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In Vitro Analysis of Albendazole Sulfoxide Enantiomers Shows that (+)-(*R*)-Albendazole Sulfoxide Is the Active Enantiomer against *Taenia solium*

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Albendazole is an anthelmintic drug widely used in the treatment of neurocysticercosis (NCC), an infection of the brain with *Taenia solium* cysts. However, drug levels of its active metabolite, albendazole sulfoxide (ABZSO), are erratic, likely resulting in decreased efficacy and suboptimal cure rates in NCC. Racemic albendazole sulfoxide is composed of ABZSO (+)-(*R*)- and (−)-(*S*) enantiomers that have been shown to differ in pharmacokinetics and activity against other helminths. The antiparasitic activities of racemic ABZSO and its (+)-(*R*)- and (−)-(*S*) enantiomers against *T. solium* cysts were evaluated *in vitro*. Parasites were collected from naturally infected pigs, cultured, and exposed to the racemic mixture or to each enantiomer (range, 10 to 500 ng/ml) or to praziquantel as a reference drug. The activity of each compound against cysts was assayed by measuring the ability to evaginate and inhibition of alkaline phosphatase (AP) and parasite antigen release. (+)-(*R*)-ABZSO was significantly more active than (−)-(*S*)-ABZSO in suppressing the release of AP and antigen into the supernatant in a dose- and time-dependent manner, indicating that most of the activity of ABZSO resides in the (+)-(*R*) enantiomer. Use of this enantiomer alone may lead to increased efficacy and/or less toxicity compared to albendazole.

Neurocysticercosis (NCC) is a major cause of adult-onset seizures and epilepsy in many areas of the developing world. It is an infection with the larval cyst form of the cestode tapeworm *Taenia solium* acquired through the accidental ingestion of ova shed in the feces of human tapeworm carriers. The released oncospheres find their way to the bloodstream and are carried throughout the body but develop mostly in the muscles, subcutaneous tissues, and brain. Almost all the symptoms are due to brain involvement (1).

Two drugs, the benzimidazole albendazole (ABZ) and the pyrazinoisoquinoline praziquantel (PZQ), are commonly used to treat neurocysticercosis, usually with accompanying immunosuppressive/anti-inflammatory medication (2–4). Albendazole is frequently preferred to praziquantel because it is more widely available, has a lower cost, and normally requires shorter courses of treatment (and hence represents lower hospitalization-associated expenses) for higher efficacy (2, 5–7).

In susceptible parasites, the main effect of ABZ is the inhibition of tubulin polymerization into microtubules, but the drug also causes biochemical changes such as inhibition of mitochondrial fumarate reductase, reduced glucose transport, and uncoupling of oxidative phosphorylation, impairing ATP generation (8, 9). Although ABZ has been used since the 1970s (10, 11), it is not completely effective in killing cysts; 12% and 45% of total cysts remain after the usual dosing regimens used with albendazole and praziquantel, respectively (12). Cure rates are unacceptably low for both drugs at about 40% to 50%, so retreatments are often required.

After oral administration, the nonchiral ABZ is oxidized in the liver to its active metabolite, albendazole sulfoxide (ABZSO), with one chiral center in the sulfoxide group. The two possible enantiomers, (+)-(*R*)- and (−)-(*S*)-ABZSO (13), show different pharmacokinetics and affinities for cytosolic proteins from different parasites (28). Kinetic parameters of (+)-(*R*)- and (−)-(*S*)-ABZSO enantiomers in NCC patients treated with ABZ differ in renal excretion (14), accumulation in plasma (15), and, most importantly, distribution in the cerebrospinal fluid (12). Even though the levels of effectiveness of the two enantiomers against a number of other helminths differ, the effect of the isolated enantiomers against *T. solium* metacestodes (cysts) has not been reported.

A sensitive *in vitro* system to quantify drug effects on cysts of *T. solium* has been recently reported (16). In this system, drug sensitivity to both ABZSO and PZQ correlates with the degree of inhibition of alkaline phosphatase (AP) release from cysts, obtained from naturally infected pigs and cultured *in vitro* (16). Using this system, we compared the anthelmintic effect of the (+)-(*R*)- and (−)-(*S*)-ABZSO enantiomers to effects of the ABZSO racemic mixture and of PZQ. Here we show that the antiparasitic effect of the drug is primarily attributable to the (+)-(*R*)-ABZSO enantiomer.

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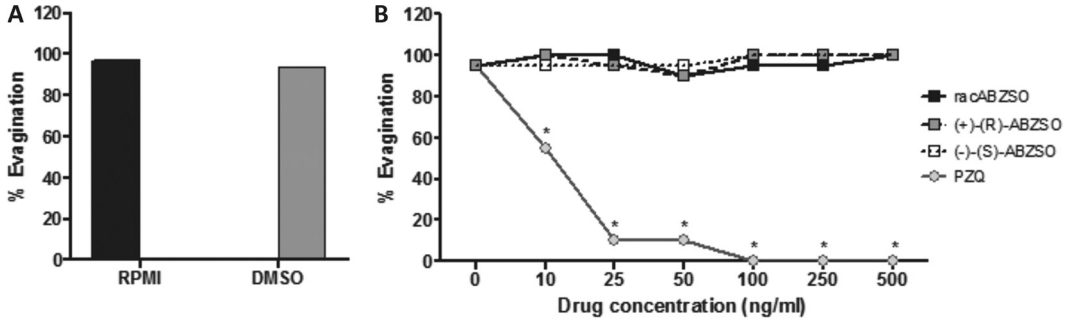

FIG 1 Effect of anthelmintic treatment on *in vitro* bile-stimulated evagination of *T. solium* cysts. Percentages of complete bile-induced evagination after 72 h of culture in medium alone (RPMI) and medium plus DMSO as negative controls (A) and after treatment with PZQ as a positive control, racABZSO, (+)-(*R*)-ABZSO, and (−)-(*S*)-ABZSO at 10 to 500 ng/ml (B) are shown. The symbols represent the means of three measurements; error bars indicate the standard errors of the means (SEM). Values that are significantly different from the untreated control values are indicated with asterisks.

MATERIALS AND METHODS

Reagents and drugs. Tissue culture media and supplements were purchased from Sigma (St. Louis, MO). Praziquantel (PZQ) tablets were obtained from Merck (Merck KGaA, Darmstadt, Germany) and used in powdered form, and ABZSO was purchased from Toronto Reseach Chemicals Inc. (North York, Ontario, Canada). The commercial ABZSO had identical contents with respect to each enantiomer (50:50), which was determined by optical rotation analysis in an automatic Autopol V Polarimeter (serial number 81161; Rudolph Research Analytical, Hackettstown, NJ) and is here called racemic ABZSO (racABZSO). Simulated moving-bed chromatography with variable zones (Varicol process) was used to isolate highly purified enantiomers from racABZSO [percentages of pure enantiomers, 99.5% for (+)-(*R*)-ABZSO and 99.0% for (−)-(*S*)-ABZSO, as described previously (13)]. For *in vitro* treatment of cysts, stock solutions of each drug were prepared in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) and diluted in culture medium (RPMI 1640 medium supplemented with 10 mM HEPES [Gibco], 2 mM glutamine, and antibiotic solution; cRPMI) to the desired concentration.

***In vitro* culture of *T. solium* cysts.** *T. solium* cysts were collected from naturally infected pigs, allowed to equilibrate in culture medium, and tested for drug effects *in vitro* as described previously (16). Cysts were washed and transported in phosphate-buffered saline (PBS; pH 7.4) supplemented with an antibiotic solution containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Gibco-Invitrogen, Gaithersburg, MD). Parasites were then washed twice in PBS and once in cRPMI and equilibrated in the same medium for 18 h at 37°C in 5% CO_2. For drug treatment, cysts were cultured in 2 ml cRPMI in 12-well plates with 10 cysts/well in triplicate. Drug dilutions in cRPMI were added to the wells to achieve final concentrations between 10 and 500 ng/ml. Controls included cRPMI alone and cRPMI with DMSO corresponding to the highest concentration of the drug used in each assay. Parasites were incubated for 72 h at 37°C in 5% CO_2. Supernatants were collected and replaced every 24 h, and a photograph was taken of each plate. Supernatants were stored at −20°C until testing for alkaline phosphatase (AP) activity and antigen (Ag) secretion.

Evagination efficiency and cyst viability. Cyst viability was measured indirectly as described before (16) by determination of the proportion of cysts that evaginated following addition of porcine bile. After the third day in culture, cysts were placed in fresh medium containing 50% porcine bile and incubated for 18 h at 37°C in 5% CO_2. The percentage of evaginated cysts was determined for each condition.

Alkaline phosphatase activity. AP release by *T. solium* was determined by a colorimetric AP detection system (Roche Diagnostics, Branford, CT), adapted to a microassay format as previously reported (16). In short, culture supernatant and kit reagents, mixed according to the manufacturer's instructions, were incubated in the dark for 1 h at 37°C. Optical density (OD) at 492 nm was read on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). Fresh medium incubated without parasites served as a blank. Results were expressed as corrected OD using the following formula:

$$\text{corrected OD} = \text{OD}_{\text{sample}} - \text{OD}_{\text{control DMSO}}$$

Antigen secretion. The release of a specific *T. solium* cyst antigen was quantified by an antigen capture enzyme-linked immunosorbent assay (ELISA) that employs the monoclonal antibodies (MAb) B158C11A10 and biotinylated B60H8A4 as described previously (17–19). Measurements were performed on supernatants collected at 24 and 72 h of culture. Results were expressed as the ratio of a sample's OD to that of its corresponding DMSO control using the following formula:

$$\text{OD ratio} = \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{DMSO control}}}$$

Statistical analysis. Nonparametric analysis of variance (ANOVA) tests were used to compare results of different drug treatments. Where required, two-way comparisons were done using Mann-Whitney U tests. Statistical analysis was done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Ability of cysts to evaginate after *in vitro* treatment. Drug effects on cultivated cysts were observed as changes in their ability to evaginate after bile stimulation, an indirect measurement of viability, as we reported previously (16). After 72 h of culture with racABZSO, (+)-(*R*)-ABZSO, or (−)-(*S*)-ABZSO, the ability of cysts to evaginate was not impaired at any of the concentrations evaluated, indicating their metabolic competence. In contrast, the PZQ treatment used for reference showed a significant inhibition of evagination in a dose- and time-dependent manner (Fig. 1). Similarly, no differences in cyst size were seen with racABZSO, (+)-(*R*)-ABZSO, or (−)-(*S*)-ABZSO compared with untreated controls, in contrast to PZQ treatment results (data not shown).

AP activity in culture supernatants of drug-treated *T. solium* cysts. Viable, untreated *T. solium* cysts normally secrete AP with a rate of release that increases over time and is inhibited after *in vitro* treatment with PZQ and ABZSO (16). To assess AP secretion, AP activity was measured in cyst supernatants 24, 48, and 72 h after addition of racABZSO, (+)-(*R*)-ABZSO, and (−)-(*S*)-ABZSO; supernatants of wells without drugs or with DMSO were used as controls. No differences in AP secretion were observed between controls at any time point ($P > 0.05$) (Fig. 2i to iv). ABZSO and both enantiomers inhibited AP secretion after 24 h, which was more evident at 72 h. (+)-(*R*)-ABZSO caused greater inhibition at

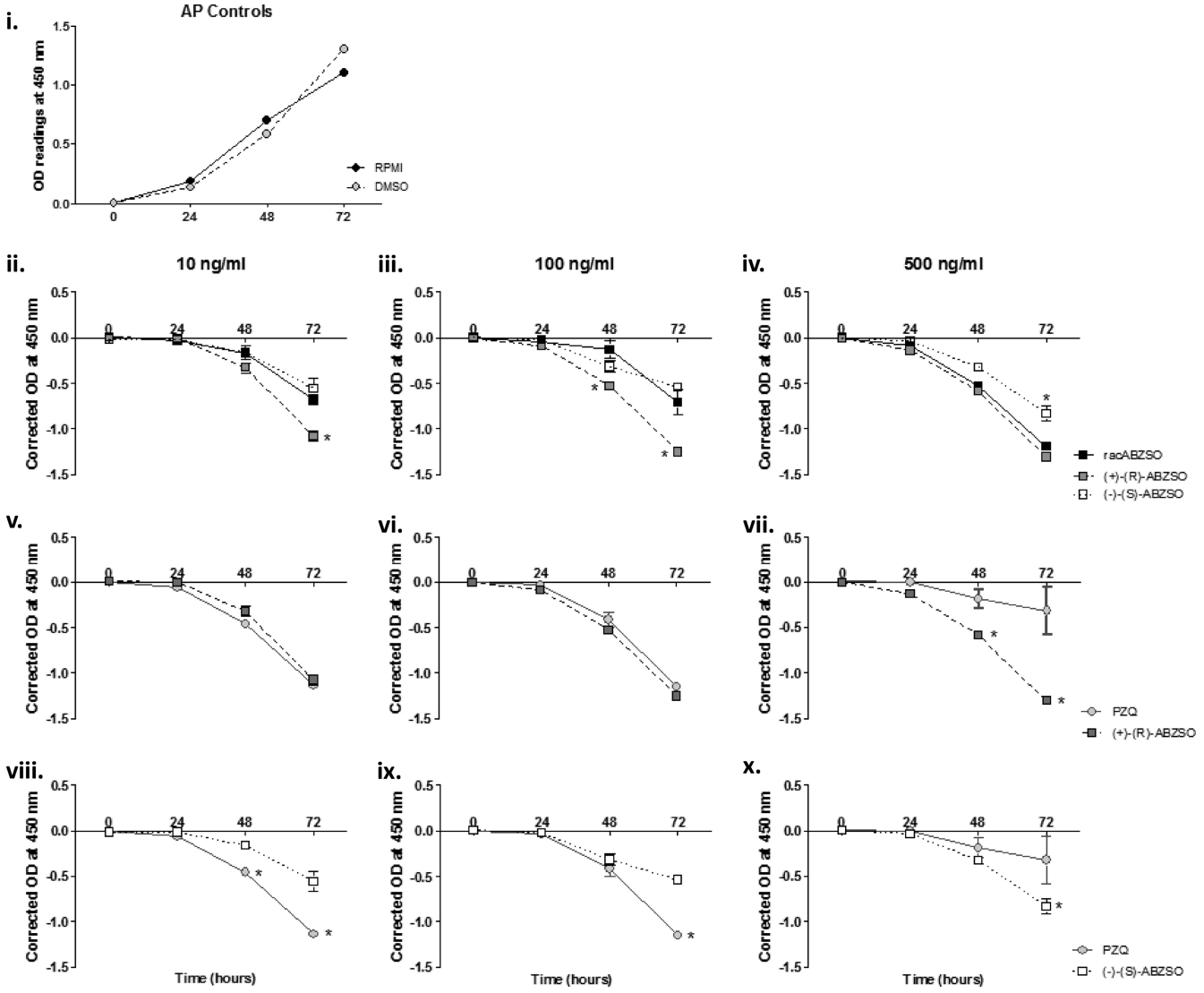

FIG 2 (i to iv) Inhibition of AP activity in culture supernatants of drug-treated *T. solium* cysts. (i) AP activity in medium alone and medium plus DMSO used as negative controls and expressed as the OD at 450 nm. (ii to iv) AP activity in the supernatants of cysts treated with racABZSO, (+)-(*R*)-ABZSO, and (−)-(*S*)-ABZSO at 10 ng/ml (ii), 100 ng/ml (iii), and 500 ng/ml (iv). Data are expressed as corrected OD at 450 nm, and symbols show the means and SEM of the results determined for triplicate wells. Measurements that are significantly different from those determined for the racemic mixture are indicated with asterisks. (v to x) Effect of ABZSO enantiomers and PZQ on inhibition of AP activity in culture supernatants of *T. solium* cysts. (v to vii) AP activity in supernatants of cysts treated with PZQ and (+)-(*R*)-ABZSO at 10 ng/ml (v), 100 ng/ml (vi), and 500 ng/ml (vii). (viii to x) AP activity in supernatants of cysts treated with PZQ and (−)-(*S*)-ABZSO at 10 ng/ml (viii), 100 ng/ml (ix), and 500 ng/ml (x). Data are expressed as corrected OD at 450 nm, showing the means and SEM of the results determined for triplicate wells. Measurements that are significantly different from those determined for PZQ are indicated with asterisks. Values for ABZSO and enantiomers are the same as those for panels i to iv, plotted here with PZQ to show how each enantiomer compares to the same doses of the reference drug PZQ.

all concentrations between 10 and 500 ng/ml (Fig. 2ii to iv). Significant differences in AP secretion between racABZSO and (+)-(*R*)-ABZSO were seen at 100 ng/ml after 48 and 72 h of treatment ($P < 0.001$ each; Fig. 2iii) as well as at 10 ng/ml after 72 h of treatment ($P < 0.001$; Fig. 2ii). Surprisingly, at the high dose of 500 ng/ml, racABZSO and (+)-(*R*)-ABZSO showed similar patterns of AP inhibition ($P > 0.05$) and the patterns of both were significantly different from that seen with (−)-(*S*)-ABZSO ($P < 0.01$; Fig. 2iv).

PZQ, at concentrations below 100 ng/ml, inhibited AP secretion at all time points evaluated; this effect was lost at 500 ng/ml after 48 h (Fig. 2viii and x). (+)-(*R*)-ABZSO had a similar pattern ($P > 0.05$), but the inhibition was maintained at 500 ng/ml (Fig. 2v to vii). (−)-(*S*)-ABZSO showed greater inhibiting activity than PZQ only at 500 ng/ml and at 72 h (Fig. 2x).

Antigen release in culture supernatants of drug-treated *T. solium* cysts. MAb B158C11A10 recognizes an antigen (Ag) secreted by living *T. solium* cysts and is used in diagnostic tests on sera and cerebrospinal fluid of patients with cysticercosis (19). Ag release was measured in supernatants from *T. solium* cysts after 24 and 72 h of culture with 10, 100, and 500 ng/ml of racABZSO, (+)-(*R*)-ABZSO, and (−)-(*S*)-ABZSO. Supernatants from cul-

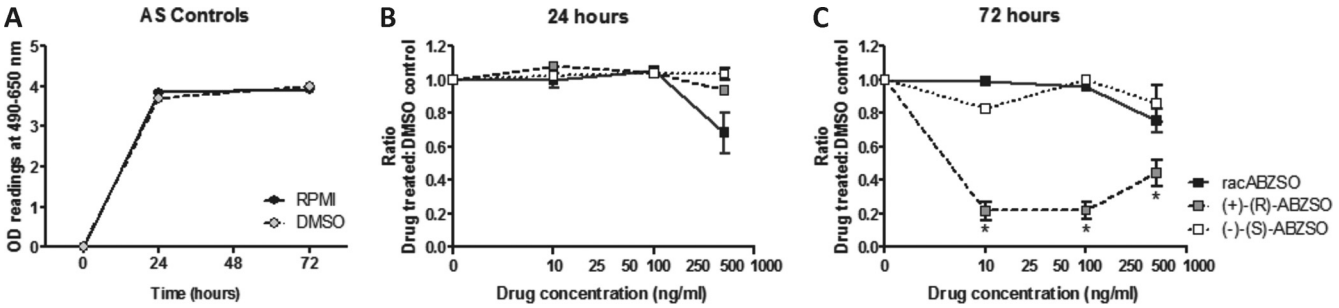

FIG 3 Inhibition of parasite antigen secretion (AS) in culture supernatants of drug-treated *T. solium* cysts. (A) AS in supernatants of cysts cultured with medium alone and medium plus DMSO solvent used as negative controls and expressed as OD at 490/650 nm. (B and C) AS in supernatants of cysts treated with racABZSO, (+)-(*R*)-ABZSO, and (−)-(*S*)-ABZSO at different doses as indicated after 24 h (B) and 72 h (C). Results are expressed as ratios of the OD at 490 and 650 nm (490−650) for each test condition and its corresponding control. Each condition was evaluated in triplicate; the symbols represent the means, and the error bars indicate the SEM. Measurements that are significantly different from those of the racemic mixture are indicated with asterisks.

tures with medium alone and DMSO showed no differences in Ag release at any of the times evaluated ($P > 0.05$; Fig. 3). (+)-(*R*)-ABZSO significantly inhibited Ag release at between 10 ng/ml and 500 ng/ml at 72 h ($P < 0.001$; Fig. 3C), while none of the concentrations of racABZSO and (−)-(*S*)-ABZSO showed significant differences compared with medium and DMSO controls ($P > 0.05$; Fig. 3B and C). The PZQ reference inhibited Ag release at between 10 and 500 ng/ml at 24 h ($P < 0.001$), but this effect was lost at 72 h (data not shown).

Antigen immunolocalization after *in vitro* drug treatment. Distributions of MAb B158C11A10 on the cysts as seen with immunohistochemistry (IHC) differed between ABZSO enantiomers. Treatment with 100 and 500 ng/ml of (+)-(*R*)-ABZSO showed strong immunoreactivity in the tegument and subtegument, mainly in microtrichia and the body of the cells (cytons), respectively. The latter could be seen as clusters, which did not appear on cysts treated with (−)-(*S*)-ABZSO; these showed strong but not localized immunoreactivity at 500 ng/ml. The distribution of the antigen after treatment with racABZSO combined characteristics of treatment with both enantiomers and showed lower immunoreactivity (see Fig. S1 in the supplemental material).

DISCUSSION

The present work characterizes the effects of highly purified enantiomers of albendazole, namely, (+)-(*R*)-ABZSO and (−)-(*S*)-ABZSO (13), against *T. solium* cysts using an established *in vitro* method to observe changes at the macroscopic and microscopic structure levels (16). We found that (+)-(*R*)-ABZSO possesses greater activity in suppressing AP secretion and inhibiting the release of an antigen than (−)-(*S*)-ABZSO, which may also decrease the anthelmintic effect of the racemic mixture. These results are the first report of the activity of ABZSO enantiomers against *T. solium* cysts.

Release of alkaline phosphatase (AP) has been previously used as a marker of drug-induced damage in different parasite *in vitro* models (20, 21), but the inhibition of this release has not been correlated to drug-associated damage. Our previous work on cultivated *T. solium* cysts showed that, in the absence of drugs, the activity of this enzyme measured in culture supernatants increases over time but that exposure to PZQ at 1 to 5 ng/ml (3 to 16 nM) and ABZSO at 50 to 75 ng/ml (177 to 268 nM) inhibits AP activity (16). This effect on *T. solium* cysts thus serves as a sensitive and quantitative measure of drug activity *in vitro*. Strikingly, an opposite effect happens in the *Echinococcus* model, where ABZSO or ABZ sulfone stimulates release of AP in a dose-dependent manner at above 10 μg/ml (21). Using AP inhibition as a marker of drug effect, changes associated with ABZSO and each of its enantiomers were detected as early as 3 days after drug exposure and at 10, 100, and 500 ng/ml (Fig. 2ii to iv), in contrast to previous reports on *T. solium* and *T. crassiceps*, where the ABZSO effect could be seen only after 11 days with doses between 65 and 556 ng/ml (22).

The drug concentrations tested were within the ranges reported in human cerebrospinal fluid (CSF) after treatment with ABZ. Concentrations detected in CSF after ABZ administration (7.5 mg/kg of body weight/12 h) ranged from 37 to 386 ng/ml for (+)-R-ABZSO, whereas those detected for (−)-S-ABZSO reached 88 ng/ml at most, with some patients showing concentrations close to the quantification limit of 5 ng/ml (12).The effects on AP secretion of both enantiomers in the racemic mixture appear to be interactive and complex. While the effect of (+)-(*R*)-ABZSO is almost maximal at 10 ng/ml at 72 h, both racABZSO and (−)-(*S*)-ABZSO show about half of the effect of (+)-(*R*)-ABZSO at this time point. At 500 ng/ml, the effect of racABZSO equals that of (+)-(*R*)-ABZSO. Since racABZSO contains equal proportions of the enantiomers, one would expect a doubling of the concentration of racABZSO to equal the effect of (+)-(*R*)-ABZSO; instead, this occurs at concentrations between 5 and 50 times higher, suggesting inhibitory effects of (−)-(*S*)-ABZSO on (+)-(*R*)-ABZSO. The nature of the activity of (−)-(*S*)-ABZSO is unclear and warrants further investigations.

MAb B158C11A10, generated against *T. saginata* excretion-secretion products, detects a cross-reacting antigen common to *T. solium* and *T. crassiceps* and is successfully used in diagnosis of NCC (18, 19). Our previous work showed that 75 ng/ml of ABZSO inhibited the release of the antigen (16). Here, antigen secretion was inhibited only by (+)-(*R*)-ABZSO in a dose- and time-dependent manner starting at 10 ng/ml, confirming the sensitivity of the assay for quantification of direct drug effects on *T. solium* cysts. The PZQ control showed inhibition of antigen release at 10 to 500 ng/ml at 24 h ($P < 0.001$), but not at 72 h, a time when the parasite is presumed to have died or to be severely damaged, as shown by the lack of evagination. We speculate that, in such a state of advanced damage, the loss of tegumental integrity and function may have resulted in uninhibited release of AP. Even at low doses, the effect of (+)-(*R*)-ABZSO at 72 h was comparable to that of PZQ at 24 h, which suggests a similar effect taking longer to occur. This is

consistent with the observed slower effect of ABZSO on cysts, as seen with AP secretion and in previous studies (16, 22).

Inhibition of molecules excreted by or secreted from *T. solium* may be a secondary effect of these drugs, as has been previously mentioned (16), but can be useful as another indicator of drug effect. Our data on antigen secretion are consistent with our observations on AP secretion with respect to the differential activities of (+)-(*R*)-ABZSO and (−)-(*S*)-ABZSO and suggest that the activity of racABZSO against *T. solium* cysts is attributable to the (+)-(*R*) enantiomer (Fig. 3). Associated with this, immunolocalization of antigen recognized by MAb B158C11A10 after treatment with (+)-(*R*)-ABZSO showed strong reactivity inside the cysts that suggested its accumulation within the cyst, consistent with inhibition of secretion in the presence of this enantiomer (see Fig. S1 in the supplemental material).

Inhibition of antigen secretion and morphological changes are consistent with inhibitory effects of (+)-(*R*)-ABZSO on AP secretion that are higher than those of (−)-(*S*)-ABZSO. In addition, the enhanced antigen reactivity noted on the surface of (+)-(*R*)-ABZSO-treated cysts at 100 ng/ml compared to (−)-(*S*)-ABZSO-treated cysts suggests that inhibition of secretion is an important mechanism of action of both enantiomers. Besides, morphological changes mainly in microtrichia and subtegumentary cells at 500 ng/ml (see Fig. S1 in the supplemental material) are similar to other reports of structural changes caused by the *in vitro* effect of ABZSO on *Taenia crassiceps* cysts (23) and of other anthelmintic drugs, such as PZQ, on *Taenia solium* cysts (11, 16, 24, 25).

The anthelmintic effect of ABZSO and its enantiomers on larvae of the nematode *Trichinella spiralis* determined using an *ex vivo* model of treatment has been previously reported; (+)-(*R*)-ABZSO was the most effective form, rendering a significant reduction of larval viability at 100 ng/ml (26). Likewise, (+)-(*R*)-ABZSO showed a significantly higher activity than (−)-(*S*)-ABZSO against *Haemonchus contortus* using a gerbil (*Meriones unguiculatus*) infection model. More recently, the *in vitro* effect of (+)-(*R*)-ABZSO on *Echinococcus granulosus* protoscoleces at 50 and 100 μg/ml was greater than that of racABZSO and (−)-(*S*)-ABZSO after 9 days of culture [99% and 100%, respectively; (−)-(*S*)-ABZSO had the lowest parasite mortality rates at 56.2% and 74.5%] (27). These observations suggest that (+)-(*R*)-ABZSO is the primary active component in the parent drug and agree with our results seen with *T. solium* cysts.

The selective effectiveness (26) is attributed to enantioselectivity in binding to proteins of different helminth parasites (28), which may explain the higher concentration of (+)-(*R*)-ABZSO in tissues of *Fasciola hepatica* (29). Also, the pharmacokinetics of the two enantiomers differ within the host (30). It has been proposed that the effectiveness of ABZ in NCC treatment is due to its ability to cross the blood-brain barrier. In the CSF, the (+)-(*R*)-ABZSO metabolite has been reported at concentrations three times higher than those of (−)-(*S*)-ABZSO (12). However, more studies are required to confirm the therapeutic role of each ABZSO enantiomer.

In summary, we found that (+)-(*R*)-ABZSO showed greater activity against *T. solium* than (−)-(*S*)-ABZSO, as demonstrated by greater inhibition of AP and antigen release as well as by histopathology. If those results are confirmed *in vivo*, the use of (+)-(*R*)-ABZSO alone offers the possibility of greater efficacy and less toxicity in the treatment of NCC and potentially other helminth infections.

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