Trovafloxacin Is Active against *Toxoplasma gondii*

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Drugs currently used for treatment of toxoplasmosis in pregnant women, congenital infections, immunocompromised patients, and patients with the ocular disease are not always effective or may be dangerous to use; therefore, there is a need for more-effective and less-toxic drugs. Recently, we examined a group of fluoroquinolones for in vitro and in vivo activities against *Toxoplasma gondii*. Among those examined in vitro (ciprofloxacin, fleroxacin, ofloxacin, temafloxacin, tosufloxacin, and trovafloxacin), only trovafloxacin significantly inhibited intracellular replication of *T. gondii* without significant toxicity for host cells. In a murine model of acute toxoplasmosis, 100 or 200 mg of trovafloxacin per kg of body weight per day for 10 days protected 100% of infected mice against death. A dose of 50 mg/kg/day protected 90% of the mice, and a dose of 25 mg/kg/day effected prolongation of time to death. The other fluoroquinolones did not have such in vivo activities. These results indicate that trovafloxacin may be useful for treatment of toxoplasmosis in humans.

The current therapy of choice for all forms of human toxoplasmosis is the synergistic combination of pyrimethamine and sulfadiazine (9, 13, 14, 36). However, use of this combination in immunocompromised patients with AIDS is frequently associated with untoward side effects (7, 8, 28, 32) that can result in discontinuation of the therapy (18, 37). In addition, it has limited usefulness and efficacy in women who acquire the infection during pregnancy, in patients with ocular toxoplasmosis, and in congenitally infected infants and children. Therefore, there is a need for therapeutic agents that have potent activity against *Toxoplasma gondii* and less toxicity than the pyrimethamine-sulfadiazine combination.

During the past several years, we have studied a number of the fluoroquinolones to determine their activity against *T. gondii* in our murine models of toxoplasmosis. None of them had activity that was comparable to those of drugs already in use, until recently when we studied trovafloxacin, a new synthetic fluoronaphthyridone with broad-spectrum antibiotic activity (1, 19, 20, 33, 34). This quinolone has rapid oral absorption (maximum concentration in serum at 1 h), an oral bioavailability of 60 to 85%, and a long half-life of elimination in healthy male volunteers (31). Here, we report results of our studies on the activity of fluoroquinolones against *T. gondii*.

MATERIALS AND METHODS

***T. gondii*.** Tachyzoites of the RH strain of *T. gondii* were obtained and used as previously described (2, 10).

Mice. Outbred, female Swiss Webster mice (Simonsen Laboratories, Gilroy, Calif.), weighing approximately 20 g at the beginning of each experiment, were used. Mice were given water and food ad libitum.

Cells. L929 cells (ATCC CCL1) were used for in vitro experiments. Cells were grown in modified Eagle's medium (Gibco BRL, Grand Island, N.Y.) containing 100 U of penicillin, 1 µg of streptomycin per ml, and 10% heat-inactivated, *T. gondii* antibody-negative donor horse serum (PAA Laboratories, Newport Beach, Calif.).

Drugs. The following quinolones were studied: ciprofloxacin (Miles Inc., West Haven, Ct.), fleroxacin (lot CH903035; Hoffmann-La Roche, Nutley, N.J.),

ofloxacin (RWJ Pharmaceutical Research Institute, Spring House, Pa.), temafloxacin and tosufloxacin (Abbott Laboratories, Abbott Park, Ill.), and trovafloxacin (Pfizer Inc., Groton, Ct.).

HPLC analysis. High-performance liquid chromatography (HPLC) assays were performed to measure trovafloxacin concentration in the drug dilutions used for the in vitro studies, with or without dimethyl sulfoxide. These assays were performed by Robert J. Polzer (Drug Metabolism Department, Pfizer Inc.). The range of trovafloxacin concentrations for the calibration assay was 0.1 to 20 µg/ml. Samples with anticipated concentrations above the upper limit of the standard calibration curve were diluted with HPLC-grade water prior to analysis. Quality control samples, including dilution quality controls, were all within 7% of their normal concentrations.

In vitro studies. Ciprofloxacin, fleroxacin, ofloxacin, temafloxacin, and tosufloxacin were tested at concentrations from 0.625 to 10 µg/ml. Trovafloxacin was tested at concentrations from 0.25 to 20 µg/ml. The same concentrations were used to determine toxicity of the drug for L929 cells. Each drug was dissolved in a small volume of dimethyl sulfoxide and brought to the required volume with modified Eagle's medium to prepare stock solutions for the in vitro assays. All dilutions were made in modified Eagle's medium. The final dimethyl sulfoxide concentration was less than 1%. Since trovafloxacin is light sensitive, all solutions were made and stored in the dark. In vitro activity was defined as the capacity of the drug to inhibit intracellular replication of *T. gondii* and was determined by a modification of the previously described [³H]uracil incorporation technique (2). Briefly, L929 cells were plated at 10⁵ cells per well in 96-well flat-bottom tissue culture microtiter plates (Costar Corp., Cambridge, Mass.) and incubated at 37°C in a 5% CO₂ incubator. After confluence, monolayers were infected with tachyzoites at a ratio of 3 tachyzoites per cell (approximately 6 × 10⁴ tachyzoites per well). Two hours following infection, the monolayers were washed to remove free parasites and various concentrations of the test drugs were added. Triplicate wells were used for each drug concentration. Addition of the drugs to the wells marked the starting time point. Four hours prior to harvesting of the cells, [³H]uracil (1 µCi per well) was added to each well, and its incorporation was determined at 24, 48, and 72 h following addition of the test drug. Cells were collected with a cell harvester, and radioactivity was counted with a scintillation counter. Infected monolayers treated with medium that contained the respective drug diluent alone served as controls. Results are reported as inhibition of replication of tachyzoites in the treated monolayers compared with replication in the untreated control monolayer. Quantification was achieved by calculating the percentage of [³H]uracil incorporation by replicating tachyzoites in treated cultures compared with incorporation in untreated controls.

Toxicity of the drugs for the L929 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell proliferation assay using a Cell Titer 96 Kit (Promega Corp., Madison, Wis.). Briefly, cells were plated in triplicate wells at 10³ cells per well. Following a 4-h incubation at 37°C in a 5% CO₂ incubator test drugs were added. Four hours before each time point at 24, 48, or 72 h, 14 µl of the dye indicator solution was added. Following an additional 4 h of incubation, 100 µl of the solubilization-stop solution was added to each well. One hour later, the plates were read for A₅₇₀ in an automatic enzyme-linked immunosorbent assay plate reader. The results are reported as

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FIG. 1. Effect of trovafloxacin on intracellular tachyzoites in vitro. Trovafloxacin was used at 0.25 to 20 µg/ml. Parasite growth was measured by [3 H]uracil incorporation into intracellularly replicating tachyzoites.

the optical density at 570 nm of cultures exposed to the drug or exposed to the respective diluent only.

In vivo studies. For the in vivo experiments, solutions of the quinolones were made according to the manufacturer's directions: ciprofloxacin, temafloxacin, and trovafloxacin were dissolved in double-distilled water; ofloxacin and tosufloxacin were dissolved in a small volume of dimethyl sulfoxide and then diluted with double-distilled water to obtain the desired concentrations; fleroxacin was dissolved in a small volume of methanol, and the desired concentrations were obtained by adding polyethylene glycol. The following doses were used: ciprofloxacin, temafloxacin, and tosufloxacin at 25, 50, and 100 mg/kg of body weight per day; fleroxacin at 10, 50, 100, and 200 mg/kg/day; ofloxacin at 50, 100, and 200 mg/kg/day; and trovafloxacin at 25, 50, 100, and 200 mg/kg/day.

Mice were infected with 2.5×10^3 tachyzoites intraperitoneally. Treatment was initiated 24 h later by gavage as a single daily oral dose and continued for 10 days. Mice were observed for 30 days from the day of infection for mortality and time to death. At the end of the 30-day period, brains of surviving mice were examined for the presence of *T. gondii* cysts as previously described (3). If cysts were not observed, portions of brain, liver, and spleen from treated mice were pooled and homogenized in sterile phosphate-buffered saline and portions of the suspensions were inoculated intraperitoneally into three normal mice (4). These subinoculated mice were observed for mortality for 30 days. Statistical analysis of the data was performed using survival tools for StatView, version 4.02 (Abacus Concepts, Berkeley, Calif.).

RESULTS

In vitro studies. Ciprofloxacin, fleroxacin, ofloxacin, temafloxacin, and tosufloxacin at concentrations from 0.625 to 10 µg/ml either did not have demonstrable in vitro activity against *T. gondii* or were too toxic to the monolayers to have useful therapeutic indices (data not shown). Results with trovafloxacin in vitro revealed a dose-dependent inhibition of *T. gondii*

(Fig. 1). Results of three separate experiments revealed that the inhibitory concentration for 50% of the population following 24 to 72 h of exposure to the drug ranged from 0.77 to 0.98 µg/ml. Figure 2 illustrates the effects of various concentrations of trovafloxacin on the growth of L929 fibroblasts. Growth inhibition of the treated fibroblasts compared with that of the untreated controls was <20% at a drug concentration of 2 µg/ml at exposure times of 24 to 72 h. However, at the same concentration, inhibition of *T. gondii* ranged from 87 to 99%.

HPLC analysis was used to determine the concentration of trovafloxacin in the dilutions used in the experiments described above. The actual concentrations of trovafloxacin were similar to the predicted concentrations.

In vivo studies. Treatment with fleroxacin, ofloxacin, temafloxacin, and tosufloxacin did not result in any protective effect against death due to *T. gondii* infection (data not shown). Treated mice all died at essentially the same time as controls. Treatment with ciprofloxacin at 100 mg/kg/day resulted in slight prolongation of time to death.

In contrast, all infected mice treated with 100 and 200 mg of trovafloxacin per kg per day survived whereas control mice all died by day 10 (Fig. 3). Ninety percent of mice treated with 50 mg/kg/day survived. The Kaplan-Meier product limited-survival analysis revealed a statistically significant difference in survival of the treated mice ($P < 0.01$, log rank test). Treatment with 25 mg/kg/day resulted in prolongation of time to death which did not quite reach statistical significance ($P =$

FIG. 2. Effect of trovafloxacin on L929 cells in vitro. Trovafloxacin was used at 0.25 to 20 µg/ml. Growth of fibroblasts was measured by the MTT cell proliferation assay. (See Materials and Methods.)

0.06). Cysts were not observed in the brains of surviving mice, and 100% of the mice subinoculated orally with 0.2 ml of the homogenates of brain, liver, and spleen obtained from surviving mice treated earlier with 50, 100, and 200 mg of trovafloxacin per kg per day survived.

DISCUSSION

The results described above reveal that trovafloxacin is active against *T. gondii* both in vitro and in vivo. The other fluoroquinolones examined for in vitro activity, ciprofloxacin, fleroxacin, ofloxacin, temafloxacin, and tosufloxacin, did not have activity or were too toxic to the host cells to allow evaluation of drug effect on *T. gondii* per se. Trovafloxacin did not have a significant toxic effect on fibroblasts at concentrations that were highly active against *T. gondii*. Whereas trovafloxacin was also remarkably active against *T. gondii* in a murine model of acute toxoplasmosis, other fluoroquinolones tested did not have such activity. Survival of mice subinoculated with tissues of mice treated with trovafloxacin revealed that a 10-day schedule of treatment with trovafloxacin at ≥ 50 mg/kg/day eliminated viable parasites from tissues, since even a few viable parasites of the RH strain will kill a mouse.

Fluoroquinolone antibiotics possess potent activity against many bacterial pathogens and are extensively used in the treatment of a wide range of gram-positive and gram-negative bacterial infections (16). The mode of action of most quinolones against bacteria is directed to the DNA gyrase, a prokaryotic type II topoisomerase (17, 38), by converting it into a cellular poison by enhancing enzyme-mediated DNA breakage (17, 23,

29). The mechanism of action of trovafloxacin against *T. gondii*, however, is unknown.

Recent studies of the pharmacokinetics of trovafloxacin in healthy adult volunteers have revealed that it is well tolerated at ≤ 300 mg administered orally (22, 31, 35) and is rapidly absorbed and that peak concentrations are achieved within 0.75 to 1 h of administration (31, 35). The half-life of elimination in humans was 10 to 12 h (6, 22, 31), and age or gender did not appear to have an effect on its pharmacokinetics (30). Mean concentrations in serum ranged from 0.3 ± 0.0 to 10.1 ± 0.5 µg/ml at dosages from 30 to 1,000 mg when administered as a single oral dose (31). In two studies, a single oral dose of 200 mg of trovafloxacin achieved concentrations in serum of 2.9 and 2.2 µg/ml (21, 35). These concentrations of the drug were active against *T. gondii* in our in vitro studies.

Pneumonia caused by *T. gondii* has been found to cause significant morbidity and mortality in patients with AIDS (11, 26, 27, 37). Thus, it is of interest to note that significant concentrations of trovafloxacin were achieved in bronchoalveolar cells following a single oral dose of 200 mg to lung transplant recipients (21). Trovafloxacin may therefore prove to be useful for treatment of toxoplasmic pneumonia.

T. gondii causes debilitating disease in congenitally infected infants and children. It was recently reported that in children from whom cerebrospinal fluid samples were obtained for various reasons (unspecified), with a median age of 4.7 years, trovafloxacin had rapid and inflammation-independent penetration into cerebrospinal fluid and significant concentrations in serum following a 1-h infusion of CP-116,517, an intravenous prodrug of trovafloxacin, at 180 mg/m² (5). Because sol-

FIG. 3. Effect of treatment with trovafloxacin (Trova) on survival of mice infected intraperitoneally with the RH strain of *T. gondii*. Treatment was by oral gavage. Mice were observed for 30 days from the day of infection. Rx, treatment.

ubility of trovafloxacin at physiological pH is nonoptimal for intravenous infusions, the prodrug, which rapidly converts to trovafloxacin with a half-life of 4.8 min in human serum (6), was used. Trovafloxacin concentrations that were active in our in vitro studies were sustained in serum for up to 24 h and in cerebrospinal fluid for up to 3 h after the infusion ended (5). Whether trovafloxacin will prove useful in treatment of infections of the central nervous system in infant and adults remains to be studied in appropriately designed studies.

Tosufloxacin also has potent activities against both gram-positive and gram-negative bacteria (12, 15, 24, 25) and is structurally similar to trovafloxacin. Trovafloxacin has a 3-azabicyclo[3.1.0]hexane substituent at position C-7 on the naphthyridine nucleus, rather than the common piperazine or 3-aminopyrrolidine substituent in most currently available fluoroquinolones. Of interest is that although tosufloxacin and trovafloxacin differ only in substitution at position C-7 on the naphthyridine ring, tosufloxacin did not have appreciable activity against *T. gondii* either in vitro or in vivo, whereas trovafloxacin was remarkably active. The loss in activity by single substitution in tosufloxacin indicates that derivatization at the C-7 position in the naphthyridine ring is critical for activity against *T. gondii*.

Pharmacokinetic parameters of trovafloxacin in humans, such as a long half-life of elimination, rapid oral absorption, a linear relationship between dose and levels in serum, and achievement of well-tolerated concentrations in serum shown to be active in our in vitro experiments, combined with excel-

lent in vitro and in vivo activity against *T. gondii* indicate that trovafloxacin may be useful in treatment of toxoplasmosis in humans.

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