Interaction of Ceftobiprole with the Low-Affinity PBP 5 of Enterococcus faecium

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Received 15 July 2009/Returned for modification 1 September 2009/Accepted 9 November 2009

Ceftobiprole is a new cephalosporin that exhibits a high level of affinity for methicillin-resistant Staphylococcus aureus PBP 2a. It was reported that ceftobiprole did not interact with a mutated form of the low-affinity protein Enterococcus faecium PBP 5 (PBP 5fm) that, when overexpressed, confers a β-lactam resistance phenotype to the bacterium. Our results show that ceftobiprole binds to unmutated PBP 5fm to form a stable acyl-enzyme and that ceftobiprole is able to efficiently kill a penicillin-resistant Enterococcus faecium strain that produces this protein.

β-Lactam antibiotics (penicillins, cephalosporins, carbapenems, and monobactams) are the most frequently prescribed antibacterial agents used to fight serious bacterial infections. They inactivate the membrane-bound D,D-transpeptidases essential for peptidoglycan synthesis by forming with them stable acyl-enzymes (9). This explains why these enzymes are generally designated penicillin-binding proteins (PBPs) (17, 19). In Gram-positive cocci, the resistance to β-lactam antibiotics is primarily conferred by the presence or the overproduction of a low-affinity PBP. In enterococci, among which is the opportunistic human pathogen Enterococcus faecium (3), the resistance to penicillin may be associated with overproduction of the intrinsic low-affinity protein PBP 5 (PBP 5fm) or to other alterations affecting PBP 5fm (15).

Ceftobiprole (BPR) (BAL9141) is a novel broad-spectrum cephalosporin that is active against Gram-positive and Gram-negative bacterial groups, including methicillin-resistant Staphylococcus aureus (MRSA) (2, 6, 10, 12) and Enterococcus faecalis (1). It has been demonstrated to be a good inhibitor of the S. aureus low-affinity protein PBP 2a (6, 8). A previous study reported that ceftobiprole had poor inhibitory activity against β-lactam-resistant E. faecium and that ceftobiprole did not bind to the mutated, low-affinity PBP 5fm protein isolated from such a strain (10). To better understand the difference between these two low-affinity PBPs with respect to ceftobiprole, we have characterized the interaction between an unmutated form of PBP 5fm and ceftobiprole.

To perform this study, we used the penicillin-sensitive Enterococcus faecium strain D63 (benzylpenicillin MIC = 5 μg/ml and ampicillin MIC = 15 μg/ml) and its laboratory-derived penicillin-resistant strain D63r (benzylpenicillin MIC = 70 μg/ml and ampicillin MIC = 125 μg/ml) (21). The PBP profile of the latter strain exhibits a 6-fold increase in quantity of PBP 5fm (21). The ceftobiprole MICs determined by the microdilution method (4) for E. faecium D63r and D63 were 8 and 2 μg/ml, respectively. These values contrasted with those reported by other workers, who have found that most ampicillin-resistant Enterococcus faecium strains have a very high penicillin MIC (>250 μg/ml) (11, 15). The ceftobiprole MICs for D63r were 70 μg/ml for benzylpenicillin and 8 μg/ml for ceftobiprole. The MICs for D63 were 5 μg/ml for benzylpenicillin and 2 μg/ml for ceftobiprole. The surviving bacteria were counted after 0, 4, and 24 h of incubation at 37°C by subculturing serial dilutions (at least 10-fold, to minimize drug carryover).

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Published ahead of print on 16 November 2009.
resistant *E. faecium* clinical isolates were resistant to ceftobiprole (13). They concluded that ceftobiprole was ineffective against ampicillin-resistant enterococcal strains.

To further study the killing effects of benzylpenicillin and ceftobiprole on *E. faecium* D63r, we exposed exponentially growing cultures of both the sensitive and the resistant strains to increasing concentrations of antibiotic corresponding to 1 and 4 times the respective MICs (Fig. 1) (18). For concentrations higher than their MICs, benzylpenicillin and ceftobiprole show killing effects (Fig. 1).

Our study was completed by the determination of the 50% inhibitory concentration (IC$_{50}$) values for ceftobiprole, benzylpenicillin, cefepime, and ceftazidime for the different PBPs of *E. faecium* D63r by using purified membrane preparations and fluorescent ampicillin (20, 21). Membrane preparations (300 µg of proteins) were first incubated (20 min at 37°C) with increasing concentrations of ceftobiprole and next incubated (with a saturating concentration of 25 µM fluorescent ampicillin) for an hour at 37°C. The titration of the PBPs by ceftobiprole (Fig. 2 and Table 1) revealed that at a 2-fold MIC, all high-molecular-mass PBPs are inhibited by the antibiotic (Fig. 2) and that the low-molecular-mass protein PBP 6, which acts as a d,d-carboxypeptidase (7), is not affected. The high-molecular-mass protein PBP 2 is the most sensitive to ceftobiprole (IC$_{50}$ = 0.2 µg/ml). PBPs 1 and 3 show similar IC$_{50}$ values (<1 µg/ml), whereas the low-affinity proteins PBP 5fm, PBP 4, and PBP 5* possess slightly higher values (≥1 µg/ml). This pattern of inhibition is completely different from that obtained with benzylpenicillin, for which the resistant protein PBP 5fm is the most insensitive PBP. The IC$_{50}$ for ceftobiprole on purified soluble PBP 5fm (sPBP 5fm) gives results similar to those observed for the membrane preparations (0.7 µg/ml). Note that PBP 4 and PBP 5* exhibit very similar IC$_{50}$ profiles (Table 1). A similar observation was made for PBP 4 and PBP 4* in *Enterococcus hirae*. PBP 4* was shown to be produced by a proteolytic cleavage of the 60 N-terminal amino acid residues of PBP 4 (11). It is very likely that, in the *E. faecium* membranes used here, PBP 5* was the result of an N-terminal truncation of PBP 4.

The kinetic parameters governing the acylation of PBP 5fm by ceftobiprole were determined by using sPBP 5fm (soluble PBP 5fm from which the N-terminal anchoring peptide was removed). It was overproduced and purified as previously described, except that the molecular sieve was eliminated (16). The pseudo-first-order equation was applied (5) (Fig. 3). The opening of the ceftobiprole β-lactam ring was measured at 319 nm with a Specord 200 spectrophotometer (Analytik Jena, Germany) at 30°C in 10 mM phosphate buffer (pH 7.0) with 10 µM sPBP 5fm. $k_a$ (the observed rate constant) was estimated by fitting with $A_f - A_i = (A_0 - A_i)e^{-k_at}$ (where $A_0$ is the initial absorbance, $A_i$ is the absorbance at time $t$, and $A_f$ is the final absorbance). The slope allowed the determination of the value of the second-order rate constant $k_+K$. A higher $k_+K$ value indicates faster acyl-enzyme formation, which means a faster inactivation of the PBP. The 110 ± 11 M$^{-1}$ s$^{-1}$ value reported for the second-order rate constant $k_+K$ obtained for ceftobiprole was 5 to 10 times higher than the value reported for benzylpenicillin (15 to 24 M$^{-1}$ s$^{-1}$), indicating that ceftobiprole inactivated sPBP 5fm faster than benzylpenicillin (21).

### TABLE 1. Inhibition of PBPs from *E. faecium* D63r and D63

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic</th>
<th>IC$_{50}$ (µg/ml) for PBP</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>D63r</td>
<td>Benzylpenicillin</td>
<td>1.6 ± 0.4</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Cefepime</td>
<td>1.7 ± 0.6</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime</td>
<td>0.9 ± 0.6</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Ceftobiprole</td>
<td>0.7 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>D63</td>
<td>Benzylpenicillin</td>
<td>0.9 ± 0.7</td>
<td>0.1 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Ceftobiprole</td>
<td>0.6 ± 0.2</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

* Concentration of the β-lactam antibiotic that inhibits 50% of the fluorescent ampicillin in comparison to the level for a control containing no drug.
this value is 70 times lower than the value obtained for S. aureus PBP 2a acylation (8.900 M\(^{-1}\) s\(^{-1}\)), produced and purified in our laboratory (14). The sPBP 5fm-ceftobiprole adduct was very stable. Indeed, no free enzyme could be detected after 4 h of incubation at 37°C.

In conclusion, ceftobiprole efficiently inhibited the low-affinity protein E. faecium PBP 5 in our penicillin-resistant strain. It demonstrated bactericidal activity against this laboratory-derived ampicillin-resistant E. faecium mutant that overproduced an unmutated PBP 5 protein. This profile is different from that observed for most E. faecium clinical isolates bearing a mutant PBP 5 protein that had reduced affinity, where resistance is reported for all β-lactams, including ceftobiprole, suggesting that simple overexpression of PBP 5 is sufficient to elevate the MIC for ceftobiprole but that amino acid substitutions in the protein are necessary for high-level resistance. Finally, ceftobiprole is, up to now, the best tool for easily determining kinetic parameters of unlabelled β-lactams or for finding new inhibitors by high-throughput screening using the purified sPBP 5fm protein, because of its rapid acylation of the protein and the ability to directly follow the cleavage of its β-lactam ring at 319 nm.

We thank Karen Bush and Anne Marie Queenan from Johnson and Johnson Pharmaceutical Research and Development, LLC, for the gift of ceftobiprole.

This research was supported in part by the Belgian Program on Interuniversity Poles of Attraction, initiated by the Belgian State (grant P6/19); the Actions de Recherche Concertées (grant 03/08-297); the Fonds de la Recherche Fondamentale Collective (grants 6 2.4511.06 and 2.4524.03); the Fonds de la Recherche en Sciences Médicales (grant 7 3.4865.08); the European Union (EUR-INTAFAR [LSHM-CT-2004-512138] and COBRA [LSHM-CT-2003-503335, 6th PCRD] projects); and the R&D of the University of Liège.

REFERENCES