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EFFICACY OF PONAZURIL IN VITRO AND IN PREVENTING AND TREATING *TOXOPLASMA GONDII* INFECTIONS IN MICE

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ABSTRACT: *Toxoplasma gondii* is an important apicomplexan parasite of humans and other warm-blooded animals. Ponazuril is a triazine anticoccidial recently approved for use in horses in the United States. We determined that ponazuril significantly inhibited *T. gondii* tachyzoite production ($P < 0.05$) at 5.0, 1.0, or 0.1 μg/ml in African green monkey kidney cells. We used outbred female CD-1 mice to determine the efficacy of ponazuril in preventing and treating acute toxoplasmosis. Each mouse was subcutaneously infected with 1,000 tachyzoites of the RH strain of *T. gondii*. Mice were weighed daily, and ponazuril was administered orally in a suspension. Mice given 10 or 20 mg/kg body weight ponazuril 1 day before infection and then daily for 10 days were completely protected against acute toxoplasmosis. Relapse did not occur after prophylactic treatments were stopped. *Toxoplasma gondii* DNA could not be detected in the brains of these mice using polymerase chain reaction (PCR). One hundred percent of mice treated with 10 or 20 mg/kg ponazuril at 3 days after infection and then daily for 10 days were protected from fatal toxoplasmosis. Sixty percent of mice treated with 10 mg/kg ponazuril at 6 days after infection and 100% of mice treated with 20 mg/kg or 50 mg ponazuril 6 days after infection and then daily for 10 days were protected from fatal toxoplasmosis. Relapse did not occur after treatments were stopped. *Toxoplasma gondii* DNA was detected in the brains of some, but not all, of these mice using PCR. The results demonstrate that ponazuril is effective in preventing and treating toxoplasmosis in mice. It should be further investigated as a safe and effective treatment for this disease in animals.

Toxoplasma gondii is an important parasite of humans and other warm-blooded animals. About 1,500,000 human cases of toxoplasmosis are reported in the United States each year, and about 15% of those infected have clinical signs (Mead et al., 1999; Jones, Kruszon-Moran et al., 2001). Congenital toxoplasmosis has long been recognized because of the devastating effects it can have on the infected fetus (Jones, Lopez et al., 2001). These include hydrocephalus, blindness, and mental retardation. Congenitally infected children who are less severely infected may suffer from a variety of neurological-related aliments throughout their lives (Roberts and Frenkel, 1990). In the United States, it is estimated that 85% of women of child-bearing age are at risk for toxoplasmosis (Jones, Kruszon-Moran et al., 2001) and that up to 4,000 cases of congenital toxoplasmosis occur each year (Jones, Lopez et al., 2001). Toxoplasmic encephalitis (TE) became recognized as an acquired immunodeficiency syndrome (AIDS)–defining illness in the early 1980s, and TE is still the most important neurological component of AIDS (Luft and Chua, 2000). Toxoplasmosis is also a frequent and fatal complication in patients who receive organ transplantation (Soave, 2001). The annual economic impact of toxoplasmosis in the human population in the United States is about $7.7 billion (Buzby and Roberts, 1996).

Ponazuril is the major metabolite of toltrazuril, a triazine anticoccidial used in the poultry industry. Ponazuril has been shown to be active against *Sarcocystis neurona* in vitro (Lindsay and Dubey, 2000) and in vivo (Franklin et al., 2003) and against *Neospora caninum* in vivo (Gottstein et al., 2001). The present study was carried out to determine the in vitro and in vivo activity of ponazuril against the RH strain of *T. gondii*.

MATERIALS AND METHODS

Cell culture

African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Manassas, Virginia) were grown to confluence in 25-cm^2 plastic cell culture flasks in growth media that consisted of 10% (v/v) fetal bovine serum in Roswell Park Memorial Institute 1640 medium, supplemented with 100 U penicillin/ml and 100 mg streptomycin/ml. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% CO_2 and 95% air.

Ponazuril and in vitro efficacy

The activity of ponazuril (lot PFA101; Bayer HealthCare Animal Health, Shawnee, Kansas) was determined in a tachyzoite production (TP) assay (Lindsay and Blagburn, 1994). Ponazuril was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 1 mg/ml. Dilutions were made from this stock solution, and the highest concentration of DMSO in any solution was 0.01% (v/v). Cell monolayers were inoculated with 2.5×10^5 RH strain *T. gondii* tachyzoites. Two hours after inoculation, the medium was removed and replaced with maintenance medium containing ponazuril at concentrations of 0.1, 1.0, or 5.0 μg/ml (Fig. 1). Control flasks received maintenance medium without ponazuril. Four flasks were used per ponazuril treatment dose. The TP assay was conducted after 4 days of treatment. The numbers of tachyzoites (mean of 16 counts/treatment [4 counts/flask]) present was determined by counting in a hemacytometer.

To determine when ponazuril acted on *T. gondii*, CV-1 cells were grown to monolayers on 22-mm^2 glass coverslips in 6-well cell culture plates. The CV-1 cells were inoculated with 1×10^5 tachyzoites, and 2 hr later the media were removed and replaced with media containing 5 μg/ml ponazuril. Replicate plates were treated with media containing 0.1% DMSO but no ponazuril. Coverslips were removed and examined 4, 9, 20, 24, and 48 hr after the addition of ponazuril-containing medium or control medium. The number of parasites in 100 host cells was determined at each observation time.

The following procedure was used to determine whether ponazuril treatments killed *T. gondii*. After the medium was collected for the TP assay, the cell monolayer was rinsed twice with maintenance medium to wash off any residual ponazuril, and 5 ml of maintenance medium was added to the flask. The flasks were then examined for 30 days for renewed growth of parasites, monolayer destruction, or both.

Statistical analysis

Mean tachyzoite counts were log transformed to stabilize variances before analysis and then back transformed for presentation. The MIXED procedure of SAS (SAS ver. 6.12, SAS Institute Inc., Cary, North Carolina) was used to perform analysis of variance. Tukey's honest significant difference ($P = 0.05$) was used to compare means.

Mice and examination for *Toxoplasma gondii*

For in vivo studies, a suspension of 50 mg ponazuril per milliliter (lot 2161AA) was obtained from Bayer HealthCare Animal Health. This

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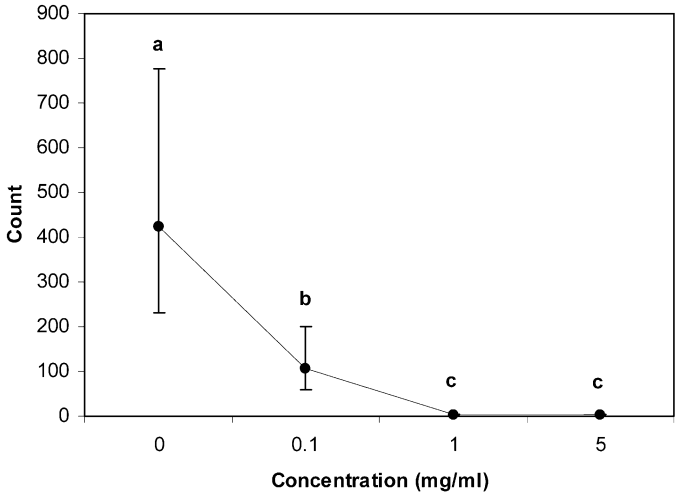

FIGURE 1. Activity of ponazuril against RH strain *Toxoplasma gondii* in CV-1 cell culture. Bars = 95% confidence intervals. Points with different letter are significantly different ($P < 0.05$) from each other.

suspension was diluted in distilled water and used for in vivo testing. Groups of 5 female CD-1 mice were used to determine the effects of treatment with ponazuril in the prevention and treatment of toxoplasmosis (Table I). All mice were inoculated subcutaneously in the dorsal scapular region with 1×10^3 tachyzoites. During the study, impression smears were made from the livers or lungs of any mice that died and were examined unstained by light microscopy for tachyzoites. At 8 wk postinoculation (PI), all surviving mice were bled from the retroorbital plexus. The serum was collected and examined for antibodies to *T. gondii* in a modified direct agglutination assay (MAT) (Dubey and Desmonts, 1987).

Toxoplasma gondii polymerase chain reaction

Brains were examined for *T. gondii* DNA using the primers described by Jauregui et al. (2001). The DNA was extracted from 0.5 g of brain tissue from mice in groups 3–9 (Table I) using a commercial DNA extraction kit (DNA Maxi Kit, Qiagen, Valencia, California). The purified DNA was diluted 1:100, and a 20-μl aliquot was taken and mixed with 200 μl of InstaGene Matrix (Bio-Rad, Hercules, California). The samples were then incubated in a 56 C water bath for 30 min. The samples were vortexed and then placed in boiling water for 8 min. The samples were vortexed and centrifuged in a microfuge for 2–3 min. A 20-μl aliquot of the supernatant was used per 50 μl polymerase chain reaction (PCR). The remaining supernatant was stored at −20 C. PCR was performed on each sample using Ready To Go PCR Beads (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey) and a Hybaid OmniGene thermocycler. The detection primers were based on the *T. gondii* ITS1 sense primer 5′-GATTTGCATTCAAGAAGCGTGATAGTAT-3′ and antisense primer 5′-AGTTTAGGAAGCAATCTGAAAGCACATC-3′. Mouse β-actin was used as a positive control for DNA isolation and PCR (sense primer 5′-TCACCCACACTGTGCCCATCTACGA-3′ and antisense primer 5′-CAGCGGAACCGCTCATTGCCAATGG-3′). Standard PCR reaction conditions were used with the following amplification parameters: 94 C for 5 min, 35 cycles at 94 C for 1 min, at 62 C for 1 min, at 72 C for 1 min, and at 72 C for 10 min. The PCR products were run on a 1% agarose gel.

RESULTS

Effects on tachyzoite production

There was a significant effect of ponazuril treatment ($P < 0.05$) on tachyzoite production. Tukey's test indicated that the 1.0 μg/ml treatment was not significantly different ($P > 0.05$) from the 5.0 μg/ml treatment, but all other pairwise comparisons were significant ($P < 0.05$) (Fig. 1).

Host CV-1 cells treated with 5 μg/ml ponazuril contained only 4 parasites at observation times of 20 hr or greater. The CV-1 cells that contained *T. gondii* and that were not treated had 8 or more tachyzoites at these observation times. Results of timed observations indicated that ponazuril inhibits *T. gondii* replication after the second division by endodyogeny approximately 20 hr after treatment.

Prevention of toxoplasmosis

All nontreated mice developed acute toxoplasmosis and died or were killed 9–11 days PI ($\bar{x}$ = 10 PI) (Table I). No mouse in group 3 or 4 given 10 or 20 mg/kg ponazuril 1 day before infection and then daily for 10 days died. None of the mouse developed acute toxoplasmosis after prophylactic treatments were stopped. Three of 5 mice in group 3 tested serologically positive for *T. gondii* using the MAT, and 1 of 5 mice in this group was positive by PCR on brain tissue (Fig. 2). All 5 mice tested serologically negative in the MAT in group 4, and *T. gondii* DNA was not detected in the brains of these mice by PCR (Table I).

TABLE I. Protocol for evaluating the effects of ponazuril against *Toxoplasma gondii* in mice.

Group	Treatment*	No. mice/no. survived†	PCR‡
1	Distilled water 3 days after infection	5/0	ND§
2	Distilled water 1 day before infection	5/0	ND
3	10 mg/kg ponazuril 1 day before infection	5/5	5/1
4	20 mg/kg ponazuril 1 day before infection	5/5	5/0
5	10 mg/kg ponazuril 3 days after infection	5/5	4/1
6	20 mg/kg ponazuril 3 days after infection	5/5	5/5
7	10 mg/kg ponazuril 6 days after infection	5/3	3/3
8	20 mg/kg ponazuril 6 days after infection	5/5	5/5
9	50 mg/kg ponazuril 6 days after infection	5/4‖	4/1

* Mice in groups 1 and 2 never received ponazuril. Mice in groups 3 and 4 received ponazuril 1 day before and on the day of infection and then daily for 10 days. Mice in groups 5–9 were treated daily for 10 days with ponazuril at the indicated day after infection.
† Number of mice inoculated/number of mice surviving infection.
‡ Results of PCR; number tested by PCR on brain/number positive by PCR on brain.
§ ND, not determined.
‖ One mouse died in this group due to aspiration pneumonia.

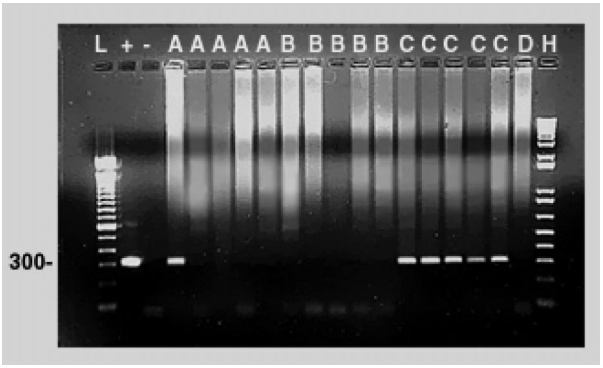

FIGURE 2. Results of *Toxoplasma gondii* ITS1 PCR on DNA from brains of mice infected with the RH strain of *T. gondii* and treated with ponazuril. (L) 100-bp ladder, (+) positive control *T. gondii* DNA, (−) negative control no DNA, (A) DNA from individual mice from group 3, (B) DNA from individual mice from group 4, (C) DNA from individual mice from group 6, (D) DNA from a mouse from group 9 and (H) 1kb+ ladder.

Treatment of acute toxoplasmosis

All nontreated mice developed acute toxoplasmosis and died or were killed 9–11 days PI ($\bar{x} = 10$ PI). Five of 5 mice (100%) in group 5 and 5 of 5 mice (100%) in group 6 were protected from fatal toxoplasmosis (Table I). All mice were serologically positive for *T. gondii* in groups 5 and 6 on the MAT. PCR was done on the brains of 4 mice in group 5, and 1 was positive, whereas PCR was done on the brains of all mice in group 6, and they were all positive.

Three of 5 mice (60%) in group 7 and 5 of 5 mice (100%) in group 8 were protected from fatal toxoplasmosis. Deaths occurred on days 9 and 12 PI in group 7. All 3 mice in group 7 and all 5 mice in group 8 tested serologically positive in the MAT. All group 7 and 8 mice tested positive for *T. gondii* by PCR. One of 5 mice in group 9 died 11 days PI. This mouse had aspiration pneumonia, and its death was probably not due to toxoplasmosis. The 4 other mice in group 9 survived until the end of the study. All surviving mice in group 9 were positive by the MAT. The brains of 4 mice in group 9 were examined by PCR, and 1 was positive. Relapse did not occur after treatments were stopped.

DISCUSSION

The present study demonstrates that ponazuril is effective in preventing and treating toxoplasmosis in mice. The lack of mortality and detection of *T. gondii* DNA in the brain of only 1 mouse treated prophylactically with 10 mg/kg and no mouse treated prophylactically with 20 mg/kg indicates that ponazuril is highly effective in the prevention of toxoplasmosis. Ponazuril at 10 or 20 mg/kg was also 100% effective in preventing mortality in mice with 3-day-old, established *T. gondii* infections but did not prevent the parasite from eventually reaching the brain in these mice as determined by PCR on brain tissue. Treatment of clinical toxoplasmosis at 6 days after infection was less effective with 10 mg/kg (60% survival) than with 20 or 50 mg/kg (100% survival; excluding 1 mouse in 50 mg/kg group that died of aspiration pneumonia).

Pyrimethamine alone or combined with sulfadiazine is the most commonly used treatment for human toxoplasmosis, whereas clindamycin and atovaquone are also frequently used (Luft and Chua, 2000). Ponazuril appears to be superior to clindamycin or atovaquone for the treatment of murine toxoplasmosis. Nikolic et al. (1999) found that treatment with 50 or 400 mg/kg clindamycin hydrochloride in the feed daily for 3 wk prevented mortality from the RH strain of *T. gondii*. Atovaquone given orally in the feed at 100 mg/kg for 14 days prevented death in 13% of the mice infected with the RH strain of *T. gondii* and examined by Djurkovic-Djakovic et al. (1999).

Diclazuril is a triazine anticoccidial related to ponazuril that has been evaluated against toxoplasmosis. Lindsay and Blagburn (1994) demonstrated that diclazuril prevented deaths from toxoplasmosis in 80 and 100% of mice treated 1 day before infection with 1 or 10 mg/kg diclazuril and then daily for 10 days after infection with RH strain of *T. gondii*. Lindsay et al. (1995) found that oral diclazuril at 10 mg/kg was 100 and 90% effective in preventing deaths in mice when given at 3 or 6 days, respectively, after infection with RH strain *T. gondii*. This activity is similar to that seen for ponazuril at 20 mg/kg in the present study.

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