

## In Vitro Susceptibilities of *Coxiella burnetii*, *Rickettsia rickettsii*, and *Rickettsia conorii* to the Fluoroquinolone Sparfloxacin

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**In vitro** susceptibilities of *Rickettsia rickettsii*, *Rickettsia conorii*, and *Coxiella burnetii* to the new fluoroquinolone sparfloxacin (AT-4140; RP 64206) were determined. Plaque and dye uptake assays were used to measure the MICs against *R. rickettsii* and *R. conorii*. The susceptibilities of *C. burnetii* Nine Mile and Q 212 were determined in two acute-infection models and in two chronic-infection models. The MICs were 0.125 to 0.25 µg/ml for *R. rickettsii* and 0.25 to 0.5 µg/ml for *R. conorii*. Sparfloxacin (1 µg/ml) cured cells recently infected with *C. burnetii* Nine Mile and Q 212 within 4 to 9 days and cured multiplying, persistently infected cells within 10 days. As previously described with other fluoroquinolones (D. Raoult, M. Drancourt, and G. Vestris, *Antimicrob. Agents Chemother.* 34:1512-1514, 1990), sparfloxacin failed to cure cells persistently infected with *C. burnetii* and blocked from dividing with cycloheximide. As determined by the dye uptake assay, no cellular toxicity was noted with sparfloxacin at up to 128 µg/ml. These results are consistent with those previously obtained with fluoroquinolones (D. Raoult, M. Yeaman, and O. Baca, *Rev. Infect. Dis.* 11[Suppl. 5]:S986, 1989), although sparfloxacin may be slightly more active.

*Rickettsia rickettsii* and *Rickettsia conorii* are the etiologic agents of Rocky Mountain spotted fever and Mediterranean spotted fever, respectively. These bacteria are antigenically (9) and genetically closely related and share the morphological characteristics and biochemical compositions of gram-negative bacteria. Both rickettsiae are strict intracellular pathogens, living free in the cytoplasm of infected host cells. Clinical studies of Rocky Mountain spotted fever and Mediterranean spotted fever led to the description of severe forms of these diseases which eventually result in death in about 2% of cases (22, 27). Apart from other host factors, a delay in appropriate antibiotic treatment is significantly associated with death. The mainstay of treatment is chloramphenicol or tetracycline and its analogs (23, 27). The fluoroquinolones also have been successfully used for treating Mediterranean spotted fever patients (3, 14). Because of their potential adverse effects, all of these compounds are not recommended during pregnancy and childhood. In these cases, some macrolides, such as josamycin, have been successfully used (2). The screening of antirickettsial activity of new antibiotics is currently done in embryonated eggs or in cultured cells. In these models, *R. rickettsii* and *R. conorii* are susceptible to chloramphenicol, tetracycline and its analogs, and rifampin (7, 8, 19). Macrolides have been found to have unpredictable efficacy (3, 4, 17), but the fluoroquinolones, including pefloxacin (18), ofloxacin (20), and ciprofloxacin (16), have been found to be very effective.

*Coxiella burnetii* is the etiological agent of Q fever, a widely distributed zoonosis. This unique bacterium multiplies within the phagolysosome of infected host cells, despite the adverse chemical (pH 4.5) conditions of this cellular compartment. In humans, *C. burnetii* elicits subclinical, acute, and chronic infections (5). Acute Q fever is a self-limiting infection which may present as prolonged fever, flu-like illness, atypical pneumonia, hepatitis, or meningoencephalitis. Tetracycline and its analogs reduce the duration

of fever (10, 24), as do the fluoroquinolones pefloxacin and ofloxacin (3). Chloramphenicol is not clinically effective. Clinical data corroborate experimental results obtained in ovo and in cells recently infected with *C. burnetii*. Chronic Q fever mainly presents as hemoculture-negative endocarditis, but vascular infections (15), osteoarticular infections (11), and possibly chronic hepatitis (26) have been described. The prognosis of chronic Q fever is poor, with a mortality rate as high as 37% for Q fever endocarditis (13). Conventional antibiotic regimens may involve several years of treatment and are not very effective, since viable *C. burnetii* organisms can be isolated from excised cardiac valves after years of antibiotic therapy (26). Promising experimental results have been obtained with the fluoroquinolones oxolinic acid, difloxacin, and ciprofloxacin (29) and pefloxacin and ofloxacin (21), which are effective in *in vitro* chronic-infection models.

Sparfloxacin (AT-4140; RP 64206) is a recently developed fluoroquinolone. In this study, the potential efficacy of this compound against rickettsial infections was tested in various cellular models.

### MATERIALS AND METHODS

**Antibiotic preparation.** Sparfloxacin (AT-4140; RP 64206) was provided by Rhone-Poulenc, Anthony, France. Twenty milligrams of sparfloxacin was dissolved in 1 ml of 0.1 N NaOH and diluted in sterile water to obtain a stock solution of 1 mg/ml (pH 8.0). The solutions were filter sterilized, and aliquots were kept at 4°C.

**Bacterial strains.** *R. rickettsii* Sheila Smith and *R. conorii* Moroccan were cultivated in Vero cells. The titer of the inoculum was determined by a plaque assay (27) and adjusted to  $4 \times 10^4$  PFU/ml in Eagle minimum essential medium (MEM). *C. burnetii* Nine Mile (acute Q fever strain) and Q 212 (chronic Q fever strain) were grown in mouse L929 fibroblast cells, either recently or persistently infected (28).

**Susceptibility tests. (i) Plaque assay.** The plaque assay was used to determine the susceptibilities of *R. rickettsii* and *R.*

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*conorii*. A suspension of  $1.5 \times 10^4$  Vero cells per ml was prepared in Eagle MEM with 5% fetal calf serum and 2 mM glutamine. Five milliliters of this solution was dispensed in each tissue culture petri dish (diameter, 60 mm; Corning Glass Works, Corning, N.Y.), which was incubated for 24 h at 36°C in a 5% CO<sub>2</sub> incubator. The medium was discarded, and the confluent cell monolayers were inoculated with 1 ml of a solution containing  $4 \times 10^4$  PFU of rickettsiae. After rocking inoculation for 1 h at 22°C, infected cells were overlaid with 5 ml of Eagle MEM containing 2% fetal calf serum and 0.5% agar. Sparfloxacin was added to a final concentration of 0 (positive control), 0.125, 0.25, 0.5, or 1 µg/ml. The dishes were incubated for 4 days at 36°C in a 5% CO<sub>2</sub> incubator and stained by the addition of a second overlay of Eagle MEM containing 0.5% agar and 0.01% neutral red dye. Three experiments were carried out at each sparfloxacin concentration.

(ii) **Dye uptake assay.** The dye uptake assay was used to determine the susceptibilities of *R. rickettsii* and *R. conorii*. One hundred milliliters of a suspension of  $1.5 \times 10^4$  Vero cells per ml was dispensed in each well of a 96-well, flat-bottomed microtiter plate (CEB, Nancy, France). The inoculum of *R. rickettsii* or *R. conorii* was added at a final volume of 50 µl per well so that 2,000 PFU of rickettsiae was added to each well of the first row, 200 PFU was added per well of the second row, and 20 PFU was added per well of the third row. The fourth row was used as a control, and no rickettsiae were added. Two thousand PFU of rickettsiae was added to each well of the eight remaining rows for the antibiotic assay. Sparfloxacin was dispensed in a volume of 50 µl per well. Eight serial dilutions from 16 to 0.125 µg/ml were tested. The microtiter plate was incubated for 4 days at 36°C in a 5% CO<sub>2</sub> incubator. The medium was discarded, 50 µl of neutral red dye (0.15% in saline; pH 5.5) was placed in each well, and the plate was incubated for 1 h at 36°C. The wells were washed three times with phosphate-buffered saline, and incorporated red dye was extracted with 100 µl of phosphate-ethanol buffer (10% ethanol in phosphate-buffered saline; pH 4.2) per well. The optical density at 492 nm (OD<sub>492</sub>) of the solution was determined with a spectrophotometer (Flow Laboratories, McLean, Va.). A concentration of sparfloxacin was estimated to be efficacious if the mean OD<sub>492</sub> of the row was between those of the cell control row and the row containing 20 PFU per well.

(iii) **Model of acute infection with *C. burnetii*.** We developed a new system for testing the susceptibility of *C. burnetii* to sparfloxacin during an acute infection. To validate this system, we used various combinations of cell lines and inocula. To initiate acute *C. burnetii* infections, we incubated cycloheximide-blocked L929 or P388D<sub>1</sub> cell monolayers for 1 h at 22°C with samples of either the Nine Mile or the Q 212 strain of *C. burnetii*. Dilutions of the inocula were prepared to yield 100% infection either within 5 days (large inoculum) or within 9 days (small inoculum). At the end of the incubation time, the inoculum was removed and culture medium with cycloheximide (1 µg/ml) and sparfloxacin (1 µg/ml) was added. P388D<sub>1</sub> cells were infected with the large inoculum of the Nine Mile strain, and L929 cells were infected with the small inoculum of either the Nine Mile or the Q 212 strain. Cells were maintained for 10 days at 37°C in a 5% CO<sub>2</sub> incubator, and the culture medium containing cycloheximide and sparfloxacin was changed twice a week. The rate of cell infection in control and sparfloxacin-treated cell cultures was monitored daily by observation of Gimenez-stained smears (5). Smears were prepared on glass slides by means of a centrifugal slide maker (Cytospin II;

Shandon, Cheshire, Great Britain). Three independent experiments were done.

(iv) **Models of chronic infection with *C. burnetii*.** Two different cellular models were used to assess the efficacy of sparfloxacin during chronic infections. In the first model, L 929 cells persistently infected with phase I *C. burnetii* Nine Mile or Q 212 were maintained as described previously (1). The ratio of infected to uninfected cells was 0.95. Sparfloxacin was added to a final concentration of 1 µg/ml. Solvent without the antibiotic was added to control cultures. All cultures were incubated at 35°C and maintained via passage every 72 h, at which time appropriate amounts of sparfloxacin were added. The percentage of infected cells was measured daily on Gimenez-stained smears for 10 days (6). Smears were prepared on glass slides as described above. Three independent experiments were done. In the second model, the multiplication of P388D<sub>1</sub> cells persistently infected with *C. burnetii* was blocked with cycloheximide at 1 µg/ml as previously described (12). Sparfloxacin was added to a final concentration of 1 µg/ml. Solvent without the antibiotic was added to control cultures. All cultures were incubated at 35°C and maintained via passage every 72 h, at which time appropriate amounts of sparfloxacin and cycloheximide were added. The percentage of infected cells was determined as described above.

**Toxicity tests.** The toxicity of sparfloxacin for Vero, L929, and P388D<sub>1</sub> cells was determined with a dye uptake assay similar to that described above. A suspension of  $1.5 \times 10^4$  cells (either Vero, L929, or P388D<sub>1</sub> cells) per ml in Eagle MEM containing 5% fetal calf serum and 2 mM glutamine was prepared. One hundred microliters of the suspension was dispensed in each well of a 96-well, flat-bottomed microdilution culture plate. Sparfloxacin was added to a final volume of 100 µl per well. Eleven serial dilutions from 128 to 0.125 µg/ml were tested per plate; the first row was used as a cell control. The plate was incubated for 4 days at 36°C in a 5% CO<sub>2</sub> incubator. The medium was removed, and 50 µl of neutral red dye was dispensed in each well. The plate was incubated for 60 min at 36°C and washed three times with phosphate-buffered saline. Incorporated red dye was extracted with 100 µl of phosphate-ethanol buffer per well. The OD<sub>492</sub> of the solution was determined with a multichannel spectrophotometer. A concentration of sparfloxacin in a given row was considered toxic when the mean OD<sub>492</sub> of the row was lower than the OD<sub>492</sub> of the cell control row. The determinations were done in triplicate.

## RESULTS

Sparfloxacin was determined by the dye uptake assay to be not toxic for Vero cells, L929 cells, or P388D<sub>1</sub> cells at concentrations of up to 128 µg/ml. This result proved that the cellular toxicity of sparfloxacin did not interfere with our susceptibility tests, which were run at concentrations of 0.125 to 1 µg/ml. As determined by the plaque assay, the MICs of sparfloxacin were 0.125 µg/ml for *R. rickettsii* and 0.25 µg/ml for *R. conorii*. As determined by the dye uptake assay, the MICs were, respectively, 0.25 and 0.5 µg/ml. As previously described (19), the results obtained with the plaque assay were consistent with those obtained with the dye uptake assay. Regardless of the method used, the MIC for *R. rickettsii* was one dilution lower than that for *R. conorii*. For *C. burnetii*, only one concentration of sparfloxacin (1 µg/ml) was tested in acute- and chronic-infection models; thus, the MIC could not be determined, but sparfloxacin prevented the acute infection of cycloheximide-

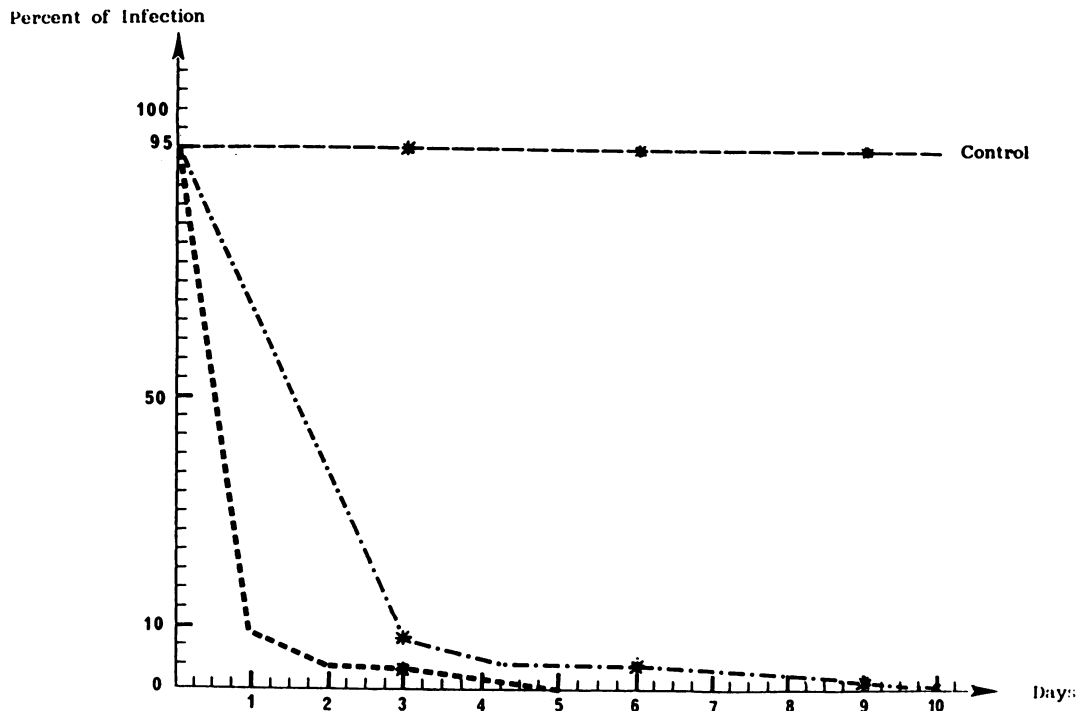


FIG. 1. Susceptibility of *C. burnetii* to sparfloracin in nonblocked, persistently infected cells. Symbols: ---, Nine Mile-infected cells; - · - · -, Q 212-infected cells. Experiments were done in triplicate.

blocked cells at this concentration. When the large inoculum of *C. burnetii* Nine Mile was used, the percentages of infection in control cells were 3% on days 1 and 2 postinfection (p.i.), 20% on day 3 p.i., 50% on day 4 p.i., and 100% from day 5 p.i. to day 10 p.i. In sparfloracin-treated cells, the percentage of infection remained stable at 3% throughout the 10 days of the experiment. When the small inoculum of *C. burnetii* Nine Mile was used, the percentages of infection in control cells rose from 3% on days 1 to 4 p.i. to 10% on day 5 p.i., 50% on day 6 p.i., and 100% from day 7 p.i. to day 10 p.i. In sparfloracin-treated cells, the percentage of infection remained stable at 3% throughout the experiment. When the small inoculum of *C. burnetii* Q 212 was used, the results for both control and treated cells were the same as those obtained with the small inoculum of the Nine Mile strain. In the chronic Q fever model, sparfloracin reduced the persistent infection of multiplying L929 cells from 95 to 100% down to 0% within 10 days (Fig. 1), whereas 95% of control cells remained infected. The reduction of the percentage of infected cells was slightly smaller with the Q 212 strain than with the Nine Mile strain. When cells persistently infected with *C. burnetii* Q 212 were blocked with cycloheximide, 95% infection was found in treated cells throughout the 10-day experiment.

#### DISCUSSION

Since rickettsiae do not multiply in axenic media (with the exception of *Rochalimea quintana*), conventional microbiological assays cannot be used for the evaluation of the antirickettsial activity of antibiotics. The embryonated-egg model and various cellular models can, however, be used for this purpose. In these systems, *R. rickettsii* and *R. conorii* are susceptible to several antibiotics: the MIC of chloramphenicol is 0.5  $\mu\text{g/ml}$ , the MIC of doxycycline is 0.06  $\mu\text{g/ml}$ ,

and the MIC of tetracycline is 0.25  $\mu\text{g/ml}$  (19). In this study, the efficacy of the new fluoroquinolone sparfloracin was tested on *R. rickettsii* and *R. conorii* in the plaque assay and the dye uptake assay. Sparfloracin was found to be highly effective, with MICs of 0.125 to 0.25  $\mu\text{g/ml}$  for *R. rickettsii* and 0.25 to 0.5  $\mu\text{g/ml}$  for *R. conorii*. These results are slightly better than those obtained with other fluoroquinolones: the MICs of ciprofloxacin are 0.25  $\mu\text{g/ml}$  for *R. conorii* and 1  $\mu\text{g/ml}$  for *R. rickettsii* (16), the MICs of pefloxacin are 0.5  $\mu\text{g/ml}$  for *R. conorii* and 1  $\mu\text{g/ml}$  for *R. rickettsii* (18), and the MICs of ofloxacin are 1  $\mu\text{g/ml}$  for both rickettsiae (20). Ciprofloxacin (14) and pefloxacin and ofloxacin (3) have been successfully used in treating Mediterranean spotted fever. However, the potential toxicities of fluoroquinolones limit their use during pregnancy and childhood.

The susceptibility of *C. burnetii* to antibiotics during acute infections has been determined in embryonated eggs. In this model, trimethoprim, rifampin, and doxycycline and its analogs are effective, whereas beta-lactams, streptomycin, chloramphenicol, and erythromycin are not (25). Recently, pefloxacin and ofloxacin were found to be more effective than antibiotics previously tested (21). We have developed a new cellular model for antibiotic bacteriostatic activity testing in which cellular multiplication is blocked by the addition of cycloheximide. In this model, sparfloracin prevents the multiplication of *C. burnetii* in both L929 cells and P388D<sub>1</sub> cells, with either a small or a large inoculum. Heterogeneity or susceptibility has been reported among strains of *C. burnetii*. Spicer et al. (25) reported that one isolate (CB-Cyprus strain, phase I) was relatively resistant to tetracycline. Likewise, the Priscilla strain was found to be less susceptible than the Nine Mile strain to ofloxacin, rifampin, and doxycycline in a model using recently infected cells (30). In our study, no difference in susceptibility to sparfloracin

was found between the Nine Mile strain and the Q 212 strain. Despite these good experimental results, the management of patients with chronic Q fever remained doubtful, and viable *C. burnetii* organisms could be isolated from excised cardiac valves after years of tetracycline therapy (26). This fact prompted Yeaman et al. (29) to use a cell line persistently infected with *C. burnetii* to evaluate antibiotic efficacies. They demonstrated that tetracycline, doxycycline, chloramphenicol, and trimethoprim were not bactericidal and that, after 10 days of exposure to physiologically relevant levels of antibiotics, more than 50% of the cells remained infected. Rifampin and the fluoroquinolones difloxacin, ciprofloxacin, and oxolinic acid were apparently bactericidal. Further study (21) showed that pefloxacin and ofloxacin were also effective, the latter fluoroquinolone being the most effective of the quinolones tested. In this model, the observed bactericidal effect may have resulted from the dilution of nondividing rickettsiae within multiplying cells because of a bacteriostatic effect. Therefore, we developed a new cellular model of chronic Q fever infection in which persistently infected cells were blocked from dividing by cycloheximide. When cellular multiplication was blocked in this way, none of the tested antibiotics could reduce the percentage of infection (12).

In this report, both models of chronic Q fever were used. Sparfloxacin was more effective at reducing persistent infection in multiplying L929 cells than ofloxacin; however, sparfloxacin did not cure persistently infected, blocked cells, as previously reported for other fluoroquinolones (12). Careful clinical studies are now required to evaluate the usefulness of sparfloxacin in the treatment of rickettsiosis.

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