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Activities of Ceftobiprole, a Novel Broad-Spectrum Cephalosporin, against *Haemophilus influenzae* and *Moraxella catarrhalis*

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Ceftobiprole, a broad-spectrum pyrrolidinone-3-ylidenemethyl cephem currently in phase III clinical trials, had MICs between 0.008 μg/ml and 8.0 μg/ml for 321 clinical isolates of *Haemophilus influenzae* and between ≤0.004 μg/ml and 1.0 μg/ml for 49 clinical isolates of *Moraxella catarrhalis*. Ceftobiprole MIC_{50} and MIC_{90} values for *H. influenzae* were 0.06 μg/ml and 0.25 μg/ml for β-lactamase-positive strains ($n = 262$), 0.03 μg/ml and 0.25 μg/ml for β-lactamase-negative strains ($n = 40$), and 0.5 μg/ml and 2.0 μg/ml for β-lactamase-negative ampicillin-resistant strains ($n = 19$), respectively. Ceftobiprole MIC_{50} and MIC_{90} values for β-lactamase-positive *M. catarrhalis* strains ($n = 40$) were 0.12 μg/ml and 0.5 μg/ml, respectively, whereas the ceftobiprole MIC range for β-lactamase-negative *M. catarrhalis* strains ($n = 9$) was ≤0.004 to 0.03 μg/ml. Ceftriaxone MICs usually were generally at least twofold lower than those of ceftobiprole, whereas amoxicillin-clavulanate MICs usually were higher than those of ceftobiprole. Azithromycin and telithromycin had unimodal MIC distributions against *H. influenzae*, with MIC_{90} values of azithromycin and telithromycin of 2 μg/ml and 4 μg/ml, respectively. Except for selected quinolone-nonsusceptible *H. influenzae* strains, moxifloxacin proved highly active, with MIC_{90} values of 0.12 μg/ml. Time-kill analyses showed that ceftobiprole, ceftriaxone, cefpodoxime, amoxicillin-clavulanate, azithromycin, telithromycin, and moxifloxacin were bactericidal at 2× MIC by 24 h against all 10 *H. influenzae* strains surveyed. Only modest increases in MICs were found for *H. influenzae* or *M. catarrhalis* clones after 50 serial passages in the presence of subinhibitory concentrations of ceftobiprole, and single-passage selection showed that the selection frequency of *H. influenzae* or *M. catarrhalis* clones with elevated ceftobiprole MICs is quite low.

Haemophilus influenzae, a bacterium with demanding nutritional requirements, is a major cause (together with *Streptococcus pneumoniae* and *Moraxella catarrhalis*) of community-acquired respiratory infections such as sinusitis, otitis media, pneumonia, acute exacerbations of chronic bronchitis, and chronic obstructive pulmonary disease (11, 23, 26). In countries such as the United States, where the *H. influenzae* type b vaccine is widely used, *H. influenzae* type b strains have been replaced by untypeable *H. influenzae* strains.

Synthesis of β-lactamases (TEM-1 and rarely ROB-1) is the principal antibiotic resistance trait expressed in *H. influenzae* (9, 17, 24, 33); lack of susceptibility to β-lactams due to alternative mechanisms is rare in most parts of the world (20). A study performed during 1997 reported the incidence of β-lactamase production among 1,676 untypeable *H. influenzae* strains isolated throughout the United States to be 41.6% (17). While the incidence of β-lactamase-negative ampicillin-resistant (BLNAR) strains in the United States is <1% (17, 25), the incidence of BLNAR strains in Japan and in some parts of France (9, 24) approaches 30%.

Almost all clinical strains of *H. influenzae* are fluoroquinolone susceptible (17), but nonsusceptibility towards this antibiotic class has been described previously (12), with one fatality recorded for a patient whose infecting *H. influenzae* strain had not been tested initially for quinolone susceptibility (2). Colonization with a quinolone-nonsusceptible clone of *H. influenzae* was recently reported in a long-term care facility in New York City, N.Y. (29). The prevalence of *H. influenzae* with reduced susceptibility to quinolones is very low (3) but will undoubtedly increase as quinolones are more frequently prescribed for respiratory tract infections.

Cefixime and cefpodoxime are the oral β-lactam antibiotics considered most efficacious against *H. influenzae* in terms of both MICs and pharmacokinetic/pharmacodynamic properties, followed by amoxicillin-clavulanate and cefuroxime (17). Among the macrolides azithromycin has the lowest MICs against *H. influenzae*, followed by erythromycin and clarithromycin (8, 21, 30). However, the presence of a macrolide efflux pump in *H. influenzae* strains classified as susceptible according to CLSI (formerly NCCLS) breakpoints (31, 32), the pharmacokinetic/pharmacodynamic properties of macrolides (16), and results of double-tap otitis media studies (10) all raise questions about the clinical efficacy of this antibiotic class against *H. influenzae* infections. MICs of telithromycin approximate those of azithromycin (21), but the pharmacological properties of telithromycin against *H. influenzae* infections are yet to be fully explored (W. A. Craig, personal communication).

M. catarrhalis is an aerobic diplococcus frequently found as a commensal of the upper respiratory tract. It is an important cause of upper respiratory tract infections in otherwise healthy children and elderly people and of lower respiratory tract infections in adults with chronic obstructive pulmonary disease (19, 27). At least 85% of *M. catarrhalis* isolates are β-lactamase producers (38).

Ceftobiprole (previously known as BAL9141) is an experi-

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mental broad-spectrum intravenous cephalosporin. Its prodrug form ceftobiprole medocaril, previously known as BAL5788, is currently in phase III clinical trials. Ceftobiprole has demonstrated antibacterial activity against gram-positive cocci (including penicillin-resistant pneumococci, methicillin- and vancomycin-resistant staphylococci, and ampicillin-susceptible enterococci) and many gram-negative bacteria (4, 14, 15, 18, 22). In this study we have compared the activities of ceftobiprole with those of amoxicillin, amoxicillin-clavulanate, ceftriaxone, cefpodoxime, azithromycin, telithromycin, moxifloxacin, and tetracycline by (i) MIC testing of 321 *H. influenzae* and 49 *M. catarrhalis* clinical isolates by the microdilution broth method; (ii) macrodilution broth and time-kill studies of the aforementioned drugs against 10 *H. influenzae* and two *M. catarrhalis* strains with differing β-lactam, macrolide, and quinolone susceptibilities; and (iii) multi- and single-passage studies of the proclivities of ceftobiprole, amoxicillin, ceftriaxone, moxifloxacin, azithromycin, and telithromycin to select for clones with elevated antibiotic MICs against eight *H. influenzae* and two *M. catarrhalis* strains.

TABLE 1. In vitro activities of selected antimicrobial agents against 321 *H. influenzae* and 49 *M. catarrhalis* strains

Antimicrobial agent	MIC (μg/ml)											
	Haemophilus influenzae								*Moraxella catarrhalis*			
	β-Lactamase positive (n = 262)			β-Lactamase negative (n = 40)			BLNAR (n = 19)		β-Lactamase positive (n = 40)			β-Lactamase negative (n = 9)
	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}
Ceftobiprole	0.015–8	0.06	0.25	0.008–1	0.03	0.25	0.03–2	0.5	2	0.03–1	0.12	0.5	≤0.004–0.03	≤0.004
Amoxicillin	4–>32	>32	>32	0.03–>32	1	4	0.5–>32	4	16	1–>32	8	16	≤0.015–0.12	≤0.015
Amoxicillin-clavulanate	0.25–8	1	4	0.03–>32	1	4	0.5–16	8	8	≤0.015–0.5	0.06	0.25	≤0.015–0.12	≤0.015
Ceftriaxone	≤0.004–0.5	≤0.004	0.015	≤0.004–0.25	≤0.004	0.03	≤0.004–0.5	0.12	0.25	0.015–2	0.06	1	≤0.004–0.015	0.008
Cefpodoxime	0.015–4	0.06	0.12	0.008–8	0.12	0.5	0.12–8	2	4	0.12–2	0.5	1	0.06–0.12	0.12
Azithromycin	0.12–>64	1	2	0.06–>64	1	2	0.5–4	1	4	≤0.03–1	≤0.03	0.06	≤0.03–0.25	≤0.03
Telithromycin	0.5–32	2	4	0.06–>64	2	4	1–8	2	8	≤0.03–1	0.12	0.25	≤0.03–1	0.12
Moxifloxacin	≤0.008–>0.25	0.015	0.03	≤0.008–>0.25	0.015	0.12	≤0.008–>0.25	0.015	>0.25	0.06–>0.25	0.06	0.12	0.06–0.12	0.12
Tetracycline	≤1–8	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1–>8	≤1	2	≤1–2	≤1

MATERIALS AND METHODS

MIC determinations. A total of 321 strains of *H. influenzae* were surveyed. These were isolated between 1999 and 2002, predominantly from sputum, bronchial aspirate, blood, and cerebrospinal fluid (the last two sources from countries which do not use the *H. influenzae* type b vaccine), and were comprised of 262 β-lactamase-positive strains (including two β-lactamase-positive amoxicillin-clavulanate-resistant [BLPACR] strains), 40 β-lactamase-negative strains, and 19 BLNAR strains. The *H. influenzae* panel also included nine quinolone-nonsusceptible strains, two macrolide-hypersusceptible strains, and one macrolide-hyperresistant strain (31, 32). The 49 *M. catarrhalis* strains were comprised of 40 β-lactamase producers and nine β-lactamase nonproducers. Strains were stored at −70°C in double-strength skim milk (Difco Laboratories, Detroit, Mich.) before MIC testing and periodically examined for purity by cultivation and Gram staining.

β-Lactamase testing was performed using nitrocefin disks (Cefinase; BBL Microbiology Systems, Inc., Cockeysville, Md.).

Susceptibility testing was performed by the microdilution broth method according to CLSI guidelines (28) using freshly prepared *Haemophilus* test medium (HTM) in 96-well microtiter plates manufactured by TREK, Inc. (Westlake, Ohio). Ceftobiprole powder was a gift from Basilea Pharmaceutica AG (Basel, Switzerland), whereas other compounds were obtained from their respective manufacturers. Inocula, prepared from 18-h chocolate agar plates (BBL, Cockeysville, Md.) by direct colony suspension, contained 3×10^4 to 7×10^4 CFU/well. Quality control strains *H. influenzae* ATCC 10211, *H. influenzae* ATCC 49247, and *H. influenzae* ATCC 49766 were included in each set of experiments. Microtiter plates were incubated in ambient air at 35°C.

Time-kill studies. For time-kill profiling 10 *H. influenzae* strains with the following phenotypes were selected: four β-lactamase positive, two β-lactamase negative, two BLPACR, and two BLNAR. Of these, six strains with amoxicillin MICs of >32 μg/ml were not tested with amoxicillin. Two β-lactamase-positive *M. catarrhalis* strains also were examined by time-kill assays.

Glass tubes containing 5 ml of freshly prepared HTM containing doubling antibiotic concentrations were inoculated with 5×10^5 to 5×10^6 CFU/ml and incubated at 35°C in a shaking water bath. Viability counts of antibiotic-containing suspensions and controls lacking antibiotic were obtained at 0, 3, 6, 12, and 24 h by plating 10-fold dilutions (HTM) of 0.1-ml aliquots from each tube onto chocolate agar plates, which were incubated for up to 48 h in 5% CO_2 at 35°C. Colony counts were performed on plates yielding 30 to 300 colonies; the lower limit of sensitivity of colony counts was 300 CFU/ml. The number of strains yielding a $\Delta(\log_{10}$ CFU/ml) of −1 (corresponding to 90% killing), −2 (99% killing), and −3 (99.9% killing) at 3, 6, 12, and 24 h relative to the inoculum size was determined. A given concentration of antibiotic (expressed as a multiplicity of MIC) was considered bactericidal if it reduced the inoculum viable count by ≥3 $\log_{10}$ CFU/ml or bacteriostatic if it reduced the inoculum viable count by <3 $\log_{10}$ CFU/ml during a specified time period.

Multistep selection studies. For resistance selection studies eight *H. influenzae* isolates (six β-lactamase producers including a BLPACR strain and two β-lac-

TABLE 2. In vitro susceptibilities of 12 strains tested by time-kill methodology

Drug	MIC (μg/ml)											
	Haemophilus influenzae										*Moraxella catarrhalis*	
	Bla$^+$						Bla$^-$		BLNAR			
	1	2	3	4	5	6	7	8	9	10	11	12
Ceftobiprole	0.06	0.06	0.06	0.06	1	0.5	0.06	0.06	0.25	0.12	0.5	0.25
Amoxicillin	>32	>32	>32	>32	>32	>32	0.5	0.5	8	2	8	8
Amoxicillin-clavulanate	1	1	0.5	1	8	8	0.5	0.5	8	2	0.5	0.06
Ceftriaxone	0.008	0.004	0.008	0.008	0.25	0.06	0.004	0.008	0.06	0.008	2	0.12
Cefpodoxime	0.12	0.12	0.06	0.06	2	1	0.06	0.06	1	0.25	1	0.25
Azithromycin	2	2	2	4	1	1	0.12	2	2	1	0.03	0.06
Telithromycin	2	2	2	4	1	2	0.12	1	2	4	0.03	0.06
Moxifloxacin	0.03	0.015	0.015	0.06	0.015	0.015	0.004	0.06	0.015	0.03	0.03	0.06
Tetracycline	1	0.5	1	0.5	16	64	0.5	1	8	0.25	0.5	1

tamase nonproducers including a BLNAR strain) and two *M. catarrhalis* isolates (both β-lactamase positive) were chosen. Serial passage of each strain (initial inoculum size, ~10^8 CFU/ml) was performed daily using freshly prepared HTM supplemented with increasing subinhibitory concentrations of a given antibiotic. For each subsequent daily passage the inoculum was taken from the tube nearest the MIC, usually 1 or 2 $\log_2$ dilution steps below the MIC, which had approximately the same turbidity as antibiotic-free controls. Strains were passaged until MICs of >64 μg/ml were obtained, up to a maximum of 50 serial passages, at which time subculturing in the presence of antibiotic was discontinued, and selected resistant/nonsusceptible clones underwent 10 daily passages on chocolate agar without antibiotics. The identity of parental strains and their derived clones was confirmed by pulsed-field gel electrophoresis using a CHEF DR III apparatus (Bio-Rad, Hercules, CA). MICs for each resistant clone for each drug were confirmed by microdilution broth assay. Resistance mechanisms for some parental strains and derived clones were determined as described below.

Mechanisms of resistance. The presence of mutations in ribosomal L4 and L22 proteins and in 23S rRNA (for macrolide-resistant clones) and in quinolone resistance-determining regions (for moxifloxacin-resistant clones) was examined using primers and conditions as described previously (6, 31). After PCR amplification products were purified using a QIAquick PCR purification kit (QIAGEN, Valencia, Calif.). Nucleotide sequences were obtained using a CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

Single-step selection studies. Selection frequencies of clones resistant to 2×, 4×, and 8× the MIC of antibiotics were determined by spreading 10^9 to 10^{11} CFU (in 100 μl) onto plates of either HTM (*H. influenzae*) or Mueller-Hinton agar supplemented with 5% sheep blood (*M. catarrhalis*). After incubation at 35°C in 5% CO_2 for 48 h, resistant colonies were confirmed by replica plating onto medium containing antibiotics. Resistance frequencies were calculated as the number of resistant colonies per inoculum (21).

RESULTS

The MIC range for ceftobiprole against the 321 *H. influenzae* strains surveyed was 0.008 to 8 μg/ml; MIC_{50} and MIC_{90} values were 0.06 and 0.25 μg/ml for β-lactamase-positive strains, 0.03 and 0.25 μg/ml for β-lactamase-negative strains, and 0.5 and 2.0 μg/ml for BLNAR strains, respectively (Table 1). The MIC range for ceftobiprole towards the 49 *M.catarrhalis* strains surveyed was ≤0.004 to 1 μg/ml; MIC_{50} and MIC_{90} values were 0.12 and 0.5 μg/ml, respectively, for β-lactamase-positive strains, whereas the MIC range for the nine β-lactamase-negative strains was ≤0.004 to 0.03 μg/ml (Table 1). Of a total of 370 *H. influenzae* and *M. catarrhalis* strains examined, only four (1.1%), all β-lactamase-positive *H. influenzae* isolates susceptible to azithromycin, moxifloxacin, and tetracycline, had ceftobiprole MICs exceeding 2.0 μg/ml (MIC of 4 μg/ml, one strain; MIC of 8 μg/ml, three strains). β-Lactamase levels in enzyme-positive strains with raised ceftobiprole MICs were not quantified.

Ceftriaxone MICs for *H. influenzae* were usually at least twofold lower than those of ceftobiprole, whereas MICs of amoxicillin (with or without clavulanate) were usually two- to fourfold higher than those of ceftobiprole. Cefpodoxime MICs were similar to or slightly higher than those of ceftobiprole. Azithromycin and telithromycin yielded unimodal MIC distributions for both *H. influenzae* and *M. catarrhalis* and were active against *M. catarrhalis* at MICs of ≤1 μg/ml. Moxifloxacin was active against all strains except those *H. influenzae* isolates chosen for their fluoroquinolone nonsusceptibility, while tetracycline yielded MICs between 2 and 8 μg/ml for *H. influenzae* and between ≤1 and >8 μg/ml for *M. catarrhalis*.

Time-kill studies. Macrodilution broth MICs for the 12 strains chosen for time-kill studies are listed in Table 2, and summaries of time-kill results for the *H. influenzae* strains surveyed are presented in Table 3. Ceftobiprole was bactericidal against 7 of 10 *H. influenzae* strains at 1× MIC and against all 10 strains at 2× MIC by 24 h. Amoxicillin-clavulanate, ceftriaxone, cefpodoxime, azithromycin, telithromycin, and moxifloxacin produced similar time-kill profiles relative to their MICs. Bactericidal activity at 2× MIC after 24 h was observed for tetracycline against 6 of 10 strains and for amoxicillin against two of four strains tested.

Time-kill analyses of two β-lactamase-positive *M. catarrhalis* strains (data not shown) found that ceftobiprole and amoxicillin were bactericidal against one strain at 4× MIC by 24 h. Amoxicillin-clavulanate, ceftriaxone, cefpodoxime, azithromycin, telithromycin, and moxifloxacin were all bactericidal against both *M. catarrhalis* strains at 2× MIC after 24 h. Tetracycline was bactericidal against one strain at 2× MIC and against both *M. catarrhalis* strains at 4× MIC after 24 h.

Multipassage resistance selection studies. The initial MIC ranges for parental strains were as follows: ceftobiprole, 0.03 to 1 μg/ml; amoxicillin-clavulanate, 0.5 to 8 μg/ml; ceftriaxone, 0.004 to 0.25 μg/ml; azithromycin, 0.25 to 2 μg/ml; telithromycin, 0.5 to 2 μg/ml; and moxifloxacin, 0.016 to 0.06 μg/ml (Table 4).

After 50 serial passages in the presence of ceftobiprole MICs for the eight *H. influenzae* strains surveyed remained constant for five strains and increased 1 to 2 $\log_2$ dilution steps for the

TABLE 3. Time-kill analyses of 10 *H. influenzae* strains

Drug and concn	No. of strains tested	No. of strains killed at time point[a]:											
		3 h			6 h			12 h			24 h		
		−1	−2	−3	−1	−2	−3	−1	−2	−3	−1	−2	−3
Ceftobiprole	10												
4× MIC		2	0	0	10	4	0	10	10	5	10	10	10
2× MIC		2	0	0	10	3	0	10	8	4	10	10	10
MIC		2	0	0	9	2	0	10	6	4	10	9	7
Amoxicillin[b]	4												
4× MIC		1	0	0	4	0	0	4	3	1	4	4	3
2× MIC		1	0	0	3	0	0	4	2	1	4	4	2
MIC		1	0	0	3	0	0	4	1	1	4	4	2
Amoxicillin-clavulanate	10												
4× MIC		5	0	0	10	2	0	10	10	3	10	10	10
2× MIC		3	0	0	10	2	0	10	8	3	10	10	10
MIC		3	0	0	7	2	0	10	7	2	10	9	8
Ceftriaxone	10												
4× MIC		3	0	0	10	4	0	10	10	4	10	10	10
2× MIC		3	0	0	9	4	0	10	10	4	10	10	10
MIC		2	0	0	9	2	0	10	7	2	10	9	7
Cefpodoxime	10												
4× MIC		4	0	0	9	2	0	10	10	3	10	10	10
2× MIC		1	0	0	8	2	0	10	8	3	10	10	10
MIC		1	0	0	7	1	0	8	5	1	10	8	7
Azithromycin	10												
4× MIC		9	4	1	10	8	4	10	10	10	10	10	10
2× MIC		8	3	1	9	7	3	10	10	9	10	10	10
MIC		5	1	0	6	4	2	8	7	4	9	9	6
Telithromycin	10												
4× MIC		8	2	0	8	6	2	10	8	6	10	10	10
2× MIC		5	1	0	8	4	1	10	7	4	10	10	10
MIC		3	0	0	3	3	0	10	4	3	8	6	7
Moxifloxacin	10												
4× MIC		10	8	2	10	9	6	10	10	9	10	10	10
2× MIC		10	4	1	10	8	3	10	10	7	10	10	10
MIC		5	2	0	9	4	1	10	8	3	10	10	9
Tetracycline	10												
4× MIC		5	1	0	9	5	0	9	9	6	9	9	9
2× MIC		5	0	0	8	2	0	9	9	4	9	9	6
MIC		3	0	0	4	1	0	8	3	1	8	5	4

[a] −1, 90% killing; −2, 99% killing; −3, 99.9% killing.
[b] Six *H. influenzae* strains with amoxicillin MICs of >32 μg/ml were not tested.

other three strains; the highest MIC recorded was 1 μg/ml. Ceftriaxone selection likewise led to MIC increases of 0 to 2 $\log_2$ dilution steps, the highest ceftriaxone MIC recorded being 0.25 μg/ml. No increase in MICs was observed for strains subcultured in amoxicillin-clavulanate. During serial passage seven of eight *H. influenzae* strains gave rise to azithromycin-nonsusceptible clones according to CLSI breakpoint criteria (7); two of these clones, recovered after 34 to 37 passages, had undergone MIC increases of 1 to >64 μg/ml. In contrast, only three of eight strains evolved resistance to telithromycin (2 to 16 μg/ml, 1 to 32 μg/ml, and 0.5 to >64 μg/ml) according to CLSI breakpoint criteria. Selection with moxifloxacin produced only one clone nonsusceptible to this fluoroquinolone (0.06 to 32 μg/ml), though MIC increases as high as 32-fold were found for the remaining seven *H. influenzae* clones passaged in the presence of moxifloxacin. Mutations in 23S rRNA and/or in ribosomal proteins L4 and L22 were identified in three of six azithromycin-nonsusceptible and telithromycin-resistant clones (Table 4). Sequencing of five moxifloxacin-nonsusceptible clones (MIC, 0.5 to 32 μg/ml) revealed mutations in GyrA, GyrB, and/or ParC in three clones (Table 4).

The MIC for azithromycin of one azithromycin-nonsusceptible clone, derived from *H. influenzae* parental strain 153-008, was somewhat unstable, dropping from >64 μg/ml to 32 μg/ml after 10 passages in drug-free medium (Table 5), though MICs for other antibiotics in the drug panel either remained unchanged or rose by 1 to 2 $\log_2$ dilution steps. After 10 passages on drug-free medium, the azithromycin-nonsusceptible clone

TABLE 4. Results of multistep resistance selection in *H. influenzae* and *M. catarrhalis* with ceftobiprole, amoxicillin-clavulanate, ceftriaxone, azithromycin, telithromycin, and moxifloxacin[a]

Strain	Drug	Initial MIC (μg/ml)	Selected resistance		Mutation(s) in:					
			MIC (μg/ml)	No. of passages	23S rRNA	L4	L22	GyrA	GyrB	ParC
H. influenzae										
110-019 [Bla$^+$]	BPR	0.125	0.125	50						
	AZM	1	32	50	ND	ND	ND			
	AMC	2	2	50						
	MXF	0.06	0.5	50				ND	ND	ND
	TEL	1	32	50	ND	DEL66RA	ND			
	CRO	0.008	0.008	50						
112-048 [Bla$^+$]	BPR	0.06	0.25	50						
	AZM	1	8	50						
	AMC	2	2	50						
	MXF	0.016	0.5	50				ND	E468N	ND
	TEL	1	2	50						
	CRO	0.004	0.008	50						
621-049 [Bla$^+$]	BPR	0.06	0.125	50						
	AZM	2	16	50						
	AMC	0.5	0.5	50						
	MXF	0.06	32	50				S84A, D88Y	ND	A112V
	TEL	2	2	50						
	CRO	0.008	0.016	50						
HI 30 [Bla$^+$]	BPR	0.25	0.25	50						
	AZM	0.25	32	50	T2132A	G64D	ND			
	AMC	2	2	50						
	MXF	0.03	0.25	50						
	TEL	0.5	64	50	ND	DEL66RA	K90E			
	CRO	0.008	0.008	50						
153-008 [Bla$^-$]	BPR	0.06	0.06	50						
	AZM	1	>64	37	ND	ND	ND			
	AMC	0.5	0.5	50						
	MXF	0.03	0.06	50						
	TEL	1	2	50						
	CRO	0.004	0.016	50						
110-061 [BLNAR]	BPR	0.125	0.125	50						
	AZM	1	>64	34	ND	ND	ND			
	AMC	2	2	50						
	MXF	0.03	1	50				D88N	ND	ND
	TEL	2	4	50						
	CRO	0.008	0.03	50						
153-004 [Bla$^+$]	BPR	0.03	0.06	50						
	AZM	1	16	50						
	AMC	1	1	50						
	MXF	0.06	1	50				ND	ND	ND
	TEL	2	16	50						
	CRO	0.004	0.008	50						
HI 92 [BLPACR]	BPR	1	1	50						
	AZM	1	4	50						
	AMC	8	8	50						
	MXF	0.016	0.25	50						
	TEL	0.5	2	50						
	CRO	0.25	0.25	50						
M. catarrhalis										
36	BPR	1	1	50						
	AZM	0.03	0.03	50						
	AMC	0.25	0.25	50						
	MXF	0.06	0.06	50						
	TEL	0.016	0.03	50						
	CRO	1	2	50						
46	BPR	0.5	0.5	50						
	AZM	0.03	0.03	50						
	AMC	0.06	0.125	50						
	MXF	0.06	0.06	50						
	TEL	0.06	0.125	50						
	CRO	0.125	0.25	50						

[a] Abbreviations: BPR, ceftobiprole; AMC, amoxicillin-clavulanate; CRO, ceftriaxone; AZM, azithromycin; TEL, telithromycin; MXF, moxifloxacin; ND, none detected; Bla$^-$, β-lactamase negative; Bla$^+$, β-lactamase positive.

TABLE 5. Cross-resistance of azithromycin-nonsusceptible clones with other antibiotics following 10 serial passages in drug-free medium

H. influenzae strain	Azithromycin MIC (μg/ml)		Cross-resistance of the selected azithromycin-resistant clone		
	Original	Selected (no. of days)	Antimicrobial agent	MIC (μg/ml)	
				Before drug-free passage	After 10 drug-free passages
153-008	1	>64 (37)	Ceftobiprole	0.06	0.125
			Amoxicillin-clavulanate	0.5	0.5
			Ceftriaxone	0.016	0.008
			Azithromycin	>64	32
			Telithromycin	2	4
			Moxifloxacin	0.06	0.03
110-061	1	>64 (34)	Ceftobiprole	0.125	0.125
			Amoxicillin-clavulanate	2	1
			Ceftriaxone	0.03	0.016
			Azithromycin	>64	>64
			Telithromycin	4	>32
			Moxifloxacin	1	0.125

derived from *H. influenzae* parental strain 110-061 showed no MIC changes for azithromycin, ceftobiprole, ceftriaxone, or amoxicillin-clavulanate outside a single $\log_2$ dilution step, though its moxifloxacin MIC dropped from 1 μg/ml to 0.125 μg/ml (Table 5). During passage on drug-free medium the MIC for telithromycin of this clone rose from 4 μg/ml to >32 μg/ml. We have no explanation for this phenomenon, which is currently under investigation.

After 50 serial passages in the presence of antibiotic, MICs for the two *M. catarrhalis* strains either remained stable or increased by 1 $\log_2$ dilution step (Table 4). Selection by moxifloxacin and azithromycin did not lead to increases in MICs compared to the parental strains (0.06 μg/ml for moxifloxacin and 0.03 μg/ml for azithromycin).

Single-passage selection studies. Single-step selection did not identify any *H. influenzae* and *M. catarrhalis* clones with enhanced resistance to ceftobiprole; selection frequencies for all strains ranged between $<5.0 \times 10^{-10}$ and $<1.3 \times 10^{-11}$ at 2× MIC and at 8× MIC (Table 6). In contrast, amoxicillin-clavulanate, ceftriaxone, and moxifloxacin each selected for clones with MICs four- to eightfold higher than those of the parental strains. Selection frequencies for these drugs were as follows: amoxicillin-clavulanate, $>3.6 \times 10^{-6}$ to $<1.0 \times 10^{-10}$ (2× MIC) and $>3.6 \times 10^{-7}$ to $< 1.0 \times 10^{-10}$ (8× MIC); ceftriaxone, 7.5×10^{-7} to $<8.3 \times 10^{-11}$ (2× MIC) and $<8.3 \times 10^{-10}$ to $<8.3 \times 10^{-11}$ (8× MIC); moxifloxacin, 3.3×10^{-8} to $<3.2 \times 10^{-10}$ (2× MIC) and 3.3×10^{-9} to $<2.6 \times 10^{-10}$ (8× MIC). The greatest increases in MICs (4- to >32-fold) were observed for azithromycin and telithromycin, with selection frequencies of 1.3×10^{-8} to $<2.9 \times 10^{-10}$ (2× MIC) and 2.0×10^{-10} to $<1.3 \times 10^{-10}$ (8× MIC) for azithromycin and 1.3×10^{-8} to $<1.0 \times 10^{-10}$ (2× MIC) and 2.3×10^{-10} to $<1.0 \times 10^{-10}$ (8× MIC) for telithromycin (Table 6).

DISCUSSION

Ceftobiprole, the active component of the prodrug ceftobiprole medocaril (formerly BAL5788), is a broad-spectrum pyrrolidinone-3-ylidenemethyl cephem with well-documented in vitro and in vivo activities against most clinically relevant pathogens, including penicillin-resistant pneumococci, β-lactamase-positive *H. influenzae*, and *Enterobacteriaceae* devoid of extended-spectrum β-lactamases (1, 13, 14, 15, 18). The MICs for *H. influenzae* and *M. catarrhalis* found in this study generally mirror those reported by Hebeisen at al (14) and by Jones et al. (18). A small number of *H. influenzae* strains with high MICs, up to 8 μg/ml, was identified by another group (N. C. Issa, M. S. Rouse, K. E. Piper, J. M. Steckelberg, and R. Patel, Abstr. 43rd. Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-540, 2003). These discrepancies may reflect methodological differences or differences in the sources from which clinical isolates were collected. In our survey, which included BLNAR and BLPACR strains, ceftobiprole had MICs of ≤2 μg/ml towards 98.8% of the 321 *H. influenzae* clinical isolates examined. The highest ceftobiprole MIC, 8 μg/ml, was encountered in three strains (0.9%), all of which were β-lactamase positive and susceptible to azithromycin, moxifloxacin, and tetracycline.

Kill kinetics for ceftobiprole resembled those of other β-lactams examined, with bactericidal activity at 2× MIC by 24 h against all 10 *H. influenzae* strains profiled. Other β-lactams had similar kill kinetics relative to their different MICs, which were usually higher than those for ceftobiprole. The significance of time-kill results in the two *M. catarrhalis* strains studied awaits confirmation by examination of more strains. *M. catarrhalis* is a difficult organism to test by time-kill assay because of clumping.

Our results indicate that ceftobiprole has a low proclivity for emergence of resistance in *H. influenzae* and *M. catarrhalis*. Under intensive selective pressure (50 serial passages) cephalosporins did not promote emergence of clones with MICs exceeding four times that of parental strains; the largest observed change in ceftobiprole MIC was from 0.06 μg/ml to 0.25 μg/ml. Likewise, the single-step selection procedure failed to detect *H. influenzae* or *M. catarrhalis* clones with elevated MICs for ceftobiprole. In contrast, prolonged serial passage of *H. influenzae* in the presence of a macrolide (azithromycin or telithromycin) led to a >4-fold increase in MIC for most strains. While mutations in ribosomal proteins L4 and/or L22, as well as in domains II and/or V of 23S rRNA, can be responsible for macrolide resistance in *H. influenzae* (5, 31), we were unable to detect any such mutations in three azithromycin-nonsusceptible clones with MICs of ≥32 μg/

TABLE 6. Selection frequencies of *H. influenzae* and *M. catarrhalis* strains by single-step methodology

Strain	Drug	Frequency at MIC:		
		2× MIC	4× MIC	8× MIC
H. influenzae				
HI 92 [BLPACR]	Ceftobiprole	$<2.2 \times 10^{-10}$	$<2.2 \times 10^{-10}$	$<2.2 \times 10^{-10}$
	Amox-clav[a]			
	Moxifloxacin	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$
	Azithromycin	$\sim 2.7 \times 10^{-10}$	$<2.7 \times 10^{-10}$	$<2.7 \times 10^{-10}$
	Ceftriaxone	$<1.2 \times 10^{-10}$	$<1.2 \times 10^{-10}$	$<1.2 \times 10^{-10}$
	Telithromycin	$\sim 1.3 \times 10^{-8}$	$<2.5 \times 10^{-10}$	$<2.5 \times 10^{-10}$
112-048 [Bla$^+$]	Ceftobiprole	$<1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$
	Amox-clav	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
	Moxifloxacin	$\sim 2.0 \times 10^{-8}$	$\sim 5.7 \times 10^{-9}$	$<1.6 \times 10^{-10}$
	Azithromycin	$\sim 2.6 \times 10^{-10}$	$\sim 1.3 \times 10^{-10}$	$<1.3 \times 10^{-10}$
	Ceftriaxone	$\sim 1.0 \times 10^{-8}$	$>1.0 \times 10^{-9}$	$<1.0 \times 10^{-10}$
	Telithromycin	$\sim 2.5 \times 10^{-9}$	$\sim 2.0 \times 10^{-10}$	$<2.0 \times 10^{-10}$
621-049 [Bla$^+$]	Ceftobiprole	$<2.0 \times 10^{-10}$	$<2.0 \times 10^{-10}$	$<2.0 \times 10^{-10}$
	Amox-clav	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
	Moxifloxacin	$\sim 1.0 \times 10^{-8}$	$\sim 1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
	Azithromycin	$\sim 1.5 \times 10^{-9}$	$<2.8 \times 10^{-10}$	$<2.8 \times 10^{-10}$
	Ceftriaxone	$<2.9 \times 10^{-10}$	$<2.9 \times 10^{-10}$	$<2.9 \times 10^{-10}$
	Telithromycin	$\sim 8.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$
153-004 [Bla$^+$]	Ceftobiprole	$<3.1 \times 10^{-10}$	$<3.1 \times 10^{-10}$	$<3.1 \times 10^{-10}$
	Amox-clav	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
	Moxifloxacin	$\sim 3.3 \times 10^{-8}$	$\sim 6.7 \times 10^{-9}$	$\sim 3.3 \times 10^{-9}$
	Azithromycin	$\sim 1.3 \times 10^{-8}$	$<1.9 \times 10^{-10}$	$<1.9 \times 10^{-10}$
	Ceftriaxone	$<8.3 \times 10^{-11}$	$<8.3 \times 10^{-11}$	$<8.3 \times 10^{-11}$
	Telithromycin	$\sim 8.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$
110-019 [Bla$^+$]	Ceftobiprole	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Amox-clav	$<2.9 \times 10^{-10}$	$<2.9 \times 10^{-10}$	$<2.9 \times 10^{-10}$
	Moxifloxacin	$<4.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$
	Azithromycin	$\sim 1.5 \times 10^{-9}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Ceftriaxone	$<4.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$
	Telithromycin	$\sim 3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$
HI 30 [Bla$^+$]	Ceftobiprole	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$
	Amox-clav	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$
	Moxifloxacin	$>2.6 \times 10^{-10}$	$<2.6 \times 10^{-10}$	$<2.6 \times 10^{-10}$
	Azithromycin	$\sim 6.7 \times 10^{-10}$	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$
	Ceftriaxone	$<4.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$	$<4.0 \times 10^{-10}$
	Telithromycin	$\sim 2.2 \times 10^{-9}$	$\sim 3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$
153-008 [Bla$^-$]	Ceftobiprole	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$
	Amox-clav	$\sim 2.1 \times 10^{-7}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Moxifloxacin	$\sim 1.7 \times 10^{-8}$	$<4.2 \times 10^{-10}$	$<4.2 \times 10^{-10}$
	Azithromycin	$\sim 1.5 \times 10^{-9}$	$<3.0 \times 10^{-10}$	$<3.0 \times 10^{-10}$
	Ceftriaxone	$<8.3 \times 10^{-10}$	$<8.3 \times 10^{-10}$	$<8.3 \times 10^{-10}$
	Telithromycin	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$	$<3.8 \times 10^{-10}$
110-061 [BLNAR]	Ceftobiprole	$<3.7 \times 10^{-10}$	$<3.7 \times 10^{-10}$	$<3.7 \times 10^{-10}$
	Amox-clav	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Moxifloxacin	$<3.2 \times 10^{-10}$	$<3.2 \times 10^{-10}$	$<3.2 \times 10^{-10}$
	Azithromycin	$\sim 3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$
	Ceftriaxone	$\sim 7.5 \times 10^{-7}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$
	Telithromycin	$\sim 5.6 \times 10^{-9}$	$<3.7 \times 10^{-10}$	$<3.7 \times 10^{-10}$
M. catarrhalis				
36 [Bla$^+$]	Ceftobiprole	$<1.3 \times 10^{-11}$	$<1.3 \times 10^{-11}$	$<1.3 \times 10^{-11}$
	Amox-clav	$<3.6 \times 10^{-10}$	$<3.6 \times 10^{-10}$	$<3.6 \times 10^{-10}$
	Moxifloxacin	$\sim 1.4 \times 10^{-9}$	$<2.5 \times 10^{-11}$	$<2.5 \times 10^{-11}$
	Azithromycin	$<2.9 \times 10^{-10}$	$<2.9 \times 10^{-10}$	$<2.9 \times 10^{-10}$
	Ceftriaxone	$<6.7 \times 10^{-10}$	$<6.7 \times 10^{-10}$	$<6.7 \times 10^{-10}$
	Telithromycin	$\sim 3.4 \times 10^{-10}$	$\sim 1.1 \times 10^{-10}$	$\sim 2.3 \times 10^{-10}$
46 [Bla$^+$]	Ceftobiprole	$<4.3 \times 10^{-11}$	$<4.3 \times 10^{-11}$	$<4.3 \times 10^{-11}$
	Amox-clav	$>3.6 \times 10^{-6}$	$>3.6 \times 10^{-7}$	$>3.6 \times 10^{-7}$
	Moxifloxacin	$\sim 1.6 \times 10^{-9}$	$<2.7 \times 10^{-10}$	$<2.7 \times 10^{-10}$
	Azithromycin	$\sim 2.0 \times 10^{-9}$	$\sim 2.0 \times 10^{-10}$	$\sim 2.0 \times 10^{-10}$
	Ceftriaxone	$>2.3 \times 10^{-9}$	$>2.3 \times 10^{-8}$	$<2.3 \times 10^{-10}$
	Telithromycin	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$

[a] *H. influenzae* HI 92 is resistant to amoxicillin-clavulanate (Amox-clav) and therefore was not tested with this drug.

ml, in which case other factors probably are responsible for the observed decrease in macrolide susceptibility. Moreover, a high frequency of resistance selection was observed for moxifloxacin (Table 4), for which 7 of 10 clones experienced increases in MICs in the range of 5- to 512-fold. However, only three of six studied clones (displaying MIC increases of 32- to 512-fold compared to their parental strains) had detectable mutations in quinolone resistance-determining regions.

The results of this study, combined with the excellent antipneumococcal activity of ceftobiprole (1, 22) and favorable pharmacokinetic and safety profiles of the prodrug ceftobiprole medocaril (21, 34–37), make ceftobiprole a very promising drug for treatment of patients with community-acquired respiratory tract infections who require hospitalization.

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