Beta-Lactamase Stability of HR 756, a Novel Cephalosporin, Compared to That of Cefuroxime and Cefoxitin

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The stability to β-lactamase hydrolysis of HR 756, a new cephalosporin antibiotic, was compared to the β-lactamase stability of cefoxitin and cefuroxime. HR 756, cefoxitin, and cefuroxime were not hydrolyzed by Richmond type I, III, IV, and V β-lactamases. Antibacterial activity of HR 756 correlated well with resistance to β-lactamase hydrolysis except against Pseudomonas aeruginosa. HR 756, cefoxitin, and cefuroxime inhibited type I β-lactamases, but not type III, IV, or V enzymes. HR 756 was the most active inhibitor.

Cephalosporin antibiotics are important chemotherapeutic agents because of their broad antibacterial spectrum, which includes many species of gram-negative organisms that have become the major species causing hospital infections in the past 2 decades. Changes in the chemical structure of the cephalosporins have resulted in an increased antibacterial spectrum, more useful pharmacokinetic properties, and, in some cases, a high level of resistance to β-lactamase hydrolysis. The presence of β-lactamases in gram-negative organisms has been associated with resistance to cephalosporins, although these enzymes are not the only factor contributing to the resistance of gram-negative bacilli to β-lactam compounds (3, 9, 10, 15). There is great interest in antibiotics that are resistant to β-lactamase hydrolysis and in compounds that are inhibitors of β-lactamases (2, 5, 11). Certain β-lactamases of gram-negative bacilli are inhibited by β-lactam antibiotics such as cloxacillin or methicillin (11). Recently, naturally occurring inhibitors of β-lactamases produced by streptomycetes have been discovered (1, 14). Cefoxitin and cefuroxime, two new β-lactam compounds, have been reported to be resistant to β-lactamases of gram-negative bacilli (3, 12).

HR 756 (Fig. 1) has been shown to be active against many bacteria resistant to the older cephalosporins (6; H. C. Neu, N. Aswapokee, and K. P. Fu, manuscript in preparation). We wished to assess the β-lactamase resistance of this compound and to compare its resistance to β-lactamase hydrolysis with the resistance of cefoxitin and cefuroxime.

MATERIALS AND METHODS

Organisms. Shigella sonnei 10-101, Pseudomonas aeruginosa 3901, Proteus morganii 771, Proteus mirabilis 3378, Citrobacter freundii 2732, Klebsiella pneumoniae 3973, and P. aeruginosa 1822 were clinical isolates that had been selected for study on the basis of their resistance to one or more β-lactam antibiotics and the presence of β-lactamases (4).

Antibiotics. Cephaloridine, cefalothin, and cefazolin were gifts from Lilly Research Laboratories. Cefoxitin was supplied by Merck Sharp & Dome. Cefuroxime was a gift of Glaxo Research Ltd., England, and HR 756 was obtained from Hoechst-Roussel, New Jersey. Antibacterial activity was determined in Mueller-Hinton broth using an inoculum of 10⁶ colony-forming units. Synergy tests were determined by a checker-board method as described previously (4).

β-Lactamase preparation. The Shigella 10-101 and Pseudomonas 1822 β-lactamases were plasmid-mediated, constitutive enzymes. P. morganii 771 β-lactamase was a constitutive enzyme. Klebsiella 3973 β-lactamase was induced by 25 µg of cephalothin per ml. P. mirabilis 3378 β-lactamase was induced by 50 µg of cephaloridine per ml. Citrobacter 2732 β-lactamase was induced by 25 µg of cephaloridine per ml, and Pseudomonas 3901 β-lactamase was induced by 500 µg of penicillin G per ml. Overnight cultures were diluted 10-fold into 300 ml of brain heart infusion broth and grown for 2 h in a gyratory shaker at 37°C. Inducers were added, and the organism was grown for another 3 h. The cultures were harvested by centrifugation, washed twice with phosphate buffer (0.05 M; pH 7.0), and suspended in phosphate buffer at a 10-fold concentration. The bacteria were sonically disrupted by a Branson sonifier for three 30-s intervals in an ice bath. The extracts were centrifuged for 2 h at 4°C at 40,000 × g, and the supernatant was dialyzed against phosphate buffer. The dialyzed material was used as the β-lactamase preparation. Pseudomonas 1822 and P. morganii 771 preparations were purified to homogeneity through diethylaminoethyl-cellulose and hydroxyapatite column chromatography as described previously (8).

Assay of β-lactamase. β-Lactamase activity was determined by the spectrophotometric method in a temperature-controlled spectrophotometer at 30°C.
The reaction mixture contained 0.5 ml of 0.2 mM cephalosporin in 0.5 ml of phosphate buffer (0.05 M, pH 7.0). The decrease in optical density at 255 nm was recorded after addition of 15 to 50 μl of crude or purified β-lactamase. Inhibition of the hydrolysis of cephaloridine was determined by addition of cefoxitin, cefuroxime, or HR 756 to the reaction mixture. Enzyme was added last. Kinetic parameters were estimated from a least-squares fit to Lineweaver-Burk plots with substrate concentrations of 0.05 to 0.2 mM.

RESULTS

The in vitro susceptibility of the organisms used is given in Table 1. HR 756 was the most active cephalosporin. Only the Pseudomonas isolates, with inhibitory levels of 50 μg/ml, might be considered resistant to HR 756. All of the organisms except the Shigella 10-101 were resistant to cephaloridine, cephapenthin, and cefazolin.

![Chemical structure of HR 756](image)

**Fig. 1. Chemical structure of HR 756.**

<table>
<thead>
<tr>
<th>Organism, enzyme type</th>
<th>Minimum inhibitory concn (μg/ml) of drug:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cephaloridine</td>
</tr>
<tr>
<td><em>C. freundii</em> 2732, type I</td>
<td>100 50 50</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 3901, type I</td>
<td>&gt;800 &gt;800 &gt;800</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 771, type I</td>
<td>&gt;400 &gt;400 &gt;400</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 1822, type III</td>
<td>&gt;800 &gt;800 &gt;800</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> 3973, type V</td>
<td>200 100 50</td>
</tr>
<tr>
<td><em>P. mirabilis</em> 3378</td>
<td>200 100 200</td>
</tr>
<tr>
<td><em>S. sonnei</em> 10-101, type V</td>
<td>50 200 3.1</td>
</tr>
</tbody>
</table>

*Richmond classification.

**Table 2. β-lactamase hydrolysis of HR 756 compared with other known cephalosporins**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Type of β-lactamase</th>
<th>Cephaloridine (μmol/min)</th>
<th>Cephalothin</th>
<th>Cefazolin</th>
<th>HR 756, cefoxime, cefuroxime</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella 3973</td>
<td>Induced; type IV</td>
<td>13.7</td>
<td>8.6</td>
<td>63</td>
<td>3.8</td>
</tr>
<tr>
<td>Citrobacter 2732</td>
<td>Induced; type I</td>
<td>4.76</td>
<td>4.70</td>
<td>98</td>
<td>2.98</td>
</tr>
<tr>
<td>Pseudomonas 1822</td>
<td>Type III; plasmid</td>
<td>3.56</td>
<td>0.60</td>
<td>17</td>
<td>0.39</td>
</tr>
<tr>
<td><em>P. mirabilis</em> 3378</td>
<td>Induced</td>
<td>12.06</td>
<td>3.03</td>
<td>25</td>
<td>0.21</td>
</tr>
<tr>
<td><em>P. mirabilis</em> 771</td>
<td>Type I</td>
<td>4.9</td>
<td>1.11</td>
<td>227</td>
<td>2.58</td>
</tr>
<tr>
<td>Shigella 10-101</td>
<td>Type V; plasmid</td>
<td>3.9</td>
<td>0.91</td>
<td>23</td>
<td>0.4</td>
</tr>
<tr>
<td>Pseudomonas 3901</td>
<td>Induced</td>
<td>5.1</td>
<td>8.3</td>
<td>163</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* β-Lactamase activity was determined by the spectrophotometric method, measuring optical density decreased at 255 nm at 30°C. Reaction mixture contained 0.5 ml of 0.4 mM cephalosporin substrate in 0.5 ml of phosphate buffer (0.05 M, pH 7).

* These values do not indicate the relative activity of different organisms.

* RHR, Relative hydrolysis rate compared with cephaloridine, which is given a value of 100.
an induced Citrobacter β-lactamase. Cefoxitin, cefuroxime, and HR 756 at equimolar concentrations inhibited 50% of the hydrolysis of cephaloridine by the Pseudomonas 3901 β-lactamase. We did not find inhibition of the β-lactamase activity of Shigella 10-101 (type V), Pseudomonas 1822 (type III), and P. mirabilis 3378 by these three compounds. Cefoxitin did not inhibit hydrolysis of cephaloridine by the Klebsiella β-lactamase tested at equimolar concentrations, but cefuroxime and HR 756 inhibited 67 and 84% of the β-lactamase activity.

P. morganii 771 and Citrobacter 2732 β-lactamases were used to determine the kinetics of the inhibitory activity of cefoxitin, cefuroxime, and HR 756 (Fig. 2). HR 756 and cefuroxime were of equal effectiveness as inhibitors of the two β-lactamases: both inhibited 80% of activity at a concentration of 0.1 μM, whereas cefoxitin was approximately 50-fold less effective than the other two agents, inhibiting 80% of activity at the concentration of 5 μM. Cefoxitin and cefuroxime were competitive inhibitors of both the type I β-lactamases (Fig. 3 and 4). HR 756 was a noncompetitive inhibitor of the β-lactamase of Citrobacter (Fig. 3) and was a competitive inhibitor of the β-lactamases of P. morganii (Fig. 4). HR 756 had Ki values of $1.17 \times 10^{-3}$ μM for Proteus β-lactamase and $7.1 \times 10^{-4}$ μM for Citrobacter β-lactamase, indicating HR 756 is a potent inhibitor although not of the order of the semisynthetic penicillins. Cefoxitin had Ki of 1.42 μM for the Proteus enzyme and 1.17 μM for Citrobacter enzyme. Cefuroxime had Ki of $4 \times 10^{-4}$ μM for Proteus and $1.26 \times 10^{-2}$ μM for the Citrobacter enzyme.

To determine whether the β-lactamase inhibition produced by HR 756 was significant in intact bacteria, HR 756 was combined with ampicillin, cephalothin, and cephaloridine and tested against P. mirabilis, Citrobacter, and Klebsiella. Table 4 shows the minimal bactericidal concentrations. Although HR 756 inhibited the β-lactamase of Citrobacter, it did not act synergistically with any of the three compounds. The minimal bactericidal concentration of HR 756 against C. freundii 2732 was greater in the presence of cephalothin. HR 756 acted synergistically against P. mirabilis with ampicillin but not with cephaloridine or cephalothin. HR 756

### Table 3. Inhibition of β-lactamase hydrolysis of cephaloridine by HR 756 compared with cefoxitin and cefuroxime

<table>
<thead>
<tr>
<th>Organism</th>
<th>Type of β-lactamase</th>
<th>Cephaloridine (μmol/min)</th>
<th>Cephaloridine + cefoxitin (μmol/min)</th>
<th>Cephaloridine + cefuroxime (μmol/min)</th>
<th>Cephaloridine + HR 756 (μmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrobacter 2732</td>
<td>Type I induced</td>
<td>4.76</td>
<td>4.1</td>
<td>1.88</td>
<td>1.82</td>
</tr>
<tr>
<td><em>P.</em> morganii 771</td>
<td>Type I constitutive</td>
<td>5.11</td>
<td>4.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>P.</em> morganii 2733</td>
<td>Type III constitutive</td>
<td>5.74</td>
<td>4.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>P.</em> mirabilis 3378</td>
<td>Induced</td>
<td>6.2</td>
<td>5.4</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Klebsiella 3973</td>
<td>Type IV induced</td>
<td>5.8</td>
<td>4.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>K.</em> pneumonia 10-101</td>
<td>Type V constitutive</td>
<td>3.7</td>
<td>2.5</td>
<td>1.6</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Reaction mixture contains 0.5 ml of 0.2 mM cephaloridine plus 0.5 ml of 0.05 M phosphate buffer (pH 7) as control or plus 0.5 ml of 0.2 mM cefoxitin, cefuroxime, and HR 756.

* RHR, Relative hydrolysis rate. See Table 2, footnote c.

![Fig. 2. Inhibition of β-lactamase hydrolysis of cephaloridine by cefoxitin, cefuroxime, and HR 756. Symbols: (●) Citrobacter 2732; (A) P. morganii β-lactamases.](http://aac.asm.org/)
showed no synergy against *Klebsiella* when combined with other antibiotics.

**DISCUSSION**

HR 756, a novel cephalosporin, has been shown to be as resistant to hydrolysis by the common gram-negative \(\beta\)-lactamases as are cefoxitin and cefuroxime. These findings suggest that the 7\(\alpha\) methoxy group is not crucial in determining \(\beta\)-lactamase resistance, as has been postulated for the \(\beta\)-lactamase stability of cefoxitin (3, 7).

Darland and Birnbaum (3) and Olsson et al. (13) reported that cefoxitin was a competitive inhibitor of *Bacteroides* \(\beta\)-lactamases. Our results also indicate that cefoxitin is a competitive inhibitor of Richmond type I \(\beta\)-lactamases, but that HR 756 and cefuroxime are more potent inhibitors.

Cole et al. (2) reported that some semisynthetic penicillins highly resistant to the \(\beta\)-lactamase of *Escherichia coli* and *Klebsiella aerogenes* were not inhibitors. Although cefoxitin, cefuroxime, and HR 756 were resistant to \(\beta\)-lactamase hydrolysis of type III, IV, and V enzymes, they did not inhibit the enzymes, suggesting that there may not be a correlation between \(\beta\)-lactamase stability and effectiveness as inhibitors. Since cefoxitin, cefuroxime, and HR 756 inhibited *Proteus* and *Citrobacter* type I \(\beta\)-
Table 4. Bactericidal activity of HR 756 combined with other \(\beta\)-lactam antibiotics

<table>
<thead>
<tr>
<th>Organism</th>
<th>Minimum bactericidal conc ((\mu)g/ml) of antibiotic:</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR 756</td>
<td>Ampicillin</td>
<td>Cephalothin</td>
<td>Cephaloridine</td>
<td>HR 756 + ampicillin</td>
<td>HR 756 + cephalothin</td>
<td>HR 756 + cephaloridine</td>
</tr>
<tr>
<td>P. mirabilis 3378</td>
<td>12.5</td>
<td>(\geq 800)</td>
<td>200</td>
<td>(\geq 800)</td>
<td>1.6/1.6(^a)</td>
<td>12.5/12.5</td>
<td>6.3/6.3</td>
</tr>
<tr>
<td>K. pneumoniae 3973</td>
<td>12.5</td>
<td>(\geq 800)</td>
<td>400</td>
<td>200</td>
<td>12.5/12.5</td>
<td>12.5/0.2</td>
<td>6.3/6.3</td>
</tr>
<tr>
<td>C. freundii 2732</td>
<td>0.8</td>
<td>400</td>
<td>400</td>
<td>400</td>
<td>0.8/0.8</td>
<td>3.1/200</td>
<td>1.6/100</td>
</tr>
</tbody>
</table>

\(^a\) First number is minimum bactericidal concentration of HR 756; second is that of the other agent.

enzymes but were not substrates, it is suggested that they did bind to the active site in the enzymes, whereas with type III, IV, and V enzymes they did not bind to these types of \(\beta\)-lactamases. Since HR 756 did not act as a Klebsiella \(\beta\)-lactamase inhibitor, it was not surprising that it did not act synergistically against Klebsiella when combined with other \(\beta\)-lactam antibiotics. Its lack of synergy with \(\beta\)-lactam antibiotics against Citrobacter may be related to poor transport of the other agents to their receptor sites.

LITERATURE CITED