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Activities of Several Benzimidazoles and Tubulin Inhibitors against *Giardia* spp. In Vitro

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Previous studies in our laboratory have shown that albendazole is effective against *Giardia* spp. in vitro and in vivo, prompting an investigation of the effects of several related benzimidazoles (BZs) on the viability of this protozoan parasite. A range of BZs was tested, and their effects were compared with those of a number of microtubule inhibitors. The effects produced by the two types of drugs were markedly similar, namely, trophozoite detachment and distortion of morphology and general structure, indicating a potential antimicrotubule mode of action for BZs. Mebendazole, albendazole, and fenbendazole proved to be among the most effective BZs tested, exhibiting apparent irreversibility. Nocodazole, oxfendazole, and albendazole sulfoxide, among others, produced transient inhibitions only. Further studies are required to evaluate all available BZs and other antigiardial agents to ensure the development of the most effective and safest antigiardial agent possible.

Giardia duodenalis is one of the most commonly identified intestinal pathogens in the world (15). Although it has a global distribution (23), *G. duodenalis* is especially prevalent in developing countries and among disadvantaged groups, such as Australian Aborigines. Young children are particularly at risk since high levels of *G. duodenalis* can cause acute or chronic diarrhea, protein-calorie malnutrition, and failure to thrive (15).

Efforts to control giardiasis by using therapeutic agents have been based on a number of compounds, including quinacrine, several nitroimidazole derivatives (metronidazole and tinidazole), and a nitrofuran, furazolidone. Although therapy with these drugs is usually effective, there are still limitations to their use, because they all exhibit undesirable side effects and, in the case of metronidazole, may be carcinogenic (30). Treatment failures can sometimes occur, requiring repeated courses of therapy (9). This repeated exposure to potentially dangerous drugs, particularly in communities in which reinfection, and thus frequent therapy, is common, is an area of grave concern. There is also evidence of variable drug susceptibilities among *Giardia* isolates (10, 11) and even within laboratory-derived clonal lines of *G. duodenalis* (3).

The search for alternative antigiardial agents led to a reevaluation of the benzimidazoles (BZs) as antiprotozoan agents (22, 27, 28). The BZs have been successfully used for many years as anthelmintics (31) and are thought to target tubulin (17), a major component of *Giardia* cytoskeletal structures. Alternative theories include alteration of the bioenergetics of *Giardia* spp. by BZs, which act as lipid-soluble proton ionophores (20). Previous studies in our laboratory have shown that the BZ albendazole is a more effective antigiardial agent in vitro than either metronidazole or tinidazole (22). Other investigators (6) have reported the superior antigiardial efficacy of mebendazole over metronidazole. These findings, together with confirmation of the efficacy of albendazole in an in vivo study that used mouse and rat models (26, 27), have prompted a more extensive investigation into the effect of several related BZs and also a number of other microtubule inhibitors on the viability of *G. duodenalis* in vitro.

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MATERIALS AND METHODS

Organisms and cultures. Trophozoites of the *G. duodenalis* isolate P1c10 (a clone of the Portland isolate P1 developed in our laboratory) were cultured at 37°C in modified BI-S-33 medium containing 10% newborn bovine serum but no added antibiotics (21).

Chemotherapeutic agents. Albendazole and albendazole sulfoxide were supplied by SmithKline Beecham. Nocodazole, fenbendazole, oxfendazole, *cis*- and *trans*-tubulozole, 2-propionamido-5-benzoyl benzimidazole, thiabendazole, mebendazole, β-lumicolchicine, colchicine, griseofulvin, vinblastine, and podophyllotoxin were gifts from E. Lacey, McMaster Laboratory, Commonwealth Scientific and Industrial Research Organisation, Sydney, Australia. Solutions of BZs and tubulin inhibitors were prepared fresh in dimethyl sulfoxide. The final dimethyl sulfoxide concentration in the culture tubes was always <0.5%.

Adherence inhibition assays. The susceptibility of *G. duodenalis* in vitro to the indicated drugs was determined as described previously (22), with one modification. Briefly, trophozoites were exposed to the drug for either 4 or 24 h, after which the culture medium was replaced with drug-free medium. The centrifugation step to pellet trophozoites prior to changing the medium was omitted because healthy trophozoites attach to the culture vessel wall and the majority of unattached trophozoites form a pellet at the bottom of the tube and are not removed when the medium is replaced. Controls containing equivalent concentrations of dimethyl sulfoxide were treated similarly. All experiments were performed in duplicate. The main criterion for determining drug susceptibility was trophozoite attachment to the culture tube walls, since attachment is highly characteristic of this organism and is an important indicator of viability (6, 19). Indeed, it has been suggested that adherence inhibition may be a more efficient mechanism for host resistance to *Giardia* spp. than killing and that the adherence mechanism of *Giardia*

TABLE 1. Comparison of IB_{50}s for the inhibition of [^{3}H]mebendazole binding by preincubation of inhibitor and then the addition of [^{3}H]mebendazole to *H. contortus* L_3 crude tubulin extracts and IC_{50}s for BZ efficacy against *Giardia* spp. in vitro

Drug	MBZ IB_{50} (μM) for *H. contortus*[a]	IC_{50} (μM) for *Giardia* spp. after 24 h of exposure
Nocodazole	0.12	0.07
Mebendazole	0.19	0.19
Albendazole	0.21	0.25
Fenbendazole	0.18	0.30
Oxfendazole	1.30	4.20
Albendazole sulfoxide	2.30	3.15

[a] [^{3}H]mebendazole was added to *H. contortus* L_3 crude tubulin extracts as described previously (17).

isolates is a feasible and more efficient target for therapy (5). Morphology and motility were also taken into account. A previously described semiquantitative method (22) was used to determine trophozoite adherence over 48 h. Dose-response curves were constructed for all drugs, and the concentration that caused a 50% reduction in adherence compared with that in controls (IC_{50}) was calculated. Cultures were assessed at 4, 24, and 48 h.

Selection for albendazole resistance. Experimental selection of a drug-resistant line of *G. duodenalis* was attempted by growing isolate P1c10 in medium containing a range of sublethal albendazole concentrations (0.16, 0.32, and 0.64 μM). After 24 and 72 h of incubation, unattached cells were discarded and fresh medium was added. Trophozoites were allowed to recover for 24 h before reincubation with the drug. The isolate was maintained in this way for over 6 months.

RESULTS

Inhibition of adherence in vitro. In all cases, trophozoites exposed to drugs for either 4 or 24 h were assessed for trophozoite adherence at 4, 24, and 48 h. IC_{50}s were calculated at 48 h and were compared to previously published concentrations that cause a 50% inhibition of binding compared with that in controls (IB_{50}s) for the binding of labeled mebendazole to nematode tubulin (17) (Table 1). Of the drugs tested, nocodazole, mebendazole, albendazole, and fenbendazole were the most potent, with IC_{50}s for a 24-h exposure of 0.07, 0.19, 0.25, and 0.3 μM, respectively. IC_{50}s for a 4-h exposure were 2.56, 2.7, 3.7, and 4 μM, respectively (Fig. 1). Oxfendazole had good potency against *Giardia* isolates for 24 h of exposure, with an IC_{50} of 4.2 μM. However, it was not effective with a 4-h exposure, having an IC_{50} of 85 μM. Albendazole sulfoxide, a metabolite of albendazole, was effective against the *Giardia* isolates only at high doses, especially after 4 h of exposure, having an IC_{50} of 63.3 μM. Thiabendazole also had low potency against *Giardia* isolates, requiring high doses to be effective in vitro, with IC_{50}s of 30.4 μM for a 24-h exposure and 203.2 μM for a 4-h exposure. The compound 2-propionamido-5-benzoyl benzimidazole did not exhibit any antigiardial effects at 24 h of exposure at the highest concentration tested (20 μM). With mebendazole, albendazole, and fenbendazole, inhibition of *Giardia* growth in vitro was maintained beyond 72 to 96 h. Drug-affected cells became totally distorted in shape and failed to complete cellular division (22). Transmission electron microscopy of drug-affected cells also confirmed

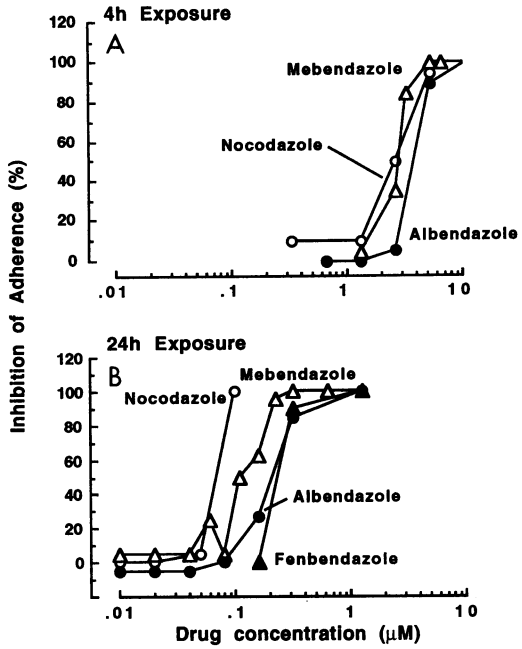

FIG. 1. In vitro efficacies of nocodazole, mebendazole, albendazole, and fenbendazole against *Giardia* isolates after 4 h (A) and 24 h (B) of exposure. Values are taken from the assessment at 48 h.

this (24). All other BZs failed to maintain their inhibition to various degrees beyond 48 h.

Of the microtubule inhibitors tested, vinblastine exhibited the greatest efficacy against *Giardia* isolates in vitro, with an IC_{50} of 0.7 μM for a 24-h exposure, but did not exhibit efficacy for 4 h of exposure at the concentrations tested (to 10 μM). Podophyllotoxin, griseofulvin, and colchicine had IC_{50}s of 4.2, 29.2, and 38.1 μM, respectively, for a 24-h exposure but, again, were not effective at the 4-h exposure at the concentrations tested (10, 50, and 50 μM, respectively). *cis*- and *trans*-tubulozole and β-lumicolchicine were not effective at the concentrations tested (2.56, 10.24, and 50 μM, respectively). Vinblastine alone maintained its inhibition beyond 72 h.

Failure to demonstrate in vitro resistance to albendazole. Efforts to select for albendazole-resistant *Giardia* isolates in vitro were unsuccessful. Resistant cells were defined as being viable and also attaching to the culture vessel wall. Drug-free passages were continued because incubation of trophozoites with the drug for 96 h or more resulted in complete inhibition of *Giardia* growth, even when the drug was used at low concentrations (0.08 μM).

DISCUSSION

The BZs are broad-spectrum drugs which have been used for many years in the treatment of helminthiasis in animals and humans (4). It is only relatively recently that their in vitro antigiardial activities have been evaluated (6, 22, 28). In the present study we assessed several related BZs and a number of microtubule inhibitors for their in vitro efficacies against *Giardia* viability.

Of the BZs tested, nocodazole was the most potent. Nocodazole, however, did not maintain its antigiardial activity beyond 48 h, which is in sharp contrast to the activities of mebendazole, albendazole, and fenbendazole, with which

inhibition was maintained. In addition, nocodazole is not a widely used anthelmintic agent, and information on its potential toxicity and side effects is limited. Nocodazole is also a potent inhibitor of mammalian tubulin (13), which would indicate potential toxicity to humans.

Mebendazole, albendazole, and fenbendazole are commonly used drugs with broad spectra of activity against a range of different parasites which includes gastrointestinal nematodes, lung and other tissue nematodes, cestodes, and trematodes (16). The toxicities of these agents are low, and they cause few side effects, with only slight side effects reported in some individuals treated with these drugs. Albendazole, mebendazole, and oxfendazole are known to produce embryotoxicity and teratogenesis in laboratory animals, however, and should not be given to pregnant women (4). Flubendazole (a fluorine analog of mebendazole) and fenbendazole, however, have not been shown to exhibit these effects. In human clinical practice, only albendazole, mebendazole, thiabendazole, and flubendazole are currently in use as anthelmintic agents (14). Albendazole sulfoxide was considerably less effective than albendazole, and the apparent reversibility of its action limits its potential usefulness as an antigiardial agent. Thiabendazole had very low potency against *Giardia* spp., and because side effects are sometimes experienced with this drug (4), it is not considered to be a suitable candidate for antigiardial chemotherapy.

The microtubule inhibitor vinblastine exhibited good potency against *Giardia* isolates in vitro with a 24-h exposure and also appeared to act irreversibly, in contrast to the reversible action of podophyllotoxin, griseofulvin, and colchicine. The toxicities of these drugs, however, render them unsuitable as antigiardial agents (18).

There is increasing evidence, however, that the primary mode of action of BZs against helminths is via inhibition of tubulin (18). Additional evidence for this is that BZ resistance in *Haemonchus contortus* may be due to point mutations that cause structural changes in β-tubulin, which in turn interferes with BZ binding. Also, the deletion of a BZ-susceptible β-tubulin gene in *Caenorhabditis elegans* was found to confer BZ resistance (29). It is therefore important to determine whether BZs exert their effects against *Giardia* spp. as tubulin antagonists, similar to the mechanism in nematodes (18), or whether efficacy against *Giardia* spp. is related to some unique aspect of the drug-parasite interaction. In support of a microtubule antagonist antigiardial mode of action for the BZs, a good correlation exists between the inhibition of labeled mebendazole binding to nematode tubulin (17) and efficacy against *Giardia* spp. in vitro ($r = 0.856$, $n = 6$), suggesting a similar mode of action of the BZs in the two types of parasites. It is also possible however, that these drugs bind to *Giardia*-specific proteins called giardins (25), which are found only in the ventral disc. Presumably, the giardins play a major role in the functioning of the ventral disc and are good candidates for specific chemotherapeutic attack. It is interesting that BZ-affected *Giardia* isolates retain flagellar activity; flagellae rely on tubulin and not giardin for their integrity (6, 22).

Previous investigators (6) have also shown that BZs are effective against *Giardia* spp., noting that "treatment caused cells to detach" and that "treatment induced gross morphologic changes not seen with any other drug." Given the predominant role that microtubules play in attachment and also cell structure (7), this is further supportive evidence that microtubule antagonism is indeed the primary antigiardial mode of action of BZs. The efficacies of the tubulin inhibitors tested against *Giardia* spp. also tend to support this idea, because the effects produced by the tubulin inhibitors were very similar to those produced by the BZs. Vinblastine, podophyllotoxin, griseofulvin, and colchicine treatments all resulted in trophozoite detachment and drastic changes in the general morphologies of the trophozoites. Interestingly, 2-propionamido-5-benzoyl benzimidazole did not inhibit *Giardia* adherence at all, and this compound was specifically constructed as a noninhibitor of tubulin (17), lending further support to this theory. Previous investigators (6, 17) have reported that BZ binding appears to be reversible. Once BZ is bound, however, it may produce a cascade of direct and indirect biochemical and physiological changes (17, 18), resulting in the irreversible effects that we observed, irrespective of whether the drug is still bound or not. The mechanism of antigiardial activity of BZs is being investigated in our laboratory in order to provide a basis for the development of the most effective drug and dosing strategies.

Mebendazole, albendazole, and fenbendazole are potentially some of the most useful antigiardial agents, having high in vitro potencies against *Giardia* spp., an effect which appears to be irreversible. Although only one *Giardia* isolate was used in the present study, previous unpublished studies in our laboratory have shown that albendazole has similar potencies against 10 different *Giardia* isolates of both human and animal origin. Both albendazole and mebendazole are relatively insoluble, and a reasonable proportion of the administered dose remains in the gastrointestinal tract (12), thus optimizing contact with *Giardia* isolates. In vivo studies in mice (26, 27) support the findings that BZs are effective antigiardial agents; similarly, in rats, three doses of less than 10 mg of albendazole per kg of body weight were completely effective in clearing the small intestine of trophozoites (27). Recent studies have suggested that albendazole (33) and mebendazole (1, 2) have clinical efficacy against giardiasis. The claims for mebendazole have been disputed, however (8), and more systematic clinical trials are necessary to establish its clinical efficacy. Our lack of success in developing albendazole-resistant *Giardia* isolates in vitro can be seen as encouraging since a problem with the BZs when used as anthelmintic agents in domestic animals has been the development of resistance to the drugs (32).

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