Three Decades of β-Lactamase Inhibitors

Sarah M. Drawz and Robert A. Bonomo

Departments of Pathology, Medicine, Pharmacology, and Molecular Biology and Microbiology.
Case Western Reserve University School of Medicine, Cleveland, Ohio, and Research Service,
Louis Stokes Cleveland Department of Veterans Affairs Medical Center, Cleveland, Ohio

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* Corresponding author. Mailing address: Infectious Diseases Section, Louis Stokes Cleveland Department of Veterans Affairs Medical Center, 10701 East Blvd., Cleveland, OH 44106. Phone: (216) 791-3800, ext. 4399. Fax: (216) 231-3482. E-mail: robert.bonomo@med.va.gov.
INTRODUCTION

The development of antibiotics remains one of the most significant advances in modern medicine (364). Antibiotics have saved countless lives and continue to be a mainstay of therapy for bacterial infections. The clinical success of the first β-lactam, penicillin G (benzylpenicillin [Fig. 1, compound 1]), prompted the search for and development of additional derivatives. This quest gave rise to the β-lactam antibiotics in clinical use today (penicillins, narrow- and extended-spectrum cephalosporins, monobactams, and carbapenems [Fig. 1, compounds 1 to 7] (14). The common structural feature of these classes of antibiotics is the highly reactive four-membered β-lactam ring.

Unfortunately, β-lactamase-mediated resistance to β-lactam antibiotics emerged as a significant clinical threat to these life-saving drugs. In response to this challenge, two strategies were advanced to preserve the utility of β-lactam antibiotics: (i) discover or design β-lactam antibiotics that are able to evade bacterial enzymatic inactivation conferred by β-lactamases, or (ii) inhibit β-lactamases so the partner β-lactam can reach the penicillin binding proteins (PBPs), the target of β-lactam antibiotics.

In this review, we summarize 3 decades of investigation of β-lactamase inhibition. This perspective is framed by our background in clinical infectious diseases. First, we highlight the fundamental principles of β-lactamase enzymology. We then summarize the salient features of β-lactam–β-lactamase inhibitor combinations that are used in clinical practice. Next, we define the problem of resistance to β-lactam–β-lactamase inhibitors by explaining the important changes in class A β-lactamases that define this phenotype. With this background, we review the β-lactamase inhibitors that have been developed to this point and discuss the novel β-lactamase inhibitors that are hoped to extend the life span of our current β-lactams. We view these agents as extremely important to the future of β-lactam therapy: inhibitors not only can preserve our current armamentarium but may also be used as novel β-lactams are introduced into the clinic. Finally, we conclude with some “lessons learned.”

MECHANISM OF ACTION OF β-LACTAM ANTIBIOTICS

β-Lactam antibiotics exhibit their bactericidal effects by inhibiting enzymes involved in cell wall synthesis. The integrity of the bacterial cell wall is essential to maintaining cell shape in a hypertonic and hostile environment (249). Osmotic stability is preserved by a rigid cell wall comprised of alternating N-acetylglucosamine (NAM) and N-acetylmuramic acid (NAG) units. These glycosidic units are linked by transglycosidases. A penicillin is attached to each NAM unit, and the cross-linking of two D-alanine–D-alanine NAM pentapeptides is catalyzed by PBPs, which act as transglycosylases (142, 377). This cross-linking of adjacent glycan strands confers the rigidity of the cell wall.

The β-lactam ring is sterically similar to the D-alanine–D-alanine of the NAM pentapeptide, and PBPs “mistakenly” use the β-lactam as a “building block” during cell wall synthesis (459). This results in acylation of the PBP, which renders the enzyme unable to catalyze further transpeptidation reactions (125). As cell wall synthesis slows to a halt, constitutive peptidoglycan autolysis continues. The breakdown of the murein sacculus leads to cell wall compromise and increased permeability. Thus, the β-lactam-mediated inhibition of transpeptidation causes cell lysis, although the specific details of penicillin’s bactericidal effects are still being unraveled (20).

RESISTANCE TO β-LACTAM ANTIBIOTICS

There are four primary mechanisms by which bacteria can overcome β-lactam antibiotics (14).

(i) Production of β-lactamases enzymes is the most common and important mechanism of resistance in Gram-negative bacteria and will be the focus of this review.

(ii) Changes in the active site of PBPs can lower the affinity for β-lactam antibiotics and subsequently increase resistance to these agents, such as those seen in PBP2x of Streptococcus pneumoniae (212). Through natural transformation and recombination with DNA from other organisms, Neisseria spp. and Streptococcus spp. have acquired highly resistant, low-affinity PBPs (39, 313, 459). In a related manner, penicillin resistance in Streptococcus sanguis, Streptococcus oralis, and Streptococcus mitis developed from horizontal transfer of a PBP2b gene from Streptococcus pneumoniae (107, 348). Methicillin resistance in Staphylococcus spp. is also a significant clinical challenge. While there are many reasons for this resistance, the β-lactam resistance phenotype is also conferred by acquisition of
the mecA gene which produces PBP2a (also denoted PBP2\(\beta\)) (76). PBP2a can assemble new cell wall in the presence of high concentration of penicillins and cephalosporins.

(iii) Decreased expression of outer membrane proteins (OMPs) is another mechanism of resistance. In order to access PBPs on the inner plasma membrane, \(\beta\)-lactams must either
diffuse through or directly traverse porin channels in the outer membrane of Gram-negative bacterial cell walls. Some Enterobacteriaceae (e.g., Enterobacter spp., Klebsiella pneumoniae, and Escherichia coli) exhibit resistance to carbapenems based on loss of these OMPs; the loss of OprD is associated with imipenem resistance and reduced susceptibility to meropenem in the nonfermener Pseudomonas aeruginosa (169, 187, 236, 286, 306, 395). Resistance to imipenem and meropenem has also been associated with the loss of the CarO OMP in clinical isolates of multidrug-resistant Acinetobacter baumannii (278, 346). Point mutations or insertion sequences in porin-encoding genes can produce proteins with decreased function and thus lower permeability to β-lactams (106). Of note, the disruption of porin proteins alone is not always sufficient for producing the resistance phenotype, and typically this mechanism is found in combination with β-lactamase expression (106, 235).

(iv) Efflux pumps, as part of either an acquired or intrinsic resistance phenotype, are capable of exporting a wide range of substrates from the periplasm to the surrounding environment (347). These pumps are an important determinant of multidrug resistance in many Gram-negative pathogens, particularly P. aeruginosa and Acinetobacter spp. In P. aeruginosa, upregulation of the MexA-MexB-OprD system, in combination with the organism’s low outer membrane permeability, can contribute to decreased susceptibility to penicillins and cephalosporins, as well as quinolones, tetracycline, and chloramphenicol (224–226, 373, 389). To illustrate, an increase in the carbenicillin MIC from 32 μg/ml to 1,028 μg/ml is associated with overproduction of this efflux pump (12, 225). Additionally, an upregulated efflux pump (e.g., AdeABC, an RND-type efflux pump, in A. baumannii) can augment the carbapenem resistance conferred by a catalytically poor β-lactamase (e.g., OXA-23) (162, 333).
**β-LACTAMASES**

The first β-lactamase enzyme was identified in *Bacillus (Escherichia) coli* before the clinical use of penicillin. In a sentinel paper published nearly 70 years ago, E. P. Abraham and E. Chain described the *B. coli* “penicillinase” (1). The enzyme was not thought to be clinically relevant, since penicillin was targeted to treat staphylococcal and streptococcal infections, and Abraham, Chain, and their colleagues were unable to isolate the enzyme from these Gram-positive organisms (2, 55). It is sobering now to consider the ramifications of this observation.

Four years later, Kirby successfully extracted these cell-free “penicillin inactivators” from *Staphylococcus aureus*, which fore-shadowed the emergence of a significant clinical problem (202). The growing number of β-lactam antibiotics has since increased the selective pressure on bacteria, promoting the survival of organisms with multiple β-lactamas (249, 256). Currently, more than 850 β-lactamas are identified (K. Bush, personal communication). As authors, we speculate that the rapid replication rate, recombination rates, and high mutation frequency permit bacteria to adapt to novel β-lactams by evolution of these β-lactamas (332).

**Classification**

Two major classification schemes exist for categorizing β-lactamases: Ambler classes A through D, based on amino acid sequence homology, and Bush-Jacoby-Medeiros groups 1 through 4, based on substrate and inhibitor profile (Table 1) (10, 57). A “family portrait” reveals the structural similarity of class A, C, and D serine β-lactamas (Fig. 2). Class B β-lactamas (“a class apart”) are metallo-β-lactamas (MBLs) (52). MBLs possess either a single Zn$^{2+}$ ion or a pair of Zn$^{2+}$ ions coordinated to His/Cys/Asp residues in the active site. In this review, the Ambler classification scheme will be used.

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**TABLE 1. β-Lactamase classification schemes**

<table>
<thead>
<tr>
<th>Ambler class</th>
<th>Bush-Jacoby-Medeiros class</th>
<th>Preferred substrates</th>
<th>Inhibited by clavulanate</th>
<th>Representative enzyme(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (serine penicillinases)</td>
<td>2a</td>
<td>Penicillins</td>
<td>+</td>
<td>PC1 from <em>S. aureus</em></td>
</tr>
<tr>
<td></td>
<td>2b</td>
<td>Penicillins, narrow-spectrum cephalosporins</td>
<td>+</td>
<td>TEM-1, TEM-2, SHV-1</td>
</tr>
<tr>
<td></td>
<td>2be</td>
<td>Penicillins, narrow-spectrum and extended-spectrum cephalosporins</td>
<td>+</td>
<td>SHV-2 to SHV-6, TEM-3 to TEM-26, CTX-Ms</td>
</tr>
<tr>
<td></td>
<td>2r</td>
<td>Penicillins</td>
<td>-</td>
<td>TEM-30, SHV-72</td>
</tr>
<tr>
<td></td>
<td>2c</td>
<td>Penicillins, carbepenicillic</td>
<td>+</td>
<td>PSE-1</td>
</tr>
<tr>
<td></td>
<td>2e</td>
<td>Extended-spectrum cephalosporins</td>
<td>+</td>
<td>FEC-1, CepA</td>
</tr>
<tr>
<td></td>
<td>2f</td>
<td>Penicillins, cephalosporins, carbapenems</td>
<td>±</td>
<td>KPC-2, SME-1, NMC-A</td>
</tr>
<tr>
<td>B (metallo-β-lactamas)</td>
<td>3</td>
<td>Most β-lactams, including carbapenems</td>
<td>-</td>
<td>IMP-1, VIM-1, CerA, and BecI (B1); CphA (B2); L1(B3)</td>
</tr>
<tr>
<td>C (cephalosporinases)</td>
<td>1</td>
<td>Cephalosporins</td>
<td>-</td>
<td>AmpC, CMY-2, ACT-1</td>
</tr>
<tr>
<td>D (oxacillinases)</td>
<td>2d</td>
<td>Penicillins, cloxacinil</td>
<td>±</td>
<td>OXA-1, OXA-10</td>
</tr>
<tr>
<td>Not classified</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Based on data from reference 57. An updated classification system by Bush and Jacoby is in press (56a) at the time of this writing.

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Class A *serine β-lactamases.* In general, class A enzymes are susceptible to the commercially available β-lactamase inhibitors (clavulanate, tazobactam, and [less so] sulbactam), although the *K. pneumoniae* carbapenemase KPC may be an important exception to this generalization (319a). The first plasmid-mediated β-lactamase was identified in *E. coli* in 1963 (and reported in 1965), and was named “TEM” after the patient from whom it was isolated (100). SHV, another common β-lactamase found primarily in *K. pneumoniae*, was named from the term “sulfhydryl reagent variable.” Early studies of SHV-1 showed that *p*-chloromercurobenzoate inhibited the hydrolysis of cephaloridine but not that of benzylpenicillin (251). TEM and SHV are common β-lactamas detected in clinical isolates of *E. coli* and *K. pneumoniae*, pathogens responsible for urinary tract, hospital-acquired respiratory tract, and bloodstream infections (60, 145, 372). While SHV-1 and TEM-1 share 68% sequence homology, the active site of SHV-1 is approximately 0.7 to 1.2 Å wider than that of TEM-1, which may have important structural implications, especially related to the substrate profiles of SHV variants (421).

Although *bla*TEM and *bla*SHV may be found on plasmids, other class A enzymes are encoded on the chromosome (e.g., *bla*PCrRo from *Burkholderia pseudomallei* or on integrons (e.g., *bla*GEI-1 from *K. pneumoniae* and *bla*VEB-1 in *P. aeruginosa* and *A. baumannii*) (57, 280).

Class A ESBLs. The growing number of β-lactamas in *E. coli* and *K. pneumoniae*, as well as the emergence of these enzymes in other pathogens (e.g., *Haemophilus influenzae* and *Neisseria gonorrhoeae*), led to the development of extended-spectrum cephalosporins with an oxyimino side chain, carbapenems, cephamycins, and monobactams (71, 115, 188, 321). Upon the introduction of the penems and cephems in the early 1980s, these new agents were effective against many β-lactam resistant bacteria. However, selective pressure quickly fostered the emergence of extended-spectrum β-lactamas (ESBLs), which could hydrolyze many of the oxyimino-cephalosporins...
In a dramatic parallel to the observations of Abraham and Chain, within 2 years of the introduction of the extended-spectrum cephalosporins cefotaxime and ceftazidime, novel ESBLs in *E. coli* and *K. pneumoniae* were reported (204). Interestingly, these “new” *β*-lactamases harbored point mutations in the parent *bla*TEM-1 and *bla*SHV-1 genes that led to single amino acid changes in the *β*-lactamases. Other ESBLs, such as CTX-M, arose by plasmid transfer from preexisting chromosomal ESBL genes from *Kluyvera* spp., which typically are nonpathogenic commensal organisms (14, 32). CTX-M ESBLs now represent important enzymes found in isolates from the community and are the most commonly isolated ESBLs in many parts of the world, particularly Europe (343).

ESBLs hydrolyze penicillins, narrow- and extended-spectrum cephalosporins (including the anti-methicillin-resistant *S. aureus* [MRSA] cephalosporin ceftobiprole), and the monobactam aztreonam (11, 321, 357). In contrast, ESBLs cannot efficiently degrade cephamycins, carbapenems, and *β*-lactamase inhibitors. Since their initial description, more than 200 different ESBLs have been identified, posing a significant risk to public health and to hospitalized patients in intensive care units, where infection with an ESBL may lead to significant morbidity and mortality (up-to-date listings of verified ESBL sequences are available on the website www.lahey.org/studies/webt.asp maintained by G. A. Jacoby and K. Bush) (321). The majority of ESBLs are from the SHV, TEM, and CTX-M families; less frequently they are derived from BES,GES-1, VEB, and PER enzymes, and sometimes these enzymes do not belong to any defined family (280).

**Class A serine carbapenemases.** Class A serine carbapenemases include the nonmetallocarbenemase of class A (NMC-A), IMI, SME, and KPC. Members of this group of *β*-lactamases can hydrolyze carbapenems as well as cephalosporins, penicillins, and aztreonam (356). These carbapenem-hydrolyzing enzymes have been identified primarily in *Entero- bacter cloacae*, *Serratia marcescens*, and *K. pneumoniae*, bacteria which often harbor multiple resistance determinants, narrowing the range of treatment options (290, 291, 359, 454, 455). The *bla* gene for the former two organisms is typically found on the chromosome, while the *K. pneumoniae* carbapenemase *bla*KPC gene is carried on plasmids containing Tn4401 (292).

**Class B metallo-*β*-lactamases.** Class B enzymes are Zn^{2+}-dependent *β*-lactamases that demonstrate a hydrolytic mechanism different from that of the serine *β*-lactamases of classes A, C, and D (57). Organisms producing these enzymes usually exhibit resistance to penicillins, cephalosporins, carbapenems, and the clinically available *β*-lactamase inhibitors (432). Interestingly, the hydrolytic profile of MBLs does not typically include aztreonam. MBLs likely evolved separately from the other Ambler classes, which have serine at their active site (249). The *bla*MBL genes are located on the chromosome, plasmid, and integrons (133, 432). *P. aeruginosa*, *K. pneumoniae*, and *V. cholerae* are the most frequent, well-recognized producers of MBLs (205, 292). The most recently described metallo-*β*-lactamase, *N. gonorrhoeae* IMI (356), is an example of an emerging resistance mechanism (250, 356).

**Class C metallo-*β*-lactamases.** Class C enzymes, also known as *PER-LIKE* enzymes, are a distinct group of *β*-lactamases that hydrolyze penicillins, cephalosporins, carbapenems, and the clinically available *β*-lactamase inhibitors (432). Class C enzymes are known to be present in *P. aeruginosa*, *K. pneumoniae*, and *V. cholerae* (238, 356), and their distribution is becoming more widespread (292). The *bla*PER gene is located on the chromosome, plasmid, and integrons (133, 432). The hydrolytic profile of class C enzymes includes cephamycins, carbapenems, and *β*-lactamase inhibitors, but not aztreonam (356).

![Class A SHV-1 *β*-lactamase](image1.png)

![Class B IMP-1 *β*-lactamase](image2.png)

![Class C E.coli AmpC *β*-lactamase](image3.png)

![Class D OXA-1 *β*-lactamase](image4.png)

**FIG. 2.** “Family portrait” of *β*-lactamase enzymes. (A) Class A SHV-1; (B) class B IMP-1; (C) class C E. coli AmpC; (D) class D OXA-1. For classes A, C, and D, the active-site serine is shown in yellow; for class B, the two Zn^{2+} ions are shown. (Based on PDB entries 1SHV, 1DDK, 2BLS, and 1M6K, respectively.)
and *A. baumannii* produce class B enzymes encoded by mobile genetic elements (93, 175, 177). In contrast, *Bacillus* spp., *Chryseobacterium* spp., and *Stenotrophomonas maltophilia* possess chromosomally encoded MBLs, but the majority of these host pathogens are not frequently responsible for serious infections (432). The role that these β-lactamases in *Bacillus* spp. and *Chryseobacterium* spp. will play in the clinical arena is still unknown.

**Class C serine cephalosporinases.** Class C AmpC β-lactamases include CMY-2, P99, ACT-1, and DHA-1, which are usually encoded by *bla* genes located on the bacterial chromosome, although plasmid-borne AmpC enzymes are becoming more prevalent (340). Organisms expressing the AmpC β-lactamase are typically resistant to penicillins, β-lactam-β-lactamase inhibitor combinations, and cephalosporins, including cefoxitin, cefotetan, ceftriaxone, andcefotaxime. AmpC enzymes poorly hydrolyze cephalixin and are inhibited by cloxacillin, oxacillin, and aztreonam (57). Members of the *Enterobacteriaceae* family, such as *Enterobacter* spp. and *Citrobacter* spp., are AmpC β-lactamase producers that resist inhibition by clavulanate and sulbactam, although *Klebsiella* spp., *Salmonella* spp., and *Proteus* spp. normally do not harbor chromosomal *bla* genes (19, 184).

Production of chromosomal AmpCs in Gram-negative bacteria is at a low level ("repressed") but can be "derepressed" by induction with certain β-lactams, particularly cefoxitin (14, 24, 148). The mechanism of this regulation has been the subject of intense investigation (183). Of significant concern is the selection of mutant bacterial populations that are genetically derepressed for AmpC production, which can cause a dramatic increase in MICs during the course of β-lactam therapy (e.g., after 14 days of ceftazidime therapy, a strain of *P. aeruginosa* with MICs increased from 1 to 32 μg/ml was selected) (184, 193, 233).

**Class D serine oxacillinases.** Class D β-lactamases were initially categorized as "oxacillinases" because of their ability to hydrolyze oxacillin at a rate of at least 50% of that of benzylpenicillin, in contrast to the relatively slow hydrolysis of oxacillin by classes A and C (98). In bacteria, OXA-β-lactamases can also confer resistance to penicillins, cephalosporins, extended-spectrum cephalosporins (OXA-type ESBLs), and carbapenems (OXA-type carbapenemases). Generally speaking, OXA enzymes are resistant to inhibition by clavulanic acid, sulbactam, and tazobactam, (with some exceptions; e.g., OXA-2 and OXA-32 are inhibited by tazobactam but not sulbactam and clavulanate, and OXA-53 is inhibited by clavulanate) (98, 276, 279, 345). Interestingly, sodium chloride at concentrations of >50 to 75 mM inhibits some carbapenem-hydrolyzing oxacillinases (e.g., OXA-25 and OXA-26) (6). Site-directed mutagenesis studies suggest that susceptibility to inhibition by sodium chloride is related to the presence of a Tyr residue at position 144 (161, 279). Presumably, Tyr144 may facilitate sodium chloride binding better than the Phe residue found in resistant oxacillinases, although the molecular mechanism remains unexplained. Examples of OXA enzymes include those rapidly emerging in *A. baumannii* (e.g., OXA-23 and OXA-24/40) and constitutively expressed in *P. aeruginosa* (e.g., OXA-50) (141a, 346, 435).

**β-Lactamase Hydrolytic Mechanisms**

Serine β-lactamases acylate β-lactam antibiotics, much like PBPs, and then use strategically positioned water molecules to hydrolyze the acylated β-lactam (265). In this manner, the β-lactamase is regenerated and can inactivate additional β-lactam molecules. This enzymatic reaction may be represented by the following equation:

\[
E + S \rightarrow E:S \rightarrow E - S \rightarrow E + P
\]

where the kinetic constant, \(k\), is defined as (129)

\[K_m = k_2/k_1 + k_3\]

where the kinetic constant, \(K_m\), is (\(k_1 + k_2\))/\(k_1\). The turnover number, \(k_{cat}\), is a composite rate constant that represents multiple chemical steps and is defined as (92)

\[k_{cat} = k_3/k_1 + k_3\]

or

\[V_{max} = k_{cat}[E]\]

**Clindamycin**

Clindamycin, in the presence of *C. difficile* or *B. fragilis*, is converted to clindamycin dimethyl acetal (CDMAD) (69, 142, 277). CDMAD is a prodrug that is hydrolyzed to clindamycin (CLDA) by *C. difficile* clindamycin acetyl esterase (CDAE). In the presence of *C. difficile* or *B. fragilis*, the product of this hydrolysis is clindamycin (CLDA) (69, 142, 277).

**β-Lactamase Hydrolytic Mechanisms**

Serine β-lactamases acylate β-lactam antibiotics, much like PBPs, and then use strategically positioned water molecules to hydrolyze the acylated β-lactam (265). In this manner, the β-lactamase is regenerated and can inactivate additional β-lactam molecules. This enzymatic reaction may be represented by the following equation:

\[
E + S \rightarrow E:S \rightarrow E - S \rightarrow E + P
\]

In this scheme, E is a β-lactamase, S is a β-lactam substrate, E:S is the Michaelis complex, E - S is the acyl enzyme, and P is the product devoid of antibacterial activity. The rate constants for each step are represented by \(k_1, k_2, k_3, k_4, k_1, k_2, k_3, k_4\), and \(k_5\) are the association and dissociation rate constants for the preacylation complex, respectively; \(k_2\) is the acylation rate constant, and \(k_6\) is the deacylation rate constant. More complicated branched reaction mechanisms are seen with some β-lactamases (129). In order to be uniform, we now define basic terms that are often used to describe the kinetic behavior of β-lactamases.

The Michaelis constant, \(K_m\), is defined as (129)

\[K_m = k_2/k_1 + k_3\]

where the kinetic constant, \(K_m\), is (\(k_1 + k_2\))/\(k_1\). The turnover number, \(k_{cat}\), is a composite rate constant that represents multiple chemical steps and is defined as (92)

\[k_{cat} = k_3/k_1 + k_3\]

or

\[V_{max} = k_{cat}[E]\]
In contrast, the acylation mechanism is less clear, and several hypotheses and lines of evidence exist. The first notion proposes that Glu166, via a water molecule, deprotonates the Ser70 hydroxyl before addition to the β-lactam bond. This Glu166 general base theory is supported by quantum mechanical/molecular mechanical (QM/MM) calculations and the protonated status of Glu166 in the ultra-high-resolution (0.85-Å) structure of TEM-1 in complex with an acylation transition state analog (TSA) (Research Collaboratory for Structural Bioinformatics Protein Data Bank [PDB] entry 1M40) (163, 265). Surprisingly, substitutions at residue 166 do not prevent acylation (139, 146, 207, 392).

A second view of acylation maintains that Lys73 can serve as the general base deprotonating Ser70 (166, 261, 401). Recent QM/MM studies by Meroueh et al. demonstrate that the pathway involving Lys73 as the activating base is energetically favorable and may exist in competition with the pathway where Glu166 serves as the activating residue (261). Additional ideas on the mechanism of acylation have been proposed, including both (i) a role for the C-3 or C-4 substrate carboxylate and Ser130 in activating Ser70 and (ii) a general acid pathway where protonation of the β-lactam nitrogen is the primary event (13, 103). Further clarification of the acylation mechanism may prove useful for the design of inhibitors that capitalize on the enzymes’ native machinery.

Highly conserved structural motifs in class A enzymes include four polar regions: (i) the active-site pocket Ser70-Xaa-Xaa-Lys73, (ii) the “Ω-loop” residues 163 to 178, (iii) Ser130-Asp131-Asn132, and (iv) Lys234-Thr(Ser)235-Gly236. The roles of residues in the “SDN” loop comprised of Ser130-Asp131-Asn132 are related to maintaining the structure of the active site cavity, enzyme stability, and stabilization of the enzyme transition state, respectively (182, 305). The functional explanation for the conservation of the Lys234-Thr(Ser)235-Gly236 triad is less well understood. Removal of the hydroxyl group from the middle residue has little impact on penicillin hydrolysis in TEM (but more on the catalytic efficiency of cephalosporins), and despite what may be expected from crystal structure alignments, the study of modified β-lactams argues against an interaction between the β-lactam carbonyl and the Thr(Ser) hydroxyl group (112, 181). In SHV, the Thr235Ala substitution lowers MICs of both penicillins and cephalosporins (174). Interestingly, the expression of the structurally conserved SHV Thr235Ser variant has no effect on β-lactam MICs but does lead to an increased susceptibility to the piperacillin-tazobactam combination (174). The most common mechanism for transformation of a broad-spectrum β-lactamase to an ESBL is point mutations that result in amino acid sequence changes near the enzyme active site, facilitating hydrolysis of oxymino-cephalosporins (186, 341). However, the specific amino acid replacements and resistance mechanisms vary between enzymes (124). In TEM,
changes at Ambler positions Arg164 (-His, -Ser), Gly238 (-Ser, -Ala), and Glu240 (-Lys) result in variants that confer the ESBL phenotype (40; www.lahey.org/studies/webt.asp). Several crystallographic studies demonstrated that the "new" TEM active site is expanded or remodeled, compared to TEM-1, to accommodate the larger side chain of expanded-spectrum cephalosporins (83, 206, 298, 304). The crystal structure of SHV-2 (Gly238Ser) at 0.91 Å resolution (PDB 1N9B), compared to SHV-1, showed a displacement in the b3-strand containing residues 238 to 242 which created an expanded β-lactam binding site but preserved the positioning of the essential catalytic residues (124, 298). Similarly, the PER-1 ESBL active site is expanded by a novel fold of the Ω-loop and the insertion of four residues after Lys240 (417).

In contrast, the crystal structure of the acyl-enzyme intermediate of the ESBL Toho-1 in complex with cefotaxime did not resemble the enlarged active sites seen with the ESBL TEM and SHV β-lactamases. Rather, the Toho-1 enzyme facilitates cephalosporin binding through movement of the Ω-loop toward the active site, interactions between conserved residues Asn104 and Asp240 and the bulky side chain of cefotaxime, and contacts with Ser237 that help position the b3-β-lactam carbonyl in the oxyanion hole (176, 382). The high-resolution crystal structures of four additional CTX-M β-lactamases confirmed that the active sites of these enzymes were not enlarged compared to those of the narrow-spectrum TEM-1 and SHV-1 (83, 85). Instead, the structural basis for the extended substrate profiles in the CTX-M enzyme family appears to be specific amino acid (i.e., Ser237 and Asp104) interactions with the oximino-cephalosporins and increased mobility of the b3-β-strand (83). Overall, this increased mobility and activity "costs the enzyme" thermal stability, a theme recapitulated by the evolution of many resistance phenotypes (383).

Point mutations are not the only mechanism for the ESBL phenotype; other alterations in enzyme regulation produce resistance, such as promoter sequence changes leading to increased enzyme expression or alterations in outer membrane porins (293, 302).

At this time, the biochemical basis for class A serine carbapenemase activity is an area of active investigation. Current evidence suggests that this property rests upon a remodeling of the active site. Unlike class A ESBLs (with expanded or enhanced mobility of the b3-β-strand), the active sites of class A carbapenemases such as KPC-2, NMC-A, and SME-1 reveal that there are multiple alterations in the spatial positions of catalytic residues (200, 338, 402). We are just beginning to understand how this remodeling allows enhanced carbapenemase activity compared to that of other class A enzymes.

Class C. The prevailing evidence regarding the class C hydrolytic mechanism suggests that Tyr150 behaves as a general base by increasing the nucleophilicity of Ser64 for acylation (299). Early studies of the deacylation mechanism indicated that Tyr150 also acts as the catalytic base, accepting a proton from the deacylating water (111, 299). For example, QM/MM calculations suggested that Tyr150 interacts with Lys67 in a conjugate base manner (136). However, the crystal structure of the E. coli AmpC in complex with a deacylation transition state analog revealed that Tyr150 remains protonated throughout the reaction and therefore is unlikely to be the anionic base that deprotonates the catalytic water for hydrolysis (84). Instead, deacylation may proceed by "substrate-activated catalysis" whereby the nitrogen on the β-lactam acts as a base and transiently accepts the proton from the hydrolytic water, while the Tyr150 proton helps stabilize the water’s developing negative charge (51, 84). The crystallographic evidence and mutagenesis studies do not rule out a role of Lys67 in the coordinate base mechanism, and further studies exploring the role of Lys67 are anticipated (84, 271).

Class D. Our understanding of the hydrolytic mechanism of class D β-lactamases is based on the careful study of OXA-10, -13, and -1 (253, 310, 334, 379, 398, 399). This class of enzymes is unique because of the direct role of carboxylation of the active-site Lys70. The carbamic acid on Lys70 can ionize to yield a carbamate that hydrogen bonds with the nucleophilic Ser67 residue. In this manner, the carboxylated Lys70 may serve as the general base by activating both Ser67 for acylation and the hydrolytic water for deacylation (144, 252, 253).

Class B. In contrast to the serine β-lactamases described above, class B includes Zn2+-dependent enzymes that follow a different hydrolytic mechanism. In general, MBLs use the -OH group from a water molecule that is coordinated by Zn2+ to hydrolyze the amide bond of a β-lactam. MBLs are divided into three classes based on their Zn2+ dependency, i.e., whether they (i) are fully active with either one or two ions (subclass B1, e.g., IMP-1, VIM-2, BcII, and CcrA), (ii) require two ions (subclass B3, e.g., L1), or (iii) employ one ion and are inhibited by binding of an additional ion (subclass B2, e.g., CphA) (130, 164, 165, 325, 326). Crowder and colleagues compiled the recent structural and mechanistic data and summarized the common features for the three subclasses of MBLs (96). MBLs utilizing two Zn2+ ions for hydrolysis, such as the subclass B3 S. maltophilia L1, coordinate the β-lactam substrate by the carbonyl and carbonyl groups, bridged by a hydroxide ion. After substrate binding, one of the Zn2+ ions, in conjunction with enzyme residues, polarizes the β-lactam carbonyl for attack by the -OH group, which is hydrogen bonded to deprotonated Asp120. Nucleophilic attack by the -OH group creates a tetrahedral species which rapidly collapses into an intermediate in which the β-lactam nitrogen is anionic. Protonation of the nitrogen leads to product formation. The source of the proton is not certain, and it may come from Asp120 or a water molecule.

The B1 enzyme Bacillus cereus BcII is active in both its mononuclear and dinuclear forms, and in the resting state, the Zn2+-bound -OH group is hydrogen bonded to the deprotonated Asp120 as well as several other active-site residues (Fig. 4) (96, 440). After attack by this -OH group, the
breakdown of the tetrahedral intermediate requires protonation of the β-lactam nitrogen; the source of this proton is under investigation. In the case of the CphA B2 MBL from Aeromonas hydrophila, a second bound Zn²⁺ ion is inhibitory. The proposed mechanism includes a water molecule activated by either His118 or Asp120, rather than a Zn²⁺-bound -OH group; the singular Zn²⁺ appears to help coordinate the β-lactam nitrogen (132, 451).

**CIRCUMVENTING β-LACTAMASES**

**β-Lactamase Inhibitors: Mechanistic Considerations**

A successful strategy for combating β-lactamase-mediated resistance is the use of agents designed to bind to the active site, which are frequently β-lactams. This strategy can take two forms: (i) create substrates that reversibly and/or irreversibly bind the enzyme with high affinity but form unfavorable steric interactions as the acyl-enzyme or (ii) develop mechanism-based or irreversible “suicide inhibitors” (53). Examples of the former are extended-spectrum cephalosporins, monobactams, or carbapenems which form acyl-enzymes and adopt catalytically incompetent conformations that are poorly hydrolyzed (Fig. 1, compounds 2, 3, and 4 to 7, respectively). For reversible inhibition, the reaction can be described as was shown above in equation 1, where S represents the (very) slowly hydrolyzed substrate. An equilibrium constant, $K_i$, can be calculated from the pre-steady-state rate constants, $k_{-1}/k_1$, and yields an estimate of affinity.

Irreversible “suicide inhibitors” can permanently inactivate the β-lactamase through secondary chemical reactions in the enzyme active site. Equation 5 represents a general mechanism of irreversible inhibitors (I) leading to permanent enzyme inactivation (E → I*):

$$E + I \xrightleftharpoons[k_{-1}]{k_1} E: I \rightarrow E - I \rightarrow E - I*$$  (5)

Examples of these inactivators are the commercially available class A inhibitors clavulanic acid, sulbactam, and tazobactam (Fig. 1, compounds 8 to 10). As will be described below, these types of inactivators often display additional pathways to inhibition. Irreversible inhibitors can be characterized by first-order rate constants for inhibition ($k_{\text{inact}}$, the rate of inactivation achieved with an “infinite” concentration of inactivator) and $K_I$ values (the concentration of inactivator which yields an inactivation rate that is half the value of $k_{\text{inact}}$) (54, 92). While $K_I$ closely approximates the meaning of $K_i$ for enzyme substrates, depending on the individual rate constants comprising the reaction, the $K_I$ may or may not equal the equilibrium constant $K_i = k_{-1}/k_1$ determined under pre-steady-state conditions.

The 50% inhibitory concentration (IC₅₀) measures the amount of inhibitor required to decrease enzyme activity to 50% of its uninhibited velocity. While an IC₅₀ can reflect an inhibitor’s affinity or $k_{\text{cat}}/k_{\text{inact}}$ ratio, these parameters are not always congruent; e.g., an inhibitor can have a very poor “affinity” and acylate the enzyme slowly but still yield a low IC₅₀ because of very low deacylation rates.

**β-Lactamase Inhibitors in Clinical Practice**

Clavulanic acid, sulbactam, and tazobactam. Clavulanic acid, the first β-lactamase inhibitor introduced into clinical medicine, was isolated from Streptomyces clavuligerus in the 1970s, more than 3 decades ago (360). Clavulanate (the salt form of the acid in solution) showed little antimicrobial activity alone, but when combined with amoxicillin, clavulanate significantly lowered the amoxicillin MICs against S. aureus, K. pneumoniae, Proteus mirabilis, and E. coli (47).

Sulbactam and tazobactam are penicillamine sulfones that were later developed for the pharmaceutical industry as synthetic compounds in 1978 and 1980, respectively (117, 121).

All three β-lactamase inhibitor compounds share structural similarity with penicillin; are effective against many susceptible organisms expressing class A β-lactamas (including CTX-M and the ESBL derivatives of TEM-1, TEM-2, and SHV-1); and are generally less effective against class B, C, and D β-lactamas (53, 60, 67, 341). The activity of an inhibitor can be evaluated by the turnover number ($t_\text{cat}$) (also equivalent to the partition ratio [$k_{\text{cat}}/k_{\text{inact}}$]), defined as the number of inhibitor molecules that are hydrolyzed per unit time before one enzyme molecule is irreversibly inactivated (58). For example, S. aureus PC1 requires one clavulanate molecule to inactivate one β-lactamase enzyme, while TEM-1 needs 16 clavulanate molecules, SHV-1 requires 60, and B. cereus I requires more than 16,000 (53, 69, 113, 123, 411). For comparison, sulbactam $t_\text{cat}$s are 10,000 and 13,000 for TEM-1 and SHV-1, respectively (180, 410).

The low $K_S$ of the inhibitors for class A β-lactamas (nM to μM), the ability to occupy the active site “longer” than β-lactams (high acylation and low deacylation rates), and the failure to be hydrolyzed efficiently are integral to their efficacy (158). Clavulanate, sulbactam, and tazobactam differ from β-lactam antibiotics as they possess a leaving group at position C-1 of the five-membered ring (sulbactam and tazobactam are sulfones, while clavulanate has an enol ether oxygen at this position). The better leaving group allows for secondary ring opening and β-lactamase enzyme modification. Compared to clavulanate, the unmodified sulfone in sulbactam is a relatively poor leaving group, a property reflected in the high partition ratios for this inhibitor (e.g., for TEM-1, sulbactam $t_\text{cat} = 160$ (179, 180), Tazobactam possesses a triazole group at the C-2 position that lowers the efficiency of the PC1 β-methyl group. This modification leads to tazobactam’s improved IC₅₀s, partition ratios, and lowered MICs for representative class A and C β-lactamas (36, 58, 60).

The efficacy of the mechanism-based inhibitors can vary within and between the classes of β-lactamas (Table 2). For class A, SHV-1 is more resistant to inactivation by sulbactam than TEM-1 but more susceptible to inactivation by clavulanate (328). Comparative studies of TEM- and SHV-derived enzymes, including ESBLS, found that the IC₅₀s for clavulanate were 60- and 580-fold lower than those for sulbactam against TEM-1 and SHV-1, respectively (328). In our opinion, the explanations for these differences in inactivation chemistry are likely subtle, yet highly important, differences in the enzyme active sites. For example, Thomson et al. compared the atomic structure models of TEM-1 and SHV-1 and found that the distance between Val216 and Arg244, residues responsible for positioning of the water
molecule important in the inactivation mechanism of clavulanate, was more than 2 Å greater in SHV-1 than in TEM-1 (411). This increased distance may be too great for coordination of a water molecule, suggesting that the strategic water is positioned elsewhere in SHV-1 and may be recruited into the active site with acylation of the substrate or inhibitor. This variation underscores the notion that mechanism-based inhibitors may undergo different inactivation chemistry even in highly similar enzymes (410, 411).

**Mechanism of inhibition.** Evidence from X-ray crystallography, UV difference spectra, isoelectric focusing, mass spectrometry (MS), and Raman microscopy suggests that the inactivators of class A β-lactamases undergo complex reaction schemes with multiple branch points after formation of the acyl-enzyme (45, 78, 79, 157, 309, 396). As represented in equations 5 and 6, the acyl-enzyme intermediate can (i) undergo a reversible change that generates a transiently inhibited enzyme, a tautomer (E – T); (ii) lead to permanent inactivation as a covalent acyl-enzyme species (E – I*); or (iii) regenerate the active enzyme via hydrolysis (E + P):

\[
\text{E} \rightarrow \text{T} \quad \frac{k_4}{k_{-4}}
\]

\[
\text{E-I} \xrightarrow{k_3} \text{E} + \text{P}
\]  

(6)

\[
\text{E} \rightarrow \text{I}^*
\]

The functional inhibition of the enzyme is determined by the relative rates \((k_4, k_3, k_{-4}, \text{ and } k_{-3})\) of each of these pathways and in particular by the formation of the \(E - I^*\) species (53).

Figure 5 shows a more detailed mechanism describing clavulanate inhibition of PCI, TEM-1, or SHV-1 (78, 79, 81, 122, 179, 180). Kinetic and mass spectrometry analysis of inactivation mechanisms, combined with crystallographic studies, suggests that clavulanate, sulbactam, and tazobactam follow similar reaction pathways, beginning with formation of an acyl-enzyme species (48, 81, 307, 308). After acylation, opening of the five-membered ring leads to formation of a transient imine intermediate. This imine species is likely the common intermediate preceding the chemical conversions that lead to transient enzyme inhibition (44, 179, 197). Raman spectroscopy of SHV/inhibitor crystals show that the imine species then rearranges to form enamine intermediates (197, 307). This enamine intermediate, in either the trans or cis conformation, represents a second important intermediate in the inactivation mechanism (69, 81, 368). Depending on the properties of the enzyme and inhibitor, the reaction will ultimately proceed to decylation or irreversible (prolonged) inactivation. In the case of decylation of the enamine intermediate, the acyl-enzyme undergoes decarboxylation and ester bond hydrolysis, regenerating the active β-lactamase, albeit very slowly (260).

The duration of transient inhibition is determined, in part, by the stability of the intermediate species. Preceding acylation of the inhibitor, a persistent noncovalent Michaelis complex may account for inhibition of enzyme activity (196). Following formation of the acyl-enzyme, stabilization of the enamine intermediate is a significant factor in prolonged enzyme inhibition. Crystals of tazobactam in complex with the SHV Glu166Ala variant (PDB 1RCJ) showed that tazobactam formed stoichiometric amounts of the \(\text{trans-enzyme}\) (308). The \(\text{trans-enzyme}\) intermediate of tazobactam, as opposed to the \(\text{trans-enzyme}\) intermediates of clavulanate and sulbactam, may be stabilized by intra- and intermolecular interactions. These interactions between the sulfone and triazolyl moieties of the tazobactam intermediate and the enzyme active site may explain, in part, tazobactam’s potent \(\text{in vitro}\) and \(\text{in vivo}\) inhibition of many serine β-lactamases (36, 60, 308, 312).

Data from Raman crystallography also support the hypothesis that compared to clavulanate and sulbactam, tazobactam more readily forms the \(\text{trans-enzyme}\) intermediate (197). In this case, Raman spectroscopy allows for the identification of reaction intermediates and calculation of their rates of decay and accumulation by examining single crystals in solution. Studies of SHV-1 in complex with each of the mechanism-based inhibitors revealed that tazobactam forms a predominant population of \(\text{trans-enzyme}\), as opposed to clavulanate and sulbactam, which form a mixture of \(\text{trans-enzyme}\) and the

### Table 2. Kinetic properties of representative β-lactamases

<table>
<thead>
<tr>
<th>β-Lactamase</th>
<th>Ambler class</th>
<th>Clavulanate</th>
<th>Sulbactam</th>
<th>Tazobactam</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1 A</td>
<td>0.1</td>
<td>60</td>
<td>160</td>
<td>1.6</td>
<td>900</td>
</tr>
<tr>
<td>SHV-1 A</td>
<td>1</td>
<td>12</td>
<td>60</td>
<td>8.6</td>
<td>12,000</td>
</tr>
<tr>
<td>SHV-5 A</td>
<td>20</td>
<td>20</td>
<td>1</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>PCl</td>
<td>30</td>
<td>30</td>
<td>1</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>CTX-M-2 A</td>
<td>200</td>
<td>200</td>
<td>1</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>CcrA B</td>
<td>&gt;500,000</td>
<td>&gt;500,000</td>
<td>&gt;500,000</td>
<td>&gt;500,000</td>
<td>400,000</td>
</tr>
<tr>
<td>P99 C</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>&gt;100,000</td>
<td>8.5</td>
</tr>
<tr>
<td>CMY-2 C</td>
<td>4,365</td>
<td>101</td>
<td>1</td>
<td>5,600</td>
<td>50</td>
</tr>
<tr>
<td>OXA-1 D</td>
<td>1,800</td>
<td>4,700</td>
<td>380</td>
<td>1400</td>
<td>50</td>
</tr>
<tr>
<td>OXA-2 D</td>
<td>1,400</td>
<td>1,400</td>
<td>0.1</td>
<td>140</td>
<td>50</td>
</tr>
</tbody>
</table>

* The definition of \(K_i\) may not be uniform from laboratory to laboratory. See the cited references for the methods used in each determination.
more chemically labile cis-enamine and imine species (197).

Analysis of the inactivation reactions of PC1, TEM, and SHV \( \beta \)-lactamases using mass spectrometry has identified a series of intermediates beyond the acyl-enzyme, imine, and enamine (48, 81, 311, 396, 411). The terminal end products of inhibition with these mechanism-based inhibitors suggest that a covalent modification of the active-site Ser70 persists through the inhibition process. Mass adducts (in daltons) correspond to different inactivated enzyme species; e.g., (i) Ser70-Ser130 cross-linked enzyme or propynyl enzyme, (ii) aldehyde, (iii) hydrated aldehyde, and (iv) decarboxylated trans-enamine are some of the products (Fig. 5).

\[ \text{Irreversible inactivation products} \]

\[ \text{Decaylation and regeneration of active enzyme} \]

\[ \text{Transient inhibition intermediates} \]

**FIG. 5.** Proposed mechanism of inhibition of class A \( \beta \)-lactamases by clavulanate, showing the different acyl-enzyme fragmentation products (expressed in daltons), that have been experimentally observed. (Based on data from references 48, 108, 197, 411, and 453.)

**\( \beta \)-Lactam–\( \beta \)-lactamase inhibitor combinations: clinical use.**

Generally, the inhibitors do not inactivate PBPs, but notable exceptions include (i) the intrinsic activities of sulbactam against *Bacteroides* spp., *Acinetobacter* spp., and *N. gonorrhoeae*; (ii) clavulanate action against *Haemophilus influenzae* and *N. gonorrhoeae*; and (iii) tazobactam inhibition of PBPs in *Borrelia burgdorferi* (53, 167, 222, 285, 423, 424, 447). As these “antibacterial effects” are relatively weak, the inhibitors are always combined with \( \beta \)-lactams for clinical use. Currently, there are five \( \beta \)-lactam–\( \beta \)-lactamase inhibitor formulations available. Amoxicillin-clavulanate, ticarcillin-clavulanate, ampicillin-sulbactam, and piperacillin-tazobactam are available in the United States. Cefoperazone-sulbactam is used in several European countries, Japan, and India but is not available in the United States, and therefore we will not include this combination in this review. Table 3 summarizes selected clinical features of these available combinations.

Importantly, as in clinical usage, the \( \beta \)-lactamase inhibitors are combined with \( \beta \)-lactams for *in vitro* susceptibility testing. The composition of these formulations can have a major impact on susceptibility results, which may or may not reflect clinical efficacy. We present to the reader the following example. The ticarcillin-clavulanate preparation for oral administration is 3.0 g-0.1 g, while ampicillin-sulbactam is at a 2.0 g-1.0 g ratio. However, both combinations are tested at a 2:1 ratio in *in vitro* studies, and since clavulanate has higher “gram-for-gram” potency than sulbactam, clavulanate may “test better” in MIC profiling. However, clinically ampicillin-sulbactam may retain efficacy because of the relatively large amount of inhibitor delivered relative to the \( \beta \)-lactam and relative to the amount of \( \beta \)-lactamase in the cell. This scenario illustrates an important issue in susceptibility testing and its correlate to clinical efficacy.

(i) Amoxicillin-clavulanate. Amoxicillin-clavulanate was the first \( \beta \)-lactam–\( \beta \)-lactamase inhibitor combination introduced into clinical practice, in 1981 in the United Kingdom and in 1984 in the United States, and remains the only combination available for oral use. The activity of amoxicillin against susceptible bacteria that do not possess a \( \beta \)-lactamase (e. g., *streptococci*, *enterococci*, *E. coli*, and *Listeria* spp.) is not improved by the use of clavulanate. However, the addition of clavulanate significantly expands amoxicillin’s spectrum to include penicillinase-producing *S. aureus*, *H. influenzae*, Moraxella catarrhalis, *Bacteroides* spp., *E. coli*, *Klebsiella* spp., and *P. mirabilis* (59, 387). The oral availability of amoxicillin-clavulanate makes it well suited for the outpatient clinic, and this \( \beta \)-lactam–\( \beta \)-lactamase inhibitor combination exerted its greatest impact on the treatment of community-acquired respiratory infections (59). Additionally, amoxicillin-clavulanate is sometimes used as an oral equivalent (“step-down therapy”) of ampicillin-sulbactam or ticarcillin-clavulanate in the treatment of skin, soft tissue, abdominal, and gynecological infections. Intravenous formulations of amoxicil-
lin-clavulanate are available in Europe and have been studied primarily for their efficacy against ESBL-producing *E. coli* bacteremia (239, 370).

(ii) **Ticarcillin-clavulanate.** When introduced in 1985, ticarcillin-clavulanate was the first β-lactam–β-lactamase inhibitor combination available for parenteral administration. Similar to the case for amoxicillin, the addition of clavulanate does not increase activity against pathogens for which ticarcillin alone is effective, such as non-β-lactamase-producing *Haemophilus* spp., *E. coli*, *Proteus* spp., *Enterobacter* spp., *Morganella* spp., and *Providencia* spp., and *P. aeruginosa*. However, the combination of ticarcillin and clavulanate does increase activity against β-lactamase-producing staphylococci, *E. coli*, *H. influenzae*, *Klebsiella* spp., *P. aeruginosa*, and *S. aureus*.

### TABLE 3. Selected clinical features of available β-lactam–β-lactamase inhibitor combinations

<table>
<thead>
<tr>
<th>β-Lactam–β-lactamase inhibitor composition</th>
<th>Trade name; manufacturer</th>
<th>Route of administration, formulations, and dosing intervala</th>
<th>( t_{1/2}, \text{h} ) (% protein bound)</th>
<th>Mean peak serum levels (µg/ml)</th>
<th>Approved clinical indications</th>
<th>Proven clinical efficacy for susceptible causative organism(s):</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Piperacillin-tazobactam</strong></td>
<td>Zosyn; Wyeth</td>
<td>i.v., 2.0 g/0.25 g q6h, 3.0 g/0.375 g q6h, 4.0 g/0.5 g q6h</td>
<td>1 h after 3.375-g dose: piperacillin, 106; tazobactam 10.7</td>
<td>Appendicitis</td>
<td>Skin and soft tissue infections, including diabetic foot infections Postpartum endometritis or pelvic inflammatory disease Community-acquired pneumonia Hospital-acquired, ventilator-acquired pneumonia</td>
<td><em>E. coli</em>, Bacteroides spp. <em>S. aureus</em> <em>E. coli</em> <em>H. influenzae</em> <em>S. aureus</em>, <em>A. baumannii</em>, <em>H. influenzae</em>, <em>K. pneumoniae</em>, <em>P. aeruginosa</em></td>
</tr>
<tr>
<td><strong>Amoxicillin-clavulanate</strong></td>
<td>Unasyn; Pfizer</td>
<td>i.v./i.m., 1.0 g/0.5 g q6h, 2.0 g/1.0 g q6h Tablet, 250 mg</td>
<td>1 h after 250-mg/62.5-mg dose: amoxicillin, 6.9; clavulanate, 1.8</td>
<td>Skin and skin structure infections Intra-abdominal infections Gynecological infections</td>
<td><em>S. aureus</em>, <em>E. coli</em>, <em>Klebsiella</em> spp., <em>P. mirabilis</em>, <em>B. fragilis</em>, <em>Enterobacter</em> spp., <em>A. calcoaceticus</em> <em>E. coli</em>, <em>Klebsiella</em> spp., <em>Bacteroides</em> spp., <em>Enterobacter</em> spp., <em>Bacteroides</em> spp.</td>
<td></td>
</tr>
<tr>
<td><strong>Amoxicillin-sulbactam</strong></td>
<td>Augmentin; GlaxoSmithKline</td>
<td>Pediatric oral suspension, 600 mg/42.9 mg/5 ml q12h Chewable tablet and oral suspension, 125 mg/31.25 mg q8h, 200 mg/28.5 mg q12h, 250 mg/62.5 mg q8h, 400 mg/57.0 mg q12h Tablet, 250 mg/125 mg q8h, 500 mg/125 mg q8-12h, 875 mg/125 mg q12h Extended-release tablet, 1,000 mg/62.5 mg q12h</td>
<td>1 h after 15-min infusion of 1-g/0.5-g dose: amoxicillin, 40-71; sulbactam, 21-40</td>
<td>Skin and skin structure infections Urinary tract infections</td>
<td><em>S. pneumoniae</em>, <em>H. influenzae</em>, <em>M. catarrhalis</em>, <em>H. influenzae</em>, <em>M. catarrhalis</em>, <em>H. parainfluenzae</em>, <em>K. pneumoniae</em>, methicillin-susceptible <em>S. aureus</em>, <em>S. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td><strong>Ticarcillin-clavulanate</strong></td>
<td>Timentin; GlaxoSmithKline</td>
<td>i.v., 3.0 g/0.1 g q4-6h</td>
<td>1 h after 3.1-g dose: ticarcillin, 131; clavulanate, 1.8</td>
<td>Septicemia Lower respiratory tract infections Bone and joint infections Skin and skin structure infections Urinary tract infections</td>
<td><em>Klebsiella</em> spp., <em>E. coli</em>, <em>S. aureus</em>, <em>P. aeruginosa</em> <em>S. aureus</em>, <em>H. influenzae</em>, <em>Klebsiella</em> spp. <em>S. aureus</em>, <em>Klebsiella</em> spp., <em>E. coli</em> <em>E. coli</em>, <em>Klebsiella</em> spp., <em>P. aeruginosa</em>, <em>Citrobacter</em> spp., <em>Enterobacter cloacae</em>, <em>Serratia marcescens</em>, <em>S. aureus</em> Preotella melanogeniosus, <em>Enterobacter</em> spp., <em>E. coli</em>, <em>K. pneumoniae</em>, <em>S. aureus</em>, <em>E. coli</em>, <em>K. pneumoniae</em>, <em>B. fragilis</em></td>
<td></td>
</tr>
</tbody>
</table>

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*a* i.v., intravenous; i.m., intramuscular; q6h, every 6 h.

*b* When treated with piperacillin-tazobactam, hospital-acquired and ventilator-acquired pneumonia caused by *P. aeruginosa* should be administered with an aminoglycoside.

*c* The dose of amoxicillin-clavulanate in the pediatric oral suspension is 45 mg/kg q12h based on information available in the material supplied by the manufacturer.
spp., N. gonorrhoeae, Moraxella catarrhalis, and Bacteroides spp. (59, 127, 319). Surprisingly, ticarcillin-clavulanate exhibits activity against the multidrug-resistant, non-lactose-fermenting S. maltophilia, and the use of this combination in addition to other antimicrobials (e.g., aztreonam or trimethoprim-sulfamethoxazole) leads to synergistic killing (118, 275). This phenomenon may represent a very interesting opportunity to study the activity of ticarcillin-clavulanate against S. maltophilia.

Clavulanate induces the expression of chromosomally mediated AmpC β-lactamases in many Enterobacteriaceae and can antagonize the antibacterial effects of ticarcillin as a partner β-lactam (8, 203, 231, 237). This ticarcillin antagonism was observed in laboratory checkerboard studies with E. cloacae and Morganella morgani strains (237). However, the impact of β-lactamase induction in the clinic is very hard to measure. When testing or evaluating antibiotics in preclinical or clinical trials, β-lactamase induction may bear importance as an eventual predictor of efficacy. In our opinion, the ability of β-lactamase inhibitors to induce cephalosporinase production should be carefully examined and defined before clinical trials are performed.

In response to the potential for increased AmpC production due to β-lactam inducers (such as clavulanate, but including also cefoxitin, imipenem, and ceftazidime) as well as the relatively poor performance of amoxicillin-clavulanate and ticarcillin-clavulanate against ESBL expressors, Livermore et al. and Nikaido et al. have proposed the combination of clavulanate with a cephalosporin, such as cefepime or cepfirome, that is more stable against AmpC enzymes (239, 288). Cefepime-clavulanate and cefpirome-clavulanate combinations, with a constant concentration of 4 μg/ml clavulanate, were effective at ≤1 μg/ml against ESBL-producing Enterobacteriaceae (239). The combination of cefepime and clavulanate enjoys a particular advantage due to the inability of class C enzymes to hydrolyze cefepime and the ability of clavulanate to inhibit ESBLs. Currently, there are important obstacles to combining these agents (i.e., all three agents are at the end of patent protection, and the required trials of the formulations would be very expensive), but provided that the clinical research can be performed, these combinations may be an attractive alternative to carbapenem treatment of ESBL-producing organisms. We hasten to add that attention should be paid to the pharmacokinetics and pharmacodynamics of these novel combinations before randomized controlled trials are performed, as it is not possible to “fix” a concentration of clavulanate in vivo.

(iii) Ampicillin-sulbactam. Ampicillin shows activity against most streptococci, enterococci, Listeria spp., and strains of S. aureus, H. influenzae, E. coli, P. mirabilis, Salmonella spp., and Shigella spp. that are devoid of β-lactamases (an important exception are ampicillin-resistant strains of H. influenzae that do not contain a β-lactamase and often have substitutions in PBPs sequences) (194, 257). In combination with sulbactam, the activity extends to β-lactamase-containing S. aureus, H. influenzae, M. catarrhalis, E. coli, Proteus spp., Klebsiella spp., and anaerobes (59, 362, 444). When the combination of ampicillin at 2.0 g and sulbactam at 1.0 g was marketed in 1987, this broad-spectrum activity made ampicillin-sulbactam ideal therapy for polymicrobial infections such as abdominal and gynecological surgical infections, aspiration pneumonia, odontogenic abscesses, and diabetic foot infections. The early clinical success of this combination (and susceptibility of many pathogens to ampicillin-sulbactam and ticarcillin-clavulanate) established confidence in the role of β-lactam-β-lactamase inhibitor therapy. Unfortunately, the resistance to ampicillin-sulbactam among clinical isolates of E. coli is increasing (199).

Sulbactam is not well absorbed orally and must be administered parenterally (126). However, the intravenous ampicillin-sulbactam combination is well tolerated, with very few reported side effects (7, 66).

(iv) Piperacillin-tazobactam. Piperacillin in combination with tazobactam became available in the United States in 1993. Piperacillin is a broad-spectrum penicillin that is bactericidal against many Gram-positive and Gram-negative aerobes and anaerobes (140). As a single agent, piperacillin demonstrates activity against P. aeruginosa, pneumococci, streptococci, anaerobes, and Enterococcus faecalis, and this activity is retained in combination with tazobactam (232). Clinicians must remember that the addition of tazobactam does not always increase susceptibility of P. aeruginosa and other Gram-negative bacilli expressing AmpC β-lactamases (140). However, tazobactam does extend piperacillin’s activity against most β-lactamase producing strains of Enterobacteriaceae, H. influenzae, N. gonorrhoeae, and M. catarrhalis and has the potential to lower MICs against these strains expressing ESBLs (59, 192). The in vitro inhibition of CMY-type β-lactamases by tazobactam is reported, but the clinical relevance of this phenotype is still uncertain (34, 116a).

Several recent retrospective studies of clinical outcomes from E. coli and K. pneumoniae ESBL-producing isolates argue for the efficacy of piperacillin-tazobactam in the treatment of these infections (135, 336, 370, 420). Gavin et al. showed that when in vitro testing revealed susceptibility to piperacillin-tazobactam (≤16/4 μg/ml), successful outcomes were seen in 10 out of 11 non-urinary tract infections (135). An examination of 43 bloodstream infections caused primarily by CTX-M ESBL-producing E. coli isolates revealed that piperacillin-tazobactam, amoxicillin-clavulanate (i.v.), or carbapenem treatment led to lower mortality than with cephalosporin or fluoroquinolone treatment (9% versus 35% (370). Another study, including infections caused by SHV- and TEM-type ESBL-producing K. pneumoniae, established that empirical therapy with β-lactam–β-lactamase inhibitor combinations was validated by in vitro susceptibility testing in 75% of cases, compared to 0% for oxymenocephalosporin treatment (420). Furthermore, initial treatment with an adequate antibiotic was associated with lower mortality rates (37%) than inadequate empirical therapy (67%). While this analysis did not stratify the outcome data based on ESBL type, other authors have reported that TEM-type ESBLs are more susceptible to piperacillin-tazobactam than are SHV-type ESBLs (185, 361).

Several observational studies have strongly suggested that the replacement of extended-spectrum cephalosporins with piperacillin-tazobactam can help lower the rates of infection from Gram-negative ESBL producers and vancomycin-resistant Enterococcus spp. (16, 43, 215, 216, 323, 355, 365). While the mechanism for decreasing ESBL-producing isolates is not clear, it must be noted that the efficacy of β-lactam–β-lactamase inhibitor combinations may be reduced for organisms.
producing multiple ESBLs, particularly if they also have an AmpC β-lactamase (42, 321, 381). For this reason, we caution against the use of piperacillin-tazobactam for the treatment of ESBL-producing pathogens.

So, why do the in vitro susceptibility data and the clinically cited data suggest that piperacillin-tazobactam can be used in the treatment of ESBL producers? The reasons for this contradiction include that piperacillin, as a partner β-lactam for β-lactamase inhibitors, is relatively resistant to hydrolysis by certain plasmid-mediated β-lactamasases (e.g., TEM) compared to amoxicillin, ampicillin, or ticarcillin. This substrate stability may be due, in part, to piperacillin’s lower affinity for bacterial PBPs (234). We also propose that the pharmacokinetics and pharmacodynamics of the 3.375-g and 4.5-g formulations lead to extended periods of time when free drug concentrations (>100 μg/ml piperacillin in serum) are greater than the MIC for ≥50% of the dosing interval for many nosocomial pathogens (242). It is likely that the high serum concentrations combined with the superior activity of piperacillin and the relatively “better” inhibitory activity of tazobactam versus clavulanate or sulbactam is enough to inhibit a majority of β-lactamasases in a complex β-lactamase background (class A and C β-lactamasases) (328). However, this efficacy is not always clinically certain, and the physician must be aware that the susceptibility pattern of an infecting Gram-negative pathogen does not always reveal the number and complexity of the β-lactamasases present. Moreover, the formulation that is tested in the lab may not mirror what is administered to patients.

Inhibitory Activity of Carbapenems

It is our opinion that consideration of carbapenems, although they are not explicitly β-lactamase inhibitors, is essential to a review of β-lactamase inhibition. These thienamycin derivatives are among the “last line of defense” against many multidrug-resistant pathogens. An increasing body of evidence suggests that carbapenems are effective not just for their broad-spectrum antibacterial activity but also for their multiple β-lactamase-inhibitory mechanisms. The insight gleaned from the ability of carbapenems to inhibit serine β-lactamasases may serve as a useful starting point for intelligent inhibitor design. Also, while we await the development and release of novel β-lactamase inhibitors, it behooves us to wisely implement the β-lactams currently in our armamentarium. With this in mind, a discussion of the inhibitory activity of carbapenems follows.

Meropenem, imipenem, ertapenem, and doripenem (doripenem was released in the U.S. and European markets in 2007) are often reserved for the most difficult-to-treat Gram-negative infections (Fig. 1, compounds 4 to 7) (320). Although resistance to carbapenems is rare in the clinic, several carbapenem-hydrolyzing serine β-lactamasases are identified, such as KPC-2, NMC-A, SME-1, and IMI-1 (358, 455). These enzymes may be found in organisms that also produce additional β-lactamasases, conferring broad substrate profiles. For example, E. cloacae isolates can produce IMI-1 or NMC-A, which hydrolyze carbapenems, and also TEM and AmpC-type enzymes (359).

In addition to their broad antibacterial spectrum, carbapenems are also effective inhibitors, or “slow substrates,” of most serine β-lactamasases.

The stability of thienamycins to β-lactamase hydrolysis was noted in early studies of imipenem, including low $k_{cat}$ values for the class A β-lactamase of B. cereus and a class C P. aeruginosa β-lactamase (150, 208, 270, 366). Charnas and Knowles observed biphasic kinetics when RTEM was acylated by carbapenem compounds; an initial burst was followed by a slower steady-state reaction, strongly suggestive of a branched reaction pathway (80, 114). Further work by Mobashery’s group demonstrated that acylation of TEM-1 by imipenem occurs readily and is followed by $\Delta^2$- to $\Delta^1$-tautomerization of the pyrroline double bond in the acyl-enzyme species (Fig. 6) (407). The rate of deacylation differs for the tautomers (the $\Delta^1$ tautomer is approximately 10-fold slower than the $\Delta^2$ tautomer), and it was initially accepted that molecular rearrangements to the more stable (i.e., more slowly deacylated) species accounted for enzyme inhibition. Site-directed mutagenesis studies of TEM-1 Arg244Ser showed that the guanidinium group on Arg244 provides directly, or indirectly via a water molecule, the necessary proton for tautomerization to the $\Delta^1$-pyrroline acyl-enzyme (407, 458). Incidentally, this water appears to be the same molecule that serves as the proton donor necessary for clavulanate inhibition in class A enzymes (179).

Elucidation of the crystal structures of several β-lactamasases in complex with carbapenems has offered additional insight into the versatile inhibition mechanism of these compounds. Maveyraud et al. found that in the structure of TEM-1 complexed with imipenem (PDB 1BTS), the ester carbonyl oxygen of the acyl enzyme was not located in the oxyanion hole (255). This approximately 180° rotation of the carbonyl was also seen in the crystal structure of acyl-enzyme species of imipenem complexed via Ser64 with the AmpC β-lactamase (PDB 1LL5) (21). In contrast, the SHV-1/meropenem crystal demonstrated two conformations of the intermediate acyl species, one where the meropenem carbonyl oxygen of the acyl-enzyme is in the oxyanion hole and one where it is not (Fig. 7) (295). Proper position in this oxyanion hole, the binding pocket for the β-lactam carbonyl, is important for both initial enzyme acylation and hydrolysis of the ester bond (277, 425). A conformation which places the β-lactam carbonyl outside of this electrophilic center may be delayed in hydrolysis.

Computational models of TEM-1 with imipenem demonstrated that the covalent adduct formed first after acylation did have its carbonyl in the oxyanion hole (255). However, this conformation created a steric clash between the hydroxethyl group and residues 129 to 131, which forces the rearrangement...
of the acyl-enzyme rotating the carbonyl outside the oxyanion hole. Thus, while producing a conformational change in the enzyme, the C-6 hydroxyethyl substituent also facilitates rearrangement of the carbapenem to a more slowly deacylated form. Subsequent examination of the TEM-Asn132Ala variant demonstrated that replacement of the residue relieved the steric encumbrance of the TEM-1/imipenem acyl-enzyme and allowed the carbonyl to rotate back into the oxyanion hole (437). Furthermore, the TEM-1/imipenem structure indicated that hydrogen bonds were formed between the hydroxyl group of the C-6 hydroxyethyl substituent on imipenem and both the Glu166-as sociated hydrolytic water and the side chain oxygen of Asn132 (255). Similarly, the SHV-1/meropenem and class D OXA-13/meropenem structures (PDB 2ZD8 and 1H8Y, respectively) revealed that the putative deacylation water molecule has an additional hydrogen-bonding interaction with the -OH group of meropenem’s C-6 hydroxyethyl substituent (295, 334). This interaction weakens the nucleophilicity and/or changes the direction of the lone pair of electrons of the water molecule and results in poor turnover of the carbapenems.

As individual hydrogen atoms are difficult to resolve by X-ray crystallography, the structural data for β-lactamases and carbapenems have not been able to discriminate between the Δ1- and Δ2-pyrrolidine tautomers. In contrast, Raman spectroscopy allows the monitoring of specific bond character and compound reactivity. Recent studies of Raman difference spectra in SHV-1 reacted with meropenem, ertapenem, and imipenem propose the correlation that the Δ1-pyrrolidine tautomer corresponds to the acyl-enzyme species with the carbonyl outside the oxyanion hole and the Δ2-pyrrolidine tautomer to the acyl-enzyme with the carbonyl inside the electrophilic center (195). Further, this work suggests that in crystal form the Δ1- and Δ2-tautomers are present in equal amounts but that Δ1-pyrrolidine predominates in solution. This ratio is consistent with the kinetic data implicating the Δ1-tautomer as the primary inhibitory species.

The recent structure of OXA-1, a class D monomeric β-lactamase, inactivated by doripenem provided further support for the role of carbapenems as inactivators of serine β-lactamases (PDB 3ISG) (379a). In this structure by Schneider and colleagues, although the carbonyl oxygen is positioned in the oxyanion hole, water molecules are absent. The carbamate of Lys70 forms a hydrogen bond to the C-6 hydroxyethyl group, and Lys212 and Thr213 form a salt bridge and hydrogen bond to doripenem. Furthermore, the bond angles of the acyl-enzyme suggest the Δ1-tautomer is formed.

In addition to the imipenem-induced conformation change of class A and C β-lactamases, the (albeit slow) turnover of carbapenems leads to a stable enzyme-product species that occupies the β-lactamase active site for relatively longer than the enzyme-products of other β-lactams, such as penicillin (whose dissociation is limited by diffusion for class A enzymes) (149, 254). However, the deacylation rate was still lower than the dissociation rate for imipenem, suggesting that acyl-enzyme rearrangement accounts most significantly for inhibition. But notably, the presence of hydrolyzed inhibitor in the active site can contribute to enzyme inhibition.

FIG. 7. Molecular representation of SHV-1/meropenem acyl-enzyme, based on PDB coordinates 2ZD8. The conformation with the carbonyl of the acyl-serine bond in the oxyanion hole (formed by NH amides of Ser70 and Ala237) is colored teal, while the conformation with the carbonyl flipped out is colored purple. Residues 244, 130, and 105, which demonstrate significant orientation changes compared to the apo SHV-1 β-lactamase, are labeled. Glu166 and Asn132, important for making hydrogen bonds with the deacylation water (the approximate location is represented as a red sphere) and C-6 hydroxyethyl substituent, respectively, are shown. The large R’ carbamoyl-pyrrolidinyl group of meropenem was disordered and thus is only partially illustrated.
INHIBITOR-RESISTANT CLASS A β-LACTAMASES

Within several years of the introduction of clavulanate combinations for clinical use, resistance to amoxicillin-clavulanate and ticarcillin-clavulanate was observed in isolates of *E. coli* and *K. pneumoniae* (247, 248, 376, 446). In 1987, Martinez et al. reported resistance rates in *E. coli* in Madrid hospitals and estimated that of amoxicillin-resistant strains, 20 to 30% were co-amoxicillin-clavulanate resistant (248). Investigations in the late 1980s demonstrated that this phenotype may result from production of β-lactams not susceptible to the inhibitors (e.g., AmpCs from *Enterobacter* spp. or *P. aeruginosa* or metallo-β-lactamases) or enzyme hyperproduction (68). Hyperproduction of a β-lactamase can be mediated by mutations in the promoter region of the gene and/or high copy numbers of plasmids carrying the bla gene; both scenarios were identified for the TEM-1 enzyme (247, 248, 446, 448, 449).

Additional resistance mechanisms were concurrently being examined in research laboratories. In 1989, Oliphant and Strulh studied clavulanate and sulbactam resistance in TEM-1 by introducing a series of random substitutions into the DNA segment which coded for the enzyme’s active site (300). Through mutagenesis of a 17-amino-acid segment of the TEM-1 active site (Arg61 to Cys77) and functional selection assays, they found that enzymes with an Ile, Leu, or Val substitution at Met69 had increased resistance to ampicillin-clavulanate and ampicillin-sulbactam. This foreshadowed an important amino acid substitution in inhibitor-resistant (IR) TEM.

Three years later, Zafaralla et al. engineered by site-directed mutagenesis the Arg244Ser variant of TEM-1 to explore the role of this key amino acid and its interaction with the penicillin C-3 carboxylate (457). This work advanced the earlier hypothesis by Moews et al. that Arg244 played an important role in the recognition of the conserved substrate C-3/C-4 carboxylate (179, 267). Further studies by Mobashery’s laboratory also revealed that Arg244Ser was more resistant to inactivation by clavulanate (179). Interestingly, the first IR class A β-lactamase was isolated from a clinical isolate of *E. coli* in the same year. The enzyme was determined to be related to TEM-1 or TEM-2 and hence was designated inhibitor-resistant TEM (IRT-1) (29, 427). Sequencing information revealed an Arg-to-Cys or -Ser substitution at residue 244 (23, 427).

Since that time in the early 1990s, the number of clinically identified IRTs has expanded to include more than 35 unique enzymes (Table 4) (www.lahey.org/studies/webt.asp). In addition to amoxicillin-clavulanate-resistant *E. coli*, IRTs have been identified in *Klebsiella*, *Proteus, Shigella*, and *Citrobacter* spp. (385). IR SHVs have also been isolated from *K. pneumoniae*, with a total of five IR SHVs presently described (Table 5) (109). Operationally, inhibitor resistance refers to resistance to amoxicillin-clavulanate and may, but does not necessarily, include resistance to sulbactam and tazobactam (410). Moreover, we stress that this is a “relative resistance”; provided that sufficient clavulanate concentrations are used, these IR enzymes can be inactivated.

Currently, reports describing non-TEM, non-SHV family IR enzymes are becoming more common. The CTX-M ESBLs, which typically hydrolyze penicillins and extended-spectrum cephalosporins (preferentially cefotaxime), have not yet shown significant resistance to the β-lactamase inhibitors (32, 68). Present studies indicate that the KPC-2 β-lactamase is not inactivated by clavulanate, sulbactam, and tazobactam, as indicated by high MICs and turnover numbers of 2,500, 1,000, and 500, respectively (319a). The ability of KPC-2 to avoid inactivation by the currently available inhibitors may represent a novel class of highly IR class A enzymes. The rapid emergence of *Enterobacteriaceae* harboring these carbapenemases is a significant public health concern (290).

Epidemiology and Detection of β-Lactamase Inhibitor Resistance

Resistance to β-lactam-β-lactamase inhibitor combinations challenges the ability to successfully treat serious urinary tract, respiratory tract, and bloodstream infections (154, 219, 376). The prevalence of organisms expressing inhibitor-resistant enzymes varies throughout the world. In 1993, a French study of 2,972 *E. coli* isolates from urinary tract infections (UTIs) found that 25% and 10% of hospital and community isolates, respectively, showed amoxicillin-clavulanate MICs of >16/2 μg/ml (160). Characterization of these isolates, including MIC profiles (amoxicillin-clavulanate MIC<sub>90</sub> of >1,042 μg/ml and cephalosporin MIC of <32 μg/ml), isoelectric focusing, and DNA-DNA hybridization, suggested that 27.5% and 45% of hospital and community isolates, respectively, were TEM-1 derived and had substitutions at previously described amino acid positions that confer the IRT phenotype. In this survey, 4.9% of *E. coli* isolates from UTIs were IRT producing. In 1998, a geriatric department of a French hospital reported an outbreak of amoxicillin-clavulanate-resistant isolates which all produced the same IR TEM β-lactamase, TEM-30 (Arg244Ser) (141). In a study published in 2000, Leflon-Guibout et al. determined the molecular mechanism of amoxicillin-clavulanate resistance (defined by MICs of >16/2 μg/ml) in *E. coli* isolates from three French hospitals from 1996 to 1998 (218). The overall resistance rate was 5%, and the majority of these resistant organisms were from patients with respiratory tract infections. Production of IRTs was implicated in resistance in 30 to 41% of the isolates over the time period, which was only slightly less frequent than the hyperproduction of chromosomal AmpC enzymes. Two independent Spanish studies reported production of IRTs in 5.4% and 9.5% of amoxicillin-clavulanate-resistant *E. coli* isolates (266, 331).

The first IRT identified in the United States was not reported until 2004 in a 14-month survey of *E. coli* isolates from a north-eastern tertiary care clinical microbiology laboratory (198). Kaye et al. found that 24% of the 283 isolates classified as ampicillin-sulbactam resistant by disk diffusion and MIC testing were also resistant to amoxicillin-clavulanate as determined by disk diffusion zone diameters of ≤13 mm. Of the amoxicillin-clavulanate-resistant isolates, 83% were from community-acquired infections. Many of these 69 isolates were recovered from UTIs of outpatients, and two expressed the IR TEM-34 (Met 69Val) and one the TEM-122 (Arg275Gln) enzyme. Incidentally, also reported in the same IR TEM-β-lactamase, TEM-30 (Arg244Ser) (141). In a study published in 2000, Leflon-Guibout et al. determined the molecular mechanism of amoxicillin-clavulanate resistance (defined by MICs of >16/2 μg/ml) in *E. coli* isolates from three French hospitals from 1996 to 1998 (218). The overall resistance rate was 5%, and the majority of these resistant organisms were from patients with respiratory tract infections. Production of IRTs was implicated in resistance in 30 to 41% of the isolates over the time period, which was only slightly less frequent than the hyperproduction of chromosomal AmpC enzymes. Two independent Spanish studies reported production of IRTs in 5.4% and 9.5% of amoxicillin-clavulanate-resistant *E. coli* isolates (266, 331).

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Isolates producing IRTs are more frequently reported in Europe than in the United States. The explanation for this discrepancy is not clear and probably reflects a combination of environmental, methodological, and clinical practice fac-
The phenomenon does not appear to be related to large differences in the use of β-lactam–β-lactamase inhibitor combinations, as these agents are widely used in both Europe and the United States and likely produce similar selective pressures on bacteria (68, 159).

The detection of IRTs presents a significant number of operational challenges. Disagreements between the results of MIC testing and disk diffusion assays are often seen, especially among isolates with intermediate resistance phenotypes (68, 198, 301). Currently, an international standard for the amount of β-lactamase inhibitor that should be combined with the partner β-lactam for detection of IR enzymes is not in use, and different combinations can significantly alter the assigned results (301, 409). For example, the use of the 2:1 ratio for amoxicillin-clavulanate appears to underestimate the presence of IR β-lactamases compared to employing the fixed concentration of 2 µg/ml clavulanate (409). Further, organisms producing low levels of IR enzymes may be undetectable at the 2:1 ratio (302).

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<td>TEM-78 (IRT-22)</td>
<td>Val Arg</td>
</tr>
<tr>
<td>TEM-79</td>
<td>Gly</td>
</tr>
<tr>
<td>TEM-80 (IRT-24)</td>
<td>Val Asp</td>
</tr>
<tr>
<td>TEM-81</td>
<td>Leu Val</td>
</tr>
<tr>
<td>TEM-82</td>
<td>Val Gln</td>
</tr>
<tr>
<td>TEM-83</td>
<td>Leu Gly</td>
</tr>
<tr>
<td>TEM-84</td>
<td>Gly</td>
</tr>
<tr>
<td>TEM-103 (IRT-28)</td>
<td>Leu Gly</td>
</tr>
<tr>
<td>TEM-122</td>
<td>Met Arg</td>
</tr>
<tr>
<td>TEM-145</td>
<td>Met His</td>
</tr>
<tr>
<td>TEM-159</td>
<td>Phe Ile</td>
</tr>
<tr>
<td>TEM-160</td>
<td>Lys Val Thr</td>
</tr>
</tbody>
</table>

$^a$ Based on data from www.lahey.org/studies/webt.asp.  
$^b$ Unless otherwise indicated, the amino acid is the same as in the non-IR TEM-1.

<table>
<thead>
<tr>
<th>β-Lactamase</th>
<th>Amino acid at position$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35 69 130 140 146 187 192 193 234 238 240</td>
</tr>
<tr>
<td>SHV-1</td>
<td>Leu Met Ser Ala Ala Ala Lys Leu Lys Gly Glu</td>
</tr>
<tr>
<td>SHV-10</td>
<td></td>
</tr>
<tr>
<td>SHV-26</td>
<td></td>
</tr>
<tr>
<td>SHV-49</td>
<td>Ala Ala Lys Asn Val Leu Gly Ser Lys</td>
</tr>
<tr>
<td>SHV-56</td>
<td>Gly Arg Thr</td>
</tr>
<tr>
<td>SHV-72</td>
<td>Gln Val Arg</td>
</tr>
</tbody>
</table>

$^a$ Based on data from www.lahey.org/studies/webt.asp.  
$^b$ Unless otherwise indicated, the amino acid is the same as in the non-IR SHV-1.
result of the methodological complications of identifying IR \(\beta\)-lactamase producers, the actual IRT prevalence may be underestimated (68). Definitive identification requires specific enzyme kinetic testing, determination of isoelectric points, and molecular characterization (60, 72, 77). Routine susceptibility tests may not accurately detect strains expressing IRTs. A direct correlation between the level of resistance (MIC) and the predicted clinical outcome is not yet known.

### Substitutions in Class A Enzymes Conferring Inhibitor Resistance

The amino acid substitutions associated with clavulanate resistance are different from those that produce the ESBL phenotype, and thus IRTs have unique hydrolytic profiles compared to ESBLs. For example, the \textit{E. coli} isolates that contain IRTs may be more susceptible to cephalosporins, carbapenems, and monobactams (35). However, among TEM and SHV \(\beta\)-lactamases, substitutions that confer an IR phenotype are sometimes found in pairs or are combined with amino acid changes that also increase the ability of the enzyme to hydrolyze oxyimino-cephalosporins. These variants are designated complex mutants of TEM (CMTs) and may foreshadow the emergence of a new class of versatile \(\beta\)-lactamases with even broader hydrolytic and resistance profiles (Table 6) (369).

Table 7 summarizes kinetic properties of representative class A IR enzymes that have been identified clinically or generated in the laboratory. IRTs, when derived from mutated wild-type \textit{bla} genes, typically display a reduction in \(\beta\)-lactam catalytic efficiency \((k_{cat}/K_m)\), mediated by lower \(k_{cat}\) and increased \(K_m\) values for penicillins compared to the wild-type enzymes (35). IR \(\beta\)-lactamases have increased \(K_P\) and IC_{50}s for clavulanate and sulbactam; smaller increases are observed for tazobactam. In general, the MICs for organisms expressing IR enzymes are lowered for penicillins and increased for clavulanate and sulbactam. MICs may remain in the susceptible range for tazobactam when obtained with the combination of piperacillin-tazobactam. This susceptibility is likely due to piperacillin’s greater potency and rapid cell entry (see above) as well as the relative preservation of tazobactam’s \(K_r\) (36). Of significant interest is the recent description of the kinetic properties of SHV-72 (Ile8Phe, Ala146Val, Lys234Arg) (258). In contrast to most other IR enzymes, this unique IR SHV demonstrates increased catalytic efficiency for penicillins.

### Table 6. Clinically identified complex mutants of TEM (CMT) \(\beta\)-lactamases*

<table>
<thead>
<tr>
<th>(\beta)-Lactamase</th>
<th>Amino acid at position:*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>Gln Met Glu Ser Arg Ala Gly Ser Glu Arg Thr Arg Asn Ala</td>
</tr>
<tr>
<td>TEM-50 (CMT-1)</td>
<td>Leu Lys Ser Arg Ala Gly Ser Ser Lys Met Leu</td>
</tr>
<tr>
<td>TEM-68</td>
<td>Ser Lys Gly Ser Lys Ser Cys</td>
</tr>
<tr>
<td>TEM-89 (CMT-3)</td>
<td>Lys Leu Lys His Thr Lys Ser Asp Gly</td>
</tr>
<tr>
<td>TEM-109 (CMT-5)</td>
<td>Leu Lys Gly Ser Ser Lys Ser</td>
</tr>
<tr>
<td>TEM-121 (CMT-4)</td>
<td>Lys Leu Lys His Thr Lys Ser Asp</td>
</tr>
<tr>
<td>TEM-151</td>
<td>Val His Ser His Ser</td>
</tr>
<tr>
<td>TEM-152</td>
<td>Val His Lys</td>
</tr>
<tr>
<td>TEM-154</td>
<td>Leu Ser</td>
</tr>
<tr>
<td>TEM-158 (CMT-9)</td>
<td>Leu Ser Asp</td>
</tr>
</tbody>
</table>

* Based on data from www.lahey.org/studies/webt.asp.

* Unless otherwise indicated, the amino acid is the same as in the non-IR TEM-1.

### Table 7. Kinetic properties of representative class A inhibitor-resistant and wild-type enzymes*

<table>
<thead>
<tr>
<th>(\beta)-Lactamase</th>
<th>Clavulanate</th>
<th>Sublactam</th>
<th>Tazobactam</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-1</td>
<td>K_r ((\mu)M)</td>
<td>k_{max} (s(^{-1}))</td>
<td>t_1</td>
<td>K_r ((\mu)M)</td>
</tr>
<tr>
<td>TEM Met69Leu</td>
<td>0.1 0.02 160 1.002 10,000 0.01 0.02 140 73, 180</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM Ser130Gly</td>
<td>105 0.003 1,600 0.0009 7,000 0.20 0.004 2,900 73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM Arg244Ser</td>
<td>33 0.00001 7,800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM Asn276Asp</td>
<td>15.6 0.01 250 0.00001 7,800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM Met69Leu, Asn276Asp</td>
<td>27 0.6 386</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHV-1</td>
<td>1 0.04 60 8.6 0.056 13,000 0.07 0.1 5 153, 410, 411</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHV Arg244Ser</td>
<td>63 0.09 50 0.13 100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHV Ser130Gly</td>
<td>47 0.03 40 0.03 300</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OHI-1</td>
<td>0.4 0.01 80 0.001 40,000 0.50 0.01 400 229</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OHI Met69Ille</td>
<td>10 0.002 2,000 0.0004 200,000 18 0.0016 2,000 229</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The definition of K_r may not be uniform from laboratory to laboratory. See the cited references for the methods used in each determination.
Uranate-resistant phenotypes are different for SHV and TEM uranate (138, 411). The biochemical correlates of these clav-
not yet reached clinical attention (411). Molecular modeling of SHV Asn276Asp showed that novel electrostatic interactions are formed between Arg244N and both 276AspO81 and O82, reminiscent of the TEM Asn276Asp crystal structure (108). Additional data from both kinetic studies and timed electrospray ionization mass spectrometry (ESI-MS) of clavulanate-inhibited SHV-1 and SHV Asn276Asp suggest that the Asn276Asp phenotype is produced by decreased affinity for the C-3 carboxylate of clavulanate and not by a change in the reaction mechanism. These results are consistent with the notion advanced by Thomson et al., suggesting that clavulanate inactivation in SHV may not rely on a water molecule coordinated by Arg244 and Val216, as in TEM enzymes (191, 210, 411). These important differences in TEM and SHV mechanisms support a divergent evolution of these enzymes vis-à-vis substrate and inhibitor profiles.

**Serine 130.** Ser130 is a conserved amino acid among all class A β-lactamases but is replaced by a Gly residue in two IRTs (TEM-59 and -76) and one IR SHV enzyme (SHV-10). The multiple roles of this residue include anchoring β-lactams to and stabilizing the active site through hydrogen bonding with the C-3/C-4 carboxylate of the inhibitors and substrates and facilitating proton transfer to the β-lactam nitrogen during acylation, leading to β-lactam ring opening (179, 180, 214, 426). Ser130 also serves as a second nucelophile which attacks the imine intermediate and becomes covalently cross-linked to Ser70 in the terminal inactivation of the mechanism-based inhibitors (209). Removing the cross-linking residue seems a clear way to overcome inactivation, but inhibitors, and particularly tazobactam, retain efficacy against the Ser130Gly enzyme. Based on these mechanistic roles, how does the Ser130Gly variant maintain hydrolytic activity and why does it confer resistance to inhibitors (408)? In response to the first part of the question, evidence from X-ray crystallography of SHV and TEM Ser130Gly (PDB 1TDL and 1YT4, respectively) confirmed that a relocated water molecule, initially predicted by Helfand et al., compensates for the loss of the Ser130 hydroxyl group by donating a proton to the β-lactam nitrogen (153, 157, 180, 408, 438). The decreased catalytic efficiencies seen in the TEM and SHV variants are likely a result of perturbations caused by the repositioned water molecule. These perturbations vary somewhat between SHV and TEM Ser130Gly (e.g., reorientation of Ser70 and Lys73 in SHV and TEM Ser130Gly, respectively), but both involve changes in hydrogen bonding networks in the active site, which are modified to accommodate the new water molecule (397, 408).

In regard to the second part of the question concerning inhibitor resistance, the data on Ser130Gly variants argue that cross-linking is not essential for enzyme inactivation. Despite increased MICs for the inhibitors, the turnover numbers for clavulanate and tazobactam by SHV Ser130Gly are equivalent to those by the wild-type enzyme (153). This “paradox of inactivation” illustrates that despite replacement of a key catalytic residue, SHV Ser130Gly is still capable of inactivation by the mechanism-based inhibitors (153, 157, 311, 397). The resistant phenotype presents primarily in whole-cell assays, where bacteria are exposed to a limited inhibitor concentration. Structural perturbations created by the Ser130Gly substitution discourage formation of the precacylation complex (increased inhibitor $K_a$), which ultimately leads to fewer inactivation events (157). Thus, while the enzymatic machinery for inhibition is intact, the decreased accumulation of acyl-enzyme intermediates and inactivation products can lead to inhibitor resistance in vivo.

**Arginine 275.** Similar to observations regarding Arg244 and Asn276, the Arg275 variant has been identified only in TEM. Before description of the IR variants TEM-103 and TEM-122, which possess only the Arg275Leu and -Gln changes, respectively, substitution at 275 was seen only in combination with the Met69Val and -Leu substitutions (9, 198). Hence, the contribution of Arg275 to the IR phenotype was not appreciated, and rigorous kinetic and structural studies of this variant have not yet been completed. However, from crystal structures of the wild-type enzymes and a better understanding of properties at nearby Arg244 and Asn276, Chaib et al. postulated that substitutions at Arg275 (like those at Asn276) disrupt local electrostatic interactions and displace the water molecule that is key to inactivation by clavulanate (74).

**Lysine 234.** The recent definition of two clinical IR SHV β-lactamases with Lys234Arg substitutions draws attention to this important active-site residue (110, 258). Previously, TEM or SHV variants obtained from the clinic had not been identified as having changes at this highly conserved class A residue. Site-directed mutagenesis of Lys234 to Arg in TEM-1 leads to a 10-fold-decreased affinity for penicillins, presumably because of the hydrogen-bonding role of Lys234 to the C-3 carboxylate (220) (Fig. 3). However, for the clinically isolated SHV-72 enzyme (Ile8Phe, Ala146Val, Lys234Arg), $K_v$ values for penicillins were not significantly changed and IC$_{50}$ values for clavulanate increased 10-fold compared to those of SHV-1 (258). Interestingly, the $K_{a}$ values for the penicillins were increased, up to 5.8-fold for ticarcillin. Molecular dynamics simulations show that Lys234Arg induces movement of the Ser130 oxygen away from Ser70. Mendonca et al. proposed that the mechanism of IR in SHV-72 may be due to movement of Ser130 based on its role in both hydrolysis and terminal inactivation by cross-linking with Ser70 (258).

**THE PROMISE OF NOVEL β-LACTAMASE INHIBITORS**

This section reviews compounds that have demonstrated favorable inactivation properties against β-lactamases and introduces newer compounds showing promise as “second-generation” inhibitors. Table 8 provides $K_v$ (or $K_f$) values and IC$_{50}$ for representative inhibitors against the different β-lactamase classes to illustrate the challenge of achieving broad-spectrum inhibition.

**Monobactam Derivatives**

Monocyclic, N-sulfonated β-lactams are a family of antibiotic compounds produced by bacteria (178, 404, 405). Structure-activity studies of these monobactams led to the development of the synthetic compound aztreonam, which interacted with the PBP’s of a wide range of aerobic Gram-negative bacteria, including *P. aeruginosa*, and was introduced into clinical practice in 1984 (151, 168, 403). In addition, aztreonam (Fig. 1, compound 3) resists hydrolysis by many plasmid-mediated β-lactamases such as TEM-1 and -2, OXA-2, and SHV-1, behaving as a very poor substrate with low affinity (e.g., $K_m$ of 2.9
ing deacylation (205). The introduction of a bridge between the active-site hydrogen bond network and leads to rate-limiting bond. This rearrangement requires breaking and reforming of C-3–C-4 bond to allow a hydrolytic water access to the ester that before deacylation, aztreonam had to rotate around the (PDB 1FR1) (152, 299). Studies of the structure demonstrated that activation of the class C
plex with the class C
by the crystal structure of the monobactam aztreonam in combination with cephalosporin-resistant
A enzymes and could not restore susceptibility to cephalosporins against organisms expressing
P. aeruginosa
Klebsiella
inhibitors against different \( \beta \)-lactamase Ambler classes

<table>
<thead>
<tr>
<th>Inhibitor (compound no. in Fig. 1)</th>
<th>Kinetic parameter</th>
<th>Value (( \mu )M) for Ambler class:</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aztreonam (3)</td>
<td>( K_w ) or ( K_i )</td>
<td>200</td>
<td>56, 217, 337</td>
</tr>
<tr>
<td>BAI30972 siderophore monobactam (14)</td>
<td>( IC_{50} )</td>
<td>1.7</td>
<td>134, 418</td>
</tr>
<tr>
<td>ATM-penicillin (15)</td>
<td>( IC_{50} )</td>
<td>0.90</td>
<td>87</td>
</tr>
<tr>
<td>BRL 42715 methylidene penem (16)</td>
<td>( IC_{50} )</td>
<td>0.008</td>
<td>749/C, and the class C</td>
</tr>
<tr>
<td>BLI-489 methylidene penem</td>
<td>( IC_{50} )</td>
<td>0.009</td>
<td>294</td>
</tr>
<tr>
<td>Novel tri- and bicyclic methylidene penems (including 17 and 18)</td>
<td>( IC_{50} )</td>
<td>0.0004–0.0031, 0.014–0.260, 0.0015–0.0048</td>
<td>25, 441</td>
</tr>
<tr>
<td>LK-157 tricyclic carbapenems (20)</td>
<td>( IC_{50} )</td>
<td>0.055</td>
<td>344</td>
</tr>
<tr>
<td>Ro 48-1220 penam sulfoxone (21)</td>
<td>( IC_{50} )</td>
<td>0.05–10.77</td>
<td>367, 422</td>
</tr>
<tr>
<td>LN-1-255 penam sulfoxone (23)</td>
<td>( K_i ) or ( IC_{50} )</td>
<td>0.001</td>
<td>272, 411</td>
</tr>
<tr>
<td>Boronic acid meta-carboxyphenyl cephalosporin analog (25)</td>
<td>( K_i )</td>
<td>3.9</td>
<td>31</td>
</tr>
<tr>
<td>NXLI104 (26)</td>
<td>( IC_{50} )</td>
<td>0.008</td>
<td>281</td>
</tr>
<tr>
<td>J-111,225 1-b-methylcarpapenem (33)</td>
<td>( K_i )</td>
<td>11.1</td>
<td>4, 244</td>
</tr>
<tr>
<td>Phosphonates</td>
<td>( K_i )</td>
<td>0.08–76</td>
<td>61</td>
</tr>
<tr>
<td>C-6-mercaptomethyl penicillinate (38)</td>
<td>( IC_{50} )</td>
<td>6.8</td>
<td>63, 322; Drawz et al., unpublished data</td>
</tr>
</tbody>
</table>

* The definition of \( K_i \) may not be uniform from laboratory to laboratory. See the cited references for the methods used in each determination.

mM for TEM-2) (403). With class C enzymes, such as \( E. cloacae \)
P99, aztreonam forms a stable acyl-enzyme that is very slowly hydrolyzed (low \( k_{cat} \)) (128, 403). Aztreonam also exhibits its very low \( k_{cat} \) values for chromosomally encoded cephalosporinases (e.g., 1.9 mM for P99) and serves as a transient inacti-vator (56, 128). In vivo, where periplasmic \( \beta \)-lactamase concentrations can be in the low mM range, clinically significant catalytic efficiencies may be obtainable (128).

Further modification of the monobactam nucleus was embarked upon to capitalize on the inhibitory activity of aztreo-nam. The 1,5-dihydroxy-4-pyridone monobactam Syn 2190 (Fig. 1, compound 11) possesses a novel C-3 side chain that may utilize the iron uptake pathway to enter Gram-negative bacteria. Syn 2190 displayed IC_{50}s in the mM range for cephalosporinases (e.g., 6 mM for \( E. cloacae \) P99) and synergy with cefazidime and cephrimic in MIC testing of clinical strains, including AmpC derepressed variants of \( P. aeruginosa \) (15, 289). In addition, Syn 2190 in combination with cefazidime or cephrimic (at a 1:1 ratio) showed improved efficacy compared to cefazidime-tazobactam in murine models of systemic infections with cephalosporin-resistant \( P. aeruginosa \). Syn 2190 has also shown utility for the challenging clinical task of identifying production of plasmid-mediated AmpC \( \beta \)-lactamase in Kleb-siella spp. In combination with cotefetan, Syn 2190 achieved 100% specificity and 91% sensitivity for AmpC producers (28). However, Syn 2190 has lower affinity than tazobactam for class A enzymes and could not restore susceptibility to cephalosporin in bacteria expressing these enzymes. This limitation against class A enzymes may partly explain the absence of further development of this compound.

The design of bridged monobactam derivatives was guided by the crystal structure of the monobactam aztreonam in complex with the class C \( \beta \)-lactamase from \( Citrobacter freundii \) (PDB 1FR1) (152, 299). Studies of the structure demonstrated that before deacylation, aztreonam had to rotate around the C-3–C-4 bond to allow a hydrolytic water access to the ester bond. This rearrangement requires breaking and reforming of the active-site hydrogen bond network and leads to rate-limiting deacylation (205). The introduction of a bridge between C-3 and C-4 limits this rotation and stabilizes the acyl-enzyme against hydrolysis by AmpC enzymes (99, 171).

Heinze-Krauss et al. synthesized and evaluated a panel of bridged monobactams that exhibited very favorable inhibition of the class C \( C. freundii \) and \( P. aeruginosa \) \( \beta \)-lactamases (IC_{50}s of as low as 10 mM) but lower affinities for the class A TEM-3 (IC_{50}s of >100 mM) (152). At sufficiently high concentrations, class A \( \beta \)-lactamases were acylated rapidly, but the rate of deacylation was higher, leading to high hydrolysis rates and low active-site occupancy. Comparison of the crystal structures of class A TEM-1, PCl, the \( \beta \)-lactamase from \( Bacillus licheniformis \) 749/C, and the class C \( \beta \)-lactamase from \( C. freundii \) revealed that in class A enzymes, hydrolysis occurs on the opposite side of the bridged monobactam ester by a water molecule activated by hydrogen bond networks not present in class C enzymes (152, 166, 191, 267, 299, 392). Rotation about the C-3–C-4 bond is less critical to the inhibition mechanism in class A enzymes, and restricting this rotation does less to stabilize the acyl-enzyme. For example, the bridged monobactam Ro 48-1256 (Fig. 1, compound 12) (at 4 and 8 \( \mu \)g/ml) effectively potentiated the activity of imipenem against both inducible and derepressed class C-expressing \( P. aeruginosa \) isolates (238). The activities of piperacillin and cefazidime were also potentiated by the combination with Ro 48-1256 against the strains expressing elevated quantities of AmpC enzyme. However, Ro 48-1256 did not improve the activity of piperacillin, cefazidime, or carbapenems against organisms expressing class A, B, or D \( \beta \)-lactamases.

Modification of the monobactam structure at the \( \beta \)-lactam nitrogen has had some success against class A \( \beta \)-lactamases (50, 171). Analysis of a series of \( N \)-sulfonyloxy-\( \beta \)-lactam monobactam derivatives demonstrated rapid acylation of the TEM-1 active site and resistance to deacylation, as evident from turnover numbers ranging from 2 to 8 (50). Additional analysis of one of these derivatives (Fig. 1, compound 13) revealed that following enzyme acylation in the class A carbapenemase NMC-A, the tosylate group is eliminated and gives rise to two acyl-enzyme species (273). These inhibited enzyme species are trapped in local energy minima which yield low
Decaylation rates. Based on molecular simulations, Mourey et al. postulated that the acyl-enzyme alternates between two different conformations that move into and out of the oxyanion hole (273). This motion requires breaking and reforming of hydrogen bonds between the acyl-enzyme ester and carbonyl oxygen atoms and the oxyanion hole backbone nitrogens and Ser130 oxygen atom. In both conformations, the water implicated in the decaylation reaction is positioned poorly for hydrolysis, offering a structural explanation for the irreversible inhibition.

A series of monobactam derivatives was recently developed to target pathogens harboring multiple β-lactamases. Some of these monobactam analogues act as very effective bactericidal agents for difficult-to-treat Gram-negative pathogens, while others are bridged monobactams, such as BAL29880, that are inhibitors of AmpC enzymes (99, 102, 116a, 317). Additional derivatives include monobactams bearing a siderophore side chain which can enhance cell entry through bacterial iron uptake systems (49). Of this siderophore type, BAL19764 and BAL30072 (Fig. 1, compound 14) have potent antibacterial activity against carbapenem-resistant *P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia*, and *S. marcescens* as well as against Gram-negative bacilli expressing VIM and IMP-β-lactamases (37, 102). Additionally, BAL19764 is stable to hydrolysis by MBLs (314). Further, these siderophore compounds are potent enzyme inhibitors; BAL30072 has low μM IC₅₀ for TEM-3 (1.7 μM), and the AmpCs from *C. freundii* and *P. aeruginosa* (0.09 and 15 μM, respectively) (134). By combining BAL19764 or BAL30072 with clavulenate and a bridged monobactam (all at a fixed concentration of 4 μg/ml), the MICs of the siderophore compounds were improved for a range of Gram-negative bacteria (including *P. aeruginosa*) producing AmpC as well as carbapenemases or overexpression of efflux systems (315, 316). However, the activity of carbapenemases is still superior to that of these new monobactams for many ESBL-producing isolates (e.g., *K. pneumoniae* CTX-M and SHV-5, *E. coli*, and *E. cloacae*) (38, 102, 173). The dual antibacterial/inhibitory action of these monobactam derivatives makes them important leads, and the further development of the siderophore compound BAL30072 seems likely.

**ATMO Derivatives**

Certain cephalosporins adopt catalytically incompatible conformations in the active site, effectively “trapping” the enzyme. Evidence from the crystal structure of an AmpC enzyme complexed with ceftazidime (PDB 1IEL) suggested that the presence of the 2-amino-4-thiazolyl methoxyimino (ATMO) R₄ side group, common among extended-spectrum cephalosporins (e.g., cefotaxime and ceftazidime), destabilized the formation of the decaylation transition state (350). Synthesis of inhibitors containing this ATMO side chain on a penicillin (Fig. 1, compound 15) and carbacephem backbone led to AmpC IC₅₀ of 900 nM and 80 nM, respectively (418). Subsequent examination of the crystal structure of the ATMO-penicillin in complex with the AmpC (PDB 1LLB) showed that it resembled the acyl-enzyme formed by ceftazidime. The ATMO groups were located in very similar positions, in both cases leading to steric hindrance between the decaying carbonyl and the β-lactam ring nitrogen, accounting for inhibition. This design highlights the potential of incorporating destabilizing groups onto conserved substrate skeletons, which will facilitate recognition by the enzyme (418).

**Penems**

**BRL 42715 and Syn 1012.** Methyldiene penems are potent inhibitors in which the inhibitory properties of the penem structure are enhanced by introduction of a double bond at C-6 and a sulfide at penem position 1 (260). These compounds offer advantages over the traditional β-lactamase inhibitors, including (i) a large R₄ side chain that may aid in cell permeability and contribute to active-site affinity and (ii) a novel inactivation mechanism. The parent compound from this group, BRL 42715 C-6-(N1-methyl-1,2,3-triazolymethyl)penem (Fig. 1, compound 16), is an effective inhibitor of class A, C, and D β-lactamases (119, 250). IC₅₀ of BRL 42715 were 10- to 100-fold lower than those of clavulanate, tazobactam, and sulbactam for class A TEM-1 and SHV-1, class C P99, class D OXA-1, and the *S. aureus* β-lactamase NCTC 11561 (87). However, BRL 42715 was a substrate for class B metallo-β-lactamases from *Aeromonas hydrophila* and *S. maltophilia* and for BcII (250). In susceptibility testing, BRL 42715 concentrations of 0.25 μg/ml or lower were able to restore amoxicillin MICs to <16 μg/ml for TEM-1- and OXA-1-producing organisms (87). Further, amoxicillin-BRL 42715 combinations were more effective than amoxicillin-clavulanate for *E. coli*, *K. pneumoniae*, *H. influenzae*, *S. aureus*, and *N. gonorrhoeae* strains producing plasmid-mediated β-lactamases (mostly TEM and SHV expressors) and for *K. oxytoca*, *Proteus vulgaris*, and *P. mirabilis* strains producing chromosomal β-lactamases. Kinetic studies of BRL 42715 and NCTC 11561, TEM-1, and P99 showed rapid, irreversible inactivation with an inhibition stoichiometry of 1:1, or turnover numbers of 0 (119).

Initial studies to elucidate the BRL 42715 inactivation product using UV difference spectroscopy for both enzyme- and base-catalyzed hydrolysis of the methyldiene penem suggested the presence of a dihydrothiazepine rearrangement product (46, 250). Accordingly, mass spectrometry studies demonstrated that the methyldiene penem inhibition pathway does not involve fragmentation in the active site (119, 250, 406). When TEM-1, P99, and the class A β-lactamase from *B. cereus* I were inactivated by BRL 42715, spectra showed a mass increases equivalent to the molecular masses of the inhibitor plus the enzyme (119). The crystal structure of the class C β-lactamase *E. cloacae* 908R in complex with BRL 42715 (PDB 1Y54) confirmed the formation of a stable seven-membered thiazepine ring covalently attached to Ser64 (263). Studies of both BRL 42715 and related penems support the mechanism involving formation of an imine intermediate from the acyl-enzyme, which then undergoes rearrangement, and eventually endo-trig cyclization to form the seven-membered thiazepine ring end product of inactivation for class A, C, and D enzymes (Fig. 10) (250, 263, 294, 428).

Crystallization of BLI-489, a related heterocyclic O C-6-substituted penem with IC₅₀ of 9.0 and 6.2 μM for SHV-1 and class C GC1, respectively (see below), revealed the common seven-membered thiazepine ring when complexed with the SHV-1 and GC1 enzymes (PDB 1ONG and 1ONH, respectively) (294). Interestingly, the orientation of the ring in SHV-1 and GC1 differed by 180° about the bond to the acylated serine. This rotameric preference may be due to the relative “openness” of the class C structures.
binding site at the bottom of the b3 β-sheet. Crystallization of a tricyclic 6-methylidene derivative in complex with SHV-1 and GC1 (PDB 1Q2P and 1Q2Q, respectively) also showed the common seven-membered thiazepine ring covalently attached to Ser70 and Ser64, respectively (429). The SHV-1/penem complex was similar to what was observed with BLI-489, where the ring had R stereochemistry at the new C-7 moiety (294). However, in the GC1/penem complex, both the R and S chiral forms of the intermediate were found.

Taken together, these findings suggest that the 6-methylidene penems’ acyl-enzyme stability is likely due to the low occupancy or disorder of the hydrolytic water molecule and, in part, to the conjugation of the ester with the ring system. The nucleophilic attack of the double bond at C-6 appears to be important to the inhibition mechanism of the methylidene penems, and these novel inhibitors seem to share a similar inactivation mechanism for different β-lactamase classes (429).

Most β-lactamases appear to follow this linear pathway to inactivation by the penem compounds, i.e., formation of an acyl-envelope and rearrangement to the stable dihydrothiazepine ring adduct (119, 250). However, some class A enzymes, such as the β-lactamases from Staphylococcus albus (Staphylococcus epidermidis) and K. pneumoniae K1, exhibit more complex kinetics (46, 250). A branched pathway leads to an acyl-envelope hydrolysis product, which also rapidly rearranges to the dihydrothiazepine species.

While BRL 42715 and the structurally related investigational penem Syn 1012 showed promising in vitro kinetics against a large spectrum of Gram-positive and Gram-negative isolates, they were relatively unstable in human and mouse plasma (342). Further, serum levels of BRL 42715 and Syn 1012 were undetectable 1 h after intravenous administration in a rabbit model. Thus, the successful elements of BRL 42715 have been applied to additional drug development efforts directed at improving in vivo stability (see below).

**BRL 42715 derivatives.** Derivatives of BRL 42715, including tricyclic and bicyclic heterocyclic methylidene penems, have been the focus of a large body of recent structure-activity studies and continue to show promising inhibition properties in investigational studies. The bicyclic heterocycles are synthesized as 6-methylidene penems attached to thiophene, imidazole, and pyrazole rings (428). Extension of the second heterocyclic ring yields the tricyclic derivatives, which show improved stability in solution and increased lipophilicity in in vivo studies (429). As a group, these compounds have proven to be potent inhibitors of class A, C, and D β-lactamases (25, 156, 335, 428, 441).

A panel of one tricyclic and six bicyclic penem compounds displayed IC_{50}s of 0.4 to 3.1 nM for TEM-1 (class A), 7.8 to 72 nM for IMI-1 (class A), 1.5 to 4.8 nM for AmpC (class C), and 14 to 260 nM for a CcrA (class B) (441). Compared to tazobactam IC_{50}s, these penems were 100- to 56,000-fold more active against TEM-1 and the AmpC enzymes. Each of the seven penems, at a constant concentration of 4 μg/ml, was combined with piperacillin, which significantly lowered MICs in ESBL producers and restored susceptibility in piperacillin-resistant Enterobacter spp., Citrobacter spp., and Serratia spp. In a mouse model of infection caused by E. coli and Enterobacter aerogenes class A (including ESBL) and C β-lactamase-producing organisms, piperacillin-penem combinations decreased the overall 50% effective dose (ED_{50}) of piperacillin from 1.6- to 8-fold (441). However, class B metallo-β-lactamase producers were not reported for susceptibility tests or the murine model, and they warrant further attention for clinical translation.

Of the panel of tricyclic and bicyclic penem compounds tested, the bicyclic BLI-489 was chosen as a lead compound to be combined with piperacillin (335). With 4 μg/ml of BLI-489, 92% of non-piperacillin-susceptible clinical strains (including expressors of representative β-lactamases from all four classes) had piperacillin–BLI-489 MICs of ≤16 μg/ml, in contrast to 66% for piperacillin-tazobactam. Furthermore, the BLI-489 combination...
demonstrated improved activity over piperacillin-tazobactam against ESBL- and AmpC-expressing strains, as well as against a panel of staphylococcal and streptococcal isolates. Notably, BLI-489 did not improve the activity of piperacillin against the enterococci and penicillin-intermediate and -resistant strains of S. pneumoniae (which may reflect that the penicillin resistance mechanism is not β-lactamase mediated) (335). While these practical studies help to bring closer the possible clinical introduction of these novel inhibitors, only a small number of the isolates were KPC or IRT producers. These KPC- and IRT-producing strains also expressed up to three other β-lactamases, which complicates the interpretation of activity against these enzymes. Thus, one cannot yet endorse the efficacy of this inhibitor combination for these relevant resistance threats.

Two of these bicyclic investigational penems (Fig. 1, compounds 17 and 18), containing either a dihydropyrazolo[1,5-c][1,3]thiazole or a dihydropyrazolo[5,1-c][1,4]thiazine moiety, are extremely effective inactivators of the class D OXA-1 β-lactamase, with nM affinity and low turnover values compared to tazobactam (t<sub>50</sub> = 0 and 350, respectively) (25). Additionally, these penems demonstrate efficacy with IR class A β-lactamases. Against the SHV Arg244Ser variant, the penem inhibitors showed significantly lower K<sub>i</sub> values than clavulanate and sulbactam (penems 1 and 2, 1.0 to 4.4 μM; clavulanate and sulbactam, 63 and 240 μM, respectively) and extremely low turnover numbers (t<sub>50</sub> = 0.50, and 100 for the penems, clavulanate, and sulbactam, respectively) (410).

**Oxapenems.** Oxapenems are derivatives of clavulanic acid, possessing an oxygen atom at penem position 1. Their synthesis was first described in the 1970s, and Cherry et al. introduced an oxapenem that had improved in vitro activity over clavulanate against the E. cloacae AmpC and staphylococcal β-lactamases (86). However, the compound was unstable and did not have activity in whole-cell assays. In 1993, Pfaendler et al. improved the stability of the compounds by adding bulky substituents at the C-2 position (339). The lead compound from this panel of oxapenems, the zwiterionic AM-112 (Fig. 1, compound 19), has shown potent β-lactamase-inhibitory properties, particularly for class C and D enzymes (189, 190). Against class A enzymes, IC<sub>50</sub> were 10- to 20-fold higher for AM-112 compared to clavulanate (190). However, the IC<sub>50</sub> of AM-112 for the class C enzymes from E. cloacae, S. marcescens, and P. aeruginosa, as well as OXA-1 and OXA-5 from E. coli, were 1,000- to 100,000-fold lower than those of clavulanate. AM-112 in combination with ceftazidime at 1:1 or 1:2 reduced ceftazidime MICs (ranging from 0.030 to 0.055 μM, respectively), but that of LK-157 was over 2000-fold better for E. cloacae AmpC (136.2 μM and 0.062 μM, respectively) (344). In combination with ampicillin, LK-157 (at 30 μg/ml) restored susceptibility for the AmpC-overexpressing E. cloacae P99 strain in MIC testing (431). Against a large panel of bacteria producing class A ESBLs (excepting KPC and CTX-M) and class C β-lactamases, LK-157 in combination with cephalosporins showed improved potency compared to clavulanate, tazobactam, and sulbactam (324). The lowest MICs (ranging from ≤0.025 to 1.6 μg/ml) were achieved for cefepime or ceftirixime in combination with LK-157 at 4 μg/ml. Despite these promising data with class A and C β-lactamase-producing Gram-negative bacteria, LK-157 behaves as a substrate with the carbapenem-hydrolyzing enzymes BeII and NMC-A (431). Further, while the inhibitor displays nM affinity for and rapidly inactivates the class D OXA-10 β-lactamase, the acetyl-enzyme is unstable and rapidly hydrolyzed (431).

The crystal structure of LK-157 in complex with the E. cloacae P99 (PDB 2Q9N), together with spectroscopic data for reaction intermediates, suggests that after deacylation at the active-site Ser64, the C-4 methoxy group is probably not bulky enough to induce the conformational change seen with inhibition of class A enzymes by carbapenems, but resonance stabilization of the acyl-enzyme through the acyl carbonyl and ethyldiene group may contribute to stability of the intermediate (255, 344). Trinems are worthy of attention for the potential to serve as leads for additional structure-based inhibitor design, and Lek Pharmaceuticals continues to investigate LK-157. Developed for parental use, the half-life (t<sub>1/2</sub>) of LK-157 in rats is similar to those of other β-lactams after intravenous administration (354). Additional studies have focused on developing an oral agent with improved permeability over LK-157, and while prodrug esters have good solubility, the intestinal permeability remains low.

**1-β-Methylcarbapenems.** A panel of carbapenem derivatives bearing a methyl group at C-1 and various substituents at C-2 were screened for inhibitory activity against the MBL IMP-1 (282). The study revealed a compound, J-110,441, that had potent inhibitory activity against several MBLs but also low K<sub>i</sub> values for class A TEM and class C E. cloacae enzymes (2.54 μM and 0.037 μM, respectively). Susceptibility testing of the AmpC-expressing E. cloacae demonstrated an MIC decrease from 64 to 4 μg/ml with imipenem and J-110,411 at 4 μg/ml. Further work with these carbapenem derivatives has focused on their potential as MBL inhibitors and will be discussed below (see Inhibition of Metallo-β-Lactamases).

**Penicillin and Cephalosporin Sulfone Derivatives**

Following the success of sulbactam and tazobactam, medicinal chemists have focused much effort on the development of
penicillin and cephalosporin sulfones with functionalities that may improve inhibitor efficiency. One of the first series of investigational sulfones, the C-7 alkylidene cephems, had lead compounds that were potent inhibitors of class C β-lactamases (65). Subsequent reports have explored the roles of C-2 and C-6 penam and C-3 cephem substituents. The findings for each will be discussed in the context of the derivative group.

**C-2/C-3-substituted penicillin and cephalosporin sulfones.** β-Lactamase inhibitors with C-2 substitutions have shown efficacy against TEM- and SHV-type enzymes, including ESBLs (367, 422). The acyllactonitrile derivative Ro 48-1220 (Fig. 1, compound 21) achieves an IC_{50} for TEM-1 (0.08 μM) that is comparable to that of clavulanate (0.10 μM), but the IC_{50} for SHV-1 (0.27 μM) is slightly higher than that of clavulanate (0.05 μM) (422). Inhibition with Ro 48-1220 was as effective as tazobactam for TEM-1 and SHV-1, with IC_{50}s within 0.03 μM for both enzymes. Most class C enzymes tested were inhibited at lower concentrations of Ro 48-1220 than of tazobactam (IC_{50} range approximately 2- to 30-fold lower) (367, 422). The IC_{50}s for X. maltophilia and Bacteroides fragilis class B enzymes were 24 and 200 μM, in comparison to 4 and >10 mM for tazobactam (367). Ro 48-1220 (at 4 μg/mL) performed well in susceptibility testing, protecting the activity of ceftriaxone and cefazidime against class C- and ESBL-producing organisms (422). Central to the potency of this sulfone inhibitor was the low enzyme recovery rate compared to tazobactam, especially for class C β-lactamases from C. freundii and E. coli (367). UV absorption spectroscopy and X-ray crystallography revealed the identity of a linear enamine formed by elimination of the SO_{2} group, ring opening, and rearrangement into a linear conjugated system (367). This particularly stable intermediate presumably accounted for enhanced inhibitor potency.

The design of the C-2-substituted penicillin sulfone SA2-13 (Fig. 1, compound 22) drew on the insight that the tazobactam imine is the common intermediate, or “gatekeeper,” in both permanent interaction and enzyme regeneration (58, 309, 453). If the more stable enamine, the trans-enamine, can be favored, then the reaction will be “trapped” in transient inhibition. In fact, IR enzymes do exhibit decreased trans-enamine formation, as in the case of the SHV Met69Val/Glu166Ala variant reacted with tazobactam and clavulanate (416). SA2-13 includes a negatively charged carboxyl group attached to a linker which is designed to stabilize the trans-enamine conformation in close proximity to conserved active-site residues. Interestingly, the crystal structure of SA2-13 in complex with SHV-1 (PDB 2H5S) showed an additional salt bridge with Lys234 and hydrogen bonds with Ser130 and Thr235. Compared to SHV-1 inhibited by tazobactam, SA2-13 had a 10-fold-lower deacylation rate, presumably mediated by the stabilization of the trans-enamine species (309). However, the dissociation constant for SA2-13 was 17-fold lower than that for tazobactam (0.1 versus 1.7 μM, respectively), and there was 47% 24-hour recovery of the activity of the SA2-13/SHV-1 mixture versus complete inactivation with tazobactam.

The 6-alkylidene-2'-substituted penicillin sulfone LN-1-255 (Fig. 1, compound 23), attains mM KS for the class A SHV-1 and ESBL SHV-2 and class D oxacillinase-, ESBL-, and even carbapenemase-type OXA enzymes (63, 322) (S. M. Drawz et al., unpublished data). Moreover, LN-1-255 restores the activity of piperacillin against E. coli IR SHV enzyme producers and of cefazidime and ceftriaxome against E. coli and K. pneumoniae expressing CTX-M, OXA, and CMY β-lactamases (63, 322). Meropenem MICs were lowered from 32 to 1 μg/mL with 4 μg/mL of LN-1-255 against S. marcescens possessing SME-1. The C-3 hydrophobic catechol moiety of this investigational compound appears to improve both its entry into cells via siderophore iron channels and its affinity for β-lactamase active sites (75).

X-ray crystallography of LN-1-255 in complex with SHV-1 (PDB 3D4F) revealed that the inhibitor’s C-6 (hexaeryl) alkylidene group plays an important role in the formation of a planar bicyclic aromatic intermediate (322). The pyridyl nitrogen of the C-6 substituent acts as a base and promotes intramolecular capture, leading to the formation of a bicyclic species (Fig. 10B). The acyl-enzyme ester carbonyl of the intermediate is resonance stabilized by the conjugated π system. Additionally, the carbonyl group of the intermediate is positioned outside the oxygen hole. The decreased deacylation rates of this species are likely due to resonance stabilization and the location of the carbonyl, at an increased distance from the hydrolytic water and improperly positioned for nucleophile attack (322).

By expanding the five-membered penicillin sulfone design to a six-membered cephalosporin sulfone, Buynak et al. developed synthetic methodologies allowing modification of C-3 substituents (62, 64). The different functional groups introduced at C-3 had significant impact on whether the inhibitor was selective for either class A or class C β-lactamases, with some features, such as electronegativity, increasing inhibition of both classes. DVR-II-41S (Fig. 1, compound 24) has both the C-7 (hexaeryl)alkylidene group of LN-1-255 and a C-3 vinyl substituent, similar to nitrocefin, an excellent substrate for nearly all classes of β-lactamases (62, 64). This cephalosporin sulfone demonstrated IC_{50}s of 90 nM for class A TEM-1 and 10 nM for class C GC1. The crystal structure of GC1/DVR-II-41S (PDB 1GA0) revealed a bicyclic aromatic system reminiscent of the LN-1-255 intermediate (94). Additional stability results from the placement of the inhibitor’s anionic sulfinate group between Tyr105 and the acyl-enzyme bond, likely blocking the approach of the deacylating water molecule.

**C-6-substituted penicillin sulfones.** C-6-substituted penicillin sulfones are nM to low μM inhibitors of serine β-lactamases, with the sulfone oxidation state at the penam thiazolidine sulfur showing improved affinities over the sulfide state (27, 61, 374, 375). The stereochemistry and specific functionality of the C-6 substituents can affect the specificity of inhibition. For example, β isomer alkyl penam derivatives, as opposed to α isomer derivatives, show improved IC_{50}s for class B β-lactamases, and derivatives with thiazole rings, particularly with fluorine, amino, or hydroxyl groups, show potency for class A β-lactamases (374). Further, compounds with a mercaptomethyl group at C-6, as opposed to hydroxymethyl, are better inhibitors of class B enzymes, while C-6-mercapto-penicillanlates are relatively less active inhibitors of classes A, B, and C (61).

The C-6-hydroxyalkylpenicillanlates have been employed as tools for studying the mechanism of β-lactamase hydrolysis and inhibition (143). The crystal structure of 6-α-hydroxyalkylpenicillanlate complexed to TEM-1 (PDB 1TEM) suggests that while C-6-substituted penicillin sulfones are acylated in the same way as β-lactam substrates, the hydroxymethyl group at C-6 impedes the approach of the hydrolytic water and pro-

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longs the lifetime of the acyl-enzyme species, effectively inhibiting the enzyme (254). However, the same compound acted as a substrate for the class A NMC-A. Crystal structure determination of NMC-A complexed with 6-α-[(1-hydroxy-1-methyl)ethyl]penicillanate (PDB 1BU1) showed that the repositioning of residue Asn132 in NMC-A, compared to TEM-1, enlarged the substrate binding cavity and allowed Asn132 to make new hydrogen bonds with the C-6 substituent (274). These interactions permit approach by the hydrolytic water and facilitate deacylation of the acyl-enzyme intermediate for the 6-α-hydroxymethylpenicillanate and C-6-substituted carbapenems such as imipenem in NMC-A. Upon introduction of a bulkier C-6 hydroxpropyl group, deacylation was impeded, in a mechanism very similar to that for the inhibition of TEM-1 by imipenem (407). Generalization of these and other findings leads to the conclusion that when the hydrolytic water molecule approaches the acyl-enzyme from the “α direction,” as with class A enzymes, α-hydroxalkylpenicillanates are effective inhibitors. Conversely, β-hydroxalkylpenicillanates inhibit the “β approach” in class C enzymes (143). A group at Wyeth Research found that a 6-β-hydroxymethyl penicillin sulfone has the best activity against both class A and C enzymes, with IC_{50} of 8 nM for TEM-1 and 1.2 μM for AmpC (26). Presumably, the inhibitor worked like tazobactam or sulbactam in class A enzymes, but the specific crowding of the β-face of the ester prevents approach of the hydrolytic water in class C β-lactamases (51). The class D OXA-10 enzyme showed inhibition by both C-6 α- and β-hydroxyisopropylpenicillanates (252). While only the β-isomer could be crystallized with OXA-10 (PDB 1K54), this structure and computational models of the α-isomer suggest related, but different, mechanisms of inactivation based on the C-6 stereochemistry of the inhibitor. However, both mechanisms involved displacing the hydrolytic water necessary for deacylation.

Non-β-Lactam Inhibitors

**Boronic acid transition state analogs (TSAs).** In the 1970s, boronic acid compounds were described as forming reversible, dative covalent bonds with serine proteases and inhibiting these enzymes by assuming tetrahedral reaction intermediates (30, 230). Kiener and Waley then applied the chemistry to the class A β-lactamase from *B. cereus* and showed that boronic acid derivatives, as well as the phenyl and m-amino phenyl derivatives, were mM inhibitors of the enzyme (201). Further study by other groups demonstrated that these compounds, which lack the β-lactam motif, form adducts that resemble the geometry of the tetrahedral transition state of the β-lactamase hydrolytic reaction (22, 82, 95) (Fig. 11). By modifying these transition state analogs to contain the R$_{1-4}$ side chains of natural substrates, affinities in the nM range for both class A TEM-1 and ESBL-type SHV and class C enzymes have been determined (70, 392, 394, 412). Weston et al. used the crystallographic structure of *E. coli* AmpC β-lactamase in complex with m-amino phenylboronic acid (PDB 3BLS) as a model for designing boronic acid-based inhibitors specifically for the class C active site (425, 443). The compound showing the greatest affinity was benzoyl[β]-thiophene-2-boronic acid, which had a $K_i$ for the *E. coli* AmpC of 27 nM.

In addition to their demonstrated potential as effective inhibitors, these compounds have been used to probe the important interactions between the inhibitor and the enzyme active site, thereby informing the design of future TSAs. Using the individual crystal structures for nine TSAs and four β-lactams complexed with the *E. coli* AmpC β-lactamase, computational analyses have generated consensus binding pockets in the AmpC active site (352). The functionalities recognized by certain residues led to the development of new glycolboronic acid TSAs, including a compound containing the R$_1$ side chain of cephalothin. One of these cephalothin analogs achieved a $K_i$ of 1 nM for the *E. coli* AmpC (Fig. 1, compound 25), a 300-fold improvement over the parent compound, which lacked the meta-carboxyphenyl substituent (272). The substituent was designed to resemble both the dihydroazine ring and the C-4 carboxylate of the cephalosporin nucleus. In the crystal structure of this cephalothin meta-carboxyphenyl analog complexed with an AmpC (PDB 1MY8), the inhibitor carboxylate interacted with Asn289, a residue not previously implicated in structural or modeling studies as interacting with the cephalosporin’s C-4 carboxylate. Asn289 is not a highly conserved residue among class C β-lactamases, but the low $K_i$ is maintained when different AmpCs with other residues at position 289 are tested (e.g., $K_i$s of 29 nM for *E. cloacae* P99 and 11 nM for *A. baumannii* (107a, 241, 371). The versatility of this inhibitor may reflect an important flexibility in AmpC active sites that could affect the recognition of both substrates and inhibitors.

High-affinity inhibitors will be clinically useful only if they can effectively permeate the bacterial cell membrane and restore susceptibility to partner β-lactams. The boronic acid derivatives enter both *S. aureus* producing class A β-lactamases and *Enterobacteriaceae* such as *E. coli*, *C. freundii*, *E. cloacae*, and *P. aeruginosa* expressing class C β-lactamases, and they effectively protect ampicillin and cefazidime (70, 272, 349, 430, 436). Boronates have not yet been developed for clinical use in combination with a β-lactam, in part because of concerns about the toxicity of boron. The safety of boron-containing compounds is currently being addressed by clinical studies sponsored by Anacor Pharmaceuticals. Anacor develops boron compounds for medical uses and has current trials for boron-
based topical treatments of fungal, bacterial, and anti-inflammatory diseases, as well as research stage data on a systemic antibacterial agent.

**Phosphonates.** Phosphonate monoester derivatives can acylate the active-site serines of class A, C, and D \(\beta\)-lactamases, leading to effective inhibition (4, 223, 243, 244, 353). These compounds exhibit branched kinetic pathways, in some cases reflecting inhibition by the products that are formed. Acyl phosphonates inhibit class C enzymes, while cyclic acyl phosphonates inhibit both class A and C \(\beta\)-lactamases. For diacyl phosphonates, hydrophobic substituents decrease the inhibitor’s \(K_i\) value, and the acylation rates of these compounds follow the order class D > class C > class A (243, 244). Majumdar et al. posit that this relative inhibitor efficiency reflects the general hydrophobicity of the enzyme active sites and that rational design of diacyl phosphate side chains could lead to \(\mu\)M inhibition of all serine \(\beta\)-lactamase classes.

Crystal structures with these phosphonic acids have also offered important insight into the reaction mechanisms of class C enzymes (297). The ability of the E. cloacae GC1 \(\beta\)-lactamase to hydrolyze extended-spectrum cephalosporins was elucidated by trapping of the enzyme in complex with a phosphonate ceftoxime transition state analog (297). A three-residue insertion into the \(\Omega\)-loop of the enzyme, compared to the C. freundii \(\beta\)-lactamase/transition state analog complex, allowed for a better fit of ceftoxime’s side chain in the enzyme active site and facilitated the activation of the hydrolytic water molecule. The clinical potential of phosphonates has been limited by their poor stability in aqueous solution and susceptibility to phosphodiesterases.

**NXL104.** NXL104 (also known as AVE1330A) (Fig. 1, compound 26) is a bridged diazabicyclo[3.2.1]octanone non-\(\beta\)-lactam inhibitor with very promising activity against class A, C, and D \(\beta\)-lactamases (31, 116, 240, 390). The majority of the published work on NXL104 describes MIC testing, although IC\(_{50}\)s of 8, 38, and 80 nM have been reported for class A TEM-1 and KPC-2 and class C P99 enzymes, respectively (31, 390). A remarkable property of NXL104 is the prolonged deacylation rate. The 50% enzyme recovery time point was 7 days for both TEM-1 and P99 (compared to 7 min for TEM-1 and clavulanate and 290 min for P99 and tazobactam), suggesting the presence of a very stable and long-lived intermediate species (31). The formation of a covalent complex has been supported by an ESI-MS study of TEM-1 and NXL104 (391). Currently, the only reported crystal structure of the inhibitor is in complex with CTX-M-15, and it shows opening of the NXL104 ring upon acylation, interaction with several conserved active-site residues, and displacement of the putative deacylation water by approximately 1 Å (104).

With ceftazidime as a partner \(\beta\)-lactam in a 4:1 ratio (ceftazidime at 4 \(\mu\)g/ml and NXL104 at 1 \(\mu\)g/ml), NXL104 lowered the MICs for a series of Enterobacteriaceae strains at least 8-fold compared to those of ceftazidime alone, to ≤4 \(\mu\)g/ml for E. coli, K. pneumoniae, Citrobacter diversus, and P. mirabilis strains and to 2 \(\mu\)g/ml for E. cloacae and Serratia sp. strains (31). Against class A \(\beta\)-lactamase producers (primarily TEM and SHV types), the ceftazidime-NXL104 combination was comparable to ceftazidime-clavulanate but superior to amoxicillin-clavulanate and piperacillin-tazobactam. Ceftazidime-NXL104 was more potent than piperacillin-tazobactam for class C enzyme-producing Enterobacteriaceae, including both ceftazidime-resistant and -susceptible strains of C. freundii and E. cloacae. The NXL104 concentration of 4 \(\mu\)g/ml restored MICs into the susceptible range for a collection of clinical P. aeruginosa isolates (21% nonsusceptible to ceftazidime versus 6% nonsusceptible to ceftazidime-NXL104) (380). In \(\textit{in vivo}\) murine studies, including models of sepsis with CTX-M Enterobacteriaceae producers, pneumonia with AmpC and SHV-11 K. pneumoniae producers, and thigh infection and sepsis with KPC-2, TEM-1, and SHV-11 K. pneumoniae producers, the ceftazidime-NXL104 combination has demonstrated promising therapeutic efficacy (221, 259, 442).

NXL104 also enhanced the activity of cefotaxime in a study of Enterobacteriaceae expressing CTX-M, KPC, or OXA-48, where 4 \(\mu\)g/ml of the inhibitor lowered MICs to ≤1 \(\mu\)g/ml. For isolates with etapenem resistance due to combinations of \(\beta\)-lactamases (ESBLs or AmpCs) and impermeability, the MICs were still ≤2 \(\mu\)g/ml. While cefotaxime-NXL104 lowered the MIC of one S. marcescens isolate expressing SME-1 from 0.5 to 0.12 \(\mu\)g/ml, MICs for K. pneumoniae and E. coli expressing class B IMP and VIM metallo-\(\beta\)-lactamases were not lowered by either cephalosporin with NXL104.

Two recent studies examined more closely the ability of NXL104 to restore susceptibility to \(\beta\)-lactams for KPC enzyme-producing clinical isolates (116, 390). Stachyra et al. demonstrated that NXL104 at 4 \(\mu\)g/ml restored susceptibility of six KPC-2 or -3 producers to piperacillin, ceftazidime, ceftriaxone, and imipenem (390). Endimiani et al. tested NXL104 at various concentrations (1, 2, and 4 \(\mu\)g/ml) against a panel of 42 K. pneumoniae isolates with ≥3 bla genes each and MIC\(_{\text{max}}\) of ≥128 \(\mu\)g/ml for all \(\beta\)-lactams tested (116). All of these K. pneumoniae strains were susceptible to NXL104 at 2 \(\mu\)g/ml with cefotaxime, ceftazidime, cefepime, and aztreonam and at 4 \(\mu\)g/ml with piperacillin. Collectively, these data suggest that NXL104, in combination with several cephalosporins, aztreonam, and even imipenem, may be an excellent candidate for restoring susceptibility to difficult-to-treat emerging carbapenemase- and ESBL-producing organisms.

NXL104, manufactured by Novexel Inc., is currently in phase II clinical trials in combination with ceftazidime for treatment of complicated urinary tract and intra-abdominal infections (www.clinicaltrials.gov). Additionally, Forest Laboratories Inc. intends to begin phase I clinical trials with NXL104 in combination with the novel broad-spectrum cephalosporin ceftaroline (which is currently in phase III trials as monotherapy for complicated skin infections and community-acquired pneumonia). The results of clinical trials are awaited.

**Hydroxamates.** O-Aryloxy carbonyl hydroxamate is an irreversible inhibitor of the class C. cloacae P99 \(\beta\)-lactamase that utilizes a mechanism of action which differs from those of the currently available inhibitors (Fig. 1, compound 27) (330, 450). The proposed inhibition mechanism involves rate-limiting acylation of the hydroxamate. However, the inhibitor is stabilized not by the traditional oxyanion hole comprised of the backbone nitrogens of Ser64 and Ser318 but rather by the side chains of Tyr150 and Lys315 (330). As with clavulanate and tazobactam, the acyl-envelope proceeds to either hydrolysis or inactivation. The inactivation mechanism leads to aminolysis of Lys315 and a covalent cross-linking of Ser64 and Lys315, as
seen in the crystal structure of the inhibited P99 (Fig. 12) (PDB 2P9V) (450). TEM-2 and OXA-1 β-lactamases were also inhibited by O-aryloxycarbonyl hydroxamates, and the MBL GIM-1 was inhibited by a “reverse” hydroxamate (hydroxy-lamino replacing hydroxylamine) conjugated to a cephalosporin nucleus (131, 330). Additional mechanistic studies of hydroxamates and their derivatives, as well as in vitro activity data, will be of much interest.

Other non-β-lactam inhibitors. Work by Conrath et al. explored the β-lactamase-inhibitory potential of soluble fragments derived from the variable region of heavy-chain dromedary antibodies (89). IC₅₀S of 35 μM and 300 μM were obtained for TEM-1 and the B. cereus MBL BeII, but the development of this strategy would require innovative techniques such as increasing the permeativity of these 15-kDa polypeptides to outer membranes of Gram negative β-lactamase producers.

Inhibitors with a sulfonamide core and varied substituents were developed by structure-based optimization (351, 413). This non-β-lactam design approach is intended to avoid both hydrolysis by β-lactamases and the upregulation of β-lactamasms induced by some β-lactam-based inhibitors. Starting from a map of binding “hot spots” developed from crystal structures of 13 different ligands in complex with the E. coli AmpC β-lactamase, 200,000 commercially available small molecules were docked into the active site of the enzyme by computer simulation (351, 352). Based on these results, lead compounds were screened in vitro, and the molecule with the highest affinity (26 μM) was crystallized in complex with the AmpC (PDB 1L2S) (Fig. 1, compound 28). The structure closely resembled the prediction of the docking simulation, revealing the plasticity of the enzyme active site to recognize functional groups different from β-lactams. This catalyzed the optimization of a novel inhibitor, and replacement of the chloride substituent with a carboxylate yielded a compound with a 26-fold-improved Kᵢ (1 μM) (413). In MIC testing, the inhibitor restored the susceptibility to ceftazidime (at a 1:1 ratio) in the AmpC producers E. cloacae and C. freundii (but not P. aeruginosa) and, in contrast to clavulanate, did not upregulate β-lactamase expression in an inducible strain of E. cloacae.

β-Sultams are the sulfonyl analogs of β-lactams, and N-acyl β-sultams (Fig. 1, compound 29) inactivate the P99 enzyme (318, 419). ESI-MS studies suggest that the β-sultams sulfonylate the serine residue to form a sulfonate ester. Elimination of the sulfonate anion leads to C-O bond fission and formation of a dehydroalanine at the previous Ser64 residue (419). These compounds do not show inhibition of class B MBLs, and there are currently no studies examining their activities in class A or D enzymes.

BLIP is a 165-amino-acid protein isolated from the same organism that produces clavulanic acid, Streptomyces clavuligerus (105). Studies of BLIP’s inhibitory activity have revealed affinities for class A enzymes that vary from pM to μM (the enzymes with the highest affinities for BLIP are KPC-2 and the P. vulgaris β-lactamases) (147, 393). Many of the individual class A residues which contribute to BLIP binding have been identified through crystallography and mutagenesis (363, 456, 460). While the clinical viability of a peptide inhibitor based on this protein is limited because of protein degradation in vivo, the high-affinity interactions offer direction for the design of novel β-lactamase inhibitors.

The mixture of vanadate and catechol compounds in aqueous solution produces vanadate-catechol complexes which can inhibit class A TEM-2, class C P99, and class D OXA-1 β-lactamases with apparent Kᵢ S of 62, 0.58, and 1.5 μM, respectively (5). A crystal structure with the complex has not been attained, but molecular modeling suggests that the active-site serine is bound to a penta- or hexa-coordinated vanadium. Vandate-catechols represent another group of compounds that are useful as tools for novel inhibitory active-site interactions, but lack clear clinical potential.

**INHIBITION OF METALLO-β-LACTAMASES**

We note that designing and/or discovering inhibitors to MBLs presents special challenges. The diversity of class B enzyme subclasses (i.e., B1, B2, and B3) and the mechanistic complexities regarding the role of one or two Zn²⁺ ions have impeded effective inhibitor design. In addition, indiscriminately inhibiting or chelating metal ions can have untoward effects on natural or endogenous metalloenzymes. For example, similarities between the active-site architectures of MBLs and essential mammalian enzymes (e.g., human glyoxalase II) complicate the design of an inhibitor that would be safe for human use (97). As a class, the MBLs are resistant to all the mechanism-based inhibitors of the serine enzymes (the inhibitory activity of the methyldiene penems against class B β-lactamases may be an important exception), and few investigational inhibitors demonstrate sub-μM efficacy (335, 441). Here, we provide a brief survey of the more promising agents in development.
Thiol Derivatives

Due to catalytic dependence on Zn$^{2+}$ ions, the metalloenzymes are inhibited by thiols or chelating agents that have an affinity for the hydrophobic pocket of the enzyme active site and bind to, or interfere with, the bonding network between the hydrolytic water and the Zn$^{2+}$ ions (170, 227, 312). Thiol derivatives, such as thiomandelic acids, typically show low-$\mu$M $K_{i}$ inhibition of B1 (e.g., BcII, IMP-1, and VIM-2) and B3 (e.g., L1) subclasses but over 100-fold-higher $K_{i}$ values for the subclass B2 CphA MBL (268). Structural analysis of thiol-based inhibitors complexes with the three MBL subclasses suggests that the thiol group chelates the Zn$^{2+}$ ions for subclasses B1 and B3 (227). For example, the crystal structure of IMP-1 from P. aeruginosa bound to a thiol inhibitor (Fig. 1, compound 30) (PDB 1DD6) showed interactions with active-site residues, allowing the thiol of the inhibitor to bridge the two catalytic Zn$^{2+}$ molecules and displace the bridging water important for hydrolysis (88). A similar inhibition mechanism was resolved from the crystal structure of the B1 VIM-2 complexed to a mercaptoenolate compound (452). However, this binding mode was not observed in the crystal structure of the thiolate D-capotril bound to the subclass B2 CphA from A. hydrophila (227). Instead, the inhibitor’s main interactions with the enzyme were via the thiolate carboxylate group (227).

Mass spectrometric data suggest that mercaptoenolate esters may act as mechanism-based inhibitors for BcII by generating mercaptoenolate acid in situ and forming a disulfide linkage with a cysteine residue in the enzyme active site (327). However, as with other thiols, the IC$_{50}$ of these mercaptoenolate esters varied from low $\mu$M to mM across MBL subclasses.

Pyridine Dicarboxylates

Recent work demonstrates that pyridine carboxylates, in particular 2-picolinic and pyridine-2,4-dicarboxylic acids (Fig. 1, compound 31), are competitive inhibitors of CphA (a subclass B2 MBL), with $K_{i}$ values of 5.7 and 4.5 $\mu$M, respectively (170). The pyridine carboxylate derivatives showed limited inhibitory activity for B1 and B3 MBLs. In the crystal structure of CphA/3pyridine-2,4-dicarboxylic acid (PDB 2GKL), the catalytic Zn$^{2+}$ ion is coordinated by the nitrogen and one of the carboxylate oxygens of the inhibitor (170). The Zn$^{2+}$ ion is also engaged in electrostatic interactions with residues Asp120, Cys221, and His263. Hydrophobic interactions with Asn233, Trp87, and Val67 help to further stabilize the inhibitor in the active site. The larger and more flexible active sites of subclass B1 and B3 MBLs may not permit comparable stabilizing contacts with the pyridine carboxylates, offering an explanation for the B2 specificity.

Trifluoromethyl Ketones and Alcohols

Reactive trifluoromethyl ketones and alcohols, conjugated to a penicillin-like phenyl side chain, effectively inhibit MBLs from B. cereus, P. aeruginosa, S. maltophilia, and A. hydrophila in vitro, presumably by binding the active-site Zn$^{2+}$ ion (433, 434). The crystal structure of a biphenyl tetrazole inhibitor, L-159,061 (Fig. 1, compound 32), in complex with the B. fragilis enzyme (PDB 1A8T) indicates that the tetrazole portion of the molecule interacts directly with the active-site Zn$^{2+}$ ion (414). IC$_{50}$ and $K_{i}$ values in the low $\mu$M range were observed, and the incorporation of substituents on the phenyl groups and their interactions with specific residues were essential to the strong binding of the biphenyl tetrazoles (414). These inhibitors do appear to enter cells, as the addition of imipenem or penicillin G to biphenyl tetrazole compounds increased inhibition zones for an imipenem-resistant B. fragilis isolate (414).

Carbapenem Analogs

1-$\beta$-Methylcarbapenems with cyclic C-2 substituents, such as J-110,441, achieve sub-µM $K_{i}$s for the IMP-1, CcrA, L1, and BcII MBLs as well as representative class A and C enzymes (282). For several IMP-1-producing S. marcescens and P. aeruginosa isolates, imipenem MICs were lowered to 4 $\mu$g/ml by the addition of J-110,441. The further development of J-111,225 included a trans-3,5-disubstituted pyrrolidinylthio substituent at the C-2 position (Fig. 1, compound 33) (281). J-111,225 maintained inhibitory activity against IMP-1 ($K_{i}$ of 0.18 $\mu$M) and synergy with imipenem but had moderately improved MICs as a single agent against IMP-1-expressing S. marcescens and P. aeruginosa (MIC range of 4 to 32 $\mu$g/ml). However, J-111,225 acted as a substrate for other chromosomally encoded class B MBLs, including CcrA, L1, and BcII.

Tricyclic Natural Products

Natural product screening for inhibitors to BcII led to the discovery of extracts from Chaetomium funicola which demonstrated IC$_{50}$s ranging from 389 to 0.3 $\mu$M for BcII, P. aeruginosa IMP-1, and B. fragilis CfiA MBLs (329). The “lead compound” of the three tricyclic compounds, SB238569 (Fig. 1, compound 34), also showed low $K_{i}$ values for the same enzymes (79, 17, and 3.4 $\mu$M, respectively). In combination with meropenem, 8 $\mu$g/ml of SB238569 restored susceptibility to B. fragilis CfiA. The crystal structure of CfiA and SB236050 (PDB 1KR3) identified important polar (Lys184, Asn194, and His162) and ring-stacking (Trp49) interactions which likely contribute to the inhibitory activity. Comparison of this structure with that of IMP-1/mercaptocarboxylate (PDB 1DD6) suggests that the binding site created by the loop preceding Lys184 is shorter in IMP-1 and may leave a larger portion of the compound exposed to solvent (88). The decreased inhibitory activity of the tricyclic inhibitor for S. maltophilia L1 may also be explained by active-site variations, including differences in the positions of the Lys184 and Asn194 equivalents.

Succinate Derivatives

The IMP-1 enzyme is encoded by a transferable bla$_{IMP-1}$ gene and represents an increasing resistance threat, and thus the development of inhibitors for this target is a priority. Succinic acid compounds have been screened as IMP-1 inhibitors, and several derivatives have restored meropenem susceptibility of IMP-1-expressing E. coli (269, 415). Compound 35 (Fig. 1) belongs to a series of 2,3-(S,S)-disubstituted succinic acids which inhibit IMP-1 with nM IC$_{50}$s (415). Crystal structure
analysis revealed that the inhibitors’ carboxylate groups interact with both Zn$^{2+}$ ions and active-site residues while displacing the bridging water (PDB 1JJIE and 1JJT) (415). Disubstituted succinic acids have also been described as inhibitors of the L1 MBL and display binding modes similar to those in IMP-1 (284, 303).

IMP-1 is also inhibited by N-arylsulfonyl hydrazone compounds, with $IC_{50}$ as low as 1.6 µM (Fig. 1, compound 36) (384). Structure-activity relationship studies revealed that bulky aromatic substituents on both sides of the sulfonyl hydrazone backbone improved the activity of these inhibitors.

Liénard et al. demonstrated the ability of dynamic combinatorial mass spectrometry (DCMS) to identify potent BcII inhibitor leads (228). A meso-2,3-dimer-capsomicinic acid was bound to the MBL active site via one thiol group, while the second thiol group was linked in a disulfide bond to a thiol-containing compound from a screening library. ESI-MS revealed which compounds bound the enzyme, and subsequent structure optimization led to the development of a mercapto-carboxylate that inhibits BcII with a $K_i$ of 740 nM (Fig. 1, compound 37).

C-6-Mercaptomethyl Penicilllinate

Penicillin derivatives with C-6-mercaptomethyl substituents (Fig. 1, compound 38) show promising kinetics against class A, B, and C β-lactamases (IC$_{50}$ of 6.8, 0.10, and 10.5 µM, respectively, against representative enzymes) (61). These penicilllates, in combination with piperacillin, lowered MICs against MBL-producing strains of P. aeruginosa. Building inhibitors on the backbone of the natural substrate for all β-lactamases, the penicillin nucleus, may help bridge the gap between these diverse enzyme classes.

LESSONS LEARNED

We dedicate the penultimate section of this review to summarizing the “lessons learned” from our consideration of the β-lactamase inhibitors. From the perspective of clinicians, we propose that several important features must be regarded in order to optimize the development of β-lactamase inactivators.

(i) High affinity for the active site of the target β-lactamase. It is axiomatic that in order for an inhibitor to work efficiently, it must exhibit high affinity for its target. This principle is emphasized by the development of clavulanate resistance in TEM-1 and SHV-1 β-lactamases mediated by substitution of amino acid residues 69, 130, 244, and 276, leading to increased inhibitor $K_s$ and/or IC$_{50}$ (73, 153, 378, 411). Particularly promising are those inhibitors that maintain high affinities across different groups of β-lactamases, such as the C-2-substituted sulfone LN-1-255, the methylene penemides, and phosphonates which achieve mM inhibition of class A, C, and D (4, 25, 63, 245, 322, 441) (Drawz et al., unpublished data).

(ii) Mimicking the “natural substrate.” The available inhibitors are effective primarily against class A serine β-lactamases, and the structures of class A β-lactamase inhibitors are similar to those of penicillin, bearing a fused four- or five-membered ring system. Similarly, data on transition state analogs suggest that class C β-lactamases have high affinity for inhibitors that resemble the natural cephalosporin substrates (272). A guideline for inhibitor design may be “penicillin analogs for penicillinas, and cephalosporin analogs for cephalosporinases.” This principle also ensures that the inhibition mechanism follows the substrate mechanism—save for hydrolysis. While β-lactamases continue to develop resistance to all developed β-lactams, designing inhibitors that follow the hydrolytic mechanism may help to thwart the emergence of resistant isolates, as the enzyme must find novel ways to preserve function but evade inhibition. We hasten to add that this approach may not be practical in all cases.

(iii) Stabilizing interactions in the active site. Stabilizing or prolonging the acyl-enzyme intermediate is central to successful inactivation by the currently available class A inhibitors (81, 396). Promising novel inhibitors, such as the methylidene penems (e.g., BRL 42715) and LN-1-255 are designed to capitalize on and enhance this stabilization. One approach is to prolong the acylation step ($k_a$). Alternatively, acyl-enzyme intermediates can be stabilized by multiple interactions in the active site, as observed for SHV-1 with tazobactam and SA2-13 (308, 309). The inhibition of NMC-A by the monobactam derivative 13 (Fig. 1) is enhanced by the incorporation of chemical components that confer structural flexibility to the inhibitor, allowing the enzyme-substrate species to adopt multiple conformations and trap the enzyme in energy minima unfavorable for hydrolysis (273, 308, 445). Another viable approach is to design inhibitors that induce conformational changes in the enzyme or displace key water molecules important for hydrolysis, such as seen with the large seven-membered intermediate formed from the methylidene penem (60, 294, 429).

(iv) Reaction chemistry that slows deacylation and favors inactivation over inhibitor hydrolysis. Features of an inhibitor which slow deacylation and drive toward terminal inactivation, such as acyl-enzyme stabilization (see above) or the presence of a good leaving group at C-1, contribute to effective inhibition. Inhibitors that can delay deacylation for longer than 15 to 20 min ($k_{off}$ rate of $\leq$0.001 s$^{-1}$) may outlast the bacterial generation time, allowing the partner β-lactam time to disrupt the cell wall synthesis necessary for cell division (396, 410). For example, the bridged monobactams delay deacylation by limiting the C-3–C-4 rotation necessary for approach of the hydrolytic water in class C enzymes (152, 238). Additionally, relatively minor changes introduced by the acylation of mero-penem by SHV-1, including minor displacements to key water molecules important for hydrolysis, such as seen with the large seven-membered intermediate formed from the methylidene penem (60, 294, 429).

(v) Rapid cell penetration. β-Lactamases are typically found in the periplasmic space of Gram-negative bacteria, and the rapidity with which inhibitors can access their target is a prerequisite for successful inhibition. Certain β-lactams are zwitterionic and rapidly penetrate the bacterial outer membrane (e.g., ceftazidime). The same principle extends to the inhibitors; e.g., the enhanced cell entry of LN-1-255 and the novel monobactams BAL19764 and BAL30072 is likely due to uptake through siderophore channels (75, 316). Attention must also be paid to designing “permeable drugs” that are not hindered as they pass through porin channels or quickly returned into the medium by multidrug efflux pumps, an important
resistance determinant independent of β-lactamases (see above) (287).

(vi) Low propensity to induce β-lactamase production. Successful inhibitors need to avoid inducing expression of the enzymes that they are designed to inhibit, particularly among AmpC β-lactamases. Examples of this approach include the non-β-lactam inhibitors with sulfonamide cores designed to evade hydrolysis as well as to avoid induction of AmpC enzyme expression (413).

(vii) Identification of measureable biological correlates of β-lactamase inhibition in the cell. Regardless of how attractive an inhibitor’s laboratory characteristics may be (e.g., nM $K_i$ values, low IC$_{50}$, high $k_{inact}$, or low MICs), a successful inhibitor must prove its worth in the clinical setting. In vivo efficacy requires an integration of many complex features, including those listed above, partnership with an appropriate β-lactam, sufficient serum and periplasm concentrations, and factors less well understood (e.g., serum protein binding and individual clearance rates). Many microbiological properties (time kill, mutant prevention concentration, and synergy) are important parameters that need to be considered. There is no certain consensus on how best to identify and measure these attributes in the early research on candidate inhibitors. Previous studies with β-lactam antibiotics have demonstrated that MICs may be poorly correlated with the sensitivity of PBPs for inactivation (213). Finding reliable biological correlates of inhibition is a substantial and relevant challenge, and our current methodological outcome measurements may benefit from re-examination in this vein.

(viii) In the meantime, use what we have. While scientists and physicians continue struggling to stay ahead, or at least abreast, of the abilities of β-lactamases, we must utilize intelligently and productively the agents that are currently available. For example, we can commit to applying the research that demonstrates the potential of novel combinations of β-lactams and β-lactamase inhibitors, such as clavulanic and cepafline, cepfirome, or meropenem, to expand and enhance inhibitors’ abilities to protect partner β-lactams (172, 239). Several significant hurdles stand in the way of these novel combinations, such as corporate relationships affecting the funding of the trials necessary to demonstrate clinical efficacy, as well as optimization of the pharmacokinetics, pharmacodynamics, and safety of the components. The β-lactamase-inhibitory activity of carbapenems should not be overlooked in the face of increasing β-lactam resistance. Carbapenems, for the time being, remain effective against many difficult-to-treat infections and teach us that the distinction between an inhibitor and a slow substrate may be blurred.

A PERSPECTIVE

From our vantage point, the main challenge in β-lactamase inhibitor development is discovering novel inhibitors with activity against a broad spectrum of inhibitor-susceptible and -resistant enzymes from multiple classes (17, 410). New inhibitors must also target carbapenemases (serine and metallo-), as carbapenems are still the most potent β-lactams available for clinical use. Future advances in inhibitor design against versatile β-lactamases must incorporate strategies that address each of the key structural features of these diverse proteins, keeping in mind that mechanism-based inhibitors may engage in unique reaction chemistry with β-lactamases. Compounds with the chemical features of the “ideal inhibitor,” such as enhanced permeability through the cell membrane, affinity for the active site of the β-lactamase, formation of intermediates that “trap” the enzyme, displacement of critical water molecules, and prolonged time to enzyme recovery, many of which are illustrated by the investigational complexes highlighted herein, may have a significant impact on the development of “second-generation” inhibitors that target resistant β-lactamases.

We also acknowledge the magnitude of this challenge. In 30 years of targeted research on β-lactamase inactivation, new inhibitors have been only slowly introduced into clinical trials. There have been many starts, but few agents cross the finish line. This is a testament to the versatility and complexity of β-lactamases and the incredible evolutionary ability of the organisms harboring them. Is the perfect β-lactamase inhibitor an unattainable goal? Perhaps. Yet, we anticipate with excitement the achievements in the next 3 decades and suspect that better chemistries and combinations will ultimately be found.

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Sarah M. Drawz received her bachelor’s degree from Amherst College magna cum laude. She is currently an M.D./Ph.D. student at Case Western Reserve University School of Medicine in the Department of Pathology. Her Ph.D. dissertation work focuses on the inhibition of β-lactamase enzymes. She plans to return to her medical studies in the coming year.

Robert A. Bonomo received his M.D. from the Case Western Reserve University School of Medicine. He trained in Internal Medicine and Infectious Diseases at University Hospitals of Cleveland and received a Certificate of Added Qualification in Geriatrics. Dr. Bonomo served as section chief of the infectious disease division at the Cleveland Veterans Affairs Medical Center before becoming Director of the Veterans Integrated Service Network 10 Geriatric Research, Education, and Clinical Center (GRECC). He is a Professor of Medicine at Case Western Reserve University School of Medicine and also holds appointments in the Departments of Pharmacology and Molecular Biology and Microbiology. Dr. Bonomo’s research focuses on microbial resistance to antibiotics, especially β-lactams.