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In Vitro and In Vivo Activities of the Hydroxynaphthoquinone Atovaquone Alone or Combined with Pyrimethamine, Sulfadiazine, Clarithromycin, or Minocycline against *Toxoplasma gondii*

STÉPHANE ROMAND,[1] MARY PUDNEY,[2] AND FRANCIS DEROUIN[1]*

Laboratoire de Parasitologie-Mycologie, Hôpital Saint-Louis, 1, Avenue Claude Vellefaux, 75475 Paris Cedex 10, France,[1] and Wellcome Research Laboratories, Wellcome Foundation Ltd., Langley Court, Beckenham, Kent BR3 3BS, United Kingdom[2]

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The efficacy of atovaquone alone or combined with pyrimethamine, sulfadiazine, clarithromycin, and minocycline 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 an enzyme-linked immunosorbent assay. For in vivo studies, mice were acutely infected intraperitoneally with 10^4 tachyzoites of the virulent RH strain and then treated perorally for 10 days from day 1 postinfection. The following drug regimens were investigated: atovaquone at 100 and 50 mg/kg of body weight per day and the combinations of atovaquone at 50 mg/kg with sulfadiazine at 200 mg/kg, pyrimethamine at 12.5 mg/kg, clarithromycin at 200 mg/kg, or minocycline at 50 mg/kg. Efficacy was assessed by determination of survival rates and sequential determination of parasite burdens in blood, brain, and lungs. In vitro, atovaquone inhibited *Toxoplasma* growth at a concentration of ≥0.02 mg/liter; the 50% inhibitory concentration was estimated to be 0.023 mg/liter. No synergistic effect was observed when it was combined with sulfadiazine, clarithromycin, or minocycline, whereas a significant antagonistic effect was noted for the combination of atovaquone with pyrimethamine. In vivo, administration of atovaquone at 100 or 50 mg/kg/day for 10 days resulted in prolonged survival compared with that in untreated mice; this survival was associated with a reduction of parasite burdens in blood and tissues during the course of treatment. The combinations of atovaquone with pyrimethamine, clarithromycin, or sulfadiazine were more efficient than each drug administered alone, in terms of survival, but parasite burdens in blood and organs were not reduced compared with those in mice treated with any of the agents alone. These experimental results confirmed the activity of atovaquone against *Toxoplasma gondii*, but no marked improvement in efficacy was observed in vitro and in vivo when this drug was combined with pyrimethamine, sulfadiazine, minocycline, or clarithromycin.

Araujo et al. (1, 2) demonstrated that the hydroxynaphthoquinone atovaquone was effective against *Toxoplasma gondii* in vitro and in vivo. In vitro experiments were performed with human foreskin fibroblasts inoculated with tachyzoites of different strains of *T. gondii*, and inhibitory concentrations ranged from 4.8×10^{-9} to 4.8×10^{-6} M (1). These authors also demonstrated that prolonged incubation of isolated brain cysts of *T. gondii* with atovaquone resulted in the inactivation of intracystic parasites (bradyzoites) (2). In vivo experiments confirmed the remarkable activity of this compound, since the administration of atovaquone at 50 mg/kg of body weight per day for 10 days was protective in mice inoculated with virulent strains of *T. gondii*. Also, in chronically infected mice, long-term administration of atovaquone at 200 mg/kg/day significantly reduced the number of cysts in the brains of treated mice compared with those of controls (2). Together, these results represented a reliable basis for clinical studies involving patients with cerebral toxoplasmosis, particularly in cases of intolerance of the standard pyrimethamine-sulfadiazine therapy.

Although the number of patients that have already been treated with atovaquone is limited, there is clear evidence that this drug is effective in cases of cerebral and ocular toxoplasmosis (17, 19, 21), but some cases of failure or relapse have been observed. This result suggests that, in such cases, treatment with atovaquone could be completed by the concurrent administration of another antimicrobial agent (18). In this study, we examined the activity of atovaquone alone or combined with pyrimethamine, sulfadiazine, clarithromycin, or minocycline against *T. gondii* in vitro and for the treatment of acute experimental murine toxoplasmosis.

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MATERIALS AND METHODS

Parasites. In vitro and in vivo studies were performed with the virulent RH strain, which was maintained in mice by passage every 2 to 3 days. For each experiment, tachyzoites were collected from the peritoneal cavities of infected mice and resuspended in physiological saline.

In vitro studies. In vitro studies were carried out with MRC5 fibroblast tissue cultures as previously described (8). In brief, confluent monolayers prepared in 96-well tissue culture plates were inoculated with 1,500 tachyzoites. After 4 h, antimicrobial agents at various concentrations were added to the medium, and the cultures were incubated for an additional 72 h. *Toxoplasma* growth was assessed by an enzyme-linked immunoassay performed directly on the fixed

* Corresponding author.

cultures; the method was a modification of our previously described method (8). Each culture well was filled with 200 μl of phosphate-buffered saline containing 0.5% gelatin and then incubated for 60 min at 37°C. Plates were washed twice for 5 min each time with Tris buffer (10 mM Tris, 0.3 M NaCl, 0.1% Tween 20 [pH 7.6]). To each well, 50 μl of a solution of peroxidase-labelled monoclonal antibody directed against the P30 membrane antigen of *T. gondii* (Diagnostics Pasteur, Marnes, France) and diluted at 1/500 in Tris buffer–1% bovine serum albumin was added and incubated for 60 min at 37°C. Plates were washed four times, and then 100 μl of the substrate (*ortho*-phenylenediamine) was added to each well. After 30 min, the reaction was stopped by the addition of 200 μl of 4 N H_2SO_4, and the optical density (OD) of the supernatant was determined for each culture well by spectrophotometric readings (λ = 405 nm). In each experiment, one tissue culture plate was stained with Giemsa stain for microscopic examination.

Atovaquone (Wellcome Foundation Ltd.), pyrimethamine (Sigma Chemical Co., St. Louis, Mo.), sulfadiazine (Dohms Laboratories), and clarithromycin (Abbott Laboratories) were dissolved in 50% methanol–50% acetone at a concentration of 1 mg/ml. Minocycline (Lederle Laboratories) was dissolved in distilled water. Serial dilutions of each drug were prepared in minimum essential medium (Flobio) such that the addition of 50 μl of each dilution to a culture well produced the required final concentration. Atovaquone was examined alone at the following concentrations: 0.0001, 0.0005, 0.002, 0.01, 0.05, 0.2, 1, 5, 20, and 100 mg/liter; each concentration was used in eight replicate wells. Preliminary studies indicated that the final concentrations of methanol and acetone used in the dilution of drugs did not inhibit the growth of *T. gondii*.

Combinations of three concentrations of atovaquone and four concentrations of each antiparasitic agent were tested in a two-way design. From the results of previous experiments, we selected concentrations that resulted in a range of inhibition from no inhibition to complete inhibition: atovaquone at 0.002, 0.01, and 0.05 mg/liter; pyrimethamine at 0.005, 0.02, 0.1, and 0.5 mg/liter; sulfadiazine at 0.01, 0.05, 0.2, and 1 mg/liter; clarithromycin at 0.1, 0.5, 2, and 10 mg/liter; and minocycline at 0.1, 0.5, 2, and 10 mg/liter (6, 8, 9). Each experiment comprised six replicate plates in which each drug or drug combination was tested in four replicate wells.

In vivo studies. Experiments were conducted with Swiss albino mice acutely infected intraperitoneally with 10^4 tachyzoites.

In one experiment, we examined the efficacy of atovaquone administered alone at 100 mg/kg/day for 10 days either from day 1 or from day 4 postinfection. Combination studies were performed in two separate experiments, with atovaquone administered at 50 mg/kg/day in combination with pyrimethamine or sulfadiazine (experiment 1) and clarithromycin or minocycline (experiment 2). For each drug combination, we selected a dose that was previously found to be noncurative, i.e., clarithromycin at 50 mg/kg, sulfadiazine at 200 mg/kg, pyrimethamine at 12.5 mg/kg, and minocycline at 50 mg/kg (6, 7, 20). Drug suspensions were prepared daily in a solution of methyl cellulose–carboxymethyl cellulose–0.1% Tween 20, briefly sonicated, and administered orally to mice via tube feeding. Treatments were administered each day at a fixed hour for a 10-day period.

For each experiment, mice were randomly allocated to separate groups. Thirty mice were not treated (controls), and 40 mice were used for each drug regimen. Mice were studied for 30 days after infection, and sequential examinations of parasite burdens in blood, brain, and lungs were performed at the following intervals after infection: days 4, 7, 10, 14, 21, and 30. At each time point, five mice from each group were sacrificed, and culturing of blood and organ homogenates was performed as described previously (20). In brief, from each blood and organ suspension, serial fourfold dilutions were prepared with culture medium, and then 40 μl of each dilution was inoculated into duplicate wells of tissue culture plates. Previous experiments with the same experimental procedure had shown that antimicrobial agents in ground tissue had no effect on parasite growth in cultures (20). After 72 h of incubation at 37°C, cultures were fixed and examined for *Toxoplasma* organisms by an indirect immunofluorescence assay. The presence of parasitic foci in each well was recorded; the final titer was the last dilution that allowed at least one parasitic focus. The number of parasites per gram or milliliter (parasite burden) was calculated as the reciprocal titer in tissue culture/volume (milliliters) or /weight (grams) × 1,000. Results were expressed as the log of the number of parasites per gram of tissue or per milliliter of blood.

Statistical analysis. Regression models were used to summarize the in vitro dose-effect relationship, and the equations of regression lines were determined for noninhibitory concentrations (line 1, slope not significantly different from 0) and inhibitory concentrations (line 2 and line 3). The 50% inhibitory concentration was estimated from the regression line equations. The combined effect of drugs was tested in a two-way analysis of variance, including an interaction effect (8).

For mice, survival rates were estimated by the Kaplan-Meier product limit method and compared by the log rank test. The mean value for parasite burden from five mice (±1 standard error) was calculated for each time point.

RESULTS

In vitro experiments. (i) Atovaquone alone. The in vitro activity of atovaquone at various concentrations was described by plotting the OD values for eight replicate wells against the logarithm of the concentration in each culture well (Fig. 1). A significant inhibitory effect on *Toxoplasma* growth was noted at a concentration of $\geq 10^{-2}$ mg/liter; between 10^{-2} and 5×10^{-2} mg/liter, the effect was summarized by the regression line equation OD = $-2.70 - 2.29$ log (concentration). For concentrations of $>5 \times 10^{-2}$ mg/liter, the slope of line 3 was not significantly different from 0 ($P > 0.05$), indicating that a maximum inhibitory effect was achieved for these concentrations. From line 2, the 50% inhibitory concentration was estimated to be 2.35×10^{-2} mg/liter (6.4×10^{-8} M) [range, (2.2 to 2.5) $\times 10^{-2}$ mg/liter].

An examination of Giemsa-stained cultures showed a toxic effect on the fibroblasts for concentrations of >5 mg/liter. At lower inhibitory concentrations of atovaquone, we observed mild morphological changes of the parasites: they appeared smaller, and their nuclei were more intensely stained than those in nontreated cultures.

(ii) Atovaquone in combination with other antimicrobial agents. In this experiment, the results that we previously obtained for pyrimethamine, sulfadiazine, clarithromycin, and minocycline alone were confirmed for the different concentrations that were examined (6, 8, 9). By the two-way analysis of variance that was used for testing the drug combinations, the effect of each drug and the interacting effect of drugs in combination were estimated. The interacting effect was calculated from the results obtained with the

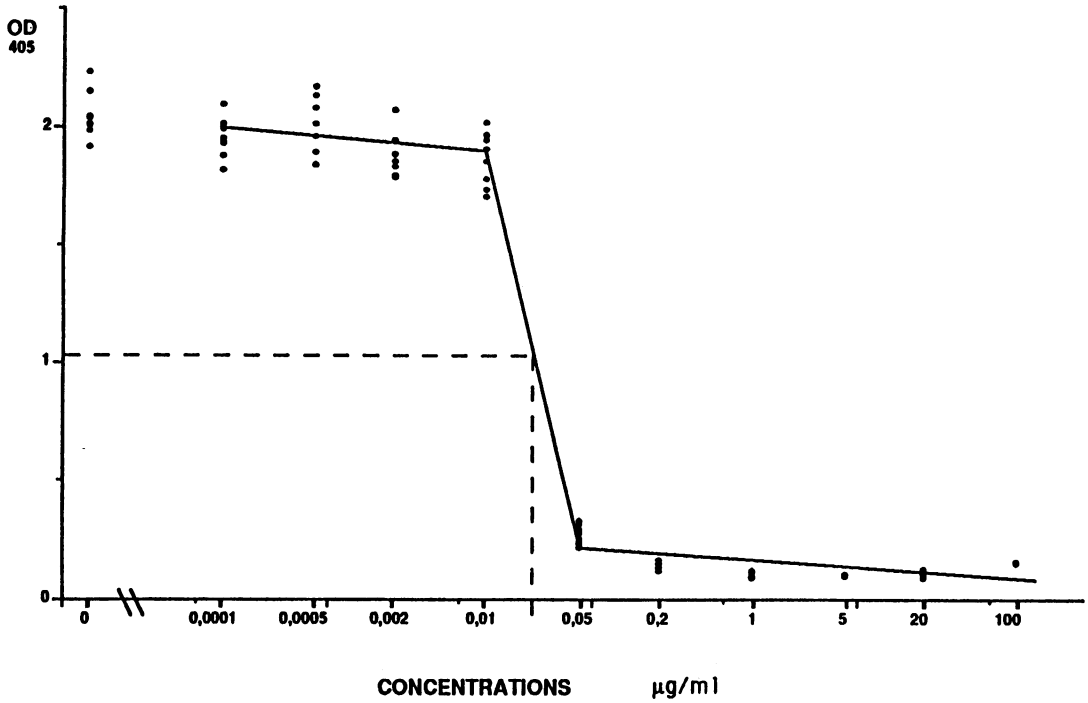

FIG. 1. In vitro effect of atovaquone on *Toxoplasma* growth. OD values in the enzyme-linked immunosorbent assay of infected cultures were plotted against concentrations of atovaquone.

four cell compartments of each two-way design experiment and took into account the individual effect of each drug. The resulting factor was significantly different from 0 when the effect of the drug combination was different from that which could be expected from the additional effect of each drug.

We analyzed the results from four replicate wells of six replicate culture plates, i.e., 24 OD values for each drug or drug combination, and found no interplate interacting effect. No significant interacting effect could be demonstrated when atovaquone was combined with sulfadiazine, minocycline, or clarithromycin at various concentrations (data not shown). For the combination of atovaquone with pyrimethamine, a significant negative interacting effect was noted at several ratios of drug concentrations (Table 1). These results were confirmed in a second experiment (data not shown).

In vivo experiments. (i) Untreated mice. All the control mice died within 7 days (range, 5 to 7 days), and the determination of parasite burden showed a predominant parasitic involvement of lungs and brain. On day 4, the parasite burdens for control mice were, respectively, 4.65 ± 0.16 log units in lungs, 2.01 ± 0.41 in brain, and 1.03 ± 0.32 in blood (mean of two experiments).

(ii) Atovaquone alone. An examination of survival rates (Table 2) and culture results indicated that atovaquone treatment at 100 mg/kg daily, started from day 1 after infection, was effective in prolonging survival, but protection was only partial, as 83% of the mice died by 30 days (Table 2). When the parasite burdens were examined, parasitic infections in the brain were found to progressively worsen from day 4, while those in the lungs briefly improved at day 10 and thereafter worsened until day 21. Transient parasitemia was noted in one of five mice at day 10 (Fig. 2A). When treatment was started from day 4 after infection, 100% of the mice died within 7 days postinfection, with high parasitic burdens in the blood and organs (Fig. 2B).

Atovaquone treatment at 50 mg/kg daily, started from day 1 postinfection, had limited efficacy in terms of survival

TABLE 1. Inhibitory effect of atovaquone combined with pyrimethamine[a]

Pyrimethamine concn (µg/ml)	Mean OD ± SEM at the following atovaquone concn (µg/ml):			
	0	2×10^{-3}	10^{-2}	5×10^{-2}
0	1.562 ± 0.056	1.540 ± 0.069	1.441 ± 0.068	0.212 ± 0.013
5×10^{-3}	1.381 ± 0.075	1.555 ± 0.062	1.523 ± 0.056[b]	0.265 ± 0.016[b]
2×10^{-2}	1.361 ± 0.053	1.623 ± 0.071[b,c]	1.670 ± 0.054[c,d]	0.261 ± 0.022[e]
0.1	0.154 ± 0.010	0.228 ± 0.021	0.338 ± 0.020[e]	0.126 ± 0.005
0.5	0.080 ± 0.003	0.077 ± 0.002	0.080 ± 0.002	0.097 ± 0.003

[a] Each value represents results from 16 (first column) or 24 (other columns) replicate wells. *P* values were calculated for the interacting effect.
[b] $P < 0.05$.
[c] Mean OD values are not significantly different from those obtained for cultures without drug (Student's *t* test; $P > 0.2$).
[d] $P < 0.001$.
[e] $P < 0.01$.

TABLE 2. In vivo activity of atovaquone, pyrimethamine, sulfadiazine, clarithromycin, and minocycline, administered alone or in combination for 10 days from day 1 postinfection

Treatment (mg/kg/day)	% Survival[a] on the indicated day after infection:						Mean survival (days)
	4	7	10	14	21	30	
Control[b]	100	0	0	0	0	0	5.5
Atovaquone (100)	100	100	73	65	52	17	21.2
Atovaquone[b] (50)	100	100	62	41.5	37	18	10.5
Pyrimethamine (12.5)	100	97	87	78	45	45	20.2
Sulfadiazine (200)	100	100	100	100	89	89	>30
Clarithromycin (200)	100	85	36	36	36	36	8.3
Minocycline (50)	100	100	79	66	66	66	>30
Atovaquone (50) + pyrimethamine (12.5)	100	100	100	100	90	69	>30
Atovaquone (50) + sulfadiazine (200)	100	100	100	100	100	93	>30
Atovaquone (50) + clarithromycin (200)	100	100	100	100	95	73	>30
Atovaquone (50) + minocycline (50)	100	100	77	60	53	53	>30

[a] Survival was estimated on the date of examination of parasitic burdens in blood and tissues.
[b] Mean of two experiments.

(Table 2). In the two experiments, the mean times to death were 5.8 and 5.9 days for control mice and 10 and 11 days for mice treated with atovaquone. In both experiments, for mice treated with atovaquone alone the parasite burden in the brain progressively increased until death, whereas the parasite burden in the lungs increased from days 7 to 10 and then remained at a high level until death. Parasites were present at a low level in the blood of one of five mice at day 7 in one experiment and in one of five mice at days 14 and 21 in the other experiment (Fig. 2C).

(iii) Combinations of atovaquone with other antimicrobial agents. Each experiment comprised a group of untreated mice and a group treated with atovaquone at 50 mg/kg/day (see results discussed above). The survival and kinetics of infection in mice treated with sulfadiazine at 200 mg/kg/day, pyrimethamine at 12.5 mg/kg/day, clarithromycin at 200 mg/kg/day, or minocycline at 50 mg/kg/day alone or combined with atovaquone at 50 mg/kg/day are presented in Table 2 and Fig. 3 to 6.

(a) Combination of atovaquone and sulfadiazine. When sulfadiazine was administered alone, 89% of the mice were alive at the end of the experiment (Table 2). Comparatively, 93% of the mice treated with the combination of atovaquone and sulfadiazine were alive at day 30. When compared by the log rank test, the survival of mice treated with the combination was significantly higher than that of mice treated with atovaquone alone ($\chi^2 = 20.22$; $P < 0.001$) but not significantly different from that of mice treated with sulfadiazine alone ($\chi^2 = 0.5$; P not significant). For mice treated with sulfadiazine (Fig. 3A), parasites were only demonstrated in the brain of one mouse at days 10 and 14. Thereafter, the parasite burden increased in the lungs and brain, with a peak level at day 21. For mice treated with the combination (Fig. 3B), parasites were not demonstrated until day 21, when the parasite burden increased in the lungs and brain, but to a level lower than that in mice treated with sulfadiazine or atovaquone alone. At day 30, parasites were only demonstrated in the brains of three or five mice.

(b) Combination of atovaquone and pyrimethamine. The survival rates in the groups of mice treated with pyrimethamine and with the combination of atovaquone and pyrimethamine were, respectively, 45 and 69% at day 30. A comparison of the survival estimates by the log rank test showed that the survival of mice treated with the combination was significantly prolonged compared with that of mice treated with atovaquone alone ($\chi^2 = 16.04$; $P < 0.001$) or pyrimethamine alone ($\chi^2 = 8.15$; $P < 0.01$). However, no marked reduction in parasite burden was noted in mice treated with the combined drugs in comparison with that observed in mice treated with pyrimethamine alone (Fig. 4). With both regimens, parasites were detected during treatment in the lungs and could be demonstrated in the lungs and brain after the cessation of therapy. With both regimens, parasitemia was undetectable throughout the follow-up period.

(c) Combination of atovaquone and clarithromycin. Although the survival of mice treated with atovaquone or clarithromycin alone was not significantly different (log rank test, not significant), the analysis of parasite kinetics showed marked differences for the two drug regimens. For mice treated with clarithromycin alone, the parasite burden decreased in the lungs but increased in the brain during and after treatment, confirming our previous observations with this drug (7) (Fig. 5A). With the combination, significant prolonged survival was noted, in comparison with that for mice treated with atovaquone alone ($\chi^2 = 20.10$; $P < 0.001$) or clarithromycin alone ($\chi^2 = 15.53$; $P < 0.001$). An examination of parasite burdens showed a marked reduction in brain and lung infections during treatment, but relapses were noted after the cessation of therapy, with a predominant brain involvement (Fig. 5B).

(d) Combination of atovaquone and minocycline. The survival of mice was not significantly different among the three groups (atovaquone, minocycline, and atovaquone plus minocycline). An examination of parasite burdens showed that the kinetics of infection were comparable in mice treated with the combination and those treated with atovaquone alone. For mice treated with atovaquone plus minocycline, the parasite burdens increased in the brain and lungs during therapy, and relapses were constantly observed after the cessation of therapy, with high parasite burdens in the brain and lungs (with the exception of the lungs at day 30) (Fig. 6).

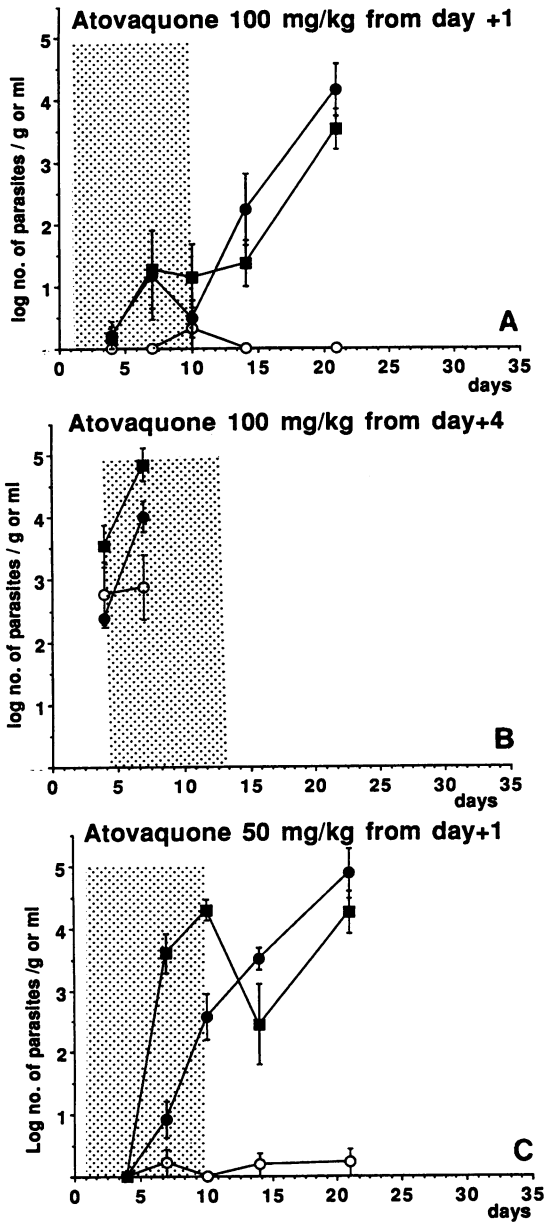

FIG. 2. Kinetics of parasite burdens in blood (○), lungs (■), and brain (●) in mice infected at day 0 with 10^4 tachyzoites of strain RH. Each point represents the mean ± standard error of the mean for five mice. Shaded areas represent the period of administration of antimicrobial agents.

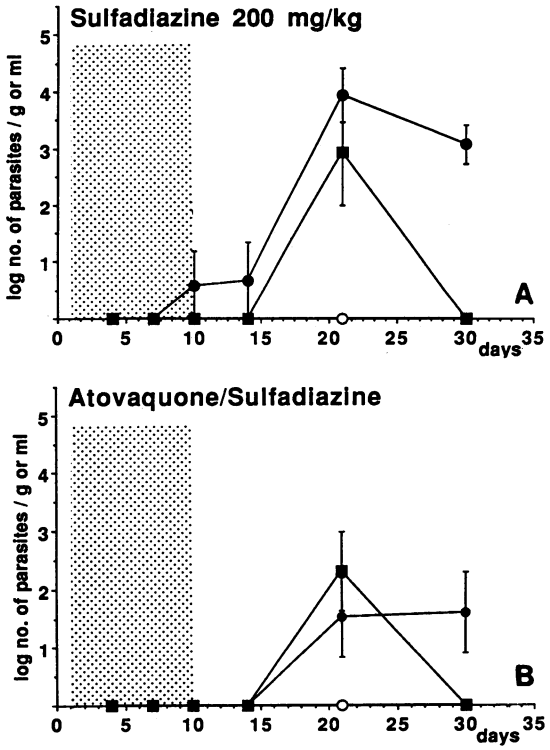

FIG. 3. Kinetics of parasite burdens with sulfadiazine alone (A) or combined with atovaquone (B) from day 1. See the legend to Fig. 2 for details.

DISCUSSION

Our results confirm that atovaquone alone is highly effective against *T. gondii* in vitro, as it could inhibit the growth of tachyzoites of the strain RH at concentrations of >0.01 mg/liter, with a 50% inhibitory concentration estimated to be 0.023 mg/liter (6.4×10^{-8} M). In the Giemsa-stained cultures, only mild morphological alterations of the parasites were noted in the presence of atovaquone. This result suggests that atovaquone has a parasitostatic rather than a parasiticidal effect on *T. gondii*, although further studies are needed to confirm this suggestion. The mode of action of atovaquone on *T. gondii* is likely to be similar to that established in other protozoa, i.e., a blockage of electron transport in the respiratory chain, which in malaria has been shown to result in a blockade of pyrimidine biosynthesis (11–14). As this mode of action represents a new pharmacological pathway for anti-*Toxoplasma* drugs, it was of interest to examine the possible synergistic effect between atovaquone and other compounds with different activities on *T. gondii*.

In vitro results were disappointing, since no synergy could be demonstrated between atovaquone and sulfadiazine, pyrimethamine, clarithromycin, or minocycline at various concentrations of each compound. On the contrary, a mild antagonistic effect was noted when atovaquone was combined with pyrimethamine. This antagonistic effect was not artifactual or due to a technical variation, as it was noted for various ratios of drug concentrations and was assessed by a two-way analysis of variance for multiple replicate tests. The reason for this apparent antagonism is unknown, as the site and mode of action of atovaquone and pyrimethamine are different: atovaquone is an inhibitor of parasite mitochondrial respiration, and pyrimethamine acts on the folate biosynthesis pathway, inhibiting dihidrofolate reductase activity and thus eventually blocking nucleic acid synthesis. However, the fact that the antagonistic effect was mild and was only evidenced by a sensitive enzyme-linked immunosorbent assay does not indicate a complete blockade of the activity of one drug by the other but rather suggests a possible interference between drugs in their penetration or concentration in cells. Equivocal interactions have also been observed with these two compounds against *Plasmodium falciparum* in vitro (20a). This negative interacting effect was not evidenced in our in vivo study, and the relevance of these results to the clinical situation is not clear. Further

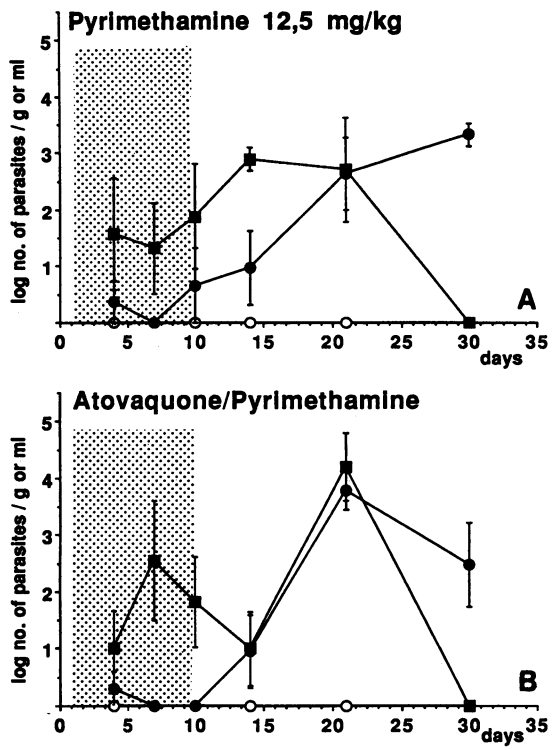

FIG. 4. Kinetics of parasite burdens with pyrimethamine alone (A) or combined with atovaquone (B) from day 1. See the legend to Fig. 2 for details.

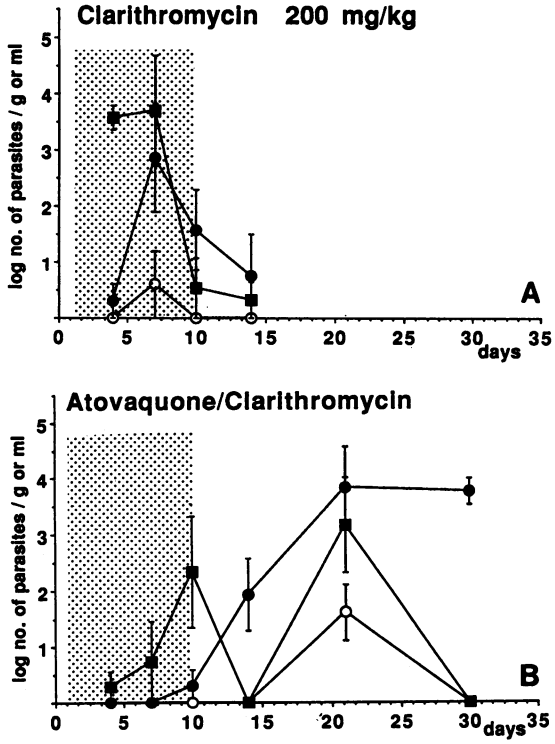

FIG. 5. Kinetics of parasite burdens with clarithromycin alone (A) or combined with atovaquone (B) from day 1. See the legend to Fig. 2 for details.

studies are warranted to examine the possible interaction of these two drugs at the cellular level and in other animal models, such as experimental pneumocystosis (15).

Experiments with mice confirmed that atovaquone is effective in the treatment of acute toxoplasmosis. Our experiments were conducted with mice infected with the highly virulent RH strain of *T. gondii* and only assessed the efficacy of atovaquone for acute infections produced by tachyzoites. In this model, the administration of 50 or 100 mg/kg daily for 10 days from day 1 postinfection prolonged the survival of treated mice in comparison with untreated control mice. However, this protection was only partial, as the parasite burden remained at a low level in the brain and lungs during treatment and thereafter progressively increased until death. The administration of atovaquone at 100 mg/kg/day from day 4 postinfection was not effective; mortality in treated mice was not significantly different from that in untreated mice, and high parasite burdens were found in the blood and organs at the time of death. Overall, these results are in agreement with those of Araujo et al. (1) for the treatment of acute toxoplasmosis when therapy is started early after the initiation of infection. However, our results differ in the degree of protection that was obtained in mice, as these authors obtained 100% protection in mice acutely infected with 3×10^3 tachyzoites and treated with atovaquone at 100 mg/kg/day for 10 days from day 1 after infection. This difference can probably be related to the inoculum size (3×10^3 tachyzoites versus 10^4 in our study), a difference that could be of major importance if atovaquone has parasitostatic activity. In addition, some differences in the virulence of the RH strain that was used in the two studies cannot be excluded, because of the thousands of repeated passages that have been made for the maintainance of the strain in each laboratory.

The fact that atovaquone has remarkable in vitro activity at a very low concentration but is only efficient in vivo when administered at a high dose is possibly related to the limited absorption of this drug when administered perorally, although previous experiments showed that a peak concentration in plasma of 16 mg/liter was reached by 8 h following oral administration of a single dose of 100 mg/kg to mice (21a). We paid particular attention to the preparation and administration of the drug suspension to reduce the individual variations in absorption that have been reported for humans and animals (10, 16, 21). In our experiments, drug levels could not be monitored during and after treatment, but we did not observe a large variability in the parasite burdens of the five mice that were sacrificed at each date of examination; such a result would have been an indication of marked differences in absorption between mice.

Experiments performed in vivo with various combinations of atovaquone with sulfadiazine, pyrimethamine, clarithromycin, or minocycline showed that any of the combinations tested had marked synergistic activity in this model of acute infection.

With the combination of atovaquone plus pyrimethamine, the mild antagonistic effect that was demonstrated between the two drugs in vitro was not observed in vivo. The survival of mice treated with atovaquone plus pyrimethamine was significantly prolonged in comparison with the survival of mice treated with each drug alone, but complete protection could not be obtained, as observed by Araujo et al. (3). An examination of the kinetics of infection in tissues showed that the beneficial effect of the combined therapy was mild,

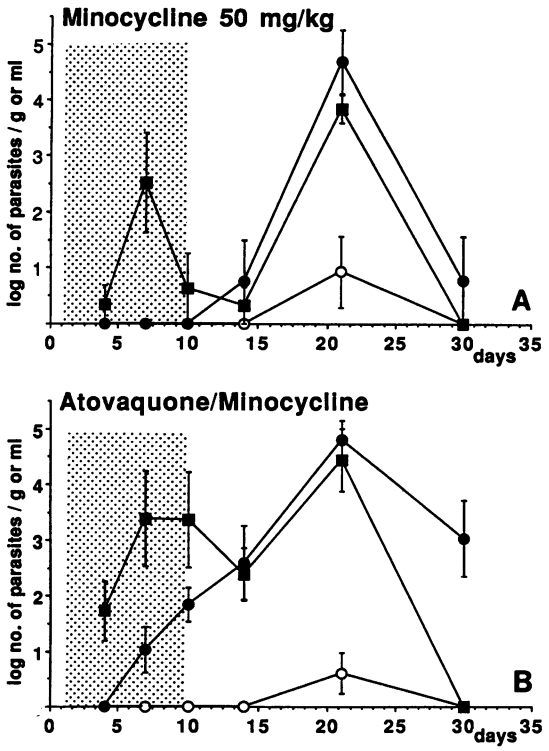

FIG. 6. Kinetics of parasite burdens with minocycline alone (A) or combined with atovaquone (B) from day 1. See the legend to Fig. 2 for details.

since parasite burdens in the lungs and brain were at comparable levels during treatment and after the cessation of therapy in mice treated with either pyrimethamine or pyrimethamine plus atovaquone.

Similarly, the survival of mice increased significantly with treatment with clarithromycin in combination with atovaquone. An examination of parasite burdens showed that this synergistic activity was limited to the period of treatment, with a marked reduction in parasite burdens in the lungs and brain, and then relapses constantly occurred after the cessation of therapy.

Araujo et al. (3) found that the combination of low doses of atovaquone and sulfadiazine was synergistic in the treatment of mice infected either intraperitoneally with 2.5×10^3 tachyzoites of the RH strain or orally with 10 cysts of the C56 strain. In our experiments, in which a larger inoculum of tachyzoites and higher doses of atovaquone and sulfadiazine were used, the survival of mice treated with the combination was higher than that of mice treated with atovaquone alone but was not different from that of mice treated with sulfadiazine alone. It is possible that the dose of sulfadiazine that was selected to make our results comparable to those obtained in previous experiments with this drug (6) may have been too high to reveal the beneficial effect of combined therapy on mortality. However, in a comparison with mice treated with sulfadiazine, we only noted a reduction of parasite burdens in the brain and lungs of mice treated with the combination, but relapses occurred after the cessation of therapy, as in mice treated with sulfadiazine alone.

In mice treated with atovaquone plus minocycline, the survival rate, the kinetics of infection, and the rate of occurrence of relapses after the cessation of therapy were similar to those observed in mice treated with atovaquone or minocycline alone.

Finally, the results obtained with these different combinations were disappointing in comparison with those obtained in similar experimental models with other combinations of antiparasitic drugs. With the combinations of dihydrofolate reductase inhibitors and sulfonamides, strong synergistic activity has been observed both in vitro and in vivo, because of the sequential activity of the drugs on the folate metabolism of the parasite (5, 8, 20). With some other combinations, marked in vivo synergy can be obtained in the absence of an in vitro interaction, possibly because of the complementary effects of the compounds on different sites of *Toxoplasma* infection (4, 6, 7). In the present study, no synergistic interaction either in vivo or in vitro could be clearly demonstrated. Improved survival was observed in vivo when drug mixtures were used, but this effect could not be demonstrated to be clearly synergistic at the drug levels used, and there was no evidence of a reduction in the parasite burden. Further research is needed to characterize in more detail the mode of action of atovaquone on *T. gondii* to identify a compound that may potentiate its activity.

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