

In Vitro Evaluation of the Antimicrobial Activity of Ceftaroline against Cephalosporin-Resistant Isolates of *Streptococcus pneumoniae*[▽]

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Increasing pneumococcal resistance to extended-spectrum cephalosporins warrants the search for novel agents with activity against such resistant strains. Ceftaroline, a parenteral cephalosporin currently in phase 3 clinical development, has demonstrated potent in vitro activity against resistant gram-positive organisms, including penicillin-resistant *Streptococcus pneumoniae*. In this study, the activity of ceftaroline was evaluated against highly cefotaxime-resistant isolates of pneumococci from the Active Bacterial Core surveillance program of the Centers for Disease Control and Prevention and against laboratory-derived cephalosporin-resistant mutants of *S. pneumoniae*. The MICs of ceftaroline and comparators were determined by broth microdilution. In total, 120 U.S. isolates of cefotaxime-resistant (MIC ≥ 4 $\mu\text{g/ml}$) *S. pneumoniae* were tested along with 18 laboratory-derived R6 strains with known penicillin-binding protein (PBP) mutations. Clinical isolates were characterized by multilocus sequence typing, and the DNAs of selected isolates were sequenced to identify mutations affecting *pbp* genes. Ceftaroline (MIC₉₀ = 0.5 $\mu\text{g/ml}$) had greater in vitro activity than penicillin, cefotaxime, or ceftriaxone (MIC₉₀ = 8 $\mu\text{g/ml}$ for all comparators) against the set of highly cephalosporin-resistant clinical isolates of *S. pneumoniae*. Ceftaroline was also more active against the defined R6 PBP mutant strains, which suggests that ceftaroline can overcome common mechanisms of PBP-mediated cephalosporin resistance. These data indicate that ceftaroline has significant potency against *S. pneumoniae* strains resistant to existing parenteral cephalosporins and support its continued development for the treatment of infections caused by resistant *S. pneumoniae* strains.

Streptococcus pneumoniae is the most common cause of community-acquired pneumonia (18, 35). While the introduction of pneumococcal conjugate vaccine led to a large decrease in the incidence of disease caused by resistant strains (21), the numbers of highly resistant pneumococcal strains not covered by the vaccine are now increasing (25); and *S. pneumoniae* strains resistant to penicillin, cephalosporins, macrolides, tetracyclines, and sulfonamides continue to be observed in the United States (2–4, 9, 13, 19).

Although the breakpoint for penicillin resistance for non-meningeal isolates has recently been increased to 8 $\mu\text{g/ml}$ (6), highly penicillin- and cephalosporin-resistant pneumococci are still emerging (25, 30), and the effectiveness of existing β -lactams in the treatment of such infections is unlikely to be successful, as determined on the basis of the pharmacokinetics and pharmacodynamics of existing agents (1, 27, 29). Ceftaroline is a novel parenteral cephalosporin with broad-spectrum in vitro antimicrobial activity against resistant gram-positive pathogens, including penicillin-resistant *S. pneumoniae* strains and methicillin-resistant *Staphylococcus aureus* strains (14, 28), as well as common gram-negative organisms.

The aim of the study described here was to evaluate the activity of ceftaroline against a panel of cefotaxime-resistant clinical isolates and laboratory mutants with defined penicillin-

binding protein (PBP) mutations. Multilocus sequence typing (MLST) analysis was used to group the isolates; and the genes encoding PBPs 1A, 2B, and 2X from selected isolates were sequenced to characterize key mutations.

(A preliminary report of these results was presented in 2007 at the 47th Interscience Conference on Antimicrobial Agents and Chemotherapy in Chicago, IL.)

MATERIALS AND METHODS

Bacterial strains. One hundred twenty invasive *S. pneumoniae* strains with cefotaxime MICs of ≥ 4 $\mu\text{g/ml}$ that were collected as part of the Centers for Disease Control and Prevention (CDC) Active Bacterial Core surveillance and Emerging Infections Programs Network from 2001 through 2006 were included. The strains were isolated from children and adults (age range, <1 to 96 years) in eight states. Eighteen strain R6 laboratory-derived mutants with defined *pbp* and *murM* gene mutations known to confer β -lactam resistance were also included in the study. These mutants were previously generated by transforming well-characterized antimicrobial-susceptible laboratory strain R6 with various combinations of *pbp* genes and the *murM* gene containing mutations known to confer resistance to β -lactam antimicrobials (11, 31, 32). Donor strains of *pbp* and *murM* genes were strain 3191, a representative of a Hungarian pneumococcal clone that was isolated during the period from 1997 to 1998 and that was found to have notably high levels of penicillin resistance (MIC, 16 $\mu\text{g/ml}$) and cefotaxime resistance (MIC, 4 $\mu\text{g/ml}$) (31, 32), and strain 72521, which was isolated in France and which has an amoxicillin MIC (8 $\mu\text{g/ml}$) higher than that of penicillin (MIC, 4 $\mu\text{g/ml}$) (11).

Antimicrobials tested. Ceftaroline (lot M599-R1001) was supplied by Forest Laboratories, Inc. (New York, NY). The other antimicrobials used in the study were purchased from commercial sources.

MIC testing. The MICs of ceftaroline, penicillin, ceftriaxone, and cefotaxime for all clinical strains and the R6 laboratory mutants were determined by the reference broth microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (5, 6). In addition, MIC data (determined by broth microdilution) for amoxicillin and meropenem were available from the

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TABLE 1. MIC ranges, MIC₅₀s, and MIC₉₀s for cefotaxime, penicillin, ceftriaxone, ceftaroline, amoxicillin, and meropenem against 120 clinical isolates

Antimicrobial	MIC (μg/ml)		
	Range	50%	90%
Cefotaxime	4–≥16	8	8
Penicillin	0.5–16	8	8
Ceftriaxone	2–≥16	8	8
Ceftaroline	0.125–2	0.5	0.5
Amoxicillin	0.25–>8	8	>8
Meropenem	0.12–2	1	2

CDC database for the clinical isolates, and the MICs for amoxicillin (determined by Etest; AB Biodisk; Solna, Sweden) were available for the R6 mutants.

MLST. MLST was performed with all clinical isolates by previously described methods (12, 15). The sequence types (STs) obtained were compared with those at the MLST website (<http://spneumoniae.mlst.net>). New alleles and allelic profiles were submitted to the MLST database for MLST assignment.

PBP sequencing. On the basis of genotypic characterization of the 120 strains by MLST, 15 strains were selected for sequencing of the *pbp1a*, *pbp2b*, and *pbp2x* genes to identify mutations. Nine isolates were selected from each of the three major STs identified, and six isolates were selected from the remaining clonal complexes. For sequencing, the PCR amplification products were purified with the ExoSAP-IT reagent (USB Corp., Cleveland, OH). The nucleotide sequences of the penicillin-binding domains of PBPs 1A, 2B, and 2X were determined by sequencing with a series of oligonucleotides that were primed at intervals of ±300 nucleotides along each gene. Cycle sequencing was performed with a BigDye Terminator (version 1.1) kit (Applied Biosystems; Foster City, CA), and electrophoresis was carried out with an ABI 3100 automated sequence analyzer. The nucleotide and deduced amino acid sequences of PBPs 1A, 2B, and 2X were aligned by using the MegAlign program (DNASTar, Madison, WI) and compared with the published sequence of penicillin-susceptible *S. pneumoniae* strain R6 (23).

RESULTS

Antimicrobial activity of ceftaroline. Ceftaroline was the most active agent tested against the 120 clinical isolates of cefotaxime-resistant *S. pneumoniae*, with MICs ranging from 0.125 μg/ml to 2 μg/ml; the MIC₉₀ was 0.5 μg/ml (Table 1). The single isolate of *S. pneumoniae* with a ceftaroline MIC of 2 μg/ml was highly resistant to cefotaxime and ceftriaxone (MICs ≥ 16 μg/ml for both agents). Overall, ceftaroline was more than 16-fold more active in vitro than cefotaxime, penicillin, ceftriaxone, amoxicillin, and meropenem.

The results in Table 2 (11, 31, 32) show that ceftaroline also exhibited potent activity against 18 laboratory strains with defined PBP and *murM* mutations known to confer resistance to β-lactams. The MICs of ceftaroline for these isolates ranged from 0.015 μg/ml to 0.25 μg/ml; the MIC₉₀ was 0.03 μg/ml, whereas the MIC₉₀s of penicillin, ceftriaxone, and amoxicillin were ≥4 μg/ml.

MLST. MLST identified 31 STs associated with the 120 strains characterized. Three STs accounted for the majority of strains (57.8%): ST320 (*n* = 41), ST13 (*n* = 14), and ST37 (*n* = 14).

DNA sequencing of penicillin-binding domains. Irrespective of the genetic background, as determined by the MLST type (Fig. 1) (23), the 15 clinical isolates selected for sequencing had similar constellations of mutations associated with resistance in their PBP 1A, 2B, and 2X genes. Isolate 2192 (MIC = 8 μg/ml for penicillin, cefotaxime, and ceftriaxone) had only single amino acid substitutions affecting PBP 2X and PBP 1A, and it remains possible that mutations in other genes affect β-lactam resistance in this strain.

DNA sequence analysis of the PBP 1A, 2B, and 2X penicillin-binding domains from the isolates revealed extensive sequence divergence compared with the sequence of strain R6.

TABLE 2. MICs of cefotaxime, penicillin, ceftriaxone, ceftaroline, and amoxicillin for 18 R6 mutants

R6 mutant ^a	MIC (μg/ml) ^b				
	CTX	PEN	CRO	CPT	AMX
R6 (parental strain)	0.03	0.015	0.03	≤0.015	≤0.015
3191 (donor strain)	4	16	8	0.25	8
72521 (donor strain)	2	4	4	0.25	8
R6 2x/3191	0.03	2	2	0.125	0.047
R6 2b/1a/mur/3191	0.03	0.03	0.06	≤0.015	0.023
R6 2x/72521	0.06	0.5	0.5	0.06	≤0.015
R6 2x/1a/3191	0.06	2	2	0.125	0.032
R6 2x/2b/72521	0.5	0.5	1	0.06	0.094
R6 2x/2b/3191	0.5	1	1	0.125	0.19
R6 2x/3191 + 2b/72521	1	2	2	0.125	0.19
R6 2x/2b/1a/72521	2	0.5	0.5	0.06	0.5
R6 2x/2b/72521 + 1a/3191	2	1	2	0.125	0.75
R6 2x/2b/1a/gDNA/72521	2	1	0.5	0.125	1
R6 2x/2b/1a/72521 + gDNA/3191	2	8	2	0.25	1
R6 2x/2b/1a/72521 + mur/3191	2	1	1	0.06	1
R6 2x/2b/3191 + 1a/72521 + mur/3191	2	8	4	0.125	1.5
R6 2x/2b/1a/72521 + 1a/3191 + gDNA/72521	4	2	2	0.125	1.5
R6 2x/2b/72521 + 1a/3191 + mur/72521	4	2	4	0.25	3
R6 2x/2b/1a/3191 + mur/72521	16	8	8	0.25	4
R6 2x/2b/1a/3191	16	4	4	0.125	4

^a Isogenic R6 transformants containing known *pbp* and *murM* mutations were generated from previous studies (11, 31, 32) by transforming parental strain R6 with various combinations of the *pbp1a*, *pbp2b*, *pbp2x*, and *murM* genes from donor strains 3191 and 72521; 2x, *pbp2x*; 2b, *pbp2b*; 1a, *pbp1a*; mur, *murM*; gDNA, genomic DNA.

^b AMX, amoxicillin; CPT, ceftaroline; CRO, ceftriaxone; CTX, cefotaxime; PEN, penicillin.

Within the STMK motif of the PBP 2X penicillin-binding domain, isolates had a Thr₃₃₈Ala substitution (Fig. 1a). Met₃₃₉Phe and Met₄₀₀Thr were common substitutions; there were no Thr₅₅₀ or Gln₅₅₂ mutations associated with cephalosporin resistance in these isolates. In the PBP 2B penicillin-binding domain, a Thr₄₄₅Ala substitution (adjacent to the S₄₄₂SN conserved motif) was present in most of the isolates (Fig. 1b). Numerous substitutions in close proximity to the PBP 2B active-site K₆₁₄TG motif were identified. In the PBP 1A penicillin-binding domain, a substitution of Thr₃₇₁ to Ser or Ala and the four consecutive substitutions at residues 574 to 577 were present in the majority of isolates (Fig. 1c). All isolates with the exception of isolate 2225 had amoxicillin MICs of ≥ 2 $\mu\text{g/ml}$; isolate 2225 had an MIC of 0.25 $\mu\text{g/ml}$.

DISCUSSION

Increases in *S. pneumoniae* resistance to cephalosporins and other β -lactam antimicrobial agents have been observed since 2005 in the United States (2–4). In view of pharmacokinetic and pharmacodynamic considerations, there may be clinical implications of very-high-level penicillin and cephalosporin resistance for patients with pneumonia. On the basis of the pharmacodynamic principle of achieving a free drug concentration greater than the MIC₉₀ for 40% to 50% of the length of the dosing interval, the clinical failure breakpoint is 2 $\mu\text{g/ml}$ for ceftriaxone given 1 g daily or cefotaxime 1 g given 3 times daily (17). The highly cephalosporin-resistant strains analyzed in this study would thus raise concern over potential clinical failure for patients with pneumonia.

In the present study, ceftaroline was more active in vitro against the set of highly cephalosporin-resistant clinical isolates of *S. pneumoniae* than either penicillin, ceftriaxone, or amoxicillin. Carbapenems are recognized to have activity against a broad range of gram-positive cocci, including penicillin-resistant *S. pneumoniae*, and meropenem was more active against the strains included in this study. Ceftaroline was also more active against laboratory R6 mutants containing *pbp* mutations known to confer resistance to other β -lactam agents. These observations suggest that ceftaroline could be clinically effective for the treatment of infections caused by *S. pneumoniae* strains resistant to the currently available antimicrobials. A clinical situation involving *S. pneumoniae* in which ceftaroline may be of value is meningitis. Current treatment guidelines advocate the use of combination therapy because of the risk of failure imposed by cephalosporin-resistant pneumococci (24). If cerebrospinal fluid penetration studies show adequate penetration of ceftaroline, then the in vitro activity demonstrated against cephalosporin-resistant strains warrants clinical trials on the use of ceftaroline as monotherapy for meningitis.

Resistance to β -lactam antimicrobial agents in *S. pneumoniae* is mediated by successive alterations in essential PBPs, with specific mutations being expressed in combination with mutations in other genes (16, 22, 37). The final resistance phenotype is therefore dependent on the collective actions of multiple PBPs. Irrespective of the genetic background, the 15 clinical isolates selected for sequence analysis had similar mutations in genes encoding PBP 1A, 2B, and 2X. Interestingly, isolate 2192, which exhibited high-level resistance to penicillin

and cefotaxime, had almost no alterations in the sequenced portions of the genes for PBP 1A, 2B, and 2X. Resistance in this strain may be associated with changes in other genes, such as *pbp2A*, *pbp1B*, or *murM* (10, 34), or may be due to a non-PBP-mediated mechanism (31).

Several of the PBP mutations observed in this study are known to be important in β -lactam resistance. The Thr₃₃₈Ala substitution in the STMK motif observed in the PBP 2X penicillin-binding domains of the clinical isolates used in this study has been shown to be important for β -lactam resistance (26). In addition, the amino acid mutations Met₃₃₉Phe and Met₄₀₀Thr, which were common substitutions in these isolates, have been shown to be important substitutions for conferring high-level cefotaxime resistance (MICs ≥ 2 $\mu\text{g/ml}$) (7).

The previously documented Thr₄₄₅Ala substitution (adjacent to the S₄₄₂SN conserved motif) in the PBP 2B penicillin-binding domain was present in most of the isolates in the current study; the importance of this conserved motif in interacting with penicillin has been noted (8). PBP 2B is not considered a target for extended-spectrum cephalosporins, but it appears to be important in the development of amoxicillin resistance; this is supported by the observation in the present study that all but one isolate had amoxicillin MICs of ≥ 2 $\mu\text{g/ml}$. Mutations in the region from residues 590 to 676 of PBP 2B, similar to the substitutions near the K₆₁₄TG motif reported in this study, have been shown to be associated with strains with higher amoxicillin MICs than penicillin MICs (11, 20).

The Thr₃₇₁-to-Ser or -Ala substitutions and the four consecutive substitutions at residues 574 to 577 that were present in the PBP 1A penicillin-binding domains in the majority of the isolates in this study have been documented previously, and the contribution of these residues to the development of penicillin resistance (MICs ≥ 0.25 $\mu\text{g/ml}$) has been confirmed (33). The substitution of Ala for Thr at residue 371 is homologous to the Thr₃₃₈Ala substitution in PBP 2X (within the Ser₃₃₇-Thr-Met-Lys conserved motif).

The activity of ceftaroline against the set of defined R6 PBP mutants in this study was superior to the activities of the comparator agents and suggests that ceftaroline may overcome common mechanisms of β -lactam resistance that are mediated by target modification. It has recently been shown that the activity of ceftaroline against methicillin-resistant *S. aureus* is associated with conformational changes to the active site of PBP 2a that are promoted by ceftaroline binding to an allosteric site (36); it is not clear if similar interactions occur between ceftaroline and PBPs in *S. pneumoniae*. The present study demonstrated that ceftaroline has significant potency against pneumococcal isolates highly resistant to existing parenteral cephalosporins. These findings support the continued development of ceftaroline for the treatment of infections caused by resistant *S. pneumoniae* strains.

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