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THE BINDING AND SUBSEQUENT INHIBITION OF TUBULIN POLYMERIZATION IN *ASCARIS SUUM* (*IN VITRO*) BY BENZIMIDAZOLE ANTHELMINTICS

MARGARET M. BARROWMAN, SUSAN E. MARRINER and JAMES A. BOGAN
Department of Veterinary Pharmacology, University of Glasgow Veterinary School, Bearsden Road, Bearsden, Glasgow G61 1QH, U.K.

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Abstract—Benzimidazole anthelmintics may act by interfering with the microtubule system in *Ascaris suum*. Binding of benzimidazole anthelmintics, and their inactive metabolites, to *A. suum* tubulin was demonstrated by the inhibition of intestinal extracts of the nematode to bind [^{3}H]colchicine. In addition, these compounds inhibited the polymerization of tubulin into microtubules *in vitro*.

It has been proposed that a possible mode of action of the anthelmintically active benzimidazole group of drugs is to bind parasite tubulin and promote disassembly of the parasite microtubular apparatus [1–3]. Kohler and Bachmann [2] have shown that mebendazole, one of the series, not only binds to parasite tubulin but has a higher affinity for parasite tubulin than host tubulin. The aim of this study was to determine whether other members of the benzimidazole series and some metabolites exhibit binding to tubulin and subsequent inhibition of tubulin polymerization into microtubules.

MATERIALS AND METHODS

Radio labelled [4-^{3}H] colchicine was obtained from Amersham International, Bucks, England and [^{14}C] albendazole was a gift from Smith, Kline & French, Philadelphia, U.S.A. All other reagents were of the best grade available commercially. The benzimidazole compounds were from the personal collections of Dr. J. A. Bogan and Dr. S. E. Marriner, and were generally gifts of the appropriate drug companies.

Preparation of tubulin extracts

(i) *Colchicine binding experiments.* The intestines were dissected from freshly collected *Ascaris suum* (or from worms stored at 4°) and homogenized in 10 mM KH_2PO_4/K_2HPO_4 buffer containing 1 mM $MgSO_4$ + 0.1 mM GTP + 25% (v/v) glycerol + 1 μM phenylmethane sulphonyl fluoride (PMSF), pH 7. The homogenate was centrifuged at 200,000 *g* for 1 hr and the pellet discarded. The resulting supernatant (crude extract) was stored at −15°, without loss of activity, until required for assay. Protein determinations were carried out according to the procedure of Lowry *et al.* [4].

(ii) *Polymerization experiments.* Crude extract was adjusted to a final concentration of 200 g l^{-1} $(NH_4)_2SO_4$ added over a period of 15 min at room temperature. The resulting precipitate was allowed to collect over the next 30 min, the solution was centrifuged at 3000 *g* for 1 hr and the pellet discarded. The supernatant was dialysed overnight against three changes of the homogenizing buffer B (GTP added at a concentration of 10^{-6} M) and the dialysate used in the polymerization assays.

This partially purified extract of *A. suum* was shown to be enriched in tubulin protein using SDS–PAGE [5].

Measurement of ^{3}H-colchicine binding

The percentage of ^{3}H-colchicine bound to *A. suum* tubulin was measured as described by Borisy and Taylor [6]. This method was also applied to the binding of ^{14}C-albendazole to *A. suum* tubulin.

Colchicine-binding inhibition assay

Crude extracts of *A. suum* intestinal tubulin were incubated with the appropriate benzimidazole solution (or control buffer) for 3 hr at 37°. The sample was incubated with [A-4-^{3}H] colchicine (0.05 mCi ml^{-1}, 0.5 μM) for 1.5 hr at 37° and then passed through a Sephadex G-100 column (20 × 1.6 cm). Fractions (1 ml) were collected and aliquots (100 μl) added to 10 ml of Fisofluor and counted in a Packard Scintillation Counter, allowing calculation of the percentage of label eluted bound to high molecular weight tubulin.

Measurement of tubulin polymerization

Tubulin polymerization was measured by following the increase of turbidity at A_{350} of tubulin solutions on heating at 37° as described by Hoebeke *et al.* [7]. The increase in absorbence reflects the extent of microtubular aggregation [8].

The tubulin samples were preincubated with the benzimidazole solutions (or control buffer) for 30 min at 37° and polymerization was initiated by addition of GTP (final concentration 10^{-3} M).

Preparation of benzimidazole solutions

All of the benzimidazole compounds were prepared as stock solutions in ethanol (final concentration 100 μg ml^{-1}) because of their low aqueous solubility. In all experiments these solutions were

diluted in the homogenizing buffer, the residual volume of ethanol being added to the control sample.

RESULTS

Binding of benzimidazole compounds to extracts of A. suum *tubulin*

Binding of benzimidazole compounds to *A. suum* tubulin was measured by the inhibition of intestinal supernatant of the nematodes to bind ^{3}H-colchicine. All of the benzimidazoles (and their metabolites) tested were shown to bind to tubulin from *A. suum* (Table 1). The degree of binding of benzimidazoles to the tubulin extracts at a concentration which gives maximum binding was not related to the known efficacy of the compounds and indeed metabolites such as albendazole sulfone and albendazole 2-amino sulfone, which are not anthelmintically active, were shown to bind almost as strongly as the parent drug.

Preincubation of tubulin extracts with albendazole and thiabendazole led to less than 50% inhibition of ^{3}H-colchicine binding and so it was possible to use this result to determine whether different benzimidazoles bound to different colchicine binding sites by preincubating tubulin extracts with the two benzimidazoles simultaneously. However, the results from Table 2 suggest that these benzimidazoles bound to the same sites as no further inhibition of ^{3}H-colchicine binding was achieved on preincubating tubulin extracts with both benzimidazoles simultaneously.

Albendazole was shown to bind to *A. suum* tubulin by following the incorporation of ^{14}C-albendazole into the high molecular weight fraction (110,000) of tubulin extracts. Preincubation of tubulin extracts with colchicine inhibited ^{14}C-albendazole binding. Although preincubation with 1 μM albendazole can only inhibit 50% of the binding of ^{3}H-colchicine (final concentration 1 μM), preincubation with 1 μM colchicine inhibits >85% of the binding of ^{14}C-albendazole to tubulin extracts. This suggests that although albendazole binds to the colchicine binding sites it does so with a lower affinity.

Table 2. Inhibition of ^{3}H-colchicine binding to *A. suum* tubulin after preincubation with mixed benzimidazoles

Sample	Control (%)	Inhibition of ^{3}H-colchicine binding (%)
Control	100	0
0.5 μM Albendazole	49.7	50.3
1 μM Albendazole	49.1	50.9
0.5 μM Thiabendazole	61.9	38.1
1 μM Thiabendazole	51.2	48.8
0.5 μM Albendazole + 0.5 μM Thiabendazole	50.3	49.7

The 100% control value for ^{3}H-colchicine represents binding of 32 pmoles colchicine (mg protein)$^{-1}$.

Tubulin sulphydryl groups have been reported as important for the structural and functional integrity of the molecule [9]. In addition, some sulphydryl compounds have been shown to prevent microtubule disassembly and even promote tubulin polymerization [10]. Thus sulphydryl and reducing agents were investigated for their ability to reverse mebendazole-induced inhibition of ^{3}H-colchicine binding to tubulin extracts (Table 3). No reversal of inhibition was achieved and, indeed, the results suggest that mercaptoethanol and levamisole, which is readily metabolised to the sulphydryl compound OMPI, mimic the effect of mebendazole suggesting that mebendazole/colchicine bind at or near sites on tubulin containing sulphydryl groups necessary for polymerization.

Table 1. Inhibition of ^{3}H-colchicine binding to tubulin extracts by preincubation with various benzimidazoles

Sample	Control binding (%)	Inhibition of ^{3}H-colchicine binding (%)
Control	100	0
0.05 μM Albendazole	86.4	13.6
0.10 μM Albendazole	75.6	24.4
0.20 μM Albendazole	67.0	33.0
0.50 μM Albendazole	52.1	47.9
1.00 μM Albendazole	50.9	49.1
0.40 μM Albendazole	57.4	42.6
0.40 μM Albendazole sulfoxide	52.4	47.6
0.40 μM Albendazole sulfone	46.2	53.8
0.40 μM Albendazole 2-amino sulfone	62.3	37.7
0.40 μM Mebendazole	11.8	88.2
0.40 μM Fenbendazole	24.5	75.5
0.40 μM Oxfendazole	28.3	71.7
0.40 μM Thiabendazole	61.8	38.2

The 100% control value for ^{3}H-colchicine represents binding of 40 pmoles colchicine (mg protein)$^{-1}$.

Table 3. Effect of sulphydryl and reducing agents on ^{3}H-colchicine binding to *A. suum* tubulin

Sample	Control binding (%)	Inhibition of ^{3}H-colchicine binding (%)
Control	100	0
Mebendazole (0.5 μM)	5	95
Levamisole (0.5 μM)	8.5	91.5
Levamisole (0.5 mM)	12.1	87.9
Mebendazole (0.5 μM) + levamisole (0.5 μM)	5	95
1 μM Mercaptoethanol	41.6	58.4
1 mM Mercaptoethanol	13.4	86.6
Mebendazole + 1 μM mercaptoethanol	8.2	91.8
Mebendazole + 10 μM mercaptoethanol	6.2	93.8
1 μM NAD	98.4	1.6
1 μM NADH	116	—
1 μM Glutathione	84.3	15.7
Mebendazole + 1 μM glutathione	6.2	93.8

The 100% control value for ^{3}H-colchicine represents binding of 95 pmoles colchicine (mg protein)$^{-1}$.

Effect of benzimidazoles on the polymerization of tubulin from A. suum

Preincubation of partially purified tubulin extracts with colchicine and a range of benzimidazole compounds (0.4 μM) inhibited the ability of tubulin to polymerize on heating to 37° (Fig. 1). Although the anthelmintically inactive albendazole metabolites were as effective as albendazole and albendazole sulfoxide, the metabolites of fenbendazole and oxfendazole were less effective as inhibitors of tubulin polymerization.

DISCUSSION

All the benzimidazoles tested were shown to inhibit ^{3}H-colchicine binding to parasite tubulin presumably by either (i) binding to colchicine binding sites or (ii) binding close to these sites and preventing access to colchicine by steric hindrance. Results showing that preincubation of *A. suum* tubulin with colchicine inhibited the binding of ^{14}C-albendazole to parasite tubulin further suggested that the benzimidazoles and colchicine bound to the same or similar sites. Since no greater degree of inhibition of colchicine binding can be achieved by preincubation of tubulin with albendazole and thiabendazole simultaneously it would appear probable that each of the benzimidazoles are binding to the same colchicine binding sites. This would suggest that the different degrees of inhibition induced by the various benzimidazoles reflects their different affinities for these binding sites.

It was perhaps surprising that the benzimidazole metabolites such as albendazole sulfone and albendazole 2-amino sulfone which are considered to be anthelmintically inactive, bound to tubulin but, it would seem likely that the colchicine binding sites recognize the core benzimidazole structure common to all the benzimidazoles as well as their different side chains. The lowered binding affinities observed for albendazole 2-amino sulfone and thiabendazole may be a consequence of the loss of the carbamate group from position two of the 5-membered ring which may be sterically analogous to the —NHCOCH$_3$ side chain on colchicine.

Albendazole sulfone and albendazole 2-amino sulfone are produced primarily in the host liver and hence may not be anthelmintically active *in vivo* since the parasite may not absorb these more polar metabolites sufficiently for anthelmintic activity.

Preincubation of *A. suum* tubulin extracts with sulphydryl reagents such as mercaptoethanol led to inhibition of colchicine binding (Table 3). These results were consistent with those of De Brabander *et al.* [10], who suggested that the antimicrotubular effects of mercaptoethanol and levamisole were due to formation of mixed disulphides with sulphydryl groups on tubulin essential for the structural and functional integrity of microtubules [9]. Since preincubation with mercaptoethanol mimicked the effects of benzimidazoles and inhibited ^{3}H-colchicine binding it may be possible that colchicine and the benzimidazoles act by binding at sites which block access to sulphydryl groups essential for polymerization. Indeed colchicine and all the benzimidazoles tested were shown to inhibit tubulin polymerization.

However, caution should be employed in interpreting the mode of anthelmintic activity from these *in vitro* experiments without similar experiments on parasites treated with benzimidazoles *in vivo*. Such *in vivo* studies are necessary before concluding that binding to tubulin and inhibition of microtubule polymerization is a possible mode of anthelmintic activity.

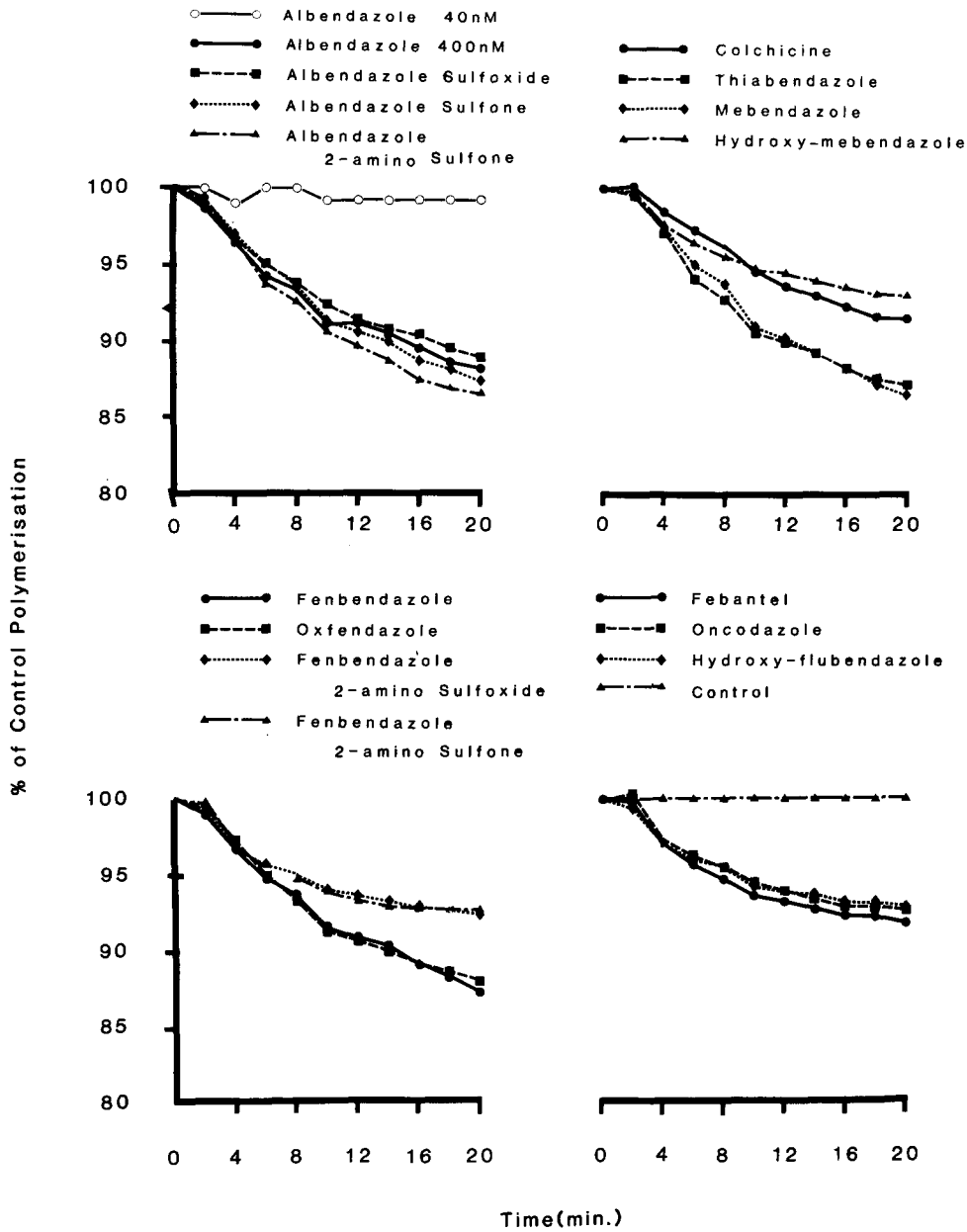

Fig. 1. Inhibition of tubulin polymerization by preincubation with benzimidazole compounds. The results represent the turbidity at each time interval expressed as a percentage of the control value. Each point is the mean of at least two independent experiments. The final concentration of the benzimidazole compounds in the preincubation mixture was 400 nM unless otherwise stated.

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