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INHIBITOR RESISTANT CLASS A BETA–LACTAMASES

Robert A. Bonomo[1,2] and Louis B. Rice[2]

[1] Geriatric CARE Center and [2] Department of Medicine, Veterans Affairs Medical Center, Cleveland, Ohio

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1. ABSTRACT

Beta-lactamase inhibitors (clavulanic acid, tazobactam, and sulbactam) greatly enhance the therapeutic efficacy of their partner antibiotics (amoxacillin, ampicillin, piperacillin, and ticarcillin) against common enteric and non-enteric organisms possessing class A beta-lactamases. Unfortunately, the number of class A enzymes being discovered that are resistant to these combinations is increasingly rapidly. The TEM and SHV class A beta-lactamases resistant to inhibitors have point mutations in critical amino acids important for catalysis. Compared to the wild type beta-lactamase, inhibitor resistant enzymes are inefficient at hydrolyzing benzylpenicillin, aminopenicillins, and cephalosporins. Nevertheless, hyper-production of these enzymes resulting from mutations in the promoter region can confer substantial levels of resistance. Understanding the microbiologic and kinetic properties of these inhibitor resistant class A beta-lactamases can lead to the design of more potent beta-lactam compounds as well as more effective inhibitors.

2. INTRODUCTION

Beta–lactamases (E.C.3.5.2.6) are the principle mechanism of resistance to beta–lactam antibiotics. Presently, there are more than 200 different beta–lactamases found in nature (1). These versatile enzymes are present in both Gram positive and Gram negative bacteria. Beta–lactamases are encoded by genes located on plasmids, transposons, and in the bacterial chromosome (2). When found on plasmids in Gram negative bacteria, these enzymes are constitutively expressed. Chromosomal beta–lactamases can be induced or constitutively expressed. A single bacterium can also possess multiple beta–lactamases (3).

The rapid evolution of beta–lactamase enzymes found in the clinic has created a major therapeutic dilemma. The synthesis and design of potent beta–lactams by the medicinal chemist has been challenged by the isolation of resistant microbes possessing novel beta–lactamase enzymes with expanded hydrolytic capacities. As a result of this struggle, two strategies have evolved. The first approach has been the synthesis of beta–lactam antibiotics resistant to hydrolysis. The penicillinase-resistant penicillins (nafcillin, oxacillin), the advanced generation cephalosporins and cephamycins (ceftriaxone, ceftazidime, and cefoxitin), monobactams (aztreonam), and carbapenems (imipenem and meropenem) are examples of this approach. The second approach has been using beta–lactamase inhibitors coupled with beta–lactam antibiotics. These enzyme inhibitors function to permanently inactivate the beta–lactamase in the periplasmic space so that the partner antibiotic can reach its target, the penicillin binding proteins (PBPs). These compounds have also been called “suicide inhibitors” or “mechanism-based” inactivators because they irreversibly acylate the beta–lactamase enzyme (4). Structurally, they resemble beta–lactam antibiotics.

Beta–lactam beta–lactamase inhibitor combination antibiotics have significantly advanced our ability to treat a variety of infections in the community and in the hospital setting. The clinical combinations available for use in the United States are ampicillin/sulbactam (IV), piperacillin/ tazobactam (IV), ticarcillin/clavulanate (IV), and amoxicillin/clavulanate (PO). Cefoperazone/sulbactam (IV) and sulbactam (IV) are available in Europe. These partner antibiotics were welcome additions to the therapeutic armamentarium since they restored the efficacy of the susceptible beta–lactam against common Class A,

plasmid determined beta–lactamases such as TEM-1 in *Escherichia coli*, SHV-1 in *Klebsiella pneumoniae.* and PC1 in *Staphylococcus aureus* (3). They also enhanced the ability of the clinician to treat complex polymicrobic (aerobic and anaerobic) infections in the gastrointestinal and respiratory tracts. Unfortunately, a number of Class A beta–lactamase have been discovered in the clinic that are resistant to inactivation by beta–lactamase inhibitors (1, 2, 5, and references therein). In this paper, we will review the classification and enzymology of beta–lactamases, recount the development of beta–lactamase inhibitors, and detail their mechanism of action. We will next discuss mechanism of resistance to inhibitors and enumerate the microbiologic and kinetic properties of beta–lactamase inhibitor resistant beta–lactamase enzymes found in nature.

3. BETA–LACTAMASE CLASSIFICATION

In order to understand the relationship of these enzymes to one another, two classification systems have been developed. In the Ambler classification scheme (based upon amino acid sequence similarity), beta–lactamase enzyme can be grouped into four major classes, class A →D (2, 6). Class A enzymes are penicillinases and cephalosporinases usually found on plasmids or transposons; class B are the metallo-beta–lactamases; class C are the chromosomal cephalosporinases; and class D are oxacillinases. Class A, C, and D have serine as the catalytic residue in the active site and are closely related to the serine protease family. Class B metallo-enzymes possess a bi-nuclear catalytic zinc in the active site. In the Bush-Jacoby-Mederios classification system, enzymes are grouped according to the substrate and inhibitor profiles (1). Substrates that define a particular group that are tested include the standard panel of beta-lactams (benzylpenicillin, cephaloridine) and the beta–lactamase inhibitors (clavulanic acid, sulbactam and EDTA). Group 1 beta–lactamases are chromosomally encoded cephalosporinases and are poorly inhibited by clavulanate. Group 2 beta-lactamases are a diverse group of beta–lactamases composed of penicillinases, cephalosporinases, oxacillinases, and carbapenemases. These are plasmid and chromosomally encoded. In general, group 2 enzymes are inhibited by clavulanate. Group 3 are metallo-enzymes-usually found in *Pseudomonas* spp.*, Bacteroides* spp. and *Serratia marcescens*. Group 4 enzymes represent a collection that is uncharacterized. Multiple sub–classifications exist in this scheme. The inhibitor resistant class A beta–lactamase are classified as group 2br (1).

4. BETA–LACTAMASES ENZYMOLOGY

Beta–lactamases hydrolyze beta–lactam antibiotics in a two step reaction. At first, the enzyme forms the Michaelis-Menten complex. The beta–lactamase is acylated. The acyl enzyme is then subsequently deacylated in a hydrolysis reaction:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E:S \xrightarrow{k_2} E\text{-}S \xrightarrow[H_2O]{k_3} E + P \quad (1)$$

In this scheme, E is a beta–lactamase, S is a beta–lactam substrate, and P is the inactive acid devoid of antibacterial activity. K_1, k_{-1}, k_2 and k_3 represent rate constants; k_2=acylation rate constant; k_3= deacylation rate constant.

The lactam bond of beta–lactam antibiotics undergoes nucleophilic attack by the serine hydroxyl of the enzyme. The ability of beta–lactam antibiotics to be efficiently hydrolyzed by the beta–lactamase depends upon the composition and structure of attached R groups. Certain R groups prevent or hinder facile entry of beta–lactam drugs into the active site. Although there is widespread acceptance of the overall reaction scheme, the molecular details still challenge our understanding.

5. STRUCTURE OF BETA–LACTAMASES

Beta–lactamases are globular proteins that possess 11 alpha helices and five beta–pleated sheets. To date the 3 dimensional structure of a number beta–lactamases has been determined: the PC1 beta–lactamase *of Staphylococcus aureus*; the 749/C beta–lactamase of *Bacillus licheniformis*; the beta–lactamase from *Bacillus cereus* 569h9; the P99 ampC beta–lactamase of *Enterobacter cloacae*; the amp C enzyme of *Citrobacter freundi* 1203; the TEM-1 beta–lactamase of *E coli*; the metallo-beta–lactamases of *Bacillus cereus* and *Bacteroides fragilis*; NMC-A beta - lactamase of *Enterobacter cloacae* NOR-1; Sme beta–lactamase from *Serratia marcescens* S6; the ampC beta-lactamase from *E. coli;* and the beta–lactamase from *Streptomyces albus* G (5, 7, and references therein, 8-10). Most recently, the 3D structure of the *Mycobacterium fortuitum* beta-lactamase, TOHO-1 beta-lactamase, and the SHV-1 beta-lactamase have been determined (11-14). In addition, the three dimensional structure of a number of other penicillin interactive enzymes has also been solved-PBP-2x of *Streptococcus pneumoniae*, DD-peptidase of *Streptomyces* R61 and the DD-peptidase of *Streptomyces* K15 (7). Based upon the 3 dimensional topology, there appears to be significant structural similarity among all these penicillin recognizing enzymes. These penicillin recognizing enzymes interact with beta–lactams by similar mechanisms (see Eq.1 and 2). A major difference between PBP and beta–lactamases are in the magnitude of k_3, the deacylation rate constant. K_3 is very slow for PBP and very fast for beta–lactamases.

$$S + PBP \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} S:PBP \xrightarrow{k_2} S\text{—}PBP \xrightarrow{k_3} P + PBP \quad (2)$$

S= beta–lactam substrate; PBP =penicillin binding protein; P =product

Another major difference between PBPs and beta–lactamases is their interaction with D-ala-D-ala peptides. PBPs have significant DD-peptidase activity (needed for bacterial cell wall synthesis). It must be stressed that class A beta–lactamases are different from class C. The increased electrophility of the oxyanion pocket (NH70 and NH 237) and the architecture of the Ω loop (Arg 164 to Asp 179) all combine with a well placed

general base (Glu 166) to make the class A enzymes highly efficient.

6. CHRONOLOGY OF BETA–LACTAMASE INHIBITORS

With the emergence of penicillinase resistant *Bacillus coli* (*E. coli) and* the rapid spread of penicillin-resistant *S. aureus*, it became eminently clear that beta–lactamase inhibition was a clinically important strategy (15). Semisynthetic penicillins (methicillin nafcillin and isoxazolyl penicillin) were discovered that could effectively inhibit beta-lactamase activity. Cloxacillin inhibited the chromosomal beta–lactamase of *E.coli* in vitro but was relatively ineffective against the plasmid-determined enzyme. BRL1437, an inhibitor developed by Beecham Pharmaceuticals with structural similarity to nafcillin, was more potent, but therapeutically this drug proved to be inadequate because it penetrated the bacterial cell wall so poorly. Olivanic acids were discovered by Beecham Pharmaceuticals in 1976. The olivanic acids (derived from *Streptomyces olivaceus*) inhibited some beta-lactamase enzymes extremely well but, as with BRL 1437, penetrated bacterial cell walls poorly and were metabolized rapidly. Although they were not initially used as beta-lactamase inhibitors, these compounds later were to serve as the source compounds for the carbapenems.

Reading and Cole soon discovered the potent beta–lactamase inhibitor, clavulanic acid (16). This compound, isolated from *Streptomyces clavuligerus*, was synergistic with amoxicillin against *S. aureus*, *K. pneumoniae*, *Neisseria gonorrhoeae, Hemophilus influenzae*, *Moraxella catarrhalis*, *B. fragilis*, and *Proteus mirabilis.* In a similar project at Pfizer, the penicillanic acid sulfone, sulbactam, was developed as a derivative of penicillanic acid. This compound was rapidly introduced into clinical practice. BRL 42715, another potent Beecham product, appeared some time later. The synthesis of tazobactam soon followed. Tazobactam, a triazole sulfone derivative, proved to be a compound as active as clavulanic acid (17,18). These efforts were later followed by the future development of the monobactams and the carbapenems. In some instances, the distinction between a beta–lactam antibiotic and a beta–lactamase inhibitor became very blurred (19). At present, clavulanate, sulbactam and tazobactam have enjoyed the most widespread clinical utility against class A beta–lactamase. More compounds are being actively studied (19).

7. MECHANISM OF ACTION OF BETA–LACTAMASE INHIBITORS

Acting as beta–lactam antibiotic, beta–lactamase inhibitors permanently acylate beta–lactamase in a complex two step reaction. The beta–lactamase inhibitor is recognized as a beta–lactam substrate by the beta–lactamase. This recognition leads to the formation of the Michaelis-Menten complex, and the enzyme is acylated. At that point, the acyl-enzyme intermediate has three fates. There can be formation of a product, E+P. An alternative route is the formation of the tautomer, E-T. This intermediate is able to equilibrate with the acyl enzyme by a process of rearrangement. The clinically desired reaction is the formation of the permanently inactive enzyme complex, E-I (4).

It is now recognized that different beta–lactamase inhibitor compounds have different affinities for beta–lactamase enzymes. As a corollary, each beta–lactamase may be inactivated by a different mechanism. The dissociation constant, K*i*, the turnover number, *tn*, the number of interactions the inhibitor has with the enzyme in order to inactivate it, may be unique for each inhibitor and enzyme (17,18). For example, it requires just one turnover for the staphylococcal beta–lactamase to be permanently inactivated by sulbactam, while it requires up to 16,000 turnovers for the *Bacillus* enzyme to be inactivated (17). As a general rule, SHV enzymes are more resistant to sulbactam than TEM, but are more susceptible to inactivation by clavulanic acid. The reason for this difference is still unclear. The chromosomal cephalosporinases are only partially inhibited by tazobactam (18).

8. RESISTANCE TO BETA–LACTAMASE INHIBITORS

At the time the beta–lactamase inhibitors were being clinically developed rare isolates of *E coli* were reported that were phenotypically resistant to amoxicillin/clavulanate (20). These reports were scattered and did not cause a major alarm. Overproduction of TEM-1 enzyme was believed to be the mechanism responsible for this resistance.

Using degenerate oligonucleotides in an attempt to understand the effect of multiple substitutions on protein activity, Oliphant and Struhl discovered that certain key substitutions in the vicinity of the active site of *E coli* RTEM conferred resistance to beta -lactam beta-lactamase inhibitor combinations (21). Using chemical mutagenesis Mavanathu, Lerner and Mobashery also discovered the TEM enzyme with single amino acid substitutions at the 244 position (Arg 244Ser and Arg 244Cys) could render *E. coli* resistant to beta–lactam beta–lactamase inhibitors combinations (22). The substitutions obtained by Manavanthu *et al.* (22) were at different amino acid positions (Arg244) than those described by Oliphant and Struhl (Arg61 to Cys77). French investigators soon found the identical mutation in the TEM enzyme at the 244 site from a neonatal bloodstream isolate of *E. coli* that conferred resistance to amoxicillin/clavulanate and ampicillin/sulbactam (23-25). These reports foreshadowed a growing microbiologic and clinical problem to be faced in the 1990s.

9. SCOPE OF BETA-LACTAM BETA-LACTAMASE INHIBITOR RESISTANCE

Table 1 lists 17 class A enzymes of the TEM and SHV variety resistant to beta–lactamase inhibitors (26).

Table 1. Genetics of inhibitor resistant beta–lactamases

A. Inhibitor resistant TEM beta–lactamases										
TEM-1	39	69	104	165	182	238	244	261	275	276
	Gln	Met	Glu	Trp	Met	Gly	Arg	Val	Arg	Asn
TEM-30							Ser			
TEM-31							Cys			
TEM-32		Ile			Thr					
TEM-33		Leu								
TEM-34		Val								
TEM-35		Leu								Asp
TEM-36		Val								Asp
TEM-37		Ile								Asp
TEM-38		Val							Leu	
TEM-39		Leu		Arg						Asp
TEM-40		Ile								
TEM-44	Lys						Ser			
TEM-45		Leu							Gln	
TEM-50		Leu	Lys			Ser				Asp
TEM-51							His			
TEM-58							Ser	Ile		
TEM-65	Lys						Cys			
The Gly238Ser and Trp165Arg mutations alone do not confer inhibitor resistance										
B. Inhibitor resistant SHV family enzymes										
SHV-1	130	140	192	238	240					
	Ser	Ala	Lys	Gly	Glu					
SHV-10	Gly	Arg	Asn	Ser	Lys					
Gly238Ser and Glu240Lys do not confer inhibitor resistance										

The TEM enzymes, TEM 30->40, 44, 45, 50, 51, 58 and 65 have been discovered in *E. coli*, *Klebsiella* spp. and *P. mirabilis* (26-35). The SHV inhibitor resistant enzyme has been found only in *E coli*. (SHV 10) (36). SHV-10 beta-lactamase is a derivative of SHV-5, an extended spectrum beta-lactamase. Clinical isolates producing inhibitor-resistant TEM beta-lactamase have been recovered in Spain, France, the United Kingdom, and Greece. So far, no isolates have yet to be described in the United States. In a survey study performed in France, TEM-30 has been the most frequent isolate followed by TEM-37 and TEM-33 (30). Clinical specimens have come from urine (predominantly), blood, sputum, and stool (human). There has been only one report of an inhibitor resistant TEM isolate from an agricultural source (37).

10. AMINO ACID SUBSTITUTIONS CONFERRING RESISTANCE TO BETA–LACTAM BETA–LACTAMASE INHIBITORS

In the TEM family of class A beta–lactamase enzymes, naturally occurring inhibitor resistant mutants have been described at positions Arg244, Met69, Trp165, Met182, Val261, Arg275 and Asn276.

Why do mutations at Arg244 confer resistance to inhibition? By studying the role of Arg244 in the turnover of substrates and inhibitors, it has been proposed that the loss of the positively charged guanidinium group of the Arg residue increases the length of a critical hydrogen bond to a conserved water molecule (38-41). Imtiaz *et al.* proposed that a nonconcerted process was responsible for the formation of the inactivating species for clavulanate and sulbactam (40-41). These complexes revealed hydrogen-bonding interactions of residues Arg244, Ser130, Ser235 and Water 673 (7). Arg 244 and Val 216 anchor Water 673 that serves as a proton donor (38). The Arg 244 Ser change results in an enzyme that does not efficiently inactivate clavulanate. Subsequent work by others has demonstrated the importance of the Arg244 position in other related beta–lactamases (42, 43). Mutants at the 276 position in TEM (Asn276Asp), SHV-1 (Asn276Asp) and OHIO-1 beta–lactamase (Asn276Gly), an SHV family enzyme, have also established the role of this position in stabilizing the guanidinium group of Arg244 in the active site (44-46). It is interesting that although the 244 site is important in resistance to inhibition, a mutant at 164 (Arg164 Ser) in TEM reverses this effect (47).

Farenzeh *et al.*. (48) have proposed that mutations at the 69 position confer resistance by displacement of the catalytic water molecule in proximity to Glu166 in TEM. They proposed that the slight movement of the backbone carbonyl is enough to displace the water molecule coordinated to the catalytic Glu166. An alternative hypothesis is that the increasing hydrophobicity of the 69 residue narrows the active site such that beta-lactamase inhibitors are more readily hydrolyzed (49). Studying clinical inhibitor-resistant mutants of TEM-1 at amino acid Met69 of TEM-1, altering the Met69 to Leu, Ile and Val, Chaibi *et al.* (50) have advanced the hypothesis that the methyl group of Ile69 and Val69 produce steric constraints with the side chain of Asn 170 as well as the main chain nitrogen of Ser70. Leu at the 69 position also exerts a hydrophobic effect.

Table 2. Biochemical characteristics of inhibitor resistant TEM enzymes

beta–lactamase	pI	IRT	Km (pen) μM/L	IC50			Ki		
				clav	sul	tazo	clav	sul	tazo
TEM-1	5.4		38	.05	1.4	.02			
TEM-30	5.2	IRT-2	335	4	81	2.3			
TEM-31	5.2	IRT-1	365	44	150	3.6			
TEM-32	5.4	IRT-3		12	160	5			
TEM-33	5.4	IRT-5	120	4	36	.4			
TEM-34	5.4	IRT-6	152	2	16	.5			
TEM-35	5.2	IRT-4	157	17	62	.7	27	49	.6
TEM-36	5.2	IRT-7		2.9	20	1.2			
TEM-37	5.2	IRT-8		>10					
TEM-38	5.2	IRT-9		>10					
TEM-39	5.4	IRT-10		>10					
TEM-40	5.4	IRT-11		5.2					
TEM-44		IRT_2-2, IRT-13							
TEM-45	5.2	IRT-14	140	22.5	104	1.48			
TEM-50	5.6	CMT-1	17	.25	.5	.04	.7	.4	.06
TEM-51	5.2	IRT-15							
TEM-58									
TEM-65	5.4								

Electrospray ionization mass spectroscopy (ESIMS) has been used by Brown *et al.* (51) to study the inactivation of TEM-2 (an enzyme similar to TEM-1 except for a single amino acid change) by clavulanic acid. As a result of inactivation by clavulanate, four major products were found. These products demonstrated increase in molecular weight of 52, 70, 88 and 155 daltons. High performance liquid chromatography, HPLC, coupled to ESMS and chemical sequencing were used to provide information regarding the chemical modifications of TEM-2 by clavulanate. The authors propose two schemes. In the first, acylation at Ser 70 and subsequent decarboxylation is followed by cross-linking with Ser 130 and the formation of a vinyl ether. In the second, reformation of TEM via Ser 70 linked to a (hydrated) aldehyde with conversion of Ser 130 to dehydro-alanyl residue occurs.

11. MICROBIOLOGIC AND KINETIC CHARACTERIZATION OF INHIBITOR RESISTANT CLASS A BETA–LACTAMASES

Although the organisms possessing these enzymes are resistant to beta–lactam beta–lactamase inhibitor combinations, they remain susceptible to narrow spectrum (cephalothin) and extended spectrum cephalosporins (ceftazidime, ceftriaxone). They also remain susceptible to carbapenems and monobactams. In most cases, the resistance to amoxicillin/clavulanate and ampicillin/sulbactam is increased greater than the resistance to piperacillin/tazobactam.

As a group, the inhibitor resistant beta–lactamases are less efficient enzymes than the wild type counterparts (table 2). In general, the K_m values follow the same trends as the minimum inhibitory concentrations, MICs. K_ms are increased for penicillins (these substrates have reduced affinity) and MICs are lower. Turnover number is also reduced (less beta–lactam gets hydrolyzed per second). The measurements of inhibition by the beta–lactamase inhibitors all show an increase in the K_i or the IC 50 for clavulanate and sulbactam. The K_i and IC50 also increase for tazobactam but the increase is less dramatic. In select cases, the retained susceptibility of piperacillin/tazobactam may be explained by the greater potency of piperacillin (as a beta–lactam antibiotic-target of PBP3) and the relative preservation of tazobactam as an inactivator (52).

12. CAN AN ENZYME ARISE IN NATURE RESISTANT TO BETA–LACTAMASE INHIBITORS AND STILL INACTIVATE THIRD GENERATION CEPHALOSPORINS?

The discovery of TEM-50, a mutant with 4 amino acid changes, demonstrated that an enzyme will evolve with the phenotype described. In brief, TEM-50 demonstrated high level resistance to penicillin/inhibitors, narrow spectrum cephalosporins, and low-level resistance to extended spectrum cephalosporins (53). It was NOT as resistant to ceftazidime as the TEM counterpart with the extended spectrum mutations. The concern herein lies in the observation that a plasmid encoded enzyme will eventually be found that is not only resistant to beta–lactamase inhibitors, but can efficiently hydrolyze first generation cephalosporins and the oxyimino-cephalosporins.

13. TREATMENT OPTIONS, FUTURE PROSPECTS AND EVOLUTIONARY CONCERNS

Given the data obtained so far, it is reasonable to anticipate that treatment of beta–lactam beta–lactamase

inhibitor resistant infections in Gram negative enterics will respond to the use of narrow spectrum cephalosporins, cephamycins, carbapenems and oxyimino-cephalosporins and monobactams. Only low level resistance to extended spectrum cephalosporins has been reported in one natural enzyme (TEM-50) (53). Two laboratory mutants of SHV have been constructed with mutations conferring resistance to inhibitors as well as extending the substrate profile to oxymino-cephalosporins (42, 45, 54, 55). To date, these double mutants have not been found in the clinic.

From examining our current understanding of the inactivation chemistry of clavulanic acid, it is possible that other important residues in the class A beta–lactamase will be involved in inhibitor resistance (Val216). Inhibitor resistant PSE beta–lactamase has been constructed by site directed mutagenesis with mutations at position 216-217-218 that confers resistance to inhibitors (56). It is unknown what phenotype/ impact these mutations will exhibit in TEM and SHV. It is also anticipated that more inhibitor resistant SHV type enzymes will evolve. Will they be at the same sites as TEM and with the same phenotype? One also wonders if the same phenomenon will occur in staphylococci, a much more ubiquitous organism. To date no inhibitor resistant staphylococcal beta–lactamase have been described. Will the selective pressures imposed by antibiotic use in conjunction with the mutagenic properties of chemotherapeutic agents accelerate this unwanted situation?

Novel compounds are being designed that are even more potent inhibitors of class A enzymes [6a-(hydroxymethyl) penicillanic acid and N sulfonyloxy beta–lactams]. There are compounds in development which inhibit class C enzymes (chromosomal beta–lactamases) (57). Further clinical testing is awaited with great anticipation.

The potential kinetic ability of these inhibitor resistant enzymes is frightening. With the first attempts at evolution, a number of enzyme substitutions have been tried-all permit the beta–lactamase to resist inactivation by the inhibitor and increase turnover of the inhibitor. This is at the expense of diminished activity against other substrates (penicillins and cephalosporins). The discovery of TEM-50 (CMT-1) in nature and the construction of the ESBL-IR mutants of SHV and OHIO-1 are significant evolutionary steps for this enzyme class. With the correct combinations of substitutions, the substrate profile is greatly enhanced and the turnover numbers for a wide variety of substrates can be increased. This scenario may be reminiscent of the evolutionary path followed by the ancestral penicillin recognizing enzymes on their way to beta-lactamase and PBPs. A major functional difference between these proteins (PBPs and beta-lactamases) is the ability to acylate and deacylate a penicillin substrate. Normally, class A enzymes do not effectively deacylate beta-lactamase inhibitors and PBPs do not deacylate beta-lactams. In contrast, inhibitor resistant enzymes may "evolve" in their ability to effectively deacylate beta-lactamase inhibitors. The next evolutionary step for the inhibitor resistant enzymes is to restore efficient acylation and deacylation of standard substrates. Furthermore, altering areas of the promoter region may permit enough "inefficient"enzyme to be synthesized that high level resistance beta–lactam beta–lactamases inhibitors and even against third generation cephalosporins might be seen (58, 59). Understanding the catalytic behavior of these penicillin recognizing enzymes and their regulation may give us important insights into future directions of enzyme evolution and permit us to anticipate threats to our antibiotic arsenal.

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Send correspondence to: Robert A. Bonomo, MD, Geriatric CARE Center, 12200 Fairhill Rd, Cleveland, OH 44120, Tel: 216-844-6370, Fax:216-844-6492, E-mail: rab14@po.cwru.edu

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