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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. 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 ailments 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 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 polymerase chain reaction

Brains were examined for *T. gondii* DNA using the primers described by Jaregui 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 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 (Amer sham Pharmacia Biotech Inc., Piscataway, New Jersey) and a Hybaid OmniGene thermocycler. The detection primers were based on the *T. gondii* ITS1 sense primer 5'-GATTTCATCAAGAAGCGTGATAGTAT-3' and antisense primer 5'-AGTTTAGGAAACGTATGAAAGCACAATC-3'. Mouse β-actin was used as a positive control for DNA isolation and PCR (sense primer 5'-TCACCCACACTTGCCCATCTACGA-3' and antisense primer 5'-CGCGGACCCGCTATGCGC-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 (♀) (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 mice 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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment*</th>
<th>No. mice/no. survived†</th>
<th>PCR‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distilled water 3 days after infection</td>
<td>5/0</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>Distilled water 1 day before infection</td>
<td>5/0</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>10 mg/kg ponazuril 1 day before infection</td>
<td>5/5</td>
<td>5/1</td>
</tr>
<tr>
<td>4</td>
<td>20 mg/kg ponazuril 1 day before infection</td>
<td>5/5</td>
<td>5/0</td>
</tr>
<tr>
<td>5</td>
<td>10 mg/kg ponazuril 3 days after infection</td>
<td>5/5</td>
<td>4/1</td>
</tr>
<tr>
<td>6</td>
<td>20 mg/kg ponazuril 3 days after infection</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>7</td>
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<td>3/3</td>
</tr>
<tr>
<td>8</td>
<td>20 mg/kg ponazuril 6 days after infection</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>9</td>
<td>50 mg/kg ponazuril 6 days after infection</td>
<td>5/3</td>
<td>4/1</td>
</tr>
</tbody>
</table>

* 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.
Treatments were stopped. The end of the study. All surviving mice in group 9 were positive for toxoplasmosis. The 4 other mice in group 9 survived until had aspiration pneumonia, and its death was probably not due PCR. One of 5 mice in group 9 died 11 days PI. This mouse ponazuril. (L) 100-bp ladder, (D) DNA from a mouse from group 9, (A) DNA from individual mice from group 6, (B) DNA from individual mice from group 4, (C) DNA from individual mice 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 (± 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 1). 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.

ACKNOWLEDGMENT

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LITERATURE CITED


Luft, B. J., and A. Chua. 2000. Central nervous system toxoplasmosis


