In Vitro Activities of Itraconazole, Methiazole, and Nitazoxanide versus *Echinococcus multilocularis* Larvae

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Albendazole (ABZ) and mebendazole are the only drugs licensed for treatment of human alveolar echinococcosis. In order to augment the armamentarium against this deadly disease, we tested a series of drugs for their efficacy against *Echinococcus multilocularis* larvae. *E. multilocularis* larvae grown intraperitoneally in Mongolian gerbils were transferred into tissue culture. Vesicles budded from the tissue blocks and after 6 weeks, drugs were added, and the effect on the vesicles was observed. We tested the following drugs at various concentrations: ABZ, artemether, caspofungin, itraconazole (ITZ), ivermectin, methiazole (MTZ), miltefosine, nitazoxanide (NTZ), rifampin, and trimethoprim-sulfamethoxazole. ABZ, ITZ, MTZ, and NTZ effectively destroyed parasite vesicles in this in vitro culture system. At high NTZ doses of 10 µg/ml, disintegration of all vesicles was observed after 7 days and was significantly more rapid than with ABZ at equal concentrations (21 days). After drug discontinuation, regrowth of vesicles occurred between 7 and 14 days for all four drugs, indicating a parasitostatic effect. Combination treatment with NTZ-ABZ at concentrations between 1 and 10 µg/ml for either 3 weeks, 3 months, or 6 months yielded no vesicle regrowth during 8 months after drug discontinuation. The treated larval tissue was injected intraperitoneally into gerbils, and no regrowth of larval tissue was observed, suggesting a parasitocidal effect after combined treatment. ITZ, MTZ, and NTZ are potent inhibitors of larval growth, although they proved to be parasitostatic only. The combination of NTZ plus ABZ was parasitocidal in vitro. Animal experiments are warranted for studies of dose, toxicity, and drug interactions.

Human alveolar echinococcosis (AE) is caused by the larval stage of *Echinococcus multilocularis*. The disease is characterized by a primary invasion of larvae into the liver and may secondarily affect other organs. Human AE is endemic in wide regions of the northern hemisphere. Although untreated AE is fatal in >90% of cases, the two available treatment options include surgery and chemotherapy (2). In most cases the diagnosis of AE is only established at an advanced stage of disease. For most of these patients, curative surgery is not an option due to diffuse parasitic infiltration of nonresectable structures. As a consequence, chemotherapy is the only means to impede further progression of the disease (19).

Benzimidazole carbamate derivatives, namely, albendazole (ABZ) and mebendazole, are the only drugs licensed for the treatment of human AE (12). Severe side effects may be observed, and the parasitostatic effect of these drugs implies that *E. multilocularis* is not depleted and may resume growth after discontinuation of treatment (3, 18). The overall success rate of benzimidazole treatment ranges between 55 and 97% (4, 9, 19). As an alternative, conventional amphotericin B desoxycholate (cAMB) effectively inhibits larval growth (20). However, its effect is only parasitostatic, and wide use is limited primarily by nephrotoxicity (17).

At this point, no reliable chemotherapeutic alternative can be offered to patients who do not tolerate or do not respond to

the therapeutic options mentioned above. Thus, it has become evident that new chemotherapeutic strategies against AE are urgently needed. A few alternative treatments have been tested (e.g., praziquantel and alpha-difluoromethylornithine in the animal model) with limited success (13, 15). Recently, a damaging potential against *E. multilocularis* was identified for nitazoxanide (NTZ) in a mouse model (22). In order to augment the armamentarium of effective drugs against this deadly disease, we tested a series of drugs for their efficacy against *E. multilocularis* larvae in vitro, focusing on the kinetics of larval destruction and the potential to kill the parasite.

MATERIALS AND METHODS

We tested the following drugs for their efficacy against *E. multilocularis* larvae (Table 1). Albendazole is known for its parasitostatic activity against *E. multilocularis*, and the drug concentrations tested were 1 and 10 µg/ml. Artemether was used at 1, 10, and 20 µg/ml and was kindly provided by Marcel Tanner and Shu-Hua Xiao (Tropical Institute of Berne, Berne, Switzerland). Artemether was used in conjunction with hemin at concentrations of 50 and 100 µg/ml.

Caspofungin was purchased from Merck, Sharp, and Dohme (Haar, Germany) and used at 7 and 70 µg/ml. Itraconazole (ITZ) was purchased from Janssen-Cilag (Neuss, Germany). It was used at a concentration of 0.7 mg/ml. Ivermectin (Sigma, Taufkirchen, Germany) was used at 0.2 mM. Methiazole (MTZ) was obtained from Latoxan (Valence, France) and was used at 1 and 10 µg/ml. Miltefosine was used at 0.5, 2.5, and 7.5 µg/ml and was obtained from Zentaris GmbH (Frankfurt, Germany). NTZ was kindly provided by Romark Laboratories (Tampa, FL) and was used at concentrations of 0.1, 1, and 10 µg/ml. Rifampin was purchased from Sigma (Taufkirchen, Germany) and tested at a concentration of 10 µg/ml. Trimethoprim-sulfamethoxazole was purchased from Ratiopharm GmbH (Ulm, Germany) and was used at concentrations of 0.01 and 0.02 mg/ml.

The harvesting and long-term cultivation of larval tissue blocks was performed as described previously (20). Briefly, larval tissue was obtained after intraperitoneal growth in Mongolian gerbils. Three tissue blocks of 0.5 cm² were trans-

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TABLE 1. Concentrations, duration of application, and efficacy of applied drugs against *E. multilocularis* larvae^a

Drug	Concn (µg/ml) ^b	Duration of application (wk)	Effect
ABZ	1, 10	4 and 6	DV after 21 days
Artemether	1, 10, 20	3	None
Artemether + hemin	1, 10, 20 + 50, 100	3	None
Caspofungin	7	2, 4, and 6	None
	70	2 and 4	None
Itraconazole	0.7 mg/ml	6	DV after 35 days
Ivermectin	0.2 mM	3	None
Methiazole	1	4	DV after 21 days
	10	4	DV after 18 days
Miltefosine	0.5, 2.5, 7.5	6	None
NTZ	0.1		DV after 17 to 21 days
	1.0	6	DV after 9–14 days
	10		DV after 7 days
NTZ + ABZ	1 + 1		DV after 9 days
	1 + 10	3, 12, and 26	DV after 9 days
	10 + 1		DV after 7 days
	10 + 10		DV after 7 days
Rifampicin	10	3	None
Trimethoprim-sulfamethoxazole	0.01 mg/ml	2 and 4	None
	0.02 mg/ml	2 and 4	None

^a Drugs are listed in alphabetical order. DV, disintegration of all vesicles.^b Concentrations are in µg/ml unless otherwise stated.

ferred into each culture flask containing HepG2 human liver cells, 20 ml of Dulbecco modified Eagle medium, 10% fetal calf serum, 200 U of penicillin/ml, 200 µg of streptomycin/ml, and 10 µg of levofloxacin/ml. Vesicles budding from the larval tissue were allowed to grow until reaching a steady state (between 140 and 180 vesicles per flask) after approximately 5 weeks. As a follow-up parameter we recorded the number of secondary vesicles growing from the tissue block. The number of vesicles was determined twice weekly by macroscopic evaluation, and all visible vesicles were counted. All experiments were performed in duplicates, and the interassay variation was <10%. Control cultures contained 40 µl of dimethyl sulfoxide per 20 ml. All drugs were added to tissue blocks cultured for more than 6 weeks. The chemotherapeutic effect was macroscopically assessed by the loss of turgidity and disintegration of larval vesicles grown in tissue culture.

In vivo viability testing was performed by injection of larval tissue into Mongolian gerbils. Larval tissue was minced through a sieve with 0.5-mm pores and resuspended in Dulbecco modified Eagle medium. Next, 0.3 ml of the suspension was injected into the peritoneal cavity of a gerbil by using a 1-ml syringe with a 20-gauge needle. Two gerbils were used for each test. After 6 weeks the gerbils were euthanized, the abdominal cavity was opened, and larval growth was assessed. The proliferation of vesicles in gerbils confirmed that the larval tissue was viable. The testing of viability by injection of tissue into rodents (in vivo viability test) is an established and reliable procedure (10, 16, 20, 24).

RESULTS

Drugs were applied at various concentrations and durations of treatment as depicted in Table 1. All experiments were performed in duplicates. No destructive effect on *E. multilocularis* vesicles was observed for artemether (with or without hemin), caspofungin, ivermectin, miltefosine, rifampin, or trimethoprim-sulfamethoxazole.

ABZ at concentrations of 1 and 10 µg/ml served as a positive control (20), and the disintegration of vesicles was complete after 21 days.

Methiazole. A first destructive effect with loss of vesicle turgor was noted after 7 days of MTZ application. Disintegration of vesicles under MTZ was complete after 18 to 21 days, depending on the drug concentrations. The dynamics of vesicle disintegration were comparable for ABZ and MTZ at concentrations of 1 and 10 µg/ml (Fig. 1A). Regrowth of the vesicles

was observed as soon as 7 days after discontinuation of MTZ and was again comparable to the time to regrowth for ABZ. The dynamics of regrowth after MTZ were dependent on the drug concentration; after treatment with MTZ at 1 µg/ml, regrowing vesicles reached a steady state at ca. 40% of the initial number of vesicles, whereas after MTZ at concentrations of 10 µg/ml the maximum number of vesicles at the steady-state level was only 3% of the initial number of vesicles before application of the drug. Drugs were again applied when vesicle growth had reached a steady state in all flasks (week 11). Again, an effective vesicle disintegration was observed for MTZ at both 1 and 10 µg/ml.

Itraconazole. The dynamics of vesicle disintegration were more delayed for ITZ compared to ABZ (Fig. 1B). A first effect on vesicles was noted after 14 days, and disintegration was complete after 5 weeks. Regrowth was observed as soon as 7 days after the discontinuation of ITZ and ABZ treatment. Compared to ABZ, the dynamics of regrowth were more rapid and more pronounced for ITZ, and 21 days after drug discontinuation the number of vesicles reached a steady state at a level of 86% of the original number of vesicles.

Drugs were again applied at week 13, and disintegration was notable after 7 days in all cultures. However, with ITZ treatment the disintegration of all vesicles took 12 weeks.

Nitazoxanide. With NTZ, the time to vesicle disintegration depended on the applied drug concentration (Fig. 1C). Although disintegration of all vesicles was observed after 7 days at concentrations 10 µg/ml, this effect was delayed until days 9 to 14 for NTZ at 1 µg/ml and was not observed until days 17 to 21 at concentrations of 0.1 µg/ml. For ABZ, the disintegration of vesicles was complete after 21 days.

The drug concentration influenced the speed of regrowth of vesicles after discontinuation of NTZ. After 6 weeks of NTZ at 0.1 µg/ml, vesicles grew as soon as 7 days after drug discontinuation and 14 to 17 days after discontinuation of NTZ at 1 µg/ml (Fig. 1C). Even after prolonged treatment for 6 months of NTZ at 1 µg/ml, regrowth of vesicles was observed after 19 days (data not shown). No regrowth of vesicles was observed after treatment with NTZ at higher concentrations of 10 µg/ml over a period of 40 weeks. However, when the treated larval tissue was reinjected into Mongolian gerbils, larval growth was observed, thereby providing proof of vital larval tissue.

The combination of NTZ plus ABZ demonstrated disintegration of all vesicles after 7 days of NTZ (10 µg/ml)–ABZ (10 µg/ml) and for NTZ (10 µg/ml)–ABZ (1 µg/ml) and after 9 days for NTZ (1 µg/ml)–ABZ (1 µg/ml) and for NTZ (1 µg/ml)–ABZ (10 µg/ml), respectively. Thus, the time to disintegration depended on the dosage of NTZ. In different assays drugs were applied for either 3 weeks, 3 months, or 6 months. With combination treatment, visible regrowth of vesicles did not occur in any experiment. The duration of observation after discontinuation of combined treatment with NTZ and ABZ was 35 weeks after any of the different combinations of dosages (NTZ and ABZ: 1 and 1 µg/ml, 10 and 1 µg/ml, 1 and 10 µg/ml, and 10 and 10 µg/ml). Hereafter, larval tissue from each experiment was injected intraperitoneally into Mongolian gerbils, and no regrowth of larval tissue was observed after 6 weeks, indicating a parasitocidal effect after combined treatment.

DISCUSSION

As described previously, larval tissue blocks with budding vesicles were maintained in culture over extended periods of time (20). This culture model constitutes a suitable test system for assessing the effect of antiparasite drugs, because the in vitro cultured larvae show strong similarities to the in vivo situation and contain all important larval structures. The larval vesicles are composed of an acellular laminated layer, which covers the entire larva (8, 11). This laminated layer protects the parasite from host defense mechanisms and consists of connective tissue, muscle cells, glycogen-containing storage cells, and undifferentiated cells. In vitro, this laminated layer is formed after 13 days (5). The tissue inside contains the multicellular germinal layer and the developing protoscoleces. The steady state of vesicle growth in vitro over prolonged periods of time resembles the chronic persistence of the parasite in the liver. Furthermore, the growth of vesicles from the tissue block in vitro resembles the centripetal expansion of larval tissue in vivo. After the addition of drugs to the culture medium, the disintegration of vesicles indicated the immediate effect of a

FIG. 1. Effect of drugs on larval vesicles grown in tissue culture. (A) Effect of MTZ at various concentrations. Symbols: dashed line, control; ○, MTZ at 1 µg/ml; ●, MTZ at 10 µg/ml; ▲, ABZ at 1 µg/ml; △, ABZ at 10 µg/ml. (B) Effect of ITZ. Symbols: dashed line, control; ■, ITZ at 0.7 µg/ml; △, ABZ at 10 µg/ml. (C) Effect of NTZ at various concentrations. Symbols: dashed line, control; ●, NTZ at 0.1 µg/ml; ◆, NTZ at 1 µg/ml; ■, NTZ at 10 µg/ml; △, ABZ at 10 µg/ml. Arrows: 1, discontinuation of drugs; 2, start of drug treatment.

drug against *E. multilocularis* larvae. However, only by prolonged cultivation of tissue blocks even after the disappearance of all budding vesicles were we able to assess the potential of a drug to effectively kill the parasite. The parasite may remain viable inside the tissue blocks long after vesicles have disintegrated. Regrowth of vesicles from the tissue block indicates persistent parasite viability and thus indicates parasitostatic drug activity rather than a parasitocidal effect. If a parasitocidal drug effect was suggested by the lack of vesicle growth, parasitic death was further corroborated by injection of larval tissue into Mongolian gerbils.

We tested a series of drugs for their effect against *E. multilocularis* larvae in this in vitro system. The rationale for the choice of drugs tested was their proven effect against other parasites (i.e., artemether, ITZ, ivermectin, miltefosine, rifampin, and trimethoprim-sulfamethoxazole) or fungi (i.e., caspofungin and ITZ). In addition, we tested MTZ, a new benzimidazole with major structural similarities to ABZ. Among the drugs tested, we found a destructive effect on *E. multilocularis* larvae for MTZ, ITZ, and NTZ.

MTZ is a new benzimidazole analogue and was used at 1 and 10 µg/ml. It constitutes a novel benzimidazole compound. ABZ and MTZ differ in the isomerism of their thioalkyl groups, the substituent being 5-propylthio in the former and 5-isopropylthio in the latter. To our knowledge, no report on the efficacy against *E. multilocularis* larvae has been published. The effect of MTZ on larval tissue was similar to that of ABZ at equal concentrations. The activity of MTZ was dependent on concentration and was only parasitostatic in the presented experiments. Figure 1A shows that drug dosage influenced the speed of regrowth of vesicles. MTZ is not licensed for human or veterinary use and, at this point, no data on toxicity or bioavailability are available.

ITZ effectively leads to the disintegration of parasite vesicles.

cles. At a dose of 0.7 mg/ml, complete disintegration was less rapid with ITZ than with ABZ (10 µg/ml). ITZ affects ergosterol and thus shares its target with cAMB. Whereas cAMB acts by direct destruction of synthesized ergosterol, ITZ inhibits sterol biosynthesis. This would explain the more delayed effect on vesicle disintegration of ITZ compared to cAMB (20). The antiparasitic effect was sustained as long as the drug was applied on the larval tissue block. A few days after drug discontinuation, the regrowth of vesicles became visible, indicating the parasitostatic potential rather than a parasitocidal effect. The confirmation of in vivo efficacy and the determination of the optimal dosage will be a task for upcoming studies in animals and humans. ITZ would offer several advantages over cAMB, which is the only alternative drug shown to be effective against AE in humans in case of intolerance of benzimidazoles (17). Nephrotoxicity might impede the long-term application of cAMB and, due to the parasitostatic effect, the discontinuation of the drug is associated with the risk of relapses. A great plus for ITZ is its oral form of application and extensive clinical experience with its use, including long-term applications (6). The main adverse effects are gastrointestinal discomfort, hypersensitivity reactions, and hepatotoxicity. In the last few years, the group of triazoles has further expanded, and new substances offer a favorable safety profile and enhanced potency against yeasts and filamentous fungi. It appears promising to investigate their effect against *E. multilocularis* larvae in future in vitro experiments.

NTZ is a nitrothiazolyl salicylamide compound that was synthesized by Jean Francois Rossignol in the 1970s, and its antiparasitic activity was first demonstrated against tapeworms (21). This discovery was followed by a large number of studies showing its effectiveness against a large variety of other parasites (1, 7, 23). Its mode of action is largely unknown. It was postulated that NTZ may interfere with the enzyme-dependent anaerobic energy metabolism of parasites (7). The present results show that NTZ is highly effective against *E. multilocularis* larvae in vitro. Both the time until larval vesicles showed first signs of alteration and the time until disintegration of all vesicles were significantly shorter for NTZ compared to ABZ at concentrations of 10 µg/ml (Fig. 1C). The present experiments demonstrate a parasitostatic potential for NTZ given as a single-drug treatment in vitro. In a murine model, Stettler et al. (22) recently studied the combined effect of NTZ and ABZ against AE. These authors were able to show that combination treatment with both drugs most effectively reduced the parasitic mass in a model of secondary infection. In addition, they found the most profound histological and ultrastructural alterations after combination treatment in comparison to single-drug treatment with either ABZ or NTZ. Although these results showed a highly deleterious effect on *E. multilocularis* larvae, a conclusion on the viability of larval tissue posttreatment could not be drawn. Our results show a combined effect of NTZ plus ABZ on *E. multilocularis* larvae in vitro. This is the first combination of drugs revealing the potential to kill the parasite in vitro. In our opinion, the promising results of combined treatment obtained by Stettler et al. (22) and ourselves warrant further testing. Although experience with long-term application of NTZ is limited, the drug has excelled due to a low degree of side effects in previous studies. It may therefore be a valuable addition to the existing armamentarium against

this deadly parasitic disease. In particular, the parasitocidal potential of the combination between NTZ and ABZ holds great promise for the definite treatment of human AE. The determination of optimal dosing and the elucidation of potential drug interactions will be a challenging task for future studies in animals.

At this point, a variety of drugs have proven effective against *E. multilocularis* either in vitro or in vivo. Interestingly, their mechanisms of action are very different. Whereas cAMB and ITZ act on ergosterol, the effect of ABZ and MTZ is attributed to the inhibition of tubulin polymerization, and the mechanism of action for NTZ has yet to be elucidated. In a recent study by Mathis et al. (14), clarithromycin was studied as yet another drug with a direct effect on *E. multilocularis* in vitro. Clarithromycin works by inhibition of mitochondrial translation. This variety of mechanisms of action against the parasite should open the way for further studies with emphasis on combination treatment. The simultaneous application of two drugs may address the persisting task to effectively kill *E. multilocularis*. A first step was made in the present study when a parasitocidal effect was shown for the combination between ABZ and NTZ in vitro.

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