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The Ketolide Antibiotics HMR 3647 and HMR 3004 Are Active against *Toxoplasma gondii* In Vitro and in Murine Models of Infection

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Ketolides are a new class of macrolide antibiotics that have been shown to be active against a variety of bacteria including macrolide-resistant bacteria and mycobacteria. We examined two ketolides, HMR 3647 and HMR 3004, for their in vitro and in vivo activities against the protozoan parasite *Toxoplasma gondii*. In vitro, both ketolides at concentrations as low as 0.05 μg/ml markedly inhibited replication of tachyzoites of the RH strain within human foreskin fibroblasts. HMR 3004 demonstrated some toxicity for host cells after they were exposed to 5 μg of the drug per ml for 72 h. In contrast, HMR 3647 did not show any significant toxicity even at concentrations as high as 25 μg/ml. In vivo, both ketolides provided remarkable protection against death in mice lethally infected intraperitoneally with tachyzoites of the RH strain or orally with tissue cysts of the C56 strain of *T. gondii*. A dosage of 100 mg of HMR 3647 per kg of body weight per day administered for 10 days protected 50% of mice infected with tachyzoites. The same dosage of HMR 3004 protected 100% of the mice. In mice infected with cysts, a dosage of 30 mg of HMR 3647 per kg per day protected 100% of the mice, whereas a dosage of 40 mg of HMR 3004 per kg per day protected 75% of the mice. These results demonstrate that HMR 3647 and HMR 3004 possess excellent activities against two different strains of *T. gondii* and may be useful for the treatment of toxoplasmosis in humans.

Toxoplasmosis remains a significant problem among organ transplant recipients, patients with AIDS who have preexisting toxoplasma antibodies (27, 28), and the fetuses of women who acquire *Toxoplasma gondii* infection during gestation (26). Although treatment of the acute infection in AIDS patients and organ transplant recipients with pyrimethamine-sulfadiazine or pyrimethamine-clindamycin has been successful in most cases, the relatively high incidence of toxicity associated with these drug combinations has frequently resulted in a lowering of the dosage or discontinuation of one or both drugs in the combination (27). The macrolide antibiotic spiramycin has been used for many years in an attempt to prevent transmission of the parasite from the newly infected mother to her fetus (26). Although spiramycin has been reported to reduce the level of such transmission by as much as 60%, this drug is not readily available in the United States, and other drugs that might be used and that have low toxicity similar to that of spiramycin for the mother and fetus but that have even greater efficacy than spiramycin have not been found. Similarly, there is great need for new, less toxic, and more effective drugs for the treatment of congenitally infected newborns and children as well as adults with ocular toxoplasmosis. For these reasons, a continued search for new therapies and new therapeutic approaches for treatment has been a high priority in our laboratories (8, 12, 23, 24). Toward this end, we have evaluated a number of new drugs for their activities against *T. gondii* in vivo in animal models of the infection or in vitro in tissue culture. Recently, we examined a new class of compounds, the ketolides. These are semisynthetic derivatives of 14-membered-ring macrolide antibiotics with in vitro activity against a variety of bacteria including macrolide-resistant bacteria and mycobacteria (15, 21, 22). Ketolides differ from erythromycin A by harboring a 3-keto group instead of a L-cladinose group. This structural change appears to increase their stability in a weakly acidic environment (1). In this report we present the results of experiments demonstrating that the ketolides HMR 3647 and HMR 3004 are highly active against *T. gondii* both in vitro in cultured cells and in vivo in murine models of the infection.

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MATERIALS AND METHODS

Mice. Female Swiss-Webster mice (Simonsen Laboratories, Gilroy, Calif.) weighing 20 g at the beginning of each experiment were used in the study. They were given water and food ad libitum throughout the experiments.

T. gondii. Tachyzoites of *T. gondii* RH and tissue cysts of *T. gondii* C56 were obtained as described previously (4, 8, 9, 13). For the in vitro and in vivo experiments, tachyzoites were obtained from the peritoneal cavities of mice previously infected for 2 days. For the in vivo experiments, each mouse was infected either intraperitoneally (i.p.) with 2.5×10^3 tachyzoites or orally with 10 tissue cysts.

Drugs. The ketolides HMR 3004 and HMR 3647 were obtained from Roussel Uclaf, Romaineville, France.

In vitro studies. In vitro activity was determined by measuring the capacity of each ketolide to inhibit intracellular replication of *T. gondii* within human foreskin fibroblasts (HFFs) (ATCC CRL 1635). The [^{3}H]uracil incorporation technique (5) was used. Briefly, both ketolides were dissolved in a small volume of dimethyl sulfoxide, and the concentrations used for the experiments, ranging from 0.025 to 25 μg/ml, were prepared in modified Eagle's medium (MEM; Gibco BRL, Grand Island, N.Y.) containing 100 U of penicillin/ml, 1 μg of streptomycin/ml, and 10% fetal bovine serum (HyClone, Logan, Utah). The concentration of dimethyl sulfoxide in the final preparation was less than 1%. HFFs were plated in 96-well flat-bottom tissue culture microtiter plates, and the plates were incubated at 37°C in an incubator with a 5% CO_2 atmosphere. After the cells reached confluence, the monolayers were infected with tachyzoites at a ratio of three parasites/cell. Two hours after infection, the monolayers were washed and the various concentrations of the ketolides were added. Quadruplicate wells were used for each concentration. The starting time point was when the drug was added to the wells. Four hours prior to harvesting the cells, [^{3}H]uracil (at 1 μCi/20 μl) was added to each well. The total amount of radioactivity incorporated into the harvested cells was determined with a scintillation counter at 24, 48, and 72 h after the addition of the test drug. Infected monolayers treated with diluent alone were used as controls.

The toxicities of the ketolides for HFF cells were determined with the Cell Titer 96 Kit (Promega Corp., Madison, Wis.) (5, 25). In this assay, a colorless tetrazolium salt is modified to a colored product by metabolically active mitochondria. Therefore, the reaction occurs only in living cells. Cells were plated at 10^3 cells/well in quadruplicate wells containing MEM, and the plates were incubated at 37°C in a incubator with a CO_2 atmosphere for 4 h. Different concentrations of each ketolide were added, and the plates were incubated as described above for the 24-, 48-, and 72-h time points. Four hours before each time point, the medium containing drug was replaced with fresh medium without drug plus the dye solution indicator. Following an additional incubation for 4 h, the cells were solubilized and the plates were read in an automatic enzyme-linked immunosorbent assay reader at a wavelength of 570 nm. Controls were cells treated with drug diluent only and cells treated with a concentration of a drug known to be cytotoxic.

In vivo studies. Each of the ketolides was added to a solution of 0.25% carboxymethyl cellulose and was sonicated for 30 s. Thereafter, they were kept at 4°C and sonicated again just before dosing the mice. Both ketolides were used as a suspension since attempts to dissolve them in a number of solvents commonly used for oral administration of drugs to mice were not successful. Controls were treated with 0.25% carboxymethyl cellulose, administered by gavage for 10 days. Four experiments were conducted with concentrations ranging from 5 to 200 mg/kg of body weight. Each ketolide was administered orally, by gavage, as a single daily dose. Treatment of mice infected with tachyzoites was initiated 24 h after inoculation of the parasites; treatment of mice infected with tissue cysts was initiated 3 days after infection. In both cases, treatment was for 10 days. Mice were observed for 30 days from the date of infection. The surviving mice were examined for residual infection by microscopy of brain tissue for the presence of *T. gondii* cysts or by i.p. subinoculation of suspensions of portions of liver and spleen into healthy mice (3).

The toxicities of the ketolides for mice were determined by treating healthy mice with dosages of each ketolide as high as 300 mg/kg/day for 10 days. These mice were weighed and observed daily for signs of toxicity such as piloerection and lethargy.

Statistical analysis of the in vitro data was done by Welch's modified *t* test, and the in vivo data were analyzed by the Mann-Whitney U test with a computer by using statistical software (Instat; GraphPad Software, Inc., San Diego, Calif.).

RESULTS

In vitro studies. A concentration of 5 μg of HMR 3004 per ml significantly inhibited replication of tachyzoites at each time period ($P < 0.01$ at 24 and 48 h and $P < 0.0075$ at 72 h) (Fig. 1A). This concentration showed toxicity (approximately 30% inhibition of the metabolic activity of HFFs compared with that for controls) only after 72 h of incubation (Fig. 2A). Lower concentrations significantly inhibited replication of tachyzoites only after 72 h of incubation ($P < 0.03$ for 0.5 μg/ml and $P < 0.04$ for 0.05 μg/ml). These concentrations were not toxic for HFF cells. A concentration of 25 μg/ml strongly inhibited replication of tachyzoites, but it was also highly toxic for HFF cells. In contrast, none of the concentrations of HMR 3647 demonstrated toxicity for HFF cells at any of the time periods of incubation (Fig. 2B), but all of them revealed significant inhibitory activity against tachyzoites, particularly after 48 and 72 h of incubation (Fig. 1B). Thus, significant inhibition of tachyzoite replication occurred after 24 h of incubation with 5 μg/ml ($P < 0.02$) and 25 μg/ml ($P < 0.01$). After 48 h of incubation the *P* values were 0.02 for 0.5 mg/ml, 0.01 for 5 μg/ml, and 0.01 for 25 μg/ml. After 72 h of incubation even a concentration as low as 0.05 μg/ml significantly inhibited replication of the tachyzoites ($P < 0.01$). The 50% inhibitory concentrations (IC_{50}) of HMR 3004 and HMR 3647 ranged from 2.47 to 0.16 μg/ml and 3.92 to 0.13 μg/ml, respectively, after 24 to 72 h of exposure. The inhibitory effects of both drugs increased with the time of exposure.

In vivo studies. Healthy mice treated with dosages of each ketolide as high as 300 mg/kg/day for 10 days to determine toxic effects did not lose weight and did not reveal any sign of toxicity.

Four experiments were conducted with concentrations of each ketolide ranging from 200 to 5 mg/kg/day. Representative results are presented in Fig. 3 and 4. All untreated control mice infected with tachyzoites died by days 8 to 9 after infection.

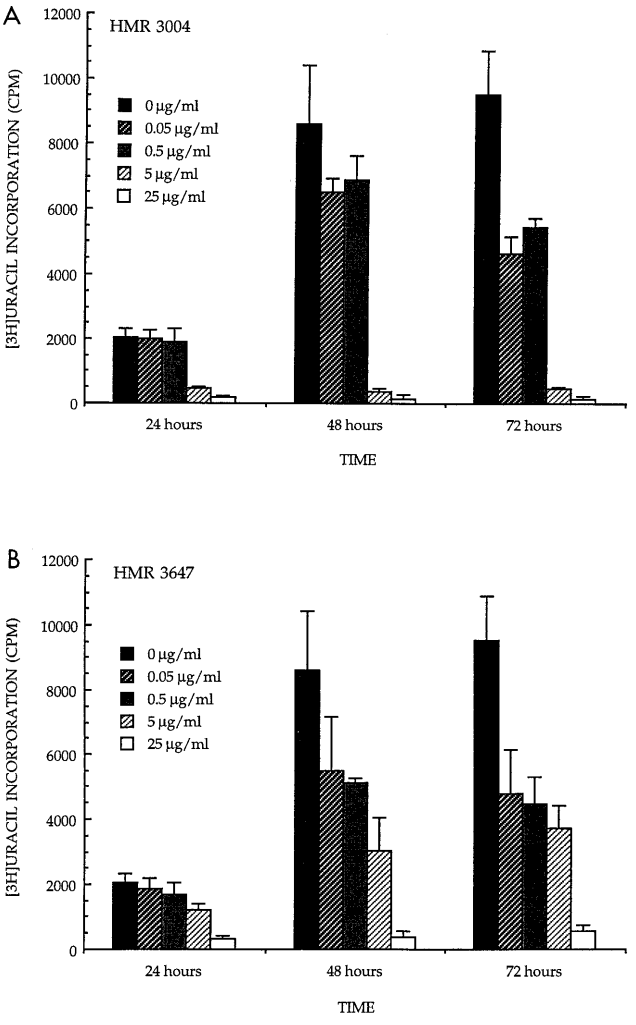

FIG. 1. Inhibition of replication of tachyzoites of the RH strain of *T. gondii* within HFF cells by ketolides HMR 3004 (A) and HMR 3647 (B). Ketolides were added 4 h after infection and were kept for the indicated time periods. Lower counts of incorporation of [^{3}H]uracil indicate inhibitory activity.

Treatment of mice infected with tachyzoites with 50 mg of HMR 3647 per kg per day for 10 days resulted in 30% survival ($P < 0.01$), whereas 100% of the mice treated with 100 or 200 mg/kg/day for 10 days survived ($P < 0.0001$) (Fig. 3A). HMR 3004 was slightly less active than HMR 3647. Treatment with a dosage of HMR 3004 of 50 mg/kg/day for 10 days resulted in 10% survival ($P < 0.07$), whereas treatment with dosages of 100 and 200 mg/kg/day resulted in 60% ($P < 0.001$) and 100% ($P < 0.001$) survivals, respectively (Fig. 3B).

Lower doses of each ketolide were needed to effectively treat mice infected orally with tissue cysts. Thus, treatment with 30 or 15 mg of HMR 3647 per kg per day resulted in 100% ($P < 0.001$) and 20% ($P < 0.02$) survivals, respectively. A dosage of 5 mg/kg/day resulted in prolongation of the time to death (Fig. 4A). Similarly, treatment with 40 or 30 mg of HMR 3004 per kg per day resulted in 70% ($P < 0.001$) and 40% ($P < 0.001$) survivals, respectively. A dosage of 20 mg/kg/day resulted in prolongation of time to death (Fig. 4B). All untreated control mice infected with tissue cysts died by day 15 of infection.

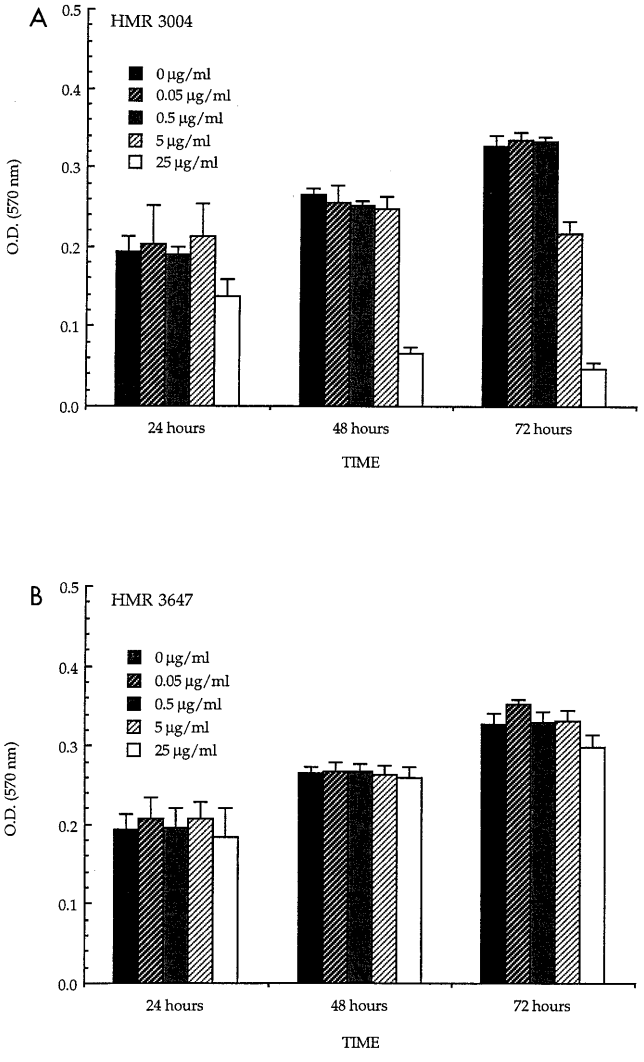

FIG. 2. Cytotoxicities of HMR 3004 (A) and HMR 3647 (B) for HFF cells determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The ketolides were added and were kept in the plates for the indicated time periods. The lower the absorbance reading the more toxic the drug. O.D., optical density.

DISCUSSION

The results presented above demonstrate that the ketolides HMR 3004 and HMR 3647 are remarkably active against *T. gondii* both in vitro and in murine models of the infection. In vivo, both ketolides were active against two different strains of *T. gondii* and were active against infections in mice infected by two different routes. Macrolide and azalide antibiotics have also been shown to be active against *T. gondii* in vitro (16, 18, 19) and in vivo (3, 11, 17, 18). The mechanism of action of ketolides in bacteria appears to be similar to that of macrolides, which consists of binding of the drug to the 50S ribosomal subunit and consequent inhibition of protein synthesis (2). The mechanisms of action of macrolide antibiotics against *T. gondii* are still unknown (14). Although the activities of macrolide and azalide antibiotics against *T. gondii* can be significantly enhanced by their combination with other drugs or with gamma interferon (7, 9, 10, 20), all macrolides and azalides examined thus far required relatively high concentrations to demonstrate in vivo activity. Thus, in our previous study, in which we used the same experimental conditions and strains of mice and *T. gondii* used in the present study, the lowest dosage of azithromycin that provided significant protection of mice against death due to acute toxoplasmosis was 200 mg/kg/day; for roxithromycin it was 500 mg/kg/day. Spiramycin was not protective even when it was used at a dosage of 400 mg/kg/day (11); clarithromycin administered at 300 mg/kg/day protected only 30% of the mice tested (7). Thus, the fact that HMR 3004 and HMR 3647 demonstrated such remarkable activity at dosages far below those required for macrolide antibiotics is of interest. The need to use higher concentrations of a therapeutic agent to achieve protection in mice does not necessarily predict a lack of efficacy in humans since the pharmacokinetics in mice and humans may differ considerably. Macrolide and azalide antibiotics have been shown to be effective against *T. gondii* when used at relatively low concentrations in combination with other drugs also active against the organism (6, 7).

Our results indicate that further studies examining the activities of ketolides in combination with other drugs as well as their effects in the treatment of toxoplasmic encephalitis are warranted.

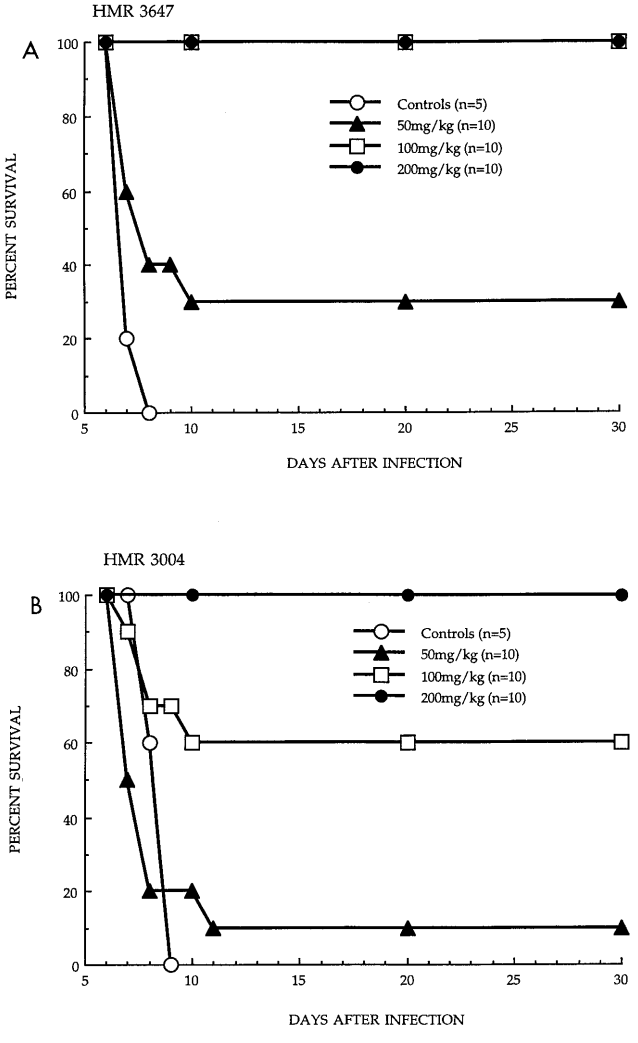

FIG. 3. Survival in mice infected i.p. with tachyzoites of the RH strain of *T. gondii* and treated with different doses of HMR 3647 (A) or HMR 3004 (B). Treatment was initiated 24 h after infection and was continued for 10 days.

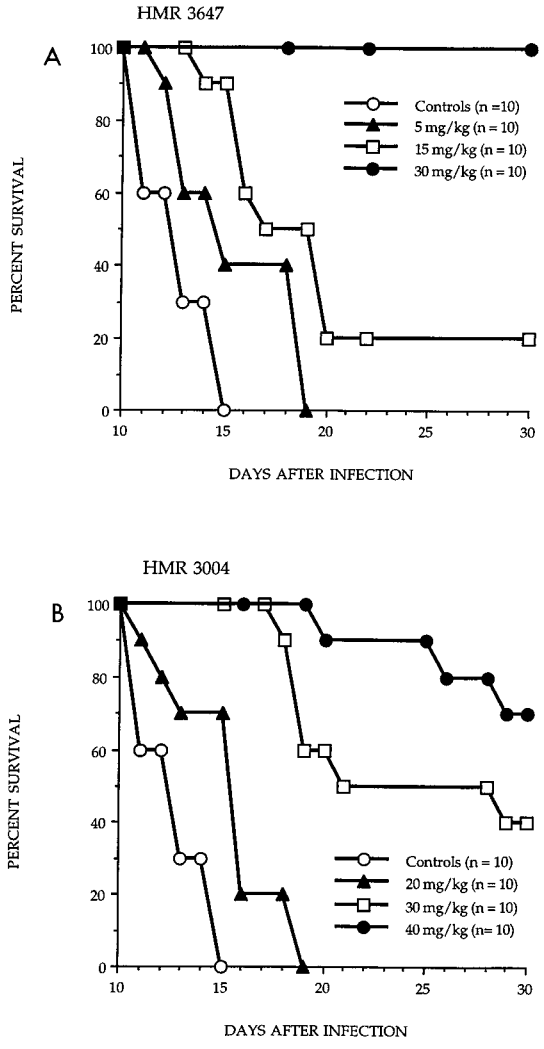

FIG. 4. Survival in mice infected orally with tissue cysts of *T. gondii* C56 and treated with different doses of HMR 3647 (A) or HMR 3004 (B). Treatment was initiated 3 days after infection and was continued for 10 days.

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