Abstract. The antimalarial peroxide, dispiro-1,2,4,5-tetraoxane WR 148999, was synergistic with chloroquine, quinine, mefloquine, and artemisinin against both D6 and W2 clones of Plasmodium falciparum. In consideration of the contrasting antagonism between artemisinin and chloroquine, these drug combination data imply that WR 148999 and artemisinin may not share a common mechanism of action. For Plasmodium berghei-infected mice given oral, subcutaneous, and intraperitoneal doses of WR 148999 ranging from 2 to 1024 mg/kg in the Thompson test, median survival times were 8.8, 11.8, and 27.5 days, respectively, compared to 8 days for control animals. Using subcutaneous administration, WR 148999 had a considerably longer duration of action than did artemisinin against P. berghei. WR 148999 did not significantly inhibit cytochrome P450 isozymes CYP 2C9, 2C19, 2D6, 2E1, or 3A4 (IC50 >500 μM) but did inhibit CYP 1A2 with an IC50 value of 36 μM, suggesting that WR 148999 may be metabolized by the latter CYP isozyme. These results combined with previous observations that formulation strategies and incorporation of polar functional groups in a series of WR 148999 analogs both failed to enhance tetraoxane oral antimalarial activity suggest that oral bioavailability of tetraoxane WR 148999 is more likely a function of extensive first-pass metabolism rather than solubility-limited dissolution.

INTRODUCTION

The apparent association between the peroxide functional group and antimalarial activity is exemplified by artemisinin and its semisynthetic derivatives which contain a unique 1,2,4-trioxane heterocycle pharmacophore (Figure 1). The importance of the pharmacophore is underscored by the fact that if the peroxide bond is reduced, antimalarial activity is lost. Numerous antimalarial synthetic 1,2,4-trioxanes have been prepared.

We reported that three dispiro-1,2,4,5-tetraoxanes, another class of antimalarial peroxides, were also active antimalarial agents, a confirmation of earlier unpublished data (Doorenbos HE and Decker DL). The most active of these was the tetraoxane WR 148999.

Artemisinin has been used clinically and is effective against multidrug-resistant Plasmodium falciparum parasites. Although artemisinin produces an initial rapid clearance of parasitemia, its clinical utility has been limited by early recrudescence of parasites. The early recrudescence may in part be related to its pharmacokinetic profile which is characterized by a short half-life, low oral bioavailability, and apparent autoinduction of metabolism. The low oral bioavailability may be a result of high first-pass metabolism and/or low solubility in the gastrointestinal tract. Therefore, the search for novel peroxide antimalarial alternatives with advantages over artemisinin has led us to further characterize the antimalarial and metabolic profile of WR 148999.

In a previous study, WR 148999 and artemisinin were shown to be approximately equipotent when administered subcutaneously to mice infected with Plasmodium berghei.

In the present study, we extend the pharmacodynamic comparison between WR 148999 and artemisinin with respect to duration of action in a mouse malaria model. In addition, we compare the antimalarial efficacy of WR 148999 in mice infected with chloroquine-sensitive or chloroquine-resistant Plasmodium berghei.

Although parenteral antimalarials are useful in the treatment of patients who are comatose or unable to tolerate oral medications, oral treatment is preferable in the vast majority of malaria patients requiring antimalarial chemotherapy. In the present study, we compare the oral and parenteral efficacy of WR 148999 administered to mice infected with Plasmodium berghei.

There is a growing concern that unless antimalarial drugs are given in combination, resistance to antimalarial drugs will continue to develop. If antimalarial drugs are to be given in combination, it is important to understand the pharmacodynamic interaction between drugs whose putative mechanisms of action differ. It is also important to be able to predict potential metabolic drug-drug interactions to guide the selection of a compatible antimalarial drug. For these reasons, we have conducted in vitro pharmacodynamic drug interaction studies and in vivo metabolism studies to guide further drug development.

MATERIALS AND METHODS

Reagents and chemicals. WR 148999 was synthesized as previously reported. Tritiated hypoxanthine (1.0 mCi/ml, specific activity 14 Ci/mmol), was obtained from NEN Life Science Products, Inc. (Boston, MA). Scintillation counting supplies were obtained from Wallac, Inc. (Gaithersburg, MD). Recombinant human CYP450 1A2, 2C9-Arg, 2C19, 2D6-Val, 2E1, 3A4 microsomes, bufuralol, 1-hydroxybufuralol, 4-hydroxydiclofenac, and S-(+)-mephentoin were obtained from Gentest Corporation (Woburn, MA). Pooled human liver microsomes were obtained from Xenotech (Kansas City, KS). All other reagents and drug metabolism standards were obtained from Sigma Chemical Company (St. Louis, MO) or Research Biochemicals International (Natick, MA). All HPLC solvents were of the highest commercial grade available and degassed prior to use.

Duration-of-action experiment. In order to determine the relative duration of antimalarial action of WR 148999 and
armesinin, the time of treatment prior to inoculation was varied in this experiment. Accordingly, CD-1 mice were administered subcutaneous 640 mg/kg doses of WR 148999 or artemisinin in peanut oil two times per day (b.i.d.) (for one day only) on Days 1 to 3 prior to infection or on the day of infection (Day 0). On Day 0, pretreated and control mice were inoculated with a chloroquine-sensitive P. berghei strain (KBG 173; MM). The number of dead mice and the day on which they died were subsequently noted. Mice living 60 days post-infection that were blood film negative were considered cured. A drug was considered toxic if the mice died before the untreated control mice. In this and studies described below, the maintenance and care of the mice complied with National Institutes of Health guidelines.

Chloroquine-sensitive versus chloroquine-resistant P. berghei experiment. In this experiment, the effect of WR 148999 on a chloroquine-sensitive (KBG 173; MM) and chloroquine resistant (C) P. berghei strains was determined. The Thompson test was used to assess in vivo activity. Accordingly, five week old CD-1 mice were inoculated intraperitoneally on Day 0 with 5 × 10⁸ (MM) or 15 × 10⁸ (C) trophozoites. The P. berghei trophozoites were obtained from an infected mouse with 60% parasitemia whose blood was subsequently diluted with uninfected mouse blood to give the appropriate trophozoite count. On Days 3, 4, and 5, groups of five or seven mice were treated orally twice daily with WR 148999 in peanut oil at a dose of 64 mg/kg. Blood films were taken one day after completion of drug treatment (Day 6) and weekly thereafter until Day 60. Parasitemia was determined from Giemsa-stained blood films. Drug activity was evaluated by suppression of parasitemia on Day 6, extension of survival time, and curative activity. The remaining details of the test are similar to that described above.

Route of administration experiment. In this experiment, oral and parenteral routes of administration of WR 148999 were compared in the Thompson Test. The MM strain of P. berghei was used in this study. WR 148999 in peanut oil was administered orally (po), subcutaneously (sc), or intraperitoneally (ip) at doses ranging from 2–1,024 mg/kg b.i.d. for three days. The remaining details of the test are similar to those described above.

In vitro antimalarial assay. In vitro activity against P. falciparum was determined using a modification of the semiautomated microdilution technique of Desjardins and others and Milhous and others. Two P. falciparum malaria parasite clones designated as Sierra Leone (D6) and Indo-china (W2) were used in susceptibility testing. The former is resistant to mefloquine, and the latter to chloroquine, pyrimethamine, sulfadoxine, and quinine. Test compounds were dissolved in dimethylsulfoxide, and solutions serially diluted with culture media. Erythrocytes with 0.25–0.5% parasitemia were added to each well of a 96-well microdilution plate to give a final hematocrit of 1.5%. Inhibition of incorporation of tritiated hypoxanthine was used as an index of antimalarial activity.

Pharmacodynamic drug interaction studies were performed by testing the two drugs at fixed ratios of concentrations proportional to their respective IC₅₀ values in ratios of 3:1, 1:1, and 1:3. Fractional inhibitory concentrations of the resulting IC₅₀ (FIC₅₀) values were used to construct isobolograms.

In vitro inhibition of human cytochrome P₄₅₀ (CYP) activities by WR 148999. In experiments involving inhibition of various CYP activities in human liver and recombinant human P450 microsomes, incubations were carried out using 0.05–0.4 mg/mL protein and were preincubated for three minutes with WR 148999 (0–500 μM) prior to the addition of selected probe substrates. For CYP2C9 enzyme incubations, a 0.1 M Tris buffer (pH = 7.5) was used. The concentrations of each probe substrate used in this study were: 7-ethoxyresorufin (2.5 μM), diclofenac (10 μM) mephenytoin (200 μM), bufuralol (10 μM), chlorozoxazone (75 μM), and testosterone (100 μM).

Incubations containing selected chemical inhibitors specific for each P450 used in this study were run in parallel as positive controls. The concentrations of each chemical inhibitor used in this study were: furylfylline (5, 50 μM), sulfaphenazole (2, 20 μM), quinidine (2, 20 μM), diethyl-dithiocarbamate (5, 50 μM), and ketoconazole (0.2, 2 μM). With the exception of quinidine and diethyl dithiocarbamate, which were dissolved in water, all inhibitors were dissolved in methanol (final methanol concentration ≤ 0.8%).

Assay methods for specific human P450 isozymes. Bufuralol 1-hydroxylase and chlorozoxazone 6-hydroxylase activities were determined by the HPLC methods of Newton and others. Quantitation of 6-β-hydroxytestosterone formation was determined using a modified HPLC method previously described. S-Mephenytoin 4-hydroxylase activity was measured by the published procedure of Meier and others. The spectrophotometric method for 7-ethoxyresorufin O-deethylase activity was determined by the method of Burke and others.

Data analysis. The effect of specific P450 inhibitors and WR 148999 on the formation of major metabolites of the various P450 probe substrates used in the study was evaluated by estimating the IC₅₀ values using the logistical dose response equation in Table Curve 2.0, Jandel Scientific (Chicago, IL).

RESULTS

Duration of action. When administered sc, WR 148999 had a considerably longer duration of action (Table 1) than did artemisinin against P. berghei. For example, when administered sc 3 days prior to infecting the mice with P. berghei, WR 148999 cured 4 of 7 mice whereas artemisinin had no effect on either life extension or mortality. In contrast
to WR 148999, artemisinin also failed to cure infected mice when it was given two days pre-infection. However, both WR 148999 and artemisinin were equally curative when administered on the day of parasite inoculation.

**Chloroquine-sensitive versus chloroquine-resistant** *P. berghei*. In another experiment, oral administration of a total dose of 192 mg/kg of WR 148999 in the Thompson test was more effective (Figure 2) against the chloroquine-resistant C-line compared to the chloroquine-sensitive MM-line of *P. berghei*. No cures were seen in the mice infected with the MM-line whereas 2 of 7 mice infected with the C-line were cured.

**Route of administration.** We had previously demonstrated[1] that WR 148999 was as effective as artemisinin in treatment of *P. berghei*-infected mice when both were administered sc twice daily on Days 3, 4, and 5 post-infection in the Thompson test.[1] We then performed parallel experiments (Table 2) to assess the antimalarial activity of tetraoxane WR 148999 using other routes of administration. In po, sc, and ip experiments, median survival times of *P. berghei*-infected mice were 8.8, 11.8, and 27.5 days, respectively, compared to 8 days for control animals. Survival curves for the various routes of administration are shown in Figure 3.

**In vitro antimalarial interaction.** To determine the pharmacodynamic interaction between WR 148999 and other antimalarials, FIC$_{50}$ values were used to construct isobolograms as shown in Figure 4. WR 148999 was synergistic in *vitro* with chloroquine, quinine, mefloquine, and artemisinin against both D6 and W2 clones of *P. falciparum* as shown in Figure 4.

**TABLE 1**

Comparision of tetraoxane WR 148999 versus artemisinin in a duration-of-action experiment in *Plasmodium berghei*-infected mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time of treatment* before infection (Days)</th>
<th>No. of dead mice/Day died</th>
<th>Cures†</th>
</tr>
</thead>
<tbody>
<tr>
<td>WR148999</td>
<td>−3</td>
<td>2/8 1/8</td>
<td>4/7</td>
</tr>
<tr>
<td></td>
<td>−2</td>
<td>1/8 1/9</td>
<td>5/7</td>
</tr>
<tr>
<td></td>
<td>−1</td>
<td>1/12 1/13 2/25</td>
<td>3/7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>7/7</td>
<td></td>
</tr>
<tr>
<td>Artemisinin</td>
<td>−3</td>
<td>1/7 6/8</td>
<td>0/7</td>
</tr>
<tr>
<td></td>
<td>−2</td>
<td>1/7 4/8 2/12</td>
<td>0/7</td>
</tr>
<tr>
<td></td>
<td>−1</td>
<td>2/8 2/9</td>
<td>3/7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>7/7</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>68/1/10</td>
<td>0/7</td>
</tr>
</tbody>
</table>

* 840 mg/kg of each compound dissolved in peanut oil was administered subcutaneously twice daily prior to or on the day of infection (Day 0) as indicated.
† The number of mice alive at Day 60 post-infection.

**TABLE 2**

Antimalarial activity of tetraoxane WR 148999 against *Plasmodium berghei* as a function of route of administration

<table>
<thead>
<tr>
<th>WR 148999 mg/kg/day</th>
<th>Oral</th>
<th>Subcutaneous</th>
<th>Intraperitoneal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of dead mice/Day died</td>
<td>Cures</td>
<td>No. of dead mice/Day died</td>
</tr>
<tr>
<td>1,024</td>
<td>1/16</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>512</td>
<td>1/18</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>256</td>
<td>3/17</td>
<td>1/24 1/27</td>
<td>1/32 1/36</td>
</tr>
<tr>
<td>128</td>
<td>3/12</td>
<td>2/13 1/20</td>
<td>1/24</td>
</tr>
<tr>
<td>64</td>
<td>1/8</td>
<td>2/9 2/10 1/12 1/16</td>
<td>0/7</td>
</tr>
<tr>
<td>32</td>
<td>4/8</td>
<td>3/9</td>
<td>2/9 1/16 1/18</td>
</tr>
<tr>
<td>16</td>
<td>1/7</td>
<td>3/8 3/9</td>
<td>3/8 2/9 2/12</td>
</tr>
<tr>
<td>8</td>
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<td>3/7 2/8 1/9</td>
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<td>4</td>
<td>4/7</td>
<td>2/8 1/9</td>
<td>2/7 2/8 1/10</td>
</tr>
<tr>
<td>2</td>
<td>3/7</td>
<td>3/8 1/9</td>
<td>3/7 2/8 1/9</td>
</tr>
<tr>
<td>Control</td>
<td>3/7</td>
<td>4/8</td>
<td>3/7 2/8 1/9</td>
</tr>
</tbody>
</table>

* WR 148999 was dissolved in peanut oil and administered twice daily to *P. berghei* (MM strain)-infected mice on Days 3, 4, and 5 post-infection. Cures are the number of mice alive at Day 60 post-infection.
† No data is available for either the 2 or 1,024 mg/kg/day doses.
hematin polymerization. Even though these considerations rationally into the relatively inert hemozoin via its inhibition of polymerization by antimalarial quinolines and for inhibiting WR 148999 was not assessed.

Experiments to test this hypothesis are in progress. Like artemisinin, WR 148999 is synergistic with mefloquine; unlike artemisinin, WR 148999 is also synergistic with chloroquine. The mechanistic basis for this synergism is unknown but could be related to the substantial binding of polar functional groups in a series of WR 148999 analogs that fail to enhance tetraoxane oral antimalarial activity. However, further studies determining the oral pharmacokinetic properties of WR 148999 are necessary to place the CYP1A2 inhibition data into perspective. We note that the potential metabolism of WR 148999 and possibly other tetraoxane analogs by CYP1A2 provides an initial basis for selecting potential drug partners and for predicting the likelihood of metabolically based drug-drug interactions.

That WR 148999 had a considerably longer duration of action than did artemisinin against P. berghei (sc administration) supports the contention that this tetraoxane is not metabolized as rapidly as is artemisinin or that its metabolite(s) possess some antimalarial activity. Interestingly, artemisinin induces its own metabolism and is metabolized principally by CYP2B6, and to a lesser extent, CYP3A4. In contrast, WR 148999 is most likely metabolized by CYP1A2. Since each compound was dissolved in peanut oil in this sc experiment, it is also possible that a depot was formed from which WR 148999 was released more slowly than artemisinin.

Notable features of WR 148999 in comparison to artemisinin are its structural simplicity, ease of synthesis with inexpensive starting materials, lack of cross-resistance with standard antimalarials, and improved single-dose efficacy and longer duration of action against P. berghei in vivo. Despite its modest antimalarial potency and low oral activity, WR 148999 illustrates the potential of tetraoxanes as a promising class of peroxide antimalarial agents. Indeed, we have since found a 5–7-fold increase in the oral antimalarial activity of three new tetraoxanes in comparison to the prototype WR 148999.

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FIGURE 4. Isobolograms showing the combinatorial antimalarial activities of tetraoxane WR 148999 and (a) chloroquine, (b) quinine, (c) mefloquine, and (d) artemisinin. WR 148999 was synergistic \textit{in vitro} with chloroquine, quinine, mefloquine, and artemisinin against both D6 and W2 clones of \textit{P. falciparum} as indicated by the concave lines. ● = clone D6; △ = clone W2. The IC\textsubscript{50}s for these drugs against the D6/ W2 clones of \textit{Plasmodium falciparum} were 3.1/58 nM for chloroquine, 28/220 nM for quinine, 18/8.7 for mefloquine, and 8.4/7.3 nM for artemisinin.

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