II
Effects of anthelmintics on second stage larvae and the second moult of *S. ratti*

Hours of incubation with drug (**)	Type of Larva	Larvae per sample (*)			
		Control mean \pm S.D.	Thiabendazole mean \pm S.D.	Mebendazole mean \pm S.D.	Cambendazole mean \pm S.D.
4	L2	4.5 \pm 2.6	3.6 \pm 2.1	5.0 \pm 3.6	0.5 \pm 0.8
24	L3	15 \pm 33	0	0	0

(*) Eight samples per group

(**) Drugs was added 24 hours after moistening the faeces

L2 = second-stage larva

L3 = third-stage (= infective) larva

Effect on viability of *S. ratti* third stage larvae

Four petri dishes each containing four groups of 4,000 larvae in 4 ml water were incubated with either no drug or 400 μ g of thiabendazole, mebendazole or cambendazole (concentration 100 μ g/ml). The viability of infective larvae was assessed 48 hours later. The anthelmintics had no effect on viability, the percen-

tage alive in control, thiabendazole, mebendazole and cambendazole groups being 90%, 94%, 92% and 90% respectively.

Effect on infectivity of *S. ratti* third stage larvae

Four petri dishes each containing 10,000 larvae in 4 ml water were incubated with either no drug or 400 μ g of thiabendazole, mebendazole

or cambendazole (concentration 100 µg/ml) for two hours. After washing three times, groups of five mice were infected percutaneously with 2,000 larvae and the numbers of larvae counted in the feces six days later. There were on signi-

ficant effects on the excretion of eggs after incubation of infective larvae with thiabendazole or mebendazole but no eggs were seen in the mice infected with third stage larvae incubated with cambendazole (Table III); this difference was highly significant ($P < 0.01$, "t" test).

T A B L E III
Effects of anthelmintics on the infectivity of *S. ratti* and *S. stercoralis*

	Control mean ± S.D.	Thiabendazole mean ± S.D.	Mebendazole mean ± S.D.	Cambendazole mean ± S.D.
<i>S. ratti</i> (*)	3,700±2,100	10,300±9,200	12,600±15,700	0
<i>S. stercoralis</i> (**)	91±70	28±43	49±34	0

(*) larvae per gram faeces. Five animals per group.

(**) larvae per gram muscle. Five animals per group.

Effect on isolated parasitic adult female *S. ratti*

Forty small petri dishes were each set up with approximately 10 worms in 2 ml PBS. To each of these was added 1 ml of PBS containing either no drug or 3 mg of thiabendazole, mebendazole or cambendazole (concentration 1 mg/ml). Ten dishes were in each group. The

numbers of viable rhabditiform larvae were counted after two hours and 24 hours after incubation. The results are shown in Table IV. No drug had any effect on the viability of parasitic female adult worms. The effect of anthelmintics on fecundity could not be assessed as very few eggs/rhabditiform larvae were released *in vitro*.

T A B L E IV
Effect of anthelmintics on the viability of isolated parasitic adult female *S. ratti*
Percentage alive (*)

Hours of incubation with drug	Controls mean ± S.D.	Thiabendazole mean ± S.D.	Mebendazole mean ± S.D.	Cambendazole mean ± S.D.
2	100±0	100±0	100±0	100±0
24	65±4	55±10	59±8	67±23

(*) Ten samples per group

Effect on development of *S. stercoralis*

Twenty four small watch glasses were set up with 1 ml homogenized feces containing approximately 100 rhabditiform larvae. To each of these was added 2 ml PBS containing either no drug or 1200 µg of thiabendazole, mebendazole or cambendazole giving a final concentration of 300 µg drug/ml. Six dishes were in each group. Development of worms was observed over the next seven days. Thousands of infective larvae were seen in each of the control dishes but all of the dishes containing thiabendazole, mebendazole or cambendazole were completely devoid of these worms; this difference was highly significant ($P = 0.001$, Fisher's Exact Test).

Effect on infectivity of *S. stercoralis* third-stage larvae

Larvae were incubated with or without drug as described earlier, then groups of five mice were infected percutaneously with 1,000 infective larvae and the numbers of larvae in the hamstring muscles were counted seven days later. There were no significant effects on the numbers of larvae in the muscles after incubation of third-stage larvae with thiabendazole or mebendazole, but no larvae were seen in mice infected with third-stage larvae incubated with cambendazole (Table III); this difference was significant ($P < 0.005$, "t" test).

Effect on isolated free-living adult *S. stercoralis*

Four petri dishes each containing 20 adult

worms in 3 ml water were incubated with either no drug or 3 mg of thiabendazole, mebendazole or cambendazole (concentration 1 mg/ml). The viability of these worms was assessed 24 hours later. The anthelmintics had no effect on viability, all worms being alive.

DISCUSSION

This study has shown that although there are many similarities in the *in vitro* effects on *Strongyloides* of the three drugs, there are also clear differences in activity, with cambendazole having the greatest number of actions and thiabendazole and mebendazole being less efficacious. Thiabendazole and cambendazole are thought to inhibit the fumarate reductase system and its associated oxidative phosphorylation reactions against some nematodes such as *Haemonchus contortus*^{18,22}. Although a similar activity could not be demonstrated with mebendazole, it was suggested that this may have reflected its use as a suspension, the drug being poorly absorbed¹⁵. Cambendazole has been observed to have enhanced potency against a number of nematodes when compared with thiabendazole, and this has been ascribed tentatively to an alteration in its mode of metabolism; substitution of an acylamino group in the C-5 position of the benzimidazole molecule prevented inactivation by metabolic hydroxylation reactions¹².

The concentrations of drug that we used varied between 100 and 1,000 µg/ml. This range was chosen because analysis of previous *in vivo* dose-response experiments in mice⁹ indicated that such concentrations were sufficient to achieve a maximal effect on the worms. For example, all worms were killed after administration of an oral dose of 2 mg mebendazole to a 20 g mouse (concentration = 100 µg/ml assuming complete absorption and even distribution of drug). Since absorption of some of these drugs is incomplete¹⁵ and distribution *in vivo* may be uneven, the *in vitro* concentrations we used are likely to be even greater than those pertaining in the tissues around worms in infected animals, thus ensuring that any differences in the effects of the three anthelmintics would be demonstrable. All three benzimidazole compounds used had no effect on the hatching of *S. ratti* eggs, and thiabendazole and mebendazole did not impair the viability of rhab-

ditiform larvae. In contrast, cambendazole reduced considerably the number of viable rhabditiform larvae after incubation of these organisms with the drug for some hours. Similar effects were seen with second-stage larvae, thus implying that cambendazole has a different mechanism of action or is dealt with differently by the parasite, and is able to interfere with essential metabolic process. The failure of cambendazole to have a similar effect on third-stage larvae does not necessarily contradict this hypothesis, for infective larvae are a non-feeding stage and therefore differ metabolically from larvae in the earlier stages of development. Thus, cambendazole could possibly inhibit the processing of ingested nutrients. A similar argument may apply in the case of *S. ratti* parasitic female adult worms. All three drugs inhibited markedly the moulting *in vitro* of *S. ratti* larvae. This is a critical time in the worms' existence and it is possible that they may then employ specific metabolic pathways which render them susceptible to all the anthelmintics.

The relevance of these findings with the *S. ratti* model to the human parasite *S. stercoralis* was confirmed by showing that all three drugs inhibited the moulting of *S. stercoralis* larvae. It was not possible, however, to examine each stage of *S. stercoralis* separately because of the complexity of the free-living developmental cycle of these worms. As with *S. ratti* adult worms, these drugs had no effect on the viability of *S. stercoralis* free-living male and female adult worms.

While these observations provide a system in which the mechanisms of action of these benzimidazole compounds could be investigated in detail, the findings which have the greatest relevance to treatment of infection were provided by the studies of the infectivity of larvae after *in vitro* incubation with the drugs. When *S. ratti* infective larvae were incubated with thiabendazole and mebendazole, then were used to infect mice, there was no effect on the development of patent infections, as evidenced by the subsequent excretion of rhabditiform larvae in the feces. When these larvae were incubated with cambendazole, however, the infection was aborted completely. Again, the relevance of the finding with *S. ratti* to *S. stercoralis* infection was confirmed by similar studies in which *S. stercoralis* larvae were incu-

bated with the drugs. *S. stercoralis* does not complete its development in mice, but infective larvae pass to the muscles and provide a system in which the anthelmintic activity of drugs against this parasite can be observed^{9,10}; a similar system has also been used to investigate the activity of anthelmintics against hookworm larvae¹⁶. When *S. stercoralis* larvae which had been incubated with thiabendazole or mebendazole were used to infect mice, no significant reduction in worm numbers in the muscles occurred. In contrast, when these larvae were incubated with cambendazole, there was a total abrogation of infection with no larvae being detectable. These results confirm earlier *in vivo* studies in which it was shown that when administered to infected mice, thiabendazole and mebendazole were ineffective but that cambendazole was efficacious⁹. Thus, incubation with cambendazole but not thiabendazole or mebendazole inhibits the ability of infective larvae to develop subsequently. The mechanisms of this action is uncertain; it could be related to the observed capacity of cambendazole to kill first- and second-stage larvae, or it might in some way block the ability to moult into fourth-stage larvae subsequently, or it could act in both ways. In favour of the first of these possibilities is the absence of *S. stercoralis* larvae in the muscles of mice, thus suggesting that they were killed by the drugs then disposed of by the host phagocytic cells.

This finding has major implications for the therapy of human strongyloidiasis. This infection is unusual among human helminthic infections in that autoinfection occurs. Thus, at any given moment, there are likely to be third-stage larvae migrating through the tissues as well as adult worms being present in the gastrointestinal tract. It is not surprising, therefore, that many patients treated with thiabendazole or mebendazole fail to respond, since their tissue larvae are unlikely to be affected by these drugs. On the other hand, it could be anticipated that cambendazole would be active against these tissue forms and thus eradicate infections. This is of the utmost importance in strongyloidiasis, for unless all the worms are eliminated, those remaining can multiply to cause a recurrence of symptoms and provide the potential for developing overwhelming strongyloidiasis should the patient become immunosuppressed.

Although cambendazole is not licensed for human use, or endorsed as such by the manufacturers, it has been used apparently successfully and without ill-effect in a number of South American studies^{1,2,12,17,20}. These studies were limited, however, in that they were short-term studies and have not evaluated whether or not infection was eradicated. This is difficult to do in the short-term as worms are often hard to find prior to treatment, and any partial reduction in worm burden often makes them impossible to detect; patients need to be followed for months to years in order to allow build-up of worms into sufficient numbers to permit their detection. The present *in vitro* observations confirm that cambendazole acts in a different manner to thiabendazole and mebendazole, and reinforces strongly the view that this drug is likely to prove the drug of choice in this potentially lethal infection, and is worthy of greater consideration.

RESUMO

Strongyloides ratti e *S. stercoralis*: efeitos de cambendazol, tiabendazol e mebendazol *in vitro*

Os efeitos da incubação de três antihelmínticos, tiabendazol, mebendazol e cambendazol sobre *Strongyloides* foram comparados. Nenhuma droga afetou a eclosão dos ovos de *S. ratti* ou a viabilidade de larvas infectantes ou vermes adultos parasitários, mas todas as três inibiram a formação de larvas de *S. ratti*. Além disso, cambendazol, mas não tiabendazol ou mebendazol, diminuiu a viabilidade de larvas de primeiro e segundo estágio de *S. ratti*. As três drogas não tiveram efeito sobre vermes adultos de vida livre isolados, de *S. stercoralis*, mas todas evitaram o desenvolvimento de larvas rabaditiformes de *S. stercoralis*. Tiabendazol e mebendazol não tiveram efeito sobre a infectividade de larvas infectantes de *S. ratti* ou de *S. stercoralis*, mas a infecção com esses vermes foi anulada por incubação prévia com cambendazol. Esses resultados indicam que cambendazol age de modo diferente das outras duas drogas. Uma vez que ele é ativo contra larvas migrando através dos tecidos, é potencialmente de muito maior valor que o tiabendazol ou mebendazol na terapêutica da strongyloidíase.

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