ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1993, p. 2454–2458
0066-4804/93/112454-05$02.00/0

Vol. 37, No. 11

Evaluation of Activity of Temafloxacin against *Bacteroides fragilis* by an In Vitro Pharmacodynamic System

RICHARD A. ZABINSKI,[1] KYLE VANCE-BRYAN,[2] ALISON J. KRINKE,[2] KARLA J. WALKER,[3] JULIA A. MOODY,[2] AND JOHN C. ROTSCHAFER[2]*

Miles Inc., West Haven, Connecticut 06516-4175[1]; College of Pharmacy, University of Minnesota, and the Antibiotic Pharmacodynamic Modeling Institute, St. Paul-Ramsey Medical Center, St. Paul, Minnesota 55101-2595[2]; and Med Tox Laboratories, New Brighton, Minnesota 55112[3]

Received 29 December 1992/Returned for modification 14 March 1993/Accepted 3 August 1993

An in vitro pharmacodynamic system has been successfully adapted to simulate in vivo antimicrobial pharmacokinetics under anaerobic conditions. This system was used to perform time-kill kinetic studies which were designed to compare the activity of temafloxacin to ciprofloxacin and cefotetan against two strains of *Bacteroides fragilis* (ATCC 25285 and ATCC 23745). All experiments were performed as single-dose, 24-h, duplicate runs. Starting bacterial inocula of 10^7 CFU/ml were exposed to starting antimicrobial concentrations of 5 μg of temafloxacin per ml, 5 μg of ciprofloxacin per ml, and 100 μg of cefotetan per ml. Terminal half-lives of 8, 4, and 4 h were simulated for each antimicrobial agent. Temafloxacin was rapidly bactericidal against *B. fragilis*. Ciprofloxacin was not bactericidal (<3 $\log_{10}$ unit decline in bacterial numbers) to either strain of *B. fragilis*. Cefotetan was bactericidal (≥3 $\log_{10}$ unit decline in bacterial numbers) to each strain but killed at a slower rate than temafloxacin. Times to 3 $\log_{10}$ unit declines of strain ATCC 25285 were 2, 4, and >24 h, whereas those of strain ATCC 23745 were 4, 4, and >24 h for temafloxacin, cefotetan, and ciprofloxacin, respectively. Total logarithmic declines of strain ATCC 25285 were >4.5, >4.5, and 2.9 $\log_{10}$ CFU/ml, whereas those of strain ATCC 23745 were 4.1, >4.5, and 1.2 $\log_{10}$ CFU/ml for each drug, respectively. These and other studies demonstrated that temafloxacin showed potential as an agent that could have been further developed for use in the treatment of anaerobic infections. However, the drug was removed from the market by its manufacturer because of toxicity issues. Although the release of newer fluoroquinolones that possess significant activity against anaerobic bacteria does not appear imminent, the time-kill studies performed in this study demonstrate that further research is warranted in the development of fluoroquinolones which possess significant antianaerobic activity.

Bacteroides fragilis is an important human pathogen in intra-abdominal and gynecologic infections. Antimicrobial regimens for infections that involve *B. fragilis* have generally been limited to certain β-lactams, clindamycin, chloramphenicol, or metronidazole. The prevalence of severe adverse reactions to chloramphenicol and the development of significant resistance among *B. fragilis* strains to clindamycin have driven the search for safe and effective therapeutic alternatives. All of the currently available fluoroquinolones possess excellent activity against aerobic and facultative gram-negative bacteria. Temafloxacin, however, has been shown to have significant in vitro activity against anaerobic bacteria while retaining excellent activity against most members of the *Enterobacteriaceae* (6, 9, 17). The in vitro evaluation of the activity of the fluoroquinolones against anaerobic bacteria has been based on standardized MIC testing. MIC testing methods possess some inherent restrictions that limit the researcher's ability to optimally evaluate the activity of a drug in vitro.

The purpose of this study was to use an in vitro pharmacodynamic system to evaluate the activity of temafloxacin, ciprofloxacin, and cefotetan against two strains of *B. fragilis*. Through time-kill curve analysis, this system can exploit the pharmacokinetic and pharmacodynamic differences between drugs and thereby evaluate their relative in vitro efficacies.

(This paper was presented in abstract form at the 4th European Congress and 2nd International Symposium on Anaerobic Bacteria and Infections, Vienna, Austria, 9 March 1992, abstr. PG6.)

* Corresponding author.

MATERIALS AND METHODS

In vitro pharmacodynamic anaerobic system. The in vitro pharmacodynamic anaerobic system (Fig. 1) is a modification of the system previously described by Garrison et al. (7). The in vitro pharmacodynamic system was placed within a Bactron IV anaerobic chamber (Sheldon Manufacturing, Cornelius, Oreg.) and consisted of each of the following: a glass vessel (volume, 1 liter) with inflow and outflow ports, connective silicone tubing (Masterflex L/S thin-wall tubing; Cole-Parmer, Chicago, Ill.), a Masterflex peristaltic pump (Cole-Parmer Instrument Co.), a fresh media reservoir, a stir/hot plate (Nuovo II; Barnstead/Thermolyne Corp., Dubuque, Iowa), a magnetic stir bar, and a thermometer.

The antimicrobial agent was injected into the system as a bolus so that targeted peak concentrations could be produced. By using the peristaltic pump, antimicrobial agent-free medium was pumped into the system such that an equal volume of antimicrobial agent-containing medium was displaced. This resulted in the simulation of a first-order, one-compartment pharmacokinetic process.

An anaerobic environment was created by placing the pharmacodynamic system within a Bactron IV anaerobic chamber (Sheldon Manufacturing). The chamber operates with anaerobic mixed gas of 5% hydrogen, 10% carbon dioxide, and 85% nitrogen. Positive pressure is maintained

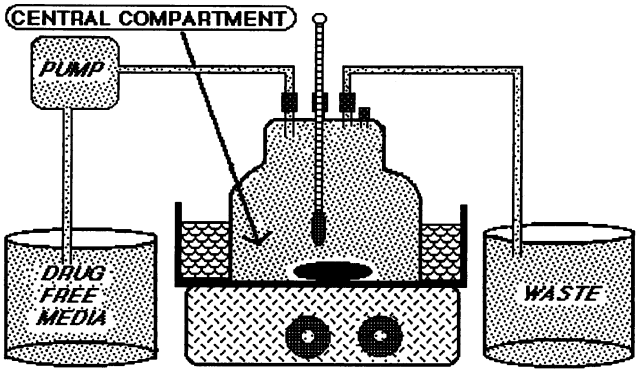

FIG. 1. In vitro pharmacodynamic anaerobic system.

within the chamber at 0.25 in. (0.64 cm) of water to prevent air from leaking into the system. BBL GasPak (Becton Dickinson, Cockeysville, Md.) indicator strips were used to ensure that anaerobiasis was maintained. The sensitivity of these indicator strips is such that they would detect oxygen concentrations of 0.5% or more (1).

Antimicrobial agents. Temafloxacin hydrochloride was provided by Abbott Pharmaceuticals, Abbott Park, Ill.; ciprofloxacin hydrochloride was provided by Miles Pharmaceuticals, West Haven, Conn.; and cefotetan was provided by ICI Pharmaceuticals, Wilmington, Del.

Organism. Experiments were performed with two American Type Culture Collection strains of *B. fragilis* (ATCC 25285 and ATCC 23745). Bacteria were reconstituted from frozen stock (−80°C) and subcultured twice before use. Standardized cultures were prepared by inoculating a 25-ml volume of Wilkins Chalgren broth with two to five colonies of *B. fragilis* that were grown on an anaerobic blood agar plate. This suspension was grown to the turbidity of a McFarland no. 1 standard, diluted 1:50, and grown again to the turbidity of a McFarland no. 1 standard. This standardized suspension was diluted 1:10 so that the initial inoculum in each experiment was 1×10^7 to 2×10^7 CFU of exponentially growing bacteria per ml.

Media. Wilkins Chalgren broth (Oxoid, Basingstoke, United Kingdom) was used as the growth medium in each experiment. Anaerobic blood agar plates (Dimed, St. Paul, Minn.) were used for viable cell counts.

Antibiotic carryover. The possibility of antibiotic carryover was evaluated for each antimicrobial agent-bacterium combination by the method of Stratton and Weeks. (18). Anaerobic blood agar plates were streaked in triplicate with 100 μl of broth spiked with temafloxacin, ciprofloxacin, or cefotetan to final concentrations of 5, 5, and 100 μg/ml, respectively. After 15 min, each plate was cross-streaked with a standardized quantity of *B. fragilis*. After 48 h of incubation, plates were visually inspected for a zone of growth inhibition and quantitatively analyzed for viable cell counts. Colony counts from drug-exposed plates were compared with those from growth control plates. Antibiotic carryover was considered significant if a zone of growth inhibition was evident and/or if drug-exposed plates contained fewer colonies than growth control plates.

MIC determination. MICs and MBCs were determined in Wilkins Chalgren broth by microtiter dilution for each antimicrobial agent-bacterium combination at an inoculum of 5×10^5 CFU/ml. Current procedural recommendations of the National Committee for Clinical Laboratory Standards were followed for testing *B. fragilis* (15, 16). Additionally, in time-kill experiments in which bacterial regrowth was observed, 24-h bacterial isolates were studied to determine whether MIC and MBC profiles had changed from the values originally established at the start of the experiments.

Time-kill kinetic studies. Time-kill kinetic studies and growth control experiments were carried out by using the in vitro pharmacodynamic anaerobic system. All experiments were single-dose studies that were simultaneously performed in duplicate. The duration of each experiment was 24 h. Target peak concentrations of 5, 5, and 100 μg/ml were used with terminal elimination half-lives of 8, 4, and 4 h for temafloxacin, ciprofloxacin, and cefotetan, respectively. All experiments were started with approximately 10^7 CFU of exponentially growing bacteria per ml. Sixteen 1-ml samples were drawn from the system over 24 h and analyzed to determine the remaining bacterial inoculum, the antimicrobial agent concentration, and the temperature. Viable bacterial counts were determined by standard serial dilution techniques at time zero; 30 s; 10, 20, 30, and 45 min; and 1, 2, 3, 4, 6, 9, 12, 18, 21, and 24 h. Since the simulation of 4- and 8-h half-lives results in a negligible dilution effect, declines in bacterial numbers due to dilution were not factored into the results. For example, simulation of a 4-h half-life over 24 h results in a logarithmic decline of only 0.016 $\log_{10}$ CFU/ml.

Bacterial enumeration. Plates were incubated at 37°C within an anaerobic (5% H_2, 10% CO_2, 85% N_2) incubator, which is a built-in feature of the Bactron IV anaerobic chamber. Plates were then quantified to determine viable bacterial counts; the dilution that revealed between 30 and 300 colonies per plate was used for constructing time-kill curves. Inoculation of a plate with 100 μl of an undiluted sample resulted in a lower limit of bacterial quantification of 3×10^2 CFU/ml.

Analysis. Time-kill curve data were plotted as logarithmic declines in CFU per milliliter versus time. Time-kill curves were evaluated by visual inspection for (i) time to a 3 $\log_{10}$ unit decline in bacterial numbers, (ii) total logarithmic decline in bacterial numbers; and (iii) total extent of regrowth. Because of small sample sizes, inferential statistics were not applied to these data. We believe that the data are best represented by being given in their entirety.

Temafloxacin, cefotetan, and ciprofloxacin concentrations were quantified by using previously validated high-pressure liquid chromatography methods (10, 12, 20). The temafloxacin assay was linear for five successive serial dilutions ranging from 0.65 to 20 μg/ml ($r^2 = 0.998$). The lower limit of quantification was 0.5 μg/ml, and the intraday coefficient of variation was 1.6%. The cefotetan assay was linear for five successive serial dilutions ranging from 0.65 to 80.33 μg/ml ($r^2 = 0.997$). The lower limit of quantification was 0.65 μg/ml, and the intraday coefficient of variation was 5.64%. The ciprofloxacin assay was linear for five successive dilutions ranging from 0.5 to 20 μg/ml ($r^2 = 0.999$). The lower limit of quantification was 0.5 μg/ml, and the intraday CV coefficient of variation was 7.4%.

RESULTS

Susceptibility testing. MICs of temafloxacin, ciprofloxacin, and cefotetan are shown for the study strains in Table 1. Preexposure MICs of temafloxacin (0.5 and 1 μg/ml) were lower than those of ciprofloxacin (2 and 4 μg/ml) for strains ATCC 25285 and ATCC 23745, respectively. MICs of cefotetan were 8 μg/ml for both strains. MBCs were within two

TABLE 1. MICs and MBCs before and after 24-h exposure to antibiotics[a]

Strain and time of measurement	Temafloxacin		Ciprofloxacin		Cefotetan	
	MIC[b]	MBC[b]	MIC	MBC	MIC	MBC
B. fragilis ATCC 25285						
Preexposure	0.5	1	2	4	8	8
Postexposure (system A)[c]	8	16	8	8	4	4
Postexposure (system B)[c]	8	16	4	4	4	4
B. fragilis ATCC 23745						
Preexposure	1	2	4	4	8	8
Postexposure (system A)	16	>16	4	4	8	8
Postexposure (system B)	8	16	4	4	ND[d]	ND

[a] Inoculum, 5×10^5 CFU/ml.
[b] MICs and MBCs are expressed in milligrams per liter.
[c] Time-kill kinetic studies were performed in duplicate and designated system A and system B.
[d] ND, no data.

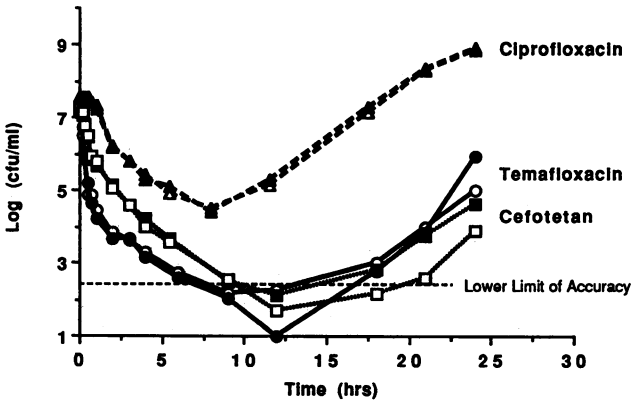

FIG. 2. Time-kill curves of *B. fragilis* ATCC 25285 from exposure to ciprofloxacin, temafloxacin, and cefotetan.

serial dilutions of MICs for each antimicrobial agent-bacterium combination.

MICs of temafloxacin for regrowth isolates from time-kill studies were significantly higher than those measured prior to antimicrobial exposure. MICs of temafloxacin increased by four dilutions (from 0.5 to 8 μg/ml) for strain ATCC 25285 and by three to four dilutions for strain ATCC 23745. MICs of ciprofloxacin for regrowth isolates of strain ATCC 25285 increased only slightly (by one to two dilutions), and MICs of ciprofloxacin to strain ATCC 23745 remained unchanged. MICs of cefotetan to regrowth isolates remained unchanged from those of preexposed strains.

Antibiotic carryover. Antibiotic carryover was not evident, since colony counts from drug-exposed plates did not differ significantly from those of growth control plates ($P < 0.01$).

Pharmacokinetic analysis. Antimicrobial concentration-versus-time data are summarized in Table 2. Actual half-lives were similar to targeted values (3.93 ± 0.22 versus 4, 4.23 ± 0.16 versus 4, and 7.95 ± 0.61 versus 8 h for ciprofloxacin, cefotetan, and temafloxacin, respectively). Actual peak concentrations were also similar to target values (4.5 ± 0.07 versus 5, 90.0 ± 7.34 versus 100, and 5.15 ± 0.36 versus 5 μg/ml, respectively).

Time-kill kinetic studies. The results of time-kill studies are plotted in Fig. 2 and 3 and are listed in Table 2. Bacteria demonstrated exponential growth during each growth control experiment, as indicated by a satisfactory fit to a log-linear relationship ($r = 0.99$).

TABLE 2. Summary of data from time-kill curves

Bacterium and antibiotic	Time to 3 $\log_{10}$ decline (h)	Total reduction in bacteria ($\log_{10}$ CFU/ml)	Extent of regrowth ($\log_{10}$ CFU/ml)
B. fragilis ATCC 25285			
Ciprofloxacin	>24[a]	2.9	8.9
Cefotetan	4	>4.5	4.3
Temafloxacin	2	>4.5	5.5
B. fragilis ATCC 23745			
Ciprofloxacin	>24[a]	1.2	9.2
Cefotetan	4	>4.5	4.3
Temafloxacin	4	4.1	5.7

[a] In time-kill studies done with ciprofloxacin, the bacteria regrew before a 3 $\log_{10}$ unit decline in bacterial numbers could be achieved.

Ciprofloxacin was not bactericidal (<3 $\log_{10}$ unit decline in bacterial numbers) against either strain. Ciprofloxacin produced average total logarithmic declines of 1.2 and 2.9 for strains ATCC 23745 and ATCC 25285, respectively. Temafloxacin produced similar total logarithmic declines compared with cefotetan for each strain. Temafloxacin produced average total logarithmic declines of 4.1 and ≥4.5, whereas cefotetan produced total logarithmic declines of ≥4.5 and ≥4.5 for *B. fragilis* ATCC 23745 and ATCC 25285, respectively. Declines of more than 4.5 logarithmic units could not be determined since 3×10^2 CFU/ml was the lower limit of accuracy.

Of the three antimicrobial agents tested, temafloxacin produced the most rapid 3 $\log_{10}$ unit decline of ATCC 25285 and was equal to cefotetan in this respect for strain ATCC 23745. Average times to 3 $\log_{10}$ unit declines of *B. fragilis* ATCC 25285 were 2 and 4 h, whereas those of *B. fragilis* ATCC 23745 were 4 and 4 h, for temafloxacin and cefotetan, respectively.

Bacterial regrowth was detected in each experiment. Bacterial populations exposed to cefotetan produced the least regrowth (4.3 $\log_{10}$ CFU/ml for each strain), whereas temafloxacin experiments resulted in slightly more regrowth (5.5 and 5.7 $\log_{10}$ CFU/ml) and ciprofloxacin experiments resulted in the most regrowth (8.9 and 9.2 $\log_{10}$ CFU/ml).

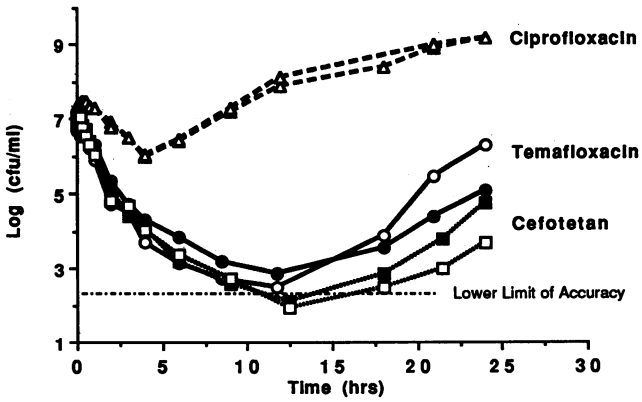

FIG. 3. Time-kill curves of *B. fragilis* ATCC 23745 from exposure to ciprofloxacin, temafloxacin, and cefotetan.

DISCUSSION

Time-kill curve analysis revealed that temafloxacin was rapidly bactericidal for *B. fragilis* and that it performed comparably to the control drug, cefotetan, in terms of time to 3 $\log_{10}$ unit decline and total logarithmic reduction. Other investigators have observed that peak-to-MIC ratios are predictive of the rate and extent to which quinolones kill bacteria that grow aerobically (2, 4, 5). In the present study, temafloxacin peak-to-MIC ratios of approximately 10 and 5 resulted in logarithmic reductions of more than 4.5 (ATCC 25285) and 4.1 (ATCC 23745) $\log_{10}$ CFU/ml. Ciprofloxacin peak-to-MIC ratios of approximately 2 and 1 resulted in total logarithmic reductions of 2.9 (ATCC 25285) and 1.2 (ATCC 23745) $\log_{10}$ CFU/ml. Although the data from the present study are limited, these limited data would suggest that peak-to-MIC ratios are predictive of the rate and extent to which quinolones kill *B. fragilis*.

Bacterial exposure to the fluoroquinolones resulted in more bacterial regrowth than did exposure to cefotetan. Since these were single-dose experiments, we can only speculate about whether a second dose of antibiotic would have had an effect on the regrowing bacteria. Blaser et al. (2) observed that bacterial regrowth occurred in the presence of enoxacin within 24 h unless the peak-to-MIC ratio exceeded 8:1. McGrath et al. (14), however, observed that the regrowth of *Pseudomonas aeruginosa* could not be prevented by multiple doses of ciprofloxacin that exceeded peak-to-MIC ratios of 8:1. Still other investigators (13) found that the combination of amikacin plus a β-lactam more effectively retarded the regrowth of *P. aeruginosa* than did various β-lactam/β-lactam combinations. Dudley et al. observed similar results with the combination of ciprofloxacin and azlocillin (5). Therefore, the bulk of available information would suggest that bacterial regrowth in neutropenic models of infection is most effectively prevented by using two antibiotics that involve different mechanisms of action. It is important to note, however, that the studies cited above were not done with *B. fragilis*, and therefore the application of these other observations to the present study is at best speculative.

Bacterial populations which regrew in the presence of temafloxacin became more resistant in terms of MIC, whereas cefotetan and ciprofloxacin postexposure MICs remained unchanged. Bacterial regrowth during exposure to a fluoroquinolone has been described by McGrath et al. (14). These investigators exposed *P. aeruginosa* to multiple doses of temafloxacin and found that the antimicrobial agent produced less killing and the bacteria regrew more readily with the administration of further doses. When the susceptibilities of the regrowth isolates were tested with graded dilutions of antibiotic-containing agar, the bacteria which regrew were found to be less sensitive than the original starting population. The investigators attributed this increase to a selection of temafloxacin-resistant subpopulations of *P. aeruginosa*.

The presence of selection-resistant subpopulations appears to be the most plausible explanation for the regrowth of *B. fragilis* seen in the present study. It is interesting, however, that the MICs of temafloxacin for regrowth isolates increased, whereas those of ciprofloxacin and cefotetan remained relatively unchanged. A half-life of 8 h was simulated for temafloxacin, whereas half-lives of 4 h were simulated for ciprofloxacin and cefotetan. Consequently, at the end of 24-h experiments, concentrations of temafloxacin, ciprofloxacin, and cefotetan were approximately 0.6, 0.07, and 1.5 μg/ml, respectively. Therefore, the most plausible explanation may be that more selective pressure was maintained by the higher residual concentrations of temafloxacin and less selective pressure was maintained by residual concentrations of ciprofloxacin and cefotetan. Support for this explanation can be found in work done by Gerber et al. (8) and McGrath et al. (13). Each of these groups of investigators found that aminoglycoside-resistant subpopulations of *P. aeruginosa* "vanished" after the selective pressure of the aminoglycoside was removed. The investigators concluded that the resistant subpopulations were overcome by more susceptible, faster-growing subpopulations when the aminoglycoside pressure was removed.

Temafloxacin is a fluoroquinolone with established in vitro activity against *B. fragilis* (6, 9, 17). However, this product was removed from the market by its manufacturer because of toxicity issues surrounding its postmarketing use (3). Other fluoroquinolones that are currently being developed are known to possess significant in vitro activity against anaerobic bacteria (11, 19). Although this study was performed with only two strains of one species of anaerobic bacteria, these data indicate that further research is warranted in the development of fluoroquinolones which possess anaerobic activity.

As further information about new fluoroquinolones becomes available, members of this class of antimicrobial agents may become valuable tools for use in the treatment of anaerobic infections and, in particular, for use as "switch therapy" as patients with mixed or anaerobic infections are discharged from the hospital for continued antimicrobial therapy as outpatients. An oral form of treatment that is effective against a broad spectrum of aerobic and anaerobic bacteria would be extremely desirable for selected infections that would otherwise require long durations of treatment with intravenous antibiotics.

ACKNOWLEDGMENTS

We are grateful to the members of the Antibiotic Pharmacodynamic Modeling Institute for their supportive input and to Deadra Johnson for the preparation of the manuscript.

At the time that the study was conducted, R. A. Zabinski and K. J. Walker were each postdoctoral fellows at the University of Minnesota and St. Paul-Ramsey Medical Center. This study was supported in part by a grant from Abbott Pharmaceuticals and the ASHP Foundation.

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