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Inhibition of *Streptococcus pneumoniae* Penicillin-Binding Protein 2x and *Actinomadura* R39 DD-Peptidase Activities by Ceftaroline

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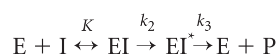
Although the rate of acylation of a penicillin-resistant form of *Streptococcus pneumoniae* penicillin-binding protein 2x (PBP2x) by ceftaroline is 80-fold lower than that of its penicillin-sensitive counterpart, it remains sufficiently high ($k_2/K = 12{,}600\ M^{-1}\ s^{-1}$) to explain the sensitivity of the penicillin-resistant strain to this new cephalosporin. Surprisingly, the *Actinomadura* R39 DD-peptidase is not very sensitive to ceftaroline.

Ceftaroline fosamil, the prodrug of ceftaroline, is a parenteral cephalosporin exhibiting broad-spectrum bactericidal *in vitro* activity against Gram-positive organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Streptococcus pneumoniae*, as well as a few common Gram-negative pathogens (1). Ceftaroline fosamil is approved in the United States for the treatment of acute bacterial skin and skin-structure infections (ABSSSI) and community-acquired bacterial pneumonia (CABP). In the European Union, ceftaroline fosamil is approved for the treatment of complicated skin and soft tissue infections (cSSTI) and community-acquired pneumonia (CAP). In *S. pneumoniae*, mutations affecting penicillin-binding protein 2x (PBP2x) appear to be particularly important in conferring β-lactam resistance (2–4). A study of pneumococcal penicillin-resistant strains (5) showed that they were sensitive to ceftaroline with MICs ranging from 0.015 to 2 mg/liter. Affinities of ceftaroline for *S. pneumoniae* PBPs were measured in binding studies, and 50% inhibitory concentration (IC_{50}) values were determined for PBP2x of one penicillin-sensitive (IC_{50}, 0.1 mg/liter) and three penicillin-resistant (IC_{50}, 0.1 to 1.0 mg/liter) strains. However, with very sensitive PBPs, the IC_{50} values can represent just 50% of the PBP concentration in the assay. In the present study, we determined the second-order rate constants (k_2/K) (see scheme I below) for acylation by ceftaroline of a sensitive PBP2x from strain R6 and a resistant form from strain 5204 (6). The k_2/K value was also determined for the reference *Actinomadura* R39 DD-peptidase, which is usually highly sensitive to β-lactam antibiotics.

The proteins were purified as previously described (7, 8). In brief, PBP2x proteins from strains R6 and 5204 were expressed as fusion proteins to glutathione *S*-transferase with a thrombin-cleavable linker (pGEX-4T1 plasmid) in the host strain *Escherichia coli* MC1061. The cell lysate was loaded onto a glutathione-Sepharose 4B affinity column (GE Healthcare) equilibrated with 50 mM Tris HCl, pH 8, 100 mM NaCl, and 1 mM EDTA. Proteins were eluted by applying one hundred units of human thrombin (Sigma) onto the column. For R39 and R6 PBP2x, the rate of inactivation was measured by the reporter substrate method (9, 10) with thioester S2d in the presence of dithiodipyridine (11, 12). The interaction of PBPs with β-lactams follows a 3-step model, designated scheme I:



$$E + I \overset{K}{\leftrightarrow} EI \overset{k_2}{\rightarrow} EI^* \overset{k_3}{\rightarrow} E + P$$

In this equation, E is the enzyme, I is the inhibitor, EI* is the acylenzyme, K is the dissociation constant, and k_2 and k_3 are first-order rate constants (see reference 9 for a complete kinetic analysis).

For R39, the pseudo-first-order rate constant k_a increased linearly with the ceftaroline concentration ($k_a = k_2\,[I]/K$), yielding a k_2/K value of $3{,}400 \pm 200\ M^{-1}\ s^{-1}$ ($K > 10\ \mu M$). For R6 PBP2x, the reaction was complete within 1 min with 0.1 μM PBP and 1 μM ceftaroline. Hydrolysis of the reporter substrate at a lower PBP concentration could not be recorded with adequate accuracy, so the experiment was performed with equimolar concentrations (0.1 μM) of ceftaroline and PBP. Under these conditions, the concentration of active enzyme ([E]) (measured via the rate of S2d thioester hydrolysis) varies with time (t) according to the equation $1/[E] = 1/E0 + (k_2/K) \times t$, where E0 is the concentration of enzyme at $t = 0$.

Figure 1 shows a plot of 1/[E] versus time for R6 PBP2x. The slope of the line yields a k_2/K value of $1 \times 10^6\ M^{-1}\ s^{-1}$ (± 10%).

Since S2d is not a sufficiently good substrate of 5204 PBP2x, the formation of EI* was followed by monitoring the quenching of the protein fluorescence at 340 nm as done previously (13, 14). With 0.5 μM PBP and 2.5 μM ceftaroline, the reaction also occurred too rapidly to allow a valid determination of k_2/K and the reaction was again studied by using equimolar concentrations (0.5 μM) of the reagents. The slope of the line 1/[E] versus time yielded a k_2/K value of $12{,}600 \pm 1{,}000\ M^{-1}\ s^{-1}$ (Fig. 2).

The deacylation rate constants (k_3) were determined at 25°C after complete inactivation of the PBPs with 100 μM ceftaroline. The residual free ceftaroline was hydrolyzed in less than 5 min by the VIM-4 β-lactamase (15), and the reactivation of PBPs was followed by measuring the recovered activity at different times up to about 150 min as previously described (16).

The k_3 values of R39 and R6 PBP2x were negligible, since no

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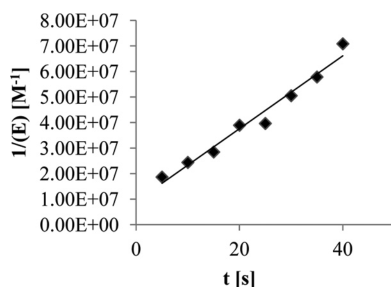

FIG 1 Plot of 1/[E] versus time for the interaction between 0.1 μM R6 PBP2x and 0.1 μM ceftaroline. Incubation was performed at 30°C in 10 mM sodium phosphate buffer, pH 7.0, in the presence of 1 mM S2d and 0.5 mM 4,4′-dithiodipyridine. The hydrolysis of S2d was monitored at 324 nm. The rate of spontaneous hydrolysis of S2d was subtracted.

significant reactivation of the enzymes was recorded over a 60-min period after eliminating excess ceftaroline (reactivation was <10% after 2 h; $k_3 < 1.5 \times 10^{-5}$ s^{-1}; half-life [$t_{1/2}$], >12 h). The k_3 value for 5204 PBP2x ($t_{1/2}$ = 53 min) was $2.2 \times 10^{-4} \pm 0.4 \times 10^{-4}$ s^{-1}. However, the k_3 value remained too low to result in a major error in the determination of the k_2/K value and is unlikely to affect the efficiency of ceftaroline as an inactivator of the resistant PBP2x (PBP2x^r). Indeed, a simple calculation indicates that at a 1 μM concentration of ceftaroline at steady state, the inactivated adduct represents 98% of the resistant PBP2x versus more than 99% of the sensitive one. Such an increase of the k_3 value was already described (17) for the adduct formed between cefotaxime and PBP2x^r from a resistant clinical isolate (CS109).

These results show that ceftaroline is a particularly efficient inactivator of the transpeptidase activity of R6 PBP2x. Indeed, a higher value of k_2/K yields an increased rate of PBP inactivation and a decreased MIC value if the PBP is an important target in the strain (Table 1). Surprisingly, the second-order inactivation rate constant for R6 is 300-fold greater than that of R39, which is generally considered a highly β-lactam-sensitive PBP and was included in the present study for this reason.

Although it is 80-fold less than that of the sensitive PBP2x, the k_2/K value of the penicillin-resistant 5204 PBP2x (12,600 M^{-1} s^{-1}) remains quite high in comparison to the value obtained by Carapito (6) for cefotaxime and the same PBP (85 M^{-1} s^{-1}). This nicely explains the much lower MIC of 5204 for ceftaroline compared to that for cefotaxime (Table 1).

In consequence, ceftaroline seems to be particularly well

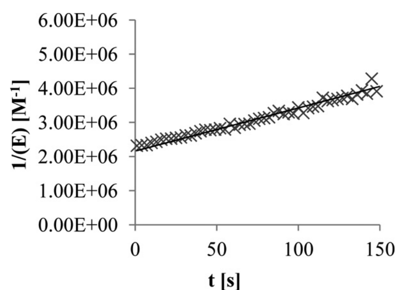

FIG 2 Plot of 1/[E] versus time for the interaction between 0.5 μM 5204 PBP2x and 0.5 μM ceftaroline. The formation of EI* was followed by monitoring the quenching of the protein fluorescence at 340 nm in 10 mM sodium phosphate buffer, pH 7.0, at 30°C.

TABLE 1 MICs and k_2/K second-order rate constants

	MIC (μg/ml) and PBP2x k_2/K (M^{-1} s^{-1}) (reference) for:			
	S. pneumoniae R6		*S. pneumoniae* 5204	
Compound	MIC	k_2/K	MIC	k_2/K
Ceftaroline	0.0125	1×10^6	0.5–0.75	12,600
Cefotaxime	<0.016 (18)	100,000 (6)	12 (18)	85 (6)
Penicillin G	0.01–0.05 (11)	110,000 (11)	6.0 (11)	104 (11)

adapted for inactivating the transpeptidase activity of *S. pneumoniae* PBP2x, even for mutants that exhibit high levels of resistance to other β-lactams. Table 1 compares the kinetics of PBP2x inactivation with MIC values of the two strains for ceftaroline and two reference compounds. The general agreement is excellent.

Finally, on the basis of the very high k_2/K value of R6 PBP2x, it can be concluded that the IC$_{50}$ for ceftaroline measured after 10 min would probably correspond to 50% of the PBP concentration in the assay. Indeed, at the IC$_{50}$ observed with penicillin-sensitive PBP2x (0.1 mg/liter, i.e., 0.17 μM) (5), the half-reaction time would be about 4 s. Even with a 10-fold-lower k_2/K value, the half-reaction time would be 40 s, and after 10 min, the reaction should be complete. This shows that the IC$_{50}$ values can underestimate the sensitivity of very sensitive PBPs.

In summary, there is a good correlation between PBP2x sensitivity and the MIC values for ceftaroline. The latter are quite low even for the β-lactam-resistant 5204 strain. Even for the 5204 PBP2x, a 1 μM ceftaroline concentration would result in a half-inactivation time of 69 s. This is certainly sufficiently rapid to kill the bacterium. A 1 μM concentration corresponds to 0.6 mg liter^{-1}, which should easily be obtained after administration of the compound.

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