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In Vitro Activity of Cefbuperazone Compared with That of Other New β-Lactam Agents Against Anaerobic Gram-Negative Bacilli and Contribution of β-Lactamase to Resistance

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Cefbuperazone was compared with other currently available and investigational antibiotics against 278 clinical isolates of anaerobic gram-negative bacilli by an agar dilution method. Cefbuperazone and cefotetan were equally active against *Bacteroides fragilis*, with 8% of the organisms tested found to be resistant to 32 μg of either drug per ml. Cefoperazone, cefotaxime, ceftriaxone, and cefmetazole were less active against these strains; cefoxitin, moxalactam, piperacillin, clindamycin, and metronidazole were more active. None of the agents were consistently active against any of the other anaerobic gram-negative bacilli except imipenem, for which the minimum concentration required to inhibit 90% of all strains tested was 4 μg/ml. A 10,000-fold increase in inoculum size caused an increase in the MIC of ceftriaxone, cefotaxime, and cefoperazone but not of cefbuperazone, cefotetan, or cefoxitin. Investigation of the mechanism of resistance to cephalosporin-like agents demonstrated a correlation between the level of resistance and β-lactamase activity. Cefbuperazone, cefotetan, and cefoxitin were not hydrolyzed, had lower MICs, and were less affected by changes in inoculum size than were cefotaxime, ceftriaxone, and cefoperazone.

This study was undertaken to evaluate the activity of cefbuperazone, several new penicillins and cephalosporins, moxalactam, and imipenem against clinically important, frequently occurring anaerobic gram-negative bacilli and compare them with currently available agents which have useful activity against anaerobic bacteria. Anaerobic gram-negative bacilli were chosen for the evaluation for the following reasons: (i) these organisms are the major pathogenic anaerobes isolated in the clinical laboratories of the Medical University of South Carolina teaching hospitals; (ii) antimicrobial susceptibility of these organisms is not frequently tested in routine hospital laboratories; and (iii) there is great confusion as to which antimicrobial agents are most effective against these organisms in vitro. MIC, inoculum size effects, stability of the compounds to *Bacteroides fragilis* β-lactamase, and contribution of β-lactamase to resistance were also investigated.

MATERIALS AND METHODS

Antibiotics. Antibiotics and their respective suppliers were as follows: cefbuperazone and cefmetazole, Bristol Laboratories, Syracuse, N.Y.; cefoperazone, Pfizer Inc., New York, N.Y.; imipenem and cefoxitin, Merck Sharp & Dohme, West Point, Pa.; cefotaxime, Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.; ceftriaxone, Hoffman-LaRoche Inc., Nutley, N.J.; cefotetan, Stuart Pharmaceuticals, Wilmington, Del.; piperacillin, Lederle Laboratories, Pearl River, N.Y.; moxalactam, penicillin G, and cephaloridine, Eli Lilly & Co., Indianapolis, Ind.; clindamycin, The Upjohn Co., Kalamazoo, Mich.; and metronidazole, G. D. Searle & Co., Chicago, Ill.

Microorganisms. Two hundred and seventy-eight strains of anaerobic gram-negative bacilli isolated and identified by the clinical microbiology laboratories of the Medical University Hospital, Veterans Administration Medical Center, and Charleston Memorial Hospital were studied. Isolates were identified according to the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* (6). One hundred and fifty-five strains were *B. fragilis*, previously *B. fragilis* subsp. *fragilis*. The other organisms of the *B. fragilis* group studied included 27 strains of *B. ovatus-thetaiotaomicron* and 35 strains of other identifiable species of the *B. fragilis* group. Thirty-one strains of *Bacteroides* species which did not fit into any particular designation were also studied. Fifteen strains of *B. melaninogenicus* and 15 strains of *Fusobacterium* species were also studied. Organisms were stored at −70°C on glass beads coated with 1% brain heart infusion agar. *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 were used throughout the study as control organisms.

Susceptibility studies. MICs for the organisms tested were determined by the agar dilution method described by Sutter et al. (14). Overnight cultures of test organisms were diluted to the optical density of a 0.5 McFarland standard. These suspensions were inoculated with a Steers-Foltz replicator onto brain heart infusion agar supplemented with cysteine, hemin, and menadione and containing serial twofold dilutions of test antibiotics (13). Results were determined after 48 h of incubation at 37°C in an anaerobic glove box. The MIC was defined as the lowest concentration of antibiotic at which there was no growth, one discrete colony, or a fine, barely visible haze as determined with the unaided eye. Comparative studies were performed in triplicate for each batch of organisms. The American Type Culture Collection control strains were included with each group.

β-Lactamase studies. Eight strains of *B. fragilis* were studied for their β-lactamase activity by a method described elsewhere (2, 5). Small volumes of overnight culture were added to larger volumes of warm, prereduced broth. The cells were harvested in the logarithmic phase of growth after 6 h of incubation. After centrifugation and suspension in

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TABLE 1. Activities of selected antimicrobial agents against 155 strains of *B. fragilis*

Antimicrobial agent	MIC (μg/ml of medium)			% Resistant[a]	Breakpoint (μg/ml of medium)[b]
	Range	50%	90%		
Cefbuperazone	2–1,024	16	32	22 (8)	16 (32)
Cefoperazone	8–1,024	64	256	97 (79)	16 (32)
Cefotaxime	0.5–512	32	256	59 (26)	16 (32)
Ceftriaxone	0.5–1,024	64	512	70 (54)	16 (32)
Cefmetazole	0.5–512	16	64	39 (21)	16 (32)
Cefoxitin	4–256	16	32	12 (4)	16 (32)
Cefotetan	0.5–1,024	8	32	15 (8)	16 (32)
Imipenem	0.06–4	0.5	0.5	0	16 (32)
Moxalactam	0.25–128	1	8	4 (3)	16 (32)
Piperacillin	1–512	8	128	12 (3)	64 (128)
Ticarcillin	8–2,048	32	512	17 (15)	64 (128)
Clindamycin	0.125–32	1	4	8 (5)	4 (8)
Metronidazole	0.125–8	0.5	1	<1 (0)	8 (16)

[a] Data show percentage of strains resistant at low break point (high break point).
[b] Breakpoint refers to regularly achievable serum levels after the administration of moderate or (in parentheses) high dosage.

phosphate buffer at 1/100 of the original volume, the cells were disrupted by intermittent sonication for a total of 3 min at 100 W and centrifuged to remove cellular debris. The protein concentration was determined spectrophotometrically by the Biuret method. Quantitative assessment of β-lactamase activity of the enzyme preparation was performed by the alkalimetric titration method at standard conditions (pH 7.0; 37°C) with an automated recording pH stat (Brinkmann Instruments Inc., Westbury, N.Y.). Enzyme activity was expressed as micromoles of substrate hydrolyzed per hour per milligram of bacterial protein.

RESULTS

The susceptibility of 155 strains of *B. fragilis* to various antimicrobial agents is shown in Table 1. Susceptibility is expressed as the range of MIC and concentration of antimicrobial agent inhibiting 50 and 90% of the strains (MIC_{50} and MIC_{90}, respectively). The activity of the antimicrobial agents is also expressed as the percentage of organisms resistant to two concentrations of antibiotic which were selected on the basis of achievable blood levels. The lower concentrations (breakpoints) generally agree with previously published guidelines (3, 9), and the higher concentrations (breakpoints) generally agree with guidelines suggested by the U.S. Food and Drug Administration. Imipenem was the most active of all agents tested. Moxalactam was found to be more active than any currently available β-lactam agent, followed (on a weight basis) by cefotetan and cefoxitin. Piperacillin was approximately twice as active as ticarcillin against the strains, and clindamycin and metronidazole were more active than any of the currently available β-lactam agents. The resistance rates of all other anaerobic gram-negative bacilli studied are shown at the low and high breakpoint concentrations in Table 2. No organisms were resistant to imipenem. Less than 30% of all of the anaerobic gram-negative bacilli tested were resistant to piperacillin or metronidazole, but the excellent to good activities of clindamycin, ticarcillin, cefoxitin, cefotetan, and cefbuperazone demonstrated against *B. fragilis* strains did not occur with the other *Bacteroides* organisms. No *Fusobacterium* species were resistant to imipenem or metronidazole at the lower breakpoint concentration. Six percent were resistant to piperacillin, and less than 30% were resistant to clindamycin and cefotaxime. To assess the reliability of the agar dilution method used in these studies, modal MICs of each antibiotic for the American Type Culture Collection control strains were calculated on the basis of 20 to 36 measurements for each antibiotic. Each determination was either equal to or within one twofold dilution of the mode for 90% of the measurements except for piperacillin and cefmetazole, which correlated with 87 and 88% of the measurements, respectively. The modes for ticarcillin, moxalactam, imipenem, cefoxitin, and clindamycin were equal to or within one twofold dilution of those determined by Sutter et al. (14) and Brown et al. (2) with the same methodology.

The relationship between the absolute rate of hydrolysis (micromoles per hour per milligram of protein) by the β-lactamase produced by individual strains and the MIC (microgram per milliliter of medium) for that strain for the

TABLE 2. Resistance rates[a] (%) of anaerobic gram-negative bacilli to selected antimicrobial agents

Organism	No. of strains	% Resistant to:[b]												
		CFB	CFP	CTX	CTR	CFM	CFX	CFT	IMI	MOX	PIP	TIC	CLN	MTZ
B. fragilis	155	22/8	97/79	59/26	70/54	39/21	12/4	15/8	0	4/3	12/3	17/15	8/5	<1/0
B. ovatus-thetaiotaomicron	27	89/86	81/70	70/56	93/89	89/86	68/54	86/86	0	82/66	19/4	33/18	14/11	0/0
B. fragilis group (other)	35	46/40	56/31	34/29	54/43	56/56	40/34	51/49	0	37/37	17/6	26/18	31/14	9/6
Bacteroides spp.	31	58/55	64/59	45/41	58/52	77/77	41/32	61/61	0	38/32	29/16	23/10	26/19	3/3
B. melaninogenicus	15	27/27	27/7	33/33	27/27	40/33	33/33	27/27	0	27/27	7/7	13/7	33/33	7/0
Fusobacterium spp.	15	53/47	47/47	27/27	60/62	53/53	47/33	37/31	0	47/26	6/6	33/20	20/6	0/0

[a] Data show percentage of strains resistant at low break point/high break point (as defined in Table 1).
[b] Abbreviations: CFB, cefbuperazone; CFP, cefoperazone; CTX, cefotaxime; CTR, ceftriaxone; CFM, cefmetazole; CFX, cefoxitin; CFT, cefotetan; IMI, imipenem; MOX, moxalactam; PIP, piperacillin; TIC, ticarcillin; CLN, clindamycin; and MTZ, metronidazole.

TABLE 3. Relationship between MIC[a] of cephalosporins and cephamycins and rate of hydrolysis[b] by *B. fragilis* β-lactamase

Antimicrobial agent	Activity against strain:															
	T-93		T-130		T-31		T-12		T-145		T-58		T-59		T-9	
	MIC	Hydrol	MIC	Hydrol	MIC	Hydrol	MIC	Hydrol	MIC	Hydrol	MIC	Hydrol	MIC	Hydrol	MIC	Hydrol
Cephaloridine	512	2,169	512	125	256	238	128	159	64	240	64	69	64	15	32	204
Cefotaxime	512	174	16	132	8	58	4	99	32	236	2	178	2	0	2	102
Cefoperazone	>512	524	64	36	64	91	16	0	64	264	32	0	32	0	4	0
Ceftriaxone	>512	92	64	10	64	37	128	0	32	0	16	0	32	0	64	0
Cefbuperazone	4	0	8	0	8	0	32	0	16	0	4	0	512	0	4	0
Cefoxitin	16	0	16	0	16	0	4	0	8	0	16	0	16	0	8	0
Cefotetan	8	0	128	0	8	0	16	0	4	0	16	0	16	0	16	0

[a] MIC expressed as micrograms per milliliter.
[b] Rate of hydrolysis (Hydrol) expressed as micromoles of substrate hydrolyzed per hour per milligram of protein.

specific substrate antibiotic is shown in Table 3. In general, among the cephalosporins there was a correlation between the degree of resistance and the amount of hydrolysis for each strain, which was especially striking for cephaloridine, cefoperazone, and ceftriaxone. Decreasing levels of resistance of these strains to the cephalosporins were accompanied by less β-lactamase activity. Cefbuperazone, cefoxitin, and cefotetan were resistant to *B. fragilis* β-lactamase. No hydrolysis of penicillin, ticarcillin, piperacillin, or moxalactam was observed.

Studies of the stability of the various cephalosporin agents to *B. fragilis* β-lactamase showed cephaloridine to be the most rapidly hydrolyzed. With the rate of hydrolysis of cephaloridine arbitrarily set at 100%, a relative rate of hydrolysis for the other β-lactam agents was calculated (Table 4). Among the hydrolyzable agents, cefotaxime, cefoperazone, and ceftriaxone showed decreasing amounts of hydrolysis, whereas cefbuperazone, cefoxitin, and cefotetan were not hydrolyzed.

The effect on MIC of increasing the inoculum of eight selected strains of *B. fragilis* (those on which hydrolysis activity is reported here) from 5×10^2 to 5×10^6 organisms is seen in Table 4. The result is expressed as the average increase in twofold dilutions for each cephalosporin tested. The most pronounced inoculum effects were seen with ceftriaxone, cefotaxime, and cefoperazone. The other agents showed a more modest increase, with cephaloridine, cefotetan, and cefoxitin changing only one twofold dilution or less. Similar results were previously found for cefoxitin, cefotaxime, and cefoperazone (2).

The effect of inoculum size on MIC can be correlated with β-lactamase susceptibility for cefotaxime, cefoperazone, and ceftriaxone, which were susceptible to hydrolysis and inoculum effect, and cefbuperazone, cefotetan, and cefoxitin, which were resistant to hydrolysis and insignificantly effected by inoculum size increases.

TABLE 4. Comparison between mean relative rate of hydrolysis of cephalosporins by β-lactamase and inoculum effect of eight strains of *B. fragilis*

Cephalosporin	Relative hydrolysis (%)[a]	Avg increase (log 2) in MIC[b]
Cephaloridine	100	1.1
Cefotaxime	76	3.4
Cefoperazone	28	2.9
Ceftriaxone	4	3.8
Cefbuperazone	0	1.4
Cefoxitin	0	0.6
Cefotetan	0	0.8

[a] Rate of hydrolysis of cephaloridine (402 μmol/h per mg of protein) = 100.
[b] When inoculum is increased from 5×10^2 to 5×10^6 CFU.

DISCUSSION

Our results showed cefbuperazone and cefotetan to be equally active against *B. fragilis* and more active than the other newer cephalosporins and cephamycins evaluated in this study. These two agents were superior in vitro against *B. fragilis* to cefoperazone, cefotaxime, ceftriaxone, cefmetazole, and ticarcillin, whereas cefoxitin, piperacillin, clindamycin, metronidazole, and moxalactam proved to be more active than cefbuperazone and cefotetan. Imipenem was clearly the most active against *B. fragilis* of the agents tested, although none of the other newer cephalosporins were much of an improvement over the older agent cefoxitin. These results are similar to those previously reported (2, 3, 10, 15). Except for imipenem, the activity of all antimicrobial agents tested was clearly poorer against other species in the *B. fragilis* group and against strains of *Bacteroides* species, *B. melaninogenicus*, and *Fusobacterium* species. The largest difference in activity against *B. fragilis* as compared with other gram-negative anaerobes was seen among the cephalosporins and moxalactam.

Increasing inoculum size caused a large increase in MIC of cefotaxime, cefoperazone, and ceftriaxone, an effect previously reported (2) and postulated to be a result of inactivation of the antibiotic by β-lactamase (11, 16). Although cefotaxime and cefoperazone were hydrolyzed by *B. fragilis* β-lactamase, other more rapidly hydrolyzed cephalosporins such as cephaloridine did not show an inoculum size effect of similar magnitude. This was previously shown for cefamandole (2). Possibly, our large inoculum, although greater than the standard 10^5 organisms, was not large enough to bring out a similar effect among other hydrolyzable agents such as cephaloridine, especially in view of the high MIC with *B. fragilis* at the low inoculum. Alternatively, inoculum size effects may be multifactorial and not entirely determined by β-lactamase inactivation.

Theoretically, no inoculum effect would be demonstrable in an organism if the antimicrobial agent had great affinity for the critical penicillin-binding protein, even in the face of a noninducible, periplasmic β-lactamase such as that of *B. fragilis*. A great affinity for nonessential penicillin-binding protein might be the explanation for the inoculum effect of a compound such as imipenem in *B. fragilis* (2), as imipenem is totally resistant to degradation by the β-lactamase of these organisms. Exceptional outer cell wall permeability for a compound such as cephaloridine might counter the activity of the noninducible periplasmic β-lactamase of *B. fragilis*, thereby eliminating an inoculum effect. These properties of anaerobic bacteria have just begun to be investigated, and

already differences in affinity of the newer cephalosporins for penicillin-binding proteins of *B. fragilis* has been reported (1), but there is not yet enough information to explain the phenomena reported here.

Our β-lactamase studies showed that resistance of *B. fragilis* to the newer agents is often related to the β-lactamase activity against the compounds. This is similar to results obtained by others for cephalosporins (4, 5, 11, 12, 16). The level of resistance to the hydrolyzable cephalosporins correlated moderately well with the rate of hydrolysis of each test strain. In some strains, resistance to a compound is not correlated with hydrolytic activity (Table 3; isolate T130 with cefotetan and isolate T59 with cefoperazone). This may reflect the limits of sensitivity of the alkalimetric titration method, or, as stated above, other factors such as decreased permeability or decreased affinity for binding proteins may be responsible for the observed in vitro resistance. These factors may be important in producing the results seen with the penicillins also.

Few data have been available regarding the stability of cefbuperazone to *B. fragilis* β-lactamase. Our results show cefbuperazone to be resistant to β-lactamase derived from eight well-characterized strains, even though some of these strains produce a large amount of β-lactamase. These same organisms were able to hydrolyze other β-lactam agents used in this study except for cefotetan and cefoxitin. Cefbuperazone, cefotetan, and cefoxitin are cephamycins with a 7α-methoxy substituent which seems important in *Bacteroides* β-lactamase resistance (4, 8).

With respect to the intensity of activity and resistance to hydrolysis, other agents, such as imipenem, moxalactam, and cefoxitin, would appear to have more promise for treatment of infections due to *B. fragilis* than cefbuperazone and cefotetan.

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