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In Vitro and In Vivo Effects of Rifabutin Alone or Combined with Atovaquone against Toxoplasma gondii

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The efficacy of rifabutin (RIFA) alone or in combination with atovaquone (ATO) was examined in vitro and in a murine model of acute toxoplasmosis. In vitro studies were performed with MRC5 fibroblast tissue cultures, with quantification of Toxoplasma growth by enzyme-linked immunosorbent assay. For in vivo studies, mice were acutely infected with 104 tachyzoites of the virulent RH strain and were then treated perorally for 10 days from day 1 or day 4 postinfection. The efficacy of each drug regimen was assessed by determination of survival rates and sequential titration of parasites in blood, brain, and lungs by a tissue culture method. In vitro, RIFA was inhibitory for Toxoplasma growth at concentrations between 0.5 and 20 μg/ml; the 50% inhibitory concentration was estimated to be 1.68 μg/ml. When RIFA and ATO were combined, synergistic effects were noted for RIFA at 20 μg/ml combined with ATO at 0.01 or 0.02 μg/ml and RIFA at 1, 2, or 3 μg/ml combined with ATO at 0.02 μg/ml. In vivo, administration of RIFA at 200 mg/kg of body weight per day from day 1 to day 10 resulted in a 100% protection during treatment, with clearance of parasites from the blood, brain, and lungs. After the cessation of therapy, relapses occurred in the brain and lungs; the mortality was 46% at the end of the experiment (day 30). Among the mice treated with RIFA at 200 mg/kg/day from day 4 to day 14, no death was recorded during the treatment period and a marked reduction in parasite burdens was observed in blood and tissues; however, relapses occurred and 10% of mice survived until day 30. Administration of RIFA at 200 mg/kg/day in combination with ATO at 100 mg/kg/day resulted in a marked prolongation of survival compared with that for mice that received ATO or RIFA alone. However, in mice receiving the combination, parasite burdens in blood and organs were similar to those in mice treated with RIFA alone. These results confirmed the activity of RIFA in the treatment of acute toxoplasmosis and the potential of the combination of RIFA-ATO since the two drugs act synergistically against Toxoplasma gondii.

Among the drugs that could be considered alternatives to folate inhibitors for the treatment of toxoplasmic encephalitis, atovaquone and rifabutin appear to be possible candidates, since both drugs have been found to be effective against Toxoplasma gondii in vitro and in vivo (1–3, 8). In murine models of acute toxoplasmosis, administration of atovaquone or rifabutin alone resulted in a significant protection of infected mice (1, 2, 8), and a synergistic effect was observed when both drugs were administered in combination (3). However, little is known about the extent of this synergistic effect, either in vitro or in vivo, with regard to the parasitic burdens in organs involved during acute toxoplasmosis.

Therefore, we examined the in vitro anti-Toxoplasma activity of rifabutin combined with atovaquone for various concentrations of each drug using a sensitive enzyme-linked immunosorbent assay (ELISA) method for the assessment of Toxoplasma growth and determination of an interacting effect between the two drugs. In addition, in vivo studies were performed with a murine model of acute toxoplasmosis, with sequential determination of parasite burdens in blood and tissues, during and after treatment with either rifabutin or atovaquone and a combination of the two drugs.

(This study was presented in part at the 7th European Congress of Clinical Microbiology and Infectious Diseases, Vienna, Austria, 26 to 30 March 1995 [10a].)

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were administered daily at a fixed hour for 10 days either from day 1 or from day 4 postinfection. These drug regimens were selected because they were previously found to be effective but not curative in the treatment of acute toxoplasmosis (3, 8) and because a preliminary experiment with 10 mice in each treatment group indicated that they were appropriate for demonstrating an interacting effect between the two drugs.

After infection, the mice were randomly allocated to separate groups. Fifteen mice were not treated (controls) and 45 mice were used for each drug regimen. At days 4, 7, 10, 14, 21, and 30 postinfection, five mice from each group were sacrificed for determination of the parasite burdens in blood, lungs, and brain by a culture-based microtitration method described previously (9). In brief, serial dilutions of blood and organ homogenates were inoculated into an MRC5 fibroblast tissue culture prepared in 96-well microplates. After 72 h of incubation at 37°C, the cultures were fixed and examined for Toxoplasma organisms by an indirect immunofluorescence assay (9).

For each sample, the titer was the last dilution which gave at least one parasitic focus in the culture. Parasite burdens were calculated as the reciprocal titer in tissue culture/volume [in microliters] or weight [in milligrams] × 1,000. The results were expressed as the log10 value of the number of parasites per gram of tissue or per milliliter of blood.

**Drug measurements.** At days 4, 7, 10, 14, 21, and 30 postinfection, the atovaquone and rifabutin concentrations in serum and brain tissue were measured by high-pressure liquid chromatography (HPLC) (two mice per time point per treatment). Atovaquone was measured as described by DeAngelis et al. (5). Brain tissue samples were crushed under liquid nitrogen and then extracted with an isooctane–n-hexane (3:100 [vol/vol]) mixture. Recovery of atovaquone from serum and brain tissues was 85%. Intra- and interassay variabilities were 10%. The limit of quantitation was 0.25 μg/ml for serum and 0.2 μg/g for brain tissue. Rifabutin was measured by a new method which was developed and validated from a previously published assay (12). Briefly, serum samples and crushed brain samples were extracted by ion-pair liquid-liquid extraction in a hexane–ethyl acetate mixture (80:20 [vol/vol]). Analytes were separated on a 5-μm Hypersil octyldecylsilane column (Shandon). The mobile phase consisted of a mixture of 0.05 M (pH 4.5) ammonium phosphate–acetonitrile-triethylamine (56:44:0.1 [vol/vol]). The detection was set at 275 nm. Intra- and interassay variabilities were 10%. The lower limits of detection were 50 μg/liter and 0.5 μg/g for plasma and brain samples, respectively.

**TABLE 1. Inhibitory effect of rifabutin combined with atovaquone with different ratios of concentrations in experiments A and B**

<table>
<thead>
<tr>
<th>Expt and atovaquone concn (μg/ml)</th>
<th>Mean ± SEM OD (103) at the following rifabutin concn (μg/ml):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Expt A</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1,728 ± 40</td>
</tr>
<tr>
<td>0.002</td>
<td>1,624 ± 38</td>
</tr>
<tr>
<td>0.01</td>
<td>1,594 ± 35</td>
</tr>
<tr>
<td>0.1</td>
<td>427 ± 17</td>
</tr>
<tr>
<td>Expt B</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>652 ± 15</td>
</tr>
<tr>
<td>0.005</td>
<td>682 ± 13</td>
</tr>
<tr>
<td>0.02</td>
<td>655 ± 14</td>
</tr>
<tr>
<td>0.1</td>
<td>72 ± 2</td>
</tr>
</tbody>
</table>

a Each value represents the results from 24 (experiment A) or 40 (experiment B) replicate wells. P values were calculated for the interacting effect in a two-way analysis of variance. A P value significantly different from 0 indicates a synergistic effect; i.e., the mean OD with the combined drugs is significantly lower than that expected from the simple additive effect of both drugs.

b P < 0.05.
c P < 0.01.
d P < 0.001.
Statistical analysis. Regression models were used to summarize the in vitro dose-effect relationship and to determine the 50% inhibitory concentration (IC\textsubscript{50}) as described previously (6). The effects of the drugs in combination were tested by two-way analysis of variance, including the estimation of an interaction factor as described for previous experiments (6). Drugs were considered to act synergistically when the interaction factor was significantly different from 0, i.e., the mean OD with the drugs combined was significantly lower than that expected from the simple additive effects of both drugs.

In mice, the survival rates were estimated by the Kaplan-Meier product limit method and were compared by the log-rank test. The mean value for the parasite burden from five mice (± standard error) was calculated for each time point.

RESULTS

In vitro experiments. Rifabutin alone inhibited Toxoplasma growth at concentrations >0.1 \textmu g/ml, with a maximum effect obtained at 20 \textmu g/ml and a toxic effect on fibroblasts obtained at 100 \textmu g/ml. The IC\textsubscript{50} was estimated to be 1.68 \textmu g/ml (range 1.63 to 1.71 \textmu g/ml) (Fig. 1).

When rifabutin was combined with atovaquone, a significant interacting effect was noted for various ratios of drug concentrations. In the first experiment, in which each ratio of the drug concentration was tested in 24 replicate wells, a significant synergistic effect was obtained when rifabutin at 20 \textmu g/ml was combined with atovaquone at 0.01 \textmu g/ml (Table 1, experiment A). In the second experiment, other ratios were examined (40 replicate test wells for each ratio), and a significant synergistic effect was observed for atovaquone at 0.02 \textmu g/ml combined with rifabutin at 1, 2, 5, and 20 \textmu g/ml (Table 1, experiment B).

In vivo experiments. The survival and kinetics of infection for 45 mice in each treatment group and 15 control mice are presented in Fig. 2 through 5. In these groups, the survival rates were comparable to those obtained previously in a preliminary experiment with 10 mice in each treatment and control group.

(i) Control mice. All untreated mice died within 9 days after infection (mean, 7.4 days; range, 6 to 9 days), with increasing parasitic burdens in the lungs and brains between day 4 and day 7 and a high level of parasitemia at day 7.

(ii) Rifabutin alone. The administration of rifabutin from day 1 after infection significantly prolonged survival compared with the survival period for control mice (P < 0.001): 100% of mice survived during the treatment period, and 54% were alive at day 30. Examination of parasitic burdens showed a complete eradication of parasites in tissues and blood during the treatment period. After the discontinuation of therapy, relapses of infection occurred in the lungs and brain, although parasitic burdens decreased in the lungs after day 21.

Starting treatment from day 4 after infection resulted in a significant protection (P < 0.001 versus controls). As observed in the preliminary experiment, all mice survived up to day 16, but only 13% were alive at day 30. Parasite burdens decreased during the treatment period but increased markedly after the cessation of therapy.

(iii) Atovaquone alone. Atovaquone treatment from day 1 after infection was effective in prolonging survival compared with the survival period for control mice (P < 0.001), and 43% of mice survived to day 30. While the mice were under therapy, parasite burdens rapidly increased in tissues and remained at high levels after treatment.

When atovaquone was administered from day 4, both survival rates and the kinetics of the parasite burdens were comparable to those observed in control mice.

(iv) Rifabutin in combination with atovaquone. The administration of combined therapy from day 1 resulted in a significant prolongation of survival compared with the survival periods for mice given atovaquone (P < 0.001) or rifabutin (P < 0.05) alone. At the end of the experiment, 95% of the mice were alive. However, no marked differences in the kinetics and
FIG. 4. Kinetics of parasite burdens in the blood (○), lungs (■), and brain (●) of mice infected at day 0 with $10^4$ tachyzoites of the RH strain of *T. gondii*. Each point represents the mean ± standard error of the mean for five mice. Shaded areas represent the period of administration of the antimicrobial agents. Mice were treated from day 1 postinfection with rifabutin at 200 mg/kg/day (A), atovaquone at 100 mg/kg/day (B), or rifabutin combined with atovaquone (C).

FIG. 5. Kinetics of parasite burdens in the blood (○), lungs (■), and brain (●) of mice infected at day 0 with $10^4$ tachyzoites of the RH strain of *T. gondii*. Each point represents the mean ± standard error of the mean for five mice. Shaded areas represent the period of administration of the antimicrobial agents. Mice were treated from day 4 postinfection with rifabutin at 200 mg/kg/day (A), atovaquone at 100 mg/kg/day (B), or rifabutin combined with atovaquone (C).
levels of tissue infection were observed between mice treated with the combination and those treated with rifabutin alone.

The survival of mice treated from day 4 with the combination therapy was significantly prolonged compared with the survival period for mice given atovaquone alone (P < 0.001), but not compared with the survival period for mice given rifabutin alone. As for treatment initiated from day 1, the kinetics and levels of parasite burdens were similar in mice treated with rifabutin alone and those treated with the combination.

(v) Drug measurements. High concentrations of atovaquone were noted during the treatment period in both groups of mice receiving atovaquone either alone or combined with rifabutin (Tables 2 and 3). After the cessation of therapy, the concentrations dropped to undetectable levels at day 14 or day 21. Rifabutin was detected at significant levels in serum and brain throughout the treatment period but was almost undetectable in the brains of mice with low levels of the drug in their serum.

DISCUSSION

Treatment of *T. gondii*-infected cells with rifabutin showed that this rifamycin S-derived compound has significant in vitro activity against the virulent RH strain, a result consistent with those reported by Olliaro et al. (8). Yet, our experimental conditions (i.e., smaller inoculum size and longer culture period) increased the sensitivity of the in vitro test, thus showing a significant inhibitory effect of a rifabutin concentration as low as 0.1 μg/ml.

In vitro, a clear synergistic activity of the combination of atovaquone and rifabutin against *T. gondii* could be demonstrated for various concentrations of both drugs. The reason for this effect remains unclear and could be related to complementary pharmacological targets of the two drugs on protozoa: although the mechanism of action of atovaquone against *T. gondii* has not been demonstrated, hydroxynaphthoquinones act on other protozoa by blocking mitochondrial electron transport, resulting in the inhibition of pyrimidine synthesis (7), whereas the mechanisms of action of rifabutin and rifamycins against sporozoan parasites are largely unknown and possibly involve the inhibition of protein synthesis in a prokaryote-type organelle (13).

Treatment of acutely infected mice showed that rifabutin alone was effective in reducing mortality, as has been observed by others (3, 8). Our study suggests that this could be related to the anti-*Toxoplasma* activity of rifabutin in tissues, resulting either in a complete clearance or a marked reduction of parasite burdens in the blood, brain, and lungs during the treatment period. However, either early or delayed administration of rifabutin failed to prevent relapses of infection after the discontinuation of treatment. Such relapses after the cessation of therapy have been observed in our experimental murine model with other drug regimens and have even been observed with the reference combination pyrimethamine-sulfadiazine (9, 10). In that respect, our short treatment schedule was found to be relevant for showing that drugs that induce a reduction or negativation of parasite burdens during the treatment period may not eradicate infection, thus suggesting an indication for a maintenance therapy.

Rifabutin combined with atovaquone significantly enhanced mouse survival compared with the protection afforded by each drug alone, consistent with the effects observed by Araujo et al. (3). In contrast, administration of atovaquone from day 4 was unable to improve the survival rate obtained with rifabutin as a single agent. Moreover, examination of the parasite burdens revealed no clear benefit of the effect of the combination regimen compared with the effect of rifabutin alone in mice treated either from day 1 or from day 4. Thus, the prolonged survival that was observed with the combined therapies cannot be attributed only to the antiparasitic activities of the drugs in tissues. This could possibly be related to an additional anti-inflammatory effect which was noted in the brain tissues of *Toxoplasma*-infected mice treated with atovaquone or rifabutin (2, 3) and which could be enhanced when both drugs are given in combination.

Although drug concentrations were measured in only two mice in each treatment group, our results have demonstrated a distribution of both atovaquone and rifabutin in the brain that achieves concentrations higher than the IC_{50} of both drugs estimated here and in another study (10). However, the levels of both drugs in the brain and serum dropped after treatment was withdrawn, which possibly accounts for the rapid recurrence of infection. Besides, concomitant use of rifabutin and atovaquone was not associated with major interactions between the bioavailabilities of the two drugs. This is in contrast to the significantly decreased levels of atovaquone observed in the plasma of humans who received combined therapy with the parent compound, rifampin (11), but it also supports the fact...

TABLE 2. Concentrations of atovaquone in serum and brain tissue of mice treated for 10 days from day 1 postinfection

<table>
<thead>
<tr>
<th>Treatment group and site</th>
<th>Conc (µg/ml or µg/g) on the following days postinfection:</th>
<th>4</th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atovaquone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>87.7</td>
<td>43.2</td>
<td>28.7</td>
<td>1.5</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>35.6</td>
<td>41.2</td>
<td>32.1</td>
<td>0.9</td>
<td>&lt;0.25</td>
<td></td>
</tr>
<tr>
<td>Atovaquone + rifabutin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>56.2</td>
<td>72.9</td>
<td>29.5</td>
<td>0.25</td>
<td>&lt;0.25</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>106.7</td>
<td>99.3</td>
<td>27.5</td>
<td>&lt;0.25</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

* Dosages were determined in two mice in each group. The concentrations in serum and brain tissue were determined by HPLC.

TABLE 3. Concentrations of rifabutin in serum and brain tissue of mice treated for 10 days from day 1 postinfection

<table>
<thead>
<tr>
<th>Treatment group and site</th>
<th>Conc (µg/ml or µg/g) on the following days postinfection:</th>
<th>4</th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>21</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifabutin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>0.86</td>
<td>7.40</td>
<td>1.00</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>5.18</td>
<td>2.98</td>
<td>0.39</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Atovaquone + rifabutin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>0.96</td>
<td>1.45</td>
<td>0.44</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>1.48</td>
<td>1.32</td>
<td>0.12</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

* ND, not determined.
that the enzyme-inducing properties of rifabutin are less pronounced than those of rifampin (4). However, data from a study with such a small sample size need to be confirmed by larger pharmacological studies.

Finally, our experimental results suggest that the use of the combination rifabutin-atovaquone may be a promising alternative to the use of folate inhibitors for the treatment or prophylaxis of acute toxoplasmosis, insofar as prolonged administration of therapy and sustained levels of drug in tissues are achieved. Thus, appropriate clinical trials are warranted to evaluate the efficacy of the combination rifabutin-atovaquone for the treatment of human toxoplasmosis.

ACKNOWLEDGMENTS

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