Interactions of Biapenem with Active-Site Serine and Metallo-β-Lactamases

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Biapenem, formerly LJC 10,627 or L-627, a carbapenem antibiotic, was studied in its interactions with 12 β-lactamases belonging to the four molecular classes proposed by R. P. Ambler (Philos. Trans. R. Soc. Lond. Biol. Sci. 289:321–331, 1980). Kinetic parameters were determined. Biapenem was readily inactivated by metallo-β-lactamases but behaved as a transient inhibitor of the active-site serine enzymes tested, although with different acylation efficiency values. Class A and class D β-lactamases were unable to confer in vitro resistance toward this carbapenem antibiotic. Surprisingly, the same situation was found in the case of class B enzymes from Aeromonas hydrophila AE036 and Bacillus cereus 5/B/6 when expressed in Escherichia coli strains.

It has been clearly shown that bacterial resistance to β-lactam antibiotics is often a multifactorial event in which outer membrane impermeability and β-lactamase production by gram-negative bacteria play central roles (39).

β-Lactamate degradation of β-lactam antibiotics represents one of the most important biochemical mechanisms of resistance to these molecules in bacteria (19). These enzymes, which are bacterial hydrolases (EC 3.5.2.6), have been classified into four different molecular classes on the basis of their primary structures and catalytic mechanisms (1). Enzymes of classes A, C, and D exert their catalytic activity by a reactive serine residue in the active site, while class B β-lactamases are metalloproteins which require a divalent transition metal ion for their activity, most often Zn2++. Antibiotic pressure has only selected bacteria able to elaborate two or more specific chromosomally encoded or plasmid-mediated β-lactamases which are active against a wide range of β-lactam compounds.

An expanded spectrum of β-lactams has been developed over the past 20 years in order to overcome bacterial resistance to molecules previously developed for clinical use. Carbapenem antibiotics are reported to be very stable in the presence of active-site serine β-lactamases, with the exception of class C enzymes produced by members of the family Enterobacteriaceae which exhibit slow antibiotic hydrolysis (25). This phenomenon, combined with a reduced permeability of the bacterial outer membrane, leads to resistance in hyperproducing strains of Enterobacter cloacae and Pseudomonas aeruginosa. These novel antibiotics also show a broad range of activity against gram-negative and gram-positive bacteria (22, 30), with the exception of Xanthomonas maltophilia, which produces a chromosomally encoded class B β-lactamase (4, 36) which is able to inactivate all β-lactam antibiotics except aztreonam (14). Moreover, carbapenem antibiotics have variable stability to hydrolysis by mammalian dehydropeptidase-I (18, 20, 21, 34). For this reason, imipenem, the first carbapenem molecule to be introduced in the clinical setting, is administered in combination with cilastatin, a dehydropeptidase-I inhibitor. Despite the antimicrobial efficacy of imipenem, it is only relatively recently that reports concerning the kinetic interaction of this molecule with β-lactamases have been published (6, 19, 29, 31).

Today, strains resistant to these drugs are isolated daily throughout the world. This has stimulated the search for new carbapenem derivatives characterized by optimal binding to penicillin-binding proteins, good permeation, and insensitivity to the action of β-lactamases.

Several recent observations have prompted us to investigate in detail the kinetics of interaction between the novel carbapenem antibiotic biapenem (8, 27, 41) and active-site serine and metallo-β-lactamases. These observations include the discovery of a plasmid-mediated metallo-β-lactamase in P. aeruginosa (40); the emergence of strains of Bacteroides spp. producing class B enzymes (10, 35, 42); the appearance of other mechanisms of resistance to imipenem, including a decline in production of the D2 outer membrane protein in Pseudomonas spp. (26); and the combined derepression of class C β-lactamase production and impaired outer membrane permeability or β-lactamase production in certain strains of the Enterobacteriaceae (25, 33).

MATERIALS AND METHODS

Bacterial strains. Citrobacter diversus ULA-27, Proteus vulgaris POP9, Acinetobacter calcoaceticus ULA-513, Pseudomonas stutzeri ULA-506, Morganella morganii MDM, and X. maltophilia ULA-511 were all clinical isolates. Mycobacterium fortuitum D516 was obtained from an American Type Culture Collection strain after nitrosoguanidine treatment (12). An Escherichia coli clone containing the plasmid pF14 encoding the TEM-1 β-lactamase was a kind gift of Tony Farmer (Smith Kline-Beecham Pharmaceuticals, Brentford, Middlesex, United Kingdom). A clone of E. coli HB101, containing the plasmid pWRT102, which was resistant to 30 μg of chloramphenicol per ml, was used to produce the Bacillus cereus 5/B/6 metallo-β-lactamase. The Aeromonas hydrophila AE036

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metal-lo-β-lactamase was produced from E. coli DH5α, containing the plasmid pA202R, which was resistant to 50 μg of tetracycline per ml (28). E. coli HB101 containing the plasmid pMON301 coding for the OXA-1 β-lactamase was a kind gift of G. Cornaglia (University of Verona, Verona, Italy). E. cloacae P99 was kindly provided by R. L. Then (Hoffman-La Roche, Basel, Switzerland). E. coli HB101 containing the plasmid bladiv, encoding the β-lactamase from C. diversus ULA-27, was from our laboratory.

Antibiotics. Biapenem was kindly provided by Cyanamid (Catania, Italy). Imipenem was obtained from Merck Sharp & Dohme (Rome, Italy). Cefazolin was obtained from Eli Lilly & Co. (Indianapolis, Ind.). Tetracycline and chloramphenicol were purchased from Sigma (Milan, Italy). Nitrocefin was purchased from Unipath (Milan, Italy). The structure of biapenem (LJC 10,627 or L-627) is shown in Fig. 1.

Purification of enzymes. The production and purification of the β-lactamases from C. diversus ULA-27 and M. fortuitum D316 were performed as described elsewhere (2, 3). The class A β-lactamase produced by P. stutzeri ULA-506 was purified according to the protocol reported by Franceschini et al. (16). The enzymes from P. vulgaris POP9 and E. cloacae P99 were purified by using a phenylboronic acid sepharose column as described by Cartwright and Waley (7). A. hydrophila AE036 (from E. coli DH5α containing the plasmid pA202R) and X. maltophilia ULA-511 metallo-β-lactamases were purified as previously described (4). The class B β-lactamase (produced by E. coli HB101 containing the plasmid pWTRH012) was purified as described by Felici and Amicosante (13). The TEM-1 β-lactamase was obtained by Fisher et al. (15). The OXA-1 β-lactamase was purified to homogeneity as previously described (24). M. morganii MDM and A. calcu-leticus ULA-513, two clinical isolates, and the purification procedure of the respective β-lactamases are described elsewhere (unpublished data). Protein concentrations were estimated by the method of Bradford (5), using bovine serum albumin (BSA) as the standard.

Assay conditions for kinetic experiments. The metallo-β-lactamases from A. hydrophila AE036 and X. maltophilia ULA-511 were assayed in the presence of 30 mM sodium cacodylate buffer, pH 6.5, containing 0.1 mM ZnCl₂, B. cereus 5/86 metallo-β-lactamase was assayed using 10 mM sodium cacodylate buffer, pH 6.0, containing 0.5 M NaCl and 0.1 mM ZnCl₂. The assays were performed at 30°C. The class A, class C, and class D enzymes used in this study were assayed in the presence of 25 mM phosphate buffer, pH 7.0, containing 0.1 M KCl, at 30°C. Enzyme concentrations below 0.1 mg/ml were assayed in the presence of 0.1 mg of BSA per ml for enzyme stability. Table 1 summarizes the experimental conditions used in the assays.

Determination of kinetic parameters for class B β-lactamases. The enzyme activity was determined by measuring the absorbance variation resulting from the hydrolysis of biapenem (λ = 293 nm; ε₂₉₃ = 7600 ± 200 M⁻¹ cm⁻¹) in a Perkin-Elmer Lambda-2 UV/VIS spectrophotometer (Perkin-Elmer, Rome, Italy) connected to an Eppon PSE 30 microcomputer via RS232C interface. Cells with 0.2- to 1.0-cm pathlengths were used, depending on the antibiotic solution concentration used in the spectrophotometric assays. kcat and k₅ values were determined either under initial-rate conditions, using the Hanes-Woolf linearization of the Michaelis-Menten equation (37), or by analyzing the complete time course of the hydrolysis of biapenem in the case of X. maltophilia ULA-511, as described by De Meester et al. (11). For all data, standard deviations were calculated by the Enzfitter program (23) and were less than 10%. The total reaction volume was 0.6 ml in all cases.

Determination of kinetics of enzyme reactivation (k₆, k₇ values). The k₆, k₇ values were obtained by monitoring the reactivation of the enzyme in the presence of 10 μM nitrocefin, at 30°C. Each enzyme was first completely inactivated, and the reaction mixture was then diluted to reduce the inactivator concentration to a negligible value. The activity was then monitored after 2 h, and a limit k₆, k₇ value was determined.

In order to have a more precise indication of enzyme reactivation, two following active-site serine β-lactamases were used as prototypes: E. coli TEM-1 (class A) and E. cloacae P99 (class C) enzymes. The β-lactamases (16 μg/ml) were incubated with biapenem at enzyme/biapenem ratios of 1:100, 1:500, and 1:1,000 until complete inactivation was observed. The reaction mixture was diluted to reduce the inactivator concentration to a negligible value, and the activity was then monitored until complete reactivation of the enzyme by determining the v₅₀ values by reference to the hydrolysis of 100 μM nitrocefin, at 30°C. In the case of complete reactivation:

\[
\frac{v_f}{v_0} = \frac{1}{1 + \frac{K_m}{[S]}}
\]

where v₀, v₅₀, and v₉₀ are the rates of the reporter substrate hydrolysis at times zero and after complete reactivation, respectively.

Determination of kinetic parameters of transient inactivation with class A, class C, and class D enzymes. With biapenem, a stable acyl enzyme (EC*) [see below] was found to accumulate, and its interaction with active-site serine β-lactamases was studied on the basis of the following model:

\[
E + C \rightarrow K_{c1} \rightarrow E + C^* \rightarrow K_{c2} \rightarrow E + P
\]

where E, C, E·C, and P represent the enzyme, inhibitor, Henri-Michaelis complex, and hydrolysis product, respectively. k₁ and k₂ are the first-order acylation and deacylation rate constants, respectively, and K represents the dissociation constant of the Henri-Michaelis complex. The values of the first-order rate constant (k₆) characterizing the rate of EC* accumulation were obtained by monitoring the hydrolysis of nitrocefin (λ = 482 nm; ε₂₉₃ = 15,000 M⁻¹ cm⁻¹) or cefazolin (λ = 260 nm; ε₂₉₃ = 7,400 M⁻¹ cm⁻¹) utilized as

\[
k_{inact} = \frac{k_{inact}}{k_{1} + K_{a}}
\]

where k₆, k₇, and k₈ values were determined by calculating the pseudo-first-order rate of inactivation (k₆) and treating the data according to the method of Frère et al. (17). For more details, see Materials and Methods.

<table>
<thead>
<tr>
<th>Source of β-lactamase and type</th>
<th>Molecular class</th>
<th>Enzyme quantity (μg/ml)</th>
<th>Biapenem concn (μM)</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli HB101(pWTRH012), BeII</td>
<td>B</td>
<td>10–30</td>
<td>50–1,000</td>
<td>1</td>
</tr>
<tr>
<td>X. maltophilia ULA-511, L1</td>
<td>B</td>
<td>5–10</td>
<td>100–300</td>
<td>2</td>
</tr>
<tr>
<td>E. coli DH5α(pAA202R), A2</td>
<td>B</td>
<td>5–10</td>
<td>50–1,500</td>
<td>1</td>
</tr>
<tr>
<td>C. diversus ULA-27</td>
<td>A</td>
<td>10–30</td>
<td>50–500</td>
<td>3</td>
</tr>
<tr>
<td>M. fortuitum D316</td>
<td>A</td>
<td>50–100</td>
<td>100–800</td>
<td>3</td>
</tr>
<tr>
<td>P. vulgaris POP9</td>
<td>A</td>
<td>100–200</td>
<td>25–200</td>
<td>3</td>
</tr>
<tr>
<td>P. stutzeri ULA-506</td>
<td>A</td>
<td>100–200</td>
<td>1–50</td>
<td>3</td>
</tr>
<tr>
<td>E. coli HB101(pPt4T), TEM-1</td>
<td>A</td>
<td>50–100</td>
<td>10–100</td>
<td>3</td>
</tr>
<tr>
<td>A. calcoaceticus ULA-513</td>
<td>A</td>
<td>100–200</td>
<td>10–200</td>
<td>3</td>
</tr>
<tr>
<td>E. cloacae P99</td>
<td>C</td>
<td>10–30</td>
<td>0.25–10</td>
<td>3</td>
</tr>
<tr>
<td>M. morganii MDM</td>
<td>C</td>
<td>20–50</td>
<td>2–25</td>
<td>3</td>
</tr>
<tr>
<td>E. coli HB101(pMON301), OXA-1</td>
<td>D</td>
<td>10–30</td>
<td>1–20</td>
<td>3</td>
</tr>
</tbody>
</table>

According to the system of R. P. Ambler (1).

Method 1: The kinetic parameters k₅ and K₅ values were determined by calculating the initial-rate value of hydrolysis (v₀) in the presence of various biapenem concentrations ([S]) and treating the obtained data by Hanes-Woolf linearization of the Michaelis-Menten equation (37). Method 2: In this case, k₆, k₇, and K₆ values were calculated by analyzing the complete curve of biapenem hydrolysis, according to the method of De Meester et al. (11). Method 3: K and k₆ values of inactivation were determined by calculating the pseudo-first-order rate of inactivation (k₆) and treating the data according to the method of Frère et al. (17). For more details, see Materials and Methods.
TABLE 2. Comparison of kinetic parameters for class B β-lactamas
determined with biapenem and with imipenem<sup>a</sup>

<table>
<thead>
<tr>
<th>Class B β-lactamase</th>
<th>Biapenem</th>
<th>Imipenem&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
<td>$k_{cat}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>B. cereus 5/B/6</td>
<td>&gt;1,500</td>
<td>&gt;100</td>
</tr>
<tr>
<td>A. hydrophila AE936</td>
<td>&gt;1,500</td>
<td>&gt;100</td>
</tr>
<tr>
<td>X. maltophilia ULA-511</td>
<td>56 ± 1</td>
<td>65</td>
</tr>
</tbody>
</table>

<sup>a</sup> For assay conditions, see Materials and Methods.

<sup>b</sup> Results for imipenem are as previously reported by Felici et al. (14).

<sup>c</sup> Only the $k_{cat}/K_m$ ratio was determined, because $v_s$ remained proportional to $S_s$ up to the highest substrate concentration which could be used.

RESULTS

Kinetic parameters. (i) Metallo-β-lactamas
es. Table 2 lists the kinetic parameters calculated for the metallo-β-lactamas
tested in this study with biapenem and compares them with those previously reported for imipenem (14). B. cereus 5/B/6 metallo-β-lactas
d showed a $K_m$ value for biapenem of the same order of magnitude as that determined for imipenem. However, the lower (about 14-fold) $k_{cat}/K_m$ ratio value indicated a reduced catalytic efficiency of B. cereus 5/B/6 metallo-

enzyme for hydrolyzing biapenem with respect to imipenem. Biapenem behaved as a good substrate for X. maltophilia ULA-511 class B β-lactas
de, with a physiologic efficiency of the same order as that observed with imipenem. The A. hy

drophi
a AE936 metalloenzyme, which can be considered a rather specific carbapenemase (13, 14), showed a reduced af

finity for biapenem and had a $k_{cat}/K_m$ ratio value that was four to five times lower than that determined for imipenem, indicating a slight difference of molecular substitutions on enzyme activity.

(ii) Class A, class C, and class D β-lactamas
es. Biapenem behaved as an inhibitor of the active-site serine β-lactamas
tested in this study. After correction for the protection by the report

substrate, the individual parameters $k_{-2}$ and $K$ were derived from the dependence of $K_i$ upon biapenem concen

tration by using the following equation (17):

$$k_i = k_{-2} \cdot \frac{[C]}{[C] + K \left( \frac{K_m + [S]}{K_m^*} \right)}$$

where $[S]$ and $K_m^*$ represent the concentration and $K_m$ value for the reporter substrate, respectively, and $[C]$ represents the inhibitor concentration.

Table 3 summarizes the values of $K$, $k_{-2}$, $k_{-3}$, and $k_{-2}/K$ calculated for the active-site serine β-lactamas
tested in the presence of biapenem. For all data, the standard deviations were less than 10% of the mean of five different measurements. Among the class A β-lactamas
es, the enzyme from P. stutzeri ULA-506 exhibited a very low $K$ value and a good acylase efficiency. The β-lactamas
es from C. diversus UL-27 and M. fortuitum D316 exhibited the lowest $k_{-2}/K$ ratio values, which were accompanied by a relatively low af

finity for biapenem. The class C β-lactamas
es from M. morganii MDM and E. cloacae P99 exhibited low $K$ values but similar $k_{-2}/K$ ratio values. The inactivation by biapenem of the unclassified (probably a class C; unpublished data) enzyme produ

ced by A. calcoaceticus ULA-513 was also studied. For this β-lactase, biapenem behaved as an inhibitor and its acylase eff

ciency was similar to that determined for some class A β-lactamas
es.

Determination of $k_{-3}$ values. Figure 2 shows the accumula

tion of product with time when the inactivated TEM-1 and E. cloacae P99 β-lactamas
es were diluted in the presence of 100 μM nitrocefin as substrate. From the curves of enzyme reac

tion, the following $k_{-3}$ values were determined, as described in Materials and Methods: (1.00 ± 0.1) × 10<sup>-3</sup> s<sup>-1</sup> for the TEM-1 enzyme (Fig. 2A) and (3.87 ± 0.1) × 10<sup>-3</sup> s<sup>-1</sup> for the β-lactas
e produced by E. cloacae P99 (Fig. 2B). Each calculated value represents the mean from three different experimen

Determination of MICs. Table 4 summarizes the in vitro ac

tivity of imipenem and biapenem against strains harboring some of the β-lactamas
tested in kinetic experiments. X. maltophilia ULA-511 was omitted because of its resistance to carbapenem antibiotics. As it can be noted, class A and class C β-lactamas
did not seem to confer resistance toward both imipenem and biapenem, because the determined MICs

TABLE 3. Kinetic parameters calculated with active-site serine β-lactamas
es, using biapenem as a transient inactivator<sup>a</sup>

<table>
<thead>
<tr>
<th>Source of β-lactamase and type</th>
<th>$K$ (μM)</th>
<th>$k_{-2}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>$k_{-3}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>$k_{-2}/K$ (M&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Molecular class&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. diversus UL-27</td>
<td>147 ± 10</td>
<td>1.10 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>&lt;1.50 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>7.48 × 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>A</td>
</tr>
<tr>
<td>P. stutzeri ULA-506</td>
<td>1.7 ± 0.1</td>
<td>5.40 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>&lt;6.30 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>3.18 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>A</td>
</tr>
<tr>
<td>E. coli(pDT4), TEM-1</td>
<td>30 ± 2</td>
<td>3.80 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>1.00 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>1.27 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>A</td>
</tr>
<tr>
<td>M. fortuitum D316</td>
<td>267 ± 5</td>
<td>3.50 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>&lt;2.20 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>1.31 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>A</td>
</tr>
<tr>
<td>P. vulgaris POP9</td>
<td>80 ± 2</td>
<td>5.05 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>&lt;9.20 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>6.31 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>A</td>
</tr>
<tr>
<td>E. coli(pMOM301), OXA-1</td>
<td>2.5 ± 0.1</td>
<td>1.20 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>&lt;9.10 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4.80 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>D</td>
</tr>
<tr>
<td>M. morganii MDM</td>
<td>5.0 ± 0.1</td>
<td>1.10 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>&lt;4.00 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>2.20 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>C</td>
</tr>
<tr>
<td>E. cloacae P99</td>
<td>0.8 ± 0.01</td>
<td>2.45 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>3.87 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>3.06 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>C</td>
</tr>
<tr>
<td>A. calcoaceticus ULA-513</td>
<td>90 ± 5</td>
<td>4.00 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>&lt;8.20 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>4.44 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>—&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> For more details see Materials and Methods.

<sup>b</sup> According to the system of R. P. Ambler (1).

<sup>c</sup> —, unclassified.
ranged between 0.06 and 8 μg/ml. In particular, when imipenem was used, slightly higher MICs were found with *E. coli* HB101 carrying the plasmid pJT4, probably due to a hyperproduction of the TEM-1 β-lactamase by this strain. Metallo-β-lactamases, produced by *E. coli* strains carrying the plasmids encoding these zinc enzymes, conferred resistance to imipenem when a higher concentration of inoculum was used. Surprisingly, the same enzymes did not allow bacteria to be resistant to biapenem.

**DISCUSSION**

In this paper we have evaluated the interaction between biapenem and β-lactamases belonging to the four molecular classes described by Ambler (1) and the effective contribution of these enzymes to the resistance mechanism of clinical isolates and laboratory strains.

A comparative analysis, using three class B enzymes, of the kinetic parameters obtained for some carbapenems indicated...
that the purified metallo-β-lactamase from *X. maltophilia* ULA-511 was the most active toward imipenem and biapenem. Although biapenem was hydrolyzed by the *B. cereus* 5B/6 and *A. hydrophila* AE036 metalloenzymes, it exhibited a low affinity and a reduced catalytic efficiency. We extended the comparison to activity in vitro, using *E. coli* strains carrying the genes coding for the above-mentioned class B β-lactamases. Biapenem was more effective than imipenem against these strains, although the kinetic parameters determined appeared to be of the same order of magnitude.

The behavior was more heterogeneous when purified active-site serine β-lactamases were used. In fact, the enzymes from *M. morganii* MD and *E. cloacae* P99 formed a relatively stable acylenzyme with a $k_{2}/k_{-2}$ value of about $2 \times 10^{4}$ to $3 \times 10^{4} \text{ M}^{-1} \text{ s}^{-1}$, whereas the chromosomal cephalosporinase (probably a class C enzyme) produced by *A. calcoaceticus* ULA-513 had a $k_{2}/k_{1}$ value that was 40-fold lower.

In general, class A β-lactamases had $k_{2}/k_{1}$ values lower than those calculated for class C enzymes, with the noticeable exception of the class A β-lactamase produced by *P. stutzeri* ULA-506. Biapenem behaved as a competitive inhibitor of this enzyme, and the $k_{-2}$ value calculated indicated the formation of a relatively stable acylenzyme. Our data are, therefore, in good agreement with those previously reported (19, 29, 31).

Analysis of the $k_{3}$ values calculated for all active-site serine β-lactamases tested suggested that the half-life of the acylenzyme was shorter than the generation time of the bacterium. The situation was somewhat improved with class C β-lactama-

ses, in which a doubling of the $k_{2}/k_{1}$ values was observed. On the basis of these observations, it would appear that biapenem behaves as a very poor substrate or as a transient inhibitor of these enzymes, which is similar to the case for imipenem.

In vitro activity of biapenem against bacterial strains producing the enzymes investigated led to different conclusions and confirmed the previously reported antimicrobial ef-

ficiency of this antibiotic (8, 9, 27). The major antibacterial activity of biapenem could be related to the higher $k_{cat}/K_{m}$ ratio values for imipenem exhibited by *B. cereus* and *A. hydrophila* metallo-β-lactamases. The differences in MIC data found between imipenem and biapenem for *C. diversus* ULA-27 cannot be explained on the basis of the kinetic parameters determined. In fact, as previously reported (3), imipenem was hy-

drolyzed with a low $k_{cat}/K_{m}$ value ($4.10 \times 10^{3} \text{ M}^{-1} \text{ s}^{-1}$). On the contrary, biapenem was not hydrolyzed by this β-lactamase; from these findings the differences observed in MICs could be attributed to a greater ability of imipenem to permeate, with respect to biapenem.

In conclusion, our study confirms the excellent stability of biapenem to β-lactamases and its powerful antibiotic activity in vitro. Interestingly, the $Zn^{2+}$-β-lactamases from *B. cereus* 5B/6 and *A. hydrophila* AE036 expressed in *E. coli* strains confer resistance to imipenem but not to biapenem, even when a high concentration of inoculum is used. As shown by Ubukata et al. (38) and by Yang et al. (41), there are not significant differences in binding to *E. coli* penicillin-binding proteins; if anything, imipenem binds better to PBP 1b as a secondary target, and permeability is actually a bit less for biapenem into *Serratia marcescens*. The possibility exists that the enzyme from *A. hydrophila* AE036 is not able to reach maximal activity because of a high $K_c$ value for biapenem with respect to imipenem.

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