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In Vitro Activities of Fleroxacin against Clinical Isolates of *Legionella* spp., Its Pharmacokinetics in Guinea Pigs, and Use To Treat Guinea Pigs with *L. pneumophila* Pneumonia

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The activities of fleroxacin against 22 clinical *Legionella* isolates were determined by agar and broth microdilution susceptibility testing. The fleroxacin MIC required to inhibit 90% of strains tested on buffered charcoal yeast extract agar medium supplemented with 0.1% α-ketoglutarate was 0.64 μg/ml and was 0.04 μg/ml when testing was done with buffered yeast extract broth supplemented with 0.1% α-ketoglutarate. Fleroxacin (0.25 μg/ml) reduced the bacterial counts of two *L. pneumophila* strains grown in guinea pig alveolar macrophages by 1 $\log_{10}$ CFU/ml, but regrowth occurred over a 3-day period; fleroxacin was significantly more active than erythromycin in this assay. Single-dose (10 mg/kg of body weight given intraperitoneally) pharmacokinetic studies performed in guinea pigs with *L. pneumophila* pneumonia revealed peak levels in plasma and lungs to be 3.3 μg/ml and 3.5 μg/g, respectively, at 0.5 h and 0.8 μg/ml and 0.8 μg/g, respectively, at 1 h. The half-life of the terminal phase of elimination from plasma and lung was ≈2 h. All 17 infected guinea pigs treated with fleroxacin (10 mg/kg/day) for 2 days survived for 14 days post-antimicrobial therapy, as did all 16 guinea pigs treated with the same dose of fleroxacin for 5 days. Only 1 of 16 animals treated with saline survived. The animals treated with fleroxacin for 2 days lost more weight and had higher temperatures than those treated with the antibiotic for 5 days. Fleroxacin is effective against *L. pneumophila* in vitro and in a guinea pig model of Legionnaires' disease. Fleroxacin should be evaluated as a treatment for human Legionnaires' disease.

Erythromycin, the current drug of choice for the treatment of Legionnaires' disease, is inhibitory only for *Legionella pneumophila* grown in macrophages. Long-duration therapy with erythromycin is needed because of the danger of relapse (17). In addition, administration of the drug is often unpleasant for patients because of gastrointestinal side effects and severe pain or phlebitis with intravenous administration. Assessment of the in vitro activities of antimicrobial agents for *L. pneumophila* is complex because of the need to measure the intracellular activities of antimicrobial agents and the inactivation of many antimicrobial agents by media optimal for the growth of the organism. The quinolone group of antimicrobial agents has been remarkably active against *L. pneumophila* in vitro and in an animal model of Legionnaires' disease (4, 8, 10–12, 18–21). We determined the activity of fleroxacin, a new quinolone antimicrobial agent, for *Legionella* using four different testing methods: agar and broth microdilution susceptibility, inhibition of bacterial growth within alveolar macrophages, and treatment of guinea pigs with *L. pneumophila* pneumonia. As a prelude to the animal treatment studies, we determined the pharmacokinetics of fleroxacin in *L. pneumophila*-infected guinea pigs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All legionellae studied were low-passage clinical isolates. The strains were identical to those used in prior studies and were composed of 2 strains each of *L. dumoffii*, *L. longbeachae*, and *L. micdadei*; 1 strain of *L. bozemanii*; and 15 strains of *L. pneumophila* (8, 10). *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as control organisms for susceptibility testing. To obtain inocula for susceptibility testing, the legionellae were grown on locally made buffered charcoal yeast extract medium supplemented with 0.1% α-ketoglutarate (BCYEα), and the nonlegionellae were grown on commercial tryptic soy agar containing 5% sheep blood (5). Incubation of all media was at 35°C in humidified air for 24 to 48 h, depending on the organism and its growth rate.

Antimicrobial agents. Standard powders of fleroxacin and erythromycin were obtained from Hoffmann-La Roche Inc., Nutley, N.J., and Abbott Laboratories, North Chicago, Ill., respectively. To prepare fleroxacin for injection, the standard powder was dissolved in sterile saline for injection (USP). The drug concentration was 4.6 mg/ml for the pharmacokinetic study and 3.2 mg/ml for the treatment study. Fleroxacin solution for injection was made fresh daily and was used within 1 h of preparation.

Antimicrobial susceptibility testing. Agar dilution susceptibility testing was performed with antimicrobial agent-containing BCYEα and Mueller-Hinton agars as described previously (8). Briefly, bacteria were inoculated onto antimicrobial agent-containing BCYEα agar with a Steers Folz Graves inoculator. The plates were incubated for 48 h, at which time the MICs were determined. Plates containing the control *S. aureus* and *E. coli* strains were incubated for 24 h. Broth microdilution susceptibility testing was performed with buffered yeast extract broth supplemented with 0.1% α-ketoglutarate (BYEα broth) (*Legionella* bacteria) or Mueller-Hinton broth (non-*Legionella* bacteria), with a final vol-

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ume of 200 μl and a final bacterial concentration of 5×10^5 CFU/ml (6). Otherwise the broth microdilution method was performed exactly as described previously for a broth macrodilution method (8). All testing was done in duplicate; in the case of disagreement, the geometric mean value was used as the MIC. Antimicrobial susceptibility testing with erythromycin for the same bacteria used in this study has been reported previously (8).

Growth inhibition in alveolar macrophages. Guinea pig pulmonary alveolar macrophages were harvested and purified as described previously (8). The final concentration of macrophages was approximately 10^5 cells per well. Incubation conditions for all macrophage studies were 5% CO_2 in air at 37°C.

L. pneumophila F889 and F2111 grown overnight on BCYEα agar were used to infect the macrophages. Approximately 10^4 bacteria were added to each well. Bacteria were incubated with macrophages for 1 h in a shaking incubator and then for 1 day in stationary culture as described previously (8). One set of replicate wells was washed (500 μl) three times with tissue culture medium and then sonicated at low energy to release intracellular bacteria, which were quantified by using BCYEα agar. The antimicrobial agents were then added to the washed, nonsonicated wells; no antimicrobial agent was added to several wells, which served as growth controls. The infected tissue cultures were then incubated for 2 days, after which time supernatant samples were taken for quantitative culture. The antimicrobial agents were then removed by washing and the experiment was continued for 4 more days, with daily quantification of *L. pneumophila* in well supernatants. All experiments were carried out in duplicate or triplicate, and quantitative plating was done in duplicate. All wells were observed microscopically daily to detect macrophage infection and to roughly quantify the numbers of macrophages in the wells. In this system, there is no extracellular growth of *L. pneumophila*, so all increases in supernatant bacterial concentration are the result of intracellular growth.

Guinea pig pneumonia model. Hartley strain male guinea pigs, ≈320 g in weight, were used for the pneumonia model as described previously (7). Animals were observed for illness 1 week prior to infection; in the case of the animals used for the treatment study, temperatures and weights were obtained during the preinfection period. The guinea pigs were infected with *L. pneumophila* serogroup 1, strain F889, which was administered intratracheally in normal saline. About 4×10^6 CFU were administered for both the pharmacokinetic and treatment studies.

Pharmacokinetic study. Fleroxacin concentrations in plasma and lung were measured in guinea pigs with *L. pneumophila* pneumonia as described previously (10). The drug was given in a single intraperitoneal dose (10 mg/kg in ≈0.9 ml) to guinea pigs 1 day after infection; the mean guinea pig weight was 410 g. At timed intervals after drug injection, anesthetized animals in groups of two to four each were exsanguinated by removal of heart blood under direct vision. The lungs were then removed, rinsed in sterile saline to remove adherent blood, blotted dry on gauze, and weighed. Heart blood was collected with a syringe and needle and was then transferred immediately to heparinized tubes (Vacutainer; Becton-Dickinson, Rutherford, N.J.). Immediately thereafter the heparinized blood was refrigerated at 5°C. Within 2 to 12 h, the plasma was separated from the cellular blood components by centrifugation at 5,000 × *g* at 5°C for 10 min. Prior to separation of the plasma, a small amount of the heparinized blood was set aside and stored at 5°C for later determination of hemoglobin concentration. The lung was homogenized in a known volume of phosphate-buffered saline (pH 7, 0.01 M). Lung homogenates were then centrifuged at 14,000 × *g* at room temperature (21 to 23°C) for 5 min. A small amount of lung homogenate supernatant was set aside and stored at 5°C for later determination of hemoglobin concentration. Blood plasma and lung homogenate supernatants were quantitatively transferred to microcentrifuge tubes and stored at −20°C until they were analyzed for fleroxacin by high-pressure liquid chromatography (HPLC). Negative controls included guinea pig plasma and lung homogenate supernatant that had been collected as described above from normal guinea pigs given identical anesthesia but no antimicrobial agent.

Hemoglobin assay. Hemoglobin determinations were performed on blood and lung homogenate specimens to determine the degree of blood contamination of the lungs. The hemoglobin content was determined by using a commercial colorimetric assay (catalog no. 525-A; Sigma Chemical, St. Louis, Mo.), according to the manufacturer's directions.

Drug assay. Fleroxacin in plasma and lung homogenate supernatants was quantified by reverse-phase ion-paired HPLC. Acetonitrile was added in equal volume to the fluids to be analyzed, and the suspension was vortexed and then centrifuged at 14,000 × *g* for 1 min. The supernatant was transferred to a fresh tube and frozen at −70°C until it was analyzed by HPLC. Samples were diluted with an equal volume of water before being injected by an automatic refrigerated (10°C) sample injector onto a PRP-1 column (25 cm long by 4.1 mm inner diameter; Hamilton, Reno, Nevada) equipped with a 3-cm precolumn composed of the same material. The solvent system was isocratic and was composed of 28% acetonitrile and 72% aqueous ion-pairing solution containing 0.015 M octanesulfonic acid and 0.050 M KH_2PO_4 (pH 3). The column flow rate was 1 ml/min. The column effluent was monitored spectrophotometrically at 280 nm. The fleroxacin peak was identified on the basis of the retention times determined with drug standards. A standardization curve for the peak area was determined by using different concentrations of fleroxacin contained in normal guinea pig plasma and lung homogenate and was found to be linear over the concentration range of 0.1 to 20.0 μg/ml for both plasma and lung homogenate. The lower limit of detection was 0.1 μg/ml. Normal guinea pig plasma contained a non-fleroxacin-containing peak that could easily be distinguished from the fleroxacin peak. No interference by normal guinea pig lung homogenate supernatant occurred.

Animal treatment study. The guinea pigs that survived surgery were randomized into three treatment groups 1 day after infection. Starting on that day, treatment was given for 2 to 5 days. One group of 17 animals received fleroxacin (10 mg/kg of body weight in 1.0 ml) each morning for 2 days (F2 group), another 16 animals received the same dose of fleroxacin each morning for 5 days (F5 group), and the last group of 16 animals received 1.0 ml of normal saline each morning. All dosing was by the intraperitoneal route. Animal weights were determined periodically during the 15-day postinfection observation period. Necropsies and quantitative lung cultures were performed on all animals that died. All animals surviving for 15 days postinfection were killed with pentobarbital; necropsies, quantitative lung cultures, and histologic examinations of the lungs were performed on all of these animals (7). Reading of the histologic slides was performed by individuals blinded with respect to the antimicrobial treatment group assignments.

Statistical analysis. Calculation of mean MICs was done by a geometric method. Comparison of nonparametric values was by the Fisher exact test, chi-square test with correction for continuity, and the Mann-Whitney test (2). The nonpaired, two-tailed Student *t* test was used to compare parametric mean values (3). A cumulative normal distribution was used to compare viable bacterial counts, which were considered to conform to a Poisson distribution for calculation of variance (3). The InStat computer program (GraphPAD, San Diego, Calif.) was used for statistical analysis. Type II errors were calculated by the method of Cohen (1). Calculation of drug pharmacokinetic parameters was performed by using the InPlot curve-fitting computer program (GraphPAD) by using a least-squares nonlinear regression method and a biexponential decay curve.

RESULTS

Agar and broth dilution susceptibility. All 22 *Legionella* strains tested by the agar dilution method were susceptible to concentrations of fleroxacin readily achievable in plasma. The average MIC, the MIC required to inhibit 50% of strains tested, the MIC required to inhibit 90% of strains tested, and the range of MICs of fleroxacin were 0.39, 0.32, 0.64, and 0.32 to 0.64 μg/ml, respectively. Fleroxacin was inhibited by BCYEα medium; the MICs of fleroxacin for the *S. aureus* and *E. coli* control strains were fourfold lower on Mueller-Hinton agar than on BCYEα agar. No data are available on the expected MICs for the control strains by agar dilution susceptibility testing; however, the observed agar and broth microdilution MICs were within the expected ranges for broth microdilution testing.

Twenty-one of the 22 *Legionella* strains tested in BCYEα broth grew well enough to obtain results; the 1 strain that did not grow well was an *L. micdadei* isolate. The average MIC, the MIC required to inhibit 50% of strains tested, the MIC required to inhibit 90% of strains tested, and the range of MICs of fleroxacin were 0.02, 0.02, 0.04, and 0.01 to 0.04 μg/ml, respectively. There was little to no evidence of fleroxacin inhibition by BCYEα broth.

Antimicrobial inhibition of intracellular growth. Both *L. pneumophila* serogroup 1 strains grown in guinea pig alveolar macrophages were significantly inhibited by both fleroxacin and erythromycin ($P < 0.0001$, two-tailed, for either drug versus the control on day 3 postinfection). Results for both strains studied were similar, so data for only strain F889 are shown (Fig. 1). Fleroxacin was significantly more inhibitory than erythromycin, with 0.25 μg of fleroxacin per ml being more active than 1.0 μg of erythromycin per ml ($P < 0.001$, two-tailed, on day 3 postinfection for either fleroxacin concentration versus erythromycin). In addition, both 0.25 and 1 μg of fleroxacin per ml caused a more prolonged postantibiotic effect than did erythromycin, especially for strain F2111. In none of the macrophage experiments did macrophage monolayers become detached before the onset of grossly evident infection with *L. pneumophila*, indicating the lack of macrophage toxicity by either antibiotic tested. A second set of independent experiments gave the same results.

Pharmacokinetic study. Pharmacokinetic data are given in Table 1. None of the negative control samples contained measurable drug. A biexponential decline in mean drug levels with time was noted, which was analyzed by using a two-compartment model. The half-lives of terminal elimination (β phase) of fleroxacin from plasma and lung were calculated to be ≈2.0 h, with the onset of the terminal phase being at ≈1 h postdose. The lung homogenate supernatants contained an average of 8% of the hemoglobin concentration of the corresponding blood specimens, with a range of 4 to 11% and a 99% confidence interval of 7 to 9%.

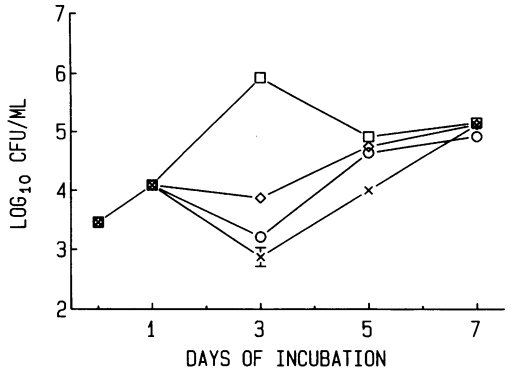

FIG. 1. Growth of *L. pneumophila* serogroup 1 strain F889 ($\log_{10}$ CFU per milliliter) in guinea pig alveolar macrophages versus day of incubation after initiation of infection. See text for experimental details. All points represent the means of triplicate wells counted in duplicate; error bars represent 95% confidence intervals, which, unless shown, were less than the height of the symbol representing the mean. Results of a representative experiment are shown; a separate, duplicate experiment gave similar results. Symbols: □, no antimicrobial agents; ◇, 1.0 μg of erythromycin per ml; ○ and ×, 0.25 and 1.0 μg of fleroxacin per ml, respectively.

Therapy in guinea pigs. All guinea pigs treated with fleroxacin for either 2 or 5 days survived, whereas 94% of the 16 guinea pigs that received saline alone died ($P < 0.0001$ by chi-square test) (Fig. 2). All lung cultures and necropsy results of animals that died before postinfection day 15 were diagnostic of *L. pneumophila* pneumonia; the mean concentration of *L. pneumophila* was 9.5 $\log_{10}$ CFU/g of lung, with a range of 8.9 to 10.0 $\log_{10}$ CFU/g. The lung of the only saline-treated survivor contained 2.0 $\log_{10}$ CFU of *L. pneumophila* per g. Two of the 17 lungs from the F2 group survivors were positive for *L. pneumophila* and contained 1.0 $\log_{10}$ CFU/g. Three of the 16 lungs from the F5 group contained *L. pneumophila*; these contained 0.9, 1.0, and 1.5 $\log_{10}$ CFU/g ($P = 1$ by the Fisher exact test for comparison of culture-positive rates of the two fleroxacin-treated groups). No significant differences in lung histology were noted between the two fleroxacin treatment groups (Table 2). Both animal weight and temperature differed significantly

TABLE 1. Concentrations of fleroxacin in plasma and lungs of guinea pigs with *L. pneumophila* pneumonia after administration of 10 mg/kg by intraperitoneal injection

Time postdose (h)	No. of animals	Concn in plasma (μg/ml)		Concn in lung (μg/g)	
		Mean	Range	Mean	Range
0.5	3	3.3	2.6–3.8	3.5	2.4–4.1
1	3	0.8	0.7–1.0	0.8	0.5–1.2[a]
2	2	0.6	0.5–0.7	0.7	0.7[b]
4	3	0.2	0.1–0.2	0.2	0.2–0.3
8	4	<0.1	<0.1–0.1[c]	<0.1	<0.1
12	3	<0.1	<0.1	<0.1	<0.1

[a] Two samples were tested.
[b] Only a single sample was tested.
[c] Two samples contained <0.1 μg/ml and two samples contained 0.1 μg/ml.

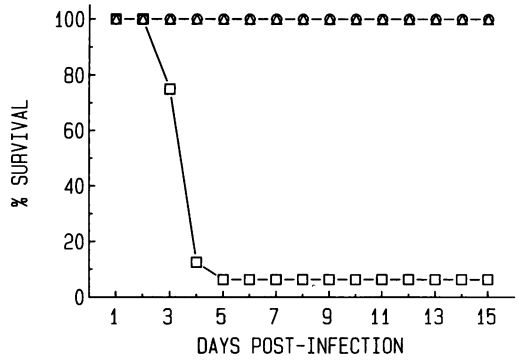

FIG. 2. Percent survival of guinea pigs with *L. pneumophila* pneumonia versus postinfection day. Animals were treated with fleroxacin (○; $n = 16$) or saline (□; $n = 16$) on postinfection days 1 to 5 or with fleroxacin on postinfection days 1 and 2 (△; $n = 17$).

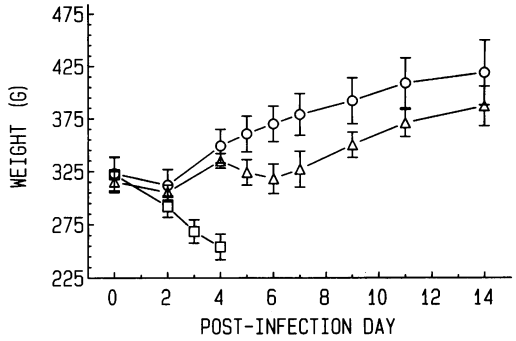

FIG. 3. Weight (in grams) versus postinfection day for animals treated with fleroxacin for either 2 days (△) or 5 days (○) or with saline for 5 days (□). Vertical bars represent 99% confidence intervals.

after postinfection day 4 between animals treated with fleroxacin for 2 or 5 days. Animal weights were significantly lower in the F2 group compared with those measured in the F5 group on days 5 to 11 postinfection ($P < 0.001$, two-tailed nonpaired t test), despite their initial equivalence ($P = 0.2$); by day 14, substantial weight gain had occurred in the F2 group, although there was still a significant difference between the two groups ($P = 0.01$) (Fig. 3). Rectal temperatures of the saline-treated animals were ≈40.5°C on days 2 and 3 postinfection, in contrast to temperatures of ≈39.5°C in the F2 and F5 groups. From days 4 to 7 postinfection, the temperatures of the F2 group were ≈39.5°C, which was 0.7 to 1.0°C higher than those of the F5 group ($P < 0.001$, two-tailed nonpaired t test). By day 9 postinfection, the temperatures of animals in the F2 group were ≈39.0°C, which were slightly lower than the temperatures of animals in the F5 group.

DISCUSSION

Fleroxacin, like other quinolone antimicrobial agents, is very active in vitro against *Legionella* spp. (4, 8, 10–12, 18–21). The agar dilution MICs obtained with BCYEα medium are falsely high, as judged by the greater MICs for the control *S. aureus* and *E. coli* strains when tested with BCYEα medium rather than Mueller-Hinton medium. The broth microdilution MICs are probably more accurate, because fleroxacin was not inhibited by BCYEα broth. Our results agree closely with those obtained previously for fleroxacin activity against *Legionella* spp. (13, 18).

For the two strains studied, fleroxacin was more active against intracellular *L. pneumophila* than was erythromycin. Prior studies have shown that erythromycin is purely inhibitory in this and other macrophage systems, even at concentrations of 5 μg/ml, and is clearly less active than azithromycin, ciprofloxacin, or WIN 57273 (8–10, 14). On the basis of historical comparisons, fleroxacin appears to be less active than some other quinolones tested in this system, such as sparfloxacin and WIN 57273 (8, 10).

TABLE 2. Lung histology results for fleroxacin-treated survivors[a]

% Consolidated lung	No. of animals with histologic finding after treatment with fleroxacin for:	
	5 days	2 days
0	1	0
≤10	5	5
11–25	8	5
26–50	2	7

[a] $P = 0.17$ by chi-square test and 0.16 by Mann-Whitney test; $1 - \beta < 0.1$, with $\alpha_2 = 0.05$.

The pharmacokinetic profile of fleroxacin in the plasma of guinea pigs is different from that measured in humans (22). Fleroxacin administered to guinea pigs was much more rapidly cleared than it is in humans; in humans the half-life of terminal elimination (β phase) in serum is about 8 to 14 h, in contrast to the 2-h half-life measured in our study. This very rapid elimination rate is greater than that observed by others in rodents, dogs, and monkeys but approximates the clearance rate measured in rabbits (15, 16). Fleroxacin lung-to-plasma ratios of ≈1.0 were observed throughout the postdose observation period, in contrast to a ratio of ≈0.5 observed previously for sparfloxacin in an identical infected guinea pig model (10). However, some other antimicrobial agents are highly concentrated in the lung in the same animal model (7). Contamination of lung with blood is not an explanation for the fleroxacin lung-to-plasma ratio of 1, because an assay for blood contamination showed levels lower than those that could account for these findings. Rather, it is likely that the intense inflammatory response in the infected lungs led to intraalveolar exudation of serum and that such an exudate contributed greatly to the fleroxacin levels in the lungs. Prior studies of fleroxacin concentrations in lungs have not been published for either infected or uninfected humans or animals. We know from the results of the present study that a single dose of fleroxacin provides concentrations in lungs above the extracellular broth MIC for 90% of the *Legionella* strains we studied for at least 8 h.

Fleroxacin was very effective for the treatment of experimental Legionnaires' disease, as are other quinolone antimicrobial agents (4, 10, 11, 19, 20). While survival was equivalent for treatment durations of either 2 or 5 days, the animals that received 2 days of fleroxacin therapy were sicker than those that received 5 days of therapy, as measured by weight loss and elevated temperatures. The lung culture and histologic findings were not significantly different between the F2 and F5 groups, implying that 2 days of therapy is sufficient to allow adequate recruitment of guinea pig host defenses and eventual cure of infection. Because of the differences in fleroxacin pharmacokinetics between humans and guinea pigs, this may mean that very short therapy with fleroxacin could be curative in humans with Legion-

naires' disease. However, comparative clinical treatment studies with other quinolone antimicrobial agents and macrolides are the only means of determining the relative efficacy of fleroxacin for human Legionnaires' disease, as well as the appropriate duration of fleroxacin therapy for this disease.

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