Affinity of Cephalosporins for β-Lactamases as a Factor in Antibacterial Efficacy

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Strains of Escherichia coli, Enterobacter aerogenes, and Enterobacter cloacae that were resistant to ceftazidime (MIC > 16 μg/ml) but susceptible to BMY 28142 (MIC < 4 μg/ml) were found to contain higher levels of β-lactamase activity (50- to 3,340-fold) than control strains of the corresponding species. Ceftazidime was at least as resistant as BMY 28142 to hydrolysis by these enzymes. However, the apparent Kᵣ values of BMY 28142 for each enzyme were lower (8- to 20-fold) than that of ceftazidime; i.e., the affinity of these enzymes for BMY 28142 appeared to be lower than that for ceftazidime. Thus, BMY 28142 was affected less than ceftazidime by a mechanism of resistance that depends, at least in part, on the relative affinities of cephalosporins for the β-lactamases of these species. These results indicate that the affinity between a β-lactamase and a cephalosporin may be a distinguishing factor in the evaluation of β-lactamase-resistant cephalosporins and suggest that affinity can play a major role in susceptibility to highly β-lactamase-resistant cephalosporins.

During routine evaluation of a new cephalosporin, BMY 28142 (S), occasional clinical isolates of Escherichia coli, Enterobacter aerogenes, and Enterobacter cloacae, as well as the P-99 strain of E. cloacae, were found to be resistant (MIC > 16 μg/ml) to ceftazidime but susceptible to BMY 28142 (MIC < 4 μg/ml). Resistance to cephalosporins that are intrinsically stable to β-lactamase hydrolysis has been correlated with high levels of β-lactamase activity (1, 2, 9-11, 14). The concept of trapping or nonhydrolytic interference was proposed to explain this apparently paradoxical phenomenon (8, 10, 11, 14, 15). Criticism of this concept, based on the premise that for this mechanism to operate the total number of β-lactamase molecules must exceed the number of β-lactam molecules (9), does not take into consideration the presence of the outer membrane barrier, as discussed by Yu and Nikaido (13). Their analysis, based on outer membrane permeability, kinetic parameters of the enzyme, affinity toward penicillin-binding proteins, and MIC, suggests that hydrolysis may be more important than nonhydrolytic binding for β-lactams such as cefoperazone, cefoxitin, and cefotaxime (13). However, the results of their calculations for ceftazidime, which is much more resistant to hydrolysis than the other three cephalosporins, were less conclusive with respect to hydrolysis versus nonhydrolytic interference as the major determinant of resistance in the single E. cloacae strain that they examined.

We therefore investigated the contribution of β-lactamase-related factors to the difference in the relative activity of ceftazidime and BMY 28142 against some of these ceftazi-
dime-resistant isolates. For each species one to three strains resistant to ceftazidime but susceptible to BMY 28142 were compared with a control strain for which MICs of BMY 28142 and ceftazidime were similar to the respective MIC₅₀₅ (MICs for 50% of the strains) of the two cephalosporins for the species (5).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. All strains whose designations start with "A" were clinical isolates; they were received from the following individuals: G. Bodey (A25049), received as a ceftazidime-resistant strain, no. 289), T. Gavan (A21160, received as culture G1320), E. Konopka (A20942), M. J. Madden (A15154), and L. Sabath (A21046, received as culture 94201). M. H. Richmond supplied strains 214, 719, and P-99. R. Bower supplied the Escherichia coli Juhl strain.

Chemicals. BMY 28142, sulfate salt, was prepared at Bristol-Myers Research Institute, Tokyo, Japan, and Bristol-Myers Co., Syracuse, N.Y. Cefazidime was generously provided by Glaxo Group Research, Ltd., Greenford, U.K. Nitrocefin was purchased from BBL Microbiology Systems, Cockeysville, Md. Cephaloridine was obtained from Sigma Chemical Co., St. Louis, Mo. All other reagents were of the highest purity commercially available.

Determination of MIC. MICs were determined on Mueller-Hinton medium by the serial twofold dilution technique as previously described (5).

Bacterial growth, β-lactamase release, and quantitation. Cells from cultures grown in brain heart infusion broth (BBL Microbiology Systems) to the late exponential phase were harvested by centrifugation (16,300 × g, 10 min, 4°C), suspended in 50 mM KH₂PO₄ (pH 7.0), sedimented again (31,000 × g, 10 min, 4°C), resuspended in 100 mM acetic acid-4 mM EDTA (pH 5.0) (0.33 g [wt wt] ml⁻¹), and frozen, using a dry ice-acetone bath. After thawing and two additional cycles of freezing and thawing, cells and debris were removed by centrifugation (31,000 × g, 15 min, 4°C). Proteases in the supernatant were inhibited with phenylmethylsulfonyl fluoride and pepstatin A added to concentrations of 0.5 mM and 2 μM, respectively. The freeze-thaw
procedure yielded β-lactamase activity equivalent to that released by sonic disruption of the cells (unpublished data) but with a 20-fold-higher specific activity. Residual cell-associated activity was not detected in those strains that have been tested. The relative activity in the freeze-thaw supernatant was determined by spectrophotometric assay (Hewlett-Packard 8430A spectrophotometer), using nitrocefin (6) as the substrate at 0.1 mM in 50 mM KH2PO4 (pH 7.0) at 25°C. Activity per cell was calculated by dividing the total activity in the crude supernatant preparation by the viable cell count determined at the time of harvesting.

Thin-layer isoelectric focusing. The freeze-thaw supernatant from each bacterial strain was analyzed by thin-layer isoelectric focusing on LKB Ampholine polyacrylamide plates, pH 3.5 to 9.5, at 4°C, using the LKB 2117 Multiphor and LKB 2103 power supply. After pre-equilibration for 15 min at 30 W, 1,800 V, and 50 mA, samples were loaded and run for 2 h in the constant-power mode at the same settings. β-Lactamase activity was visualized by soaking the gel in 1mM nitrocefin in 50 mM KH2PO4, pH 7.0.

Purification of β-lactamases. Enzymes were purified from freeze-thaw preparations by chromatography on carboxymethyl-Trisacryl M, PBE-94, and Bio-Gel-P60 D. D. Carlton, M. J. M. Hitchcock, and D. J. Phelps, manuscript in preparation). Purified enzymes (10- to 26-fold purification from freeze-thaw supernatants) were used for the determination of all kinetic parameters.

Assay of β-lactamase activity. β-Lactamase activity was measured spectrophotometrically in 50 mM KH2PO4 at pH 7.0. Relative rates of hydrolysis were determined at an initial substrate concentration of 0.1 mM. The wavelengths and molar extinction coefficients were as follows: BMY 28142, ΔE at 260 nm = 8,200; cephaloridine, ΔE at 270 nm = 8,340; and nitrocefin, ΔE at 510 nm = 19,000. All assays were run at 25°C. Km values for nitrocefin were calculated from Lineweaver-Burk plots. Apparent Ks were calculated by the method of Dixon (6) from the rates of hydrolysis of nitrocefin in the presence of various concentrations of BMY 28142 and ceftazidime. Nitrocefin and “inhibitor” were added simultaneously to yield values reflecting initial binding interactions.

High-pressure liquid chromatography. High-pressure liquid chromatography analysis was done on a Waters C18 μ-Bondapak reversed-phase column, using isocratic elution with 10% methanol in 50 mM KH2PO4, pH 7.0. An Hitachi 100-40 spectrophotometer (280-nm wavelength) was the detection instrument.

RESULTS AND DISCUSSION

The MIC of BMY 28142 was eightfold lower than that of ceftazidime for the ceftazidime-susceptible strains (Table 1, first strain of each species); for ceftazidime-resistant strains, the MIC differential was 32- to 125-fold greater. Each of these strains contained a single β-lactamase as determined by thin-layer isoelectric focusing of crude supernatant preparations. Based on their reactivity profiles (data not shown) and pl (Table 1), all were class 1 cephalosporinases (7). The pls of the enzymes from the E. cloacae strains varied between 8.2 and 9.0, but the three E. aerogenes enzymes all had a pl of 8.5 and the two Enterobacter cloacae enzymes had a pl of 9.2. Within each species, purified enzymes had similar Km values for nitrocefin. The variations could be within experimental limits or reflect minor differences in enzymes. When enzymatic degradation of 0.1 mM BMY 28142 or ceftazidime was detectable, their rates of hydrolysis were comparable and <0.25% of the rate of hydrolysis of cephaloridine (Table 1). Only with the P-99 enzyme could any significant difference in the rates of hydrolysis of BMY 28142 and ceftazidime be shown. For this one enzyme the rate of hydrolysis of ceftazidime was eightfold slower than that of BMY 28142.

According to Vu and Nikaido (13), hydrolysis rates at substrate concentrations of 0.1 to 1 μM have more physiological relevance. Spectrophotometric measurement of hydrolysis rates of BMY 28142 and ceftazidime at 1 μM in 10-cm cuvettes in the HP 8430A spectrophotometer did not give reproducible results due to physical problems. Since the major question was whether ceftazidime was hydrolyzed to a greater extent than BMY 28142 at 1 μM, we measured by high-pressure liquid chromatography the amounts of sub-

TABLE 1. Relation of antimicrobial activity to relative β-lactamase activity and affinity

<table>
<thead>
<tr>
<th>Organism/strain</th>
<th>MIC (μg/ml)</th>
<th>β-Lactamase parameters</th>
<th>Rate of hydrolysis</th>
<th>Relative activity</th>
<th>Apparent Ks (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMY</td>
<td>TAZ</td>
<td>pl</td>
<td>Ks (μM)</td>
<td>BMY</td>
</tr>
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<td>Escherichia coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Juhl 719</td>
<td>0.03</td>
<td>0.25</td>
<td>9.2</td>
<td>195</td>
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</tr>
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<td>0.25</td>
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<tr>
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<td>8.2</td>
<td>66</td>
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<tr>
<td>214</td>
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<td>&gt;125</td>
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<tr>
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<td>125</td>
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<tr>
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<td>8.5</td>
<td>145</td>
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<tr>
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<td>63</td>
<td>8.5</td>
<td>156</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* BMY, BMY 28142; TAZ, ceftazidime.

\* Spectrophotometric assay using nitrocefin (see Materials and Methods).

Results are expressed relative to the lowest specific activity (E. coli strain 1) equaled to 1.

ND, Not done.
strate and hydrolysis product in reaction mixtures of the P-99 enzyme and either cephaloradin (1 μM) incubated at 25°C for 5, 15, and 30 min. At an enzyme concentration (0.24 U mL⁻¹; 1 U = 1 μmol of nitrocefin hydrolyzed min⁻¹) sufficient to hydrolyze 1 μM cephaloridine by >50% within 5 min, no hydrolysis of BMY 28142 or ceftazidime was observed after 30 min (sample size, 2 ml; limit of detection, ∼1%). Thus, significant hydrolysis, i.e., enough to influence susceptibility of the organism to ceftazidime, did not occur at 1 μM under the standard assay conditions used. Attempts to demonstrate product inhibition with hydrolyzed BMY 28142 were unsuccessful.

A major, consistent difference between the ceftazidime-resistant and ceftazidime-susceptible strains was the large differential in β-lactamase activity per cell. β-Lactamase activities were 50- to 3,340-fold higher in the crude supernatant preparations from ceftazidime-resistant strains than in those from ceftazidime-susceptible strains (Table 1). However, since ceftazidime was at least as stable as BMY 28142 to the degradative action of these enzymes, the greater MIC differential for the ceftazidime-resistant strains did not appear to be related to amplification of small differences in the rates of hydrolysis of the two cephaloradins.

The affinity of BMY 28142 for these β-lactamases, as estimated from the apparent inhibition constants (3) for nitrocefin hydrolysis, appeared to be much lower than that of ceftazidime. The apparent K₅ for BMY 28142 (8 to 115 μM) were at least 8-fold, and in most instances 20-fold, lower than those for BMY 28142 (≥1 mM for five and 180 to 460 μM for three of the eight enzymes tested). Although the ceftazidime-resistant and ceftazidime-susceptible strains are unrelated and possibly produce minor variants of similar enzymes within each species, the consistent correlation of a larger MIC differential between ceftazidime and BMY 28142 with production of higher amounts of β-lactamases, all having lower affinities for BMY 28142 than ceftazidime in six strains of three species, as compared with randomly selected control strains, is in itself remarkable. These data are consistent with the concept of a nonhydrolytic barrier mechanism of resistance (4, 8, 10, 12, 13). The lower affinity of BMY 28142 may result in less interaction with β-lactamases and thus less limitation of access to the target binding proteins. A similar correlation of lower MIC with lower affinity for β-lactamases has been reported for cephrimyc (HR 810) in comparison to cefotaxime for E. cloacae (11). Cephrimyc had both a lower MIC for several strains of E. cloacae and lower affinity for the β-lactamases from these strains despite being slightly less stable than cefotaxime to enzymatic hydrolysis (11).

Alternately, hydrolysis could provide the dominant contribution if the rate of hydrolysis/rate of penetration quotient (at equilibrium) was significantly greater for ceftazidime than for BMY 28142. In view of the difficulties in directly measuring rates of penetration of unlabeled, slowly hydrolyzed compounds into intact, growing cells and accurately assessing the kinetic characteristics of β-lactamases in situ, i.e., within the periplasm, this is currently a moot point with respect to compounds such as ceftazidime and BMY 28142. However, the elegant studies of Nikaido and co-workers (e.g., reference 13) have demonstrated the likely predominance of hydrolysis in resistance to certain other β-lactams. Application of those techniques may lend support to the hydrolytic alternative.

Whether the permeability of the outer membrane and the array of β-lactam-binding proteins is the same in the overproducers as in the control strains, and thus whether only the high levels of β-lactamase are integral to the resistance of these strains, remains to be determined. Restricted permeability is necessary (13) regardless of whether hydrolysis or nonhydrolytic interferences has the greater influence on the difference in susceptibility to ceftazidime and BMY 28142. Higher permeability restriction per se was probably not a major factor, since the MICs of ceftazidime and BMY 28142 both were two- to fourfold higher for ompB mutants than for parental strains of Escherichia coli (kindly provided by R. Hancock); thus, the ratios of the MICs were unchanged, quite unlike the observations described above. More detailed studies with the ceftazidime-resistant strains will be required to address this question directly.

Regardless of mechanism, these results show that the activity of BMY 28142 against strains which produce high levels of β-lactamase is not reduced to the same extent as the activity of ceftazidime or cefotaxime (9). Hence, compounds that have a low affinity for chromosomally encoded β-lactamases, in addition to high resistance to enzymatic hydrolysis, may represent an important therapeutic advance beyond currently available β-lactam antibiotics.

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LITERATURE CITED

11. Then, R. L., and P. Angehrn. 1982. Trapping of nonhydrolyz-


