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Analytical Study and Developments of Different Kinds Inhibitory of Metallo Beta Lactamases

Rustam Singh Tomar[1]* Dr. Prashant Kumar Sharma[2]

[1] Research Scholar, Mewar University, Chittorgarh, Rajasthan

[2] Supervisor, Department of Chemistry, Mewar University, Chittorgarh, Rajasthan

Email: prashantsharma.chem@gmail.com

Abstract – The production of metallo-β-lactamases is the most important strategy by which pathogenic bacteria become resistant to currently known β-lactam antibiotics. The emergence of these enzymes is particularly concerning for the future treatment of bacterial infections. There are no clinically available drugs capable of inhibiting any of the metallo-β-lactamases, so there is an urgent need to find such inhibitors. In this review, an up-to-date status of the inhibitors investigated for the inhibition of metallo-β-lactamases has been given so that this rich source of structural information of presently known metallo-β-lactamases could be helpful in generating a broad-spectrum potent inhibitor of metallo-β-lactamases.

Carbapenem resistance continues to erode the effectiveness of antibiotics such as imipenem and meropenem in the clinic. Resistance mechanisms can include interplay between porin loss (membrane permeability), mutation of penicillin binding proteins necessary for cell division, and expression of class A, B and D β-lactamases. Bacterial resistance to β-lactams such as penicillin or amoxicillin has been overcome in the clinic using several strategies, including development of antibiotics not susceptible to hydrolysis by β-lactamases, or co-administration of the antibiotic with β-lactamase inhibitors. This overview will focus on progress since 2000 in identifying inhibitors of class B. or metallo- β-lactamases with the aim of reversing carbapenem resistance.

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INTRODUCTION

β-Lactams have been the most effective class of antibacterial agents used in clinical practice for the past half century, owing to their high level of activity and good tolerability profiles . However, the emergence and spread of β-lactamases have eroded their effectiveness on Gramnegative bacteria, and this antibiotic resistance currently represents a highly relevant global public health issue.

β-Lactamases can inactivate almost all β-lactam antibiotics by hydrolyzing the amide bond in the β-lactamring, which poses a great challenge in the treatment of bacterial infections . Continuous evolvement of the β-lactamases broadens their substrate spectrum and makes the situation more discouraging. Based on the primary sequence homology, β-lactamases have been grouped into four classes: classes A, B, C, and D . Class A, C and D β-lactamases are serine enzymes, which catalyze the hydrolysis of the β-lactams via a serine-bound acyl intermediate in the active site. While for class B β-lactamases (the so called metallo-β-lactamases, MβLs), one or two zinc ions in the active site are required for their activity.

To overcome β-lactamase-mediated resistance, combinations of β-lactams and β-lactamase inhibitors (such as sulbactam, tazobactam and clavulanic acid) have been widely used in the clinic. However, these combinations are only successful in the treatment of infections mediated by class A β-lactamases and none of the effective inhibitors against class have been introduced to the market.

Thus, it is of particular urgency to discover/design broadspectrum The first MBL was isolated from Bacillus cereus 569/H/9 in 1966, where EDTA was shown to inhibit the cephalosporinase activity. As Bacillus cereus is a nonpathogen organism and the isolate was the only example of these zinc-dependent enzymes, the discovery was only considered as a curiosity. In the early 80's, an increased number of MBLs was isolated from many organisms even from pathogenes such as Stenotrophomonas maltophilia or Pseudomonas aeruginosa. More frightening was the identification of a gene coding for a MBL in Bacillus anthracis. Their fast dissemination could be explained by the location of their encoding genes on mobile DNA plasmids, which allow horizontal gene transfer.

To date, a considerable number of small organic molecules have been tested for inhibition of the

MBLs. A recent review by Heinz et al., 2004 has reviewed the different classes of reported MBL inhibitors (1): tricyclic natural products, trifluoromethyl alcohols and ketones, hydroxamic acids, mercaptocarboxylates, biphenyl tetrazoles, carbapenem and penicillin derivatives, cephamycins and moxalactam, thiols, cysteinyl peptides, inhibitors derived from single-domain antibody fragment elicited in the Camelidae, thioesters derivatives, phenazines from a Streptomyces, succinic acid derivatives, sulphonyl hydrazones, disulfides, thiolsubstituted penicillin inhibitor, degradation products of cephalosporins, captopril, thiomandelic acid. Recently benzohydroxamic acids and pyridine carboxylates were also identified as potential inhibitors of MBLs.

Potent in-vitro MBL inhibitors such as succinic acid and mercaptocarboxylic acid derivatives have also been reported, displaying some inhibition constants in the low nanomolar range. Most of the inhibition studies were performed using the di-Zn forms of the MBLs, at the exception of CphA which was considered as a monozinc-enzyme. In most cases, crystal structures revealed that the MBL bound inhibitor replaces the zinc bound-water molecules and acts as new metal ligand.

MBLs are classified into three groups on the basis of amino acid homology and conservation of active site residues as B1, B2 and B3. The availability of X-ray crystal structures has greatly facilitated the classification of MBLs. In all three types of MBL (B1, B2 and B3), the active site possesses at least one but more often two zinc atoms, commonly labeled as Zn1 and Zn2.

Both zinc atoms are chelated by three different amino acids. The numbering of amino acids that coordinate with two zinc ions.

The members of subclass B1 show more than 23% identity. In the active site of subclass B1, one of the zinc ions is coordinated by three histidines (His116, His118 and His196) and a water molecule with the arrangement of the zinc ion being tetrahedral. The second zinc ion is coordinated by the carboxylate group of Asp120, the methylthiolate group of Cys221, the nitrogen atom in the imidazole group of His263 and two molecules of water. The geometry of this complex is distorted trigonal bipyramidal.

The members of subclass B2 share only 11% of identity with subclass B1. In this subclass (B2), Zn1 is coordinated by Asn116, His118, His196 and Zn2 is bonded to Asp120, Cys221, His263. In addition, some members of the B2 subclass have only one zinc atom in the active site such as 18XG and 3SD9 enzymes.

The coordination of Zn1 in subclass B3 is the same as in B1. Coordination around the Zn^2 site, however, is different. In this case one of the coordinating residues for Zn^2 has changed from Cys221 to His121. An associated change is that the Cys221 has been substituted for Ser221 which now engages in a hydrogen bond interaction with the zinc bound water.

As mentioned earlier, MBLs have the ability to hydrolytically degrade almost all of the β-lactam antibiotics including newer generation β-lactam antibiotics like cephalosporins and carbapenems. Clinical inhibitors of serine β-lactamase such as clavulanic acid, sulbactam and tazobactam are also substrates of MBLs. Additionally, none of the serine β-lactamase inhibitors are effective against MBLs. MBL substrates are collectively.

MBLs use at least one but more commonly two Zn^{2+} ions in their active site to catalyze the hydrolysis of β-lactam rings. Two mechanisms are proposed for MBLs based on mono-zinc and di-zinc ions. In the mono-zinc mechanism, the formation of hydroxide nucleophile is facilitated with interaction of both the Asp120 residue and Zn^{2+} ion. The hydroxide group attacks the electrophilic carbonyl carbon to form the tetrahedral intermediate. Finally, protonation of the tetrahedral intermediate nitrogen by the Asp120 residue results in CN bond cleavage and deactivation of penicillin.

The main characteristics of MBLs are the presence of two Zn(II) ions in the active site (although in some cases only one may be needed for catalytic activity) and an overall abba protein fold. The metal ions are proposed to assist in the binding of the antibiotic substrate and the generation of an attacking nucleophile. MBLs are classified as B1, B2 or B3 type depending on the residues ligating the metal ions in the active site. A standard numbering scheme for residues in MBLs has been developed based on sequence alignments for B1, B2 and B3 MBLs, and this scheme is used throughout this Letter. Various catalytic mechanisms have been proposed for MBLs, and were summarised in a recent review.

Although the proposed mechanistic strategies vary with respect to the role of the metal ions, the number of metal ions in the active site, the mode of substrate binding and the identity of the rate-limiting step, it is generally accepted that a nucleophilic attack by a metal ion-bound hydroxide onto the antibiotic's fourmembered lactam ring triggers ring opening and the hydrolysis of the antibiotic to a therapeutically inactive form.

The development of antibiotics remains one of the most significant advances in modern medicine. 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, 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. 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 lifesaving 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.

β -Lactam antibiotics have proven to be an overwhelming success in the treatment of bacterial infections. The dynamic nature of bacteria allows for their alarmingly facile adaptation to a changing environment. Bacteria have developed several mechanisms of resistance to antibiotics. The reduced permeability of the cell wall, alterations in target enzymes (penicillin-binding proteins) and production of various forms of β-lactamase all contribute to the diminishing effectiveness of antibiotics. β-Lactamases have been grouped into four molecular classes: classes A, C and D are serine active site β -lactamases. while class B enzymes are metalloproteins that are zinc dependent. Due to the growing number of metallo- β-lactamases (MBLs), three subclasses (Bl, B2 and B3) have been characterized based on their known sequences. MBLs expressed in Bacillus cereus (Bell).

Bacteroldes fragilis and Pseudomonas aeruginosa (IMP-1) have been characterized in greatest detail. The IMP-1 enzyme is of particular interest since it is encoded by both plasmids and integrons. These mobile fragments of DNA could be responsible for the future widespread dissemination of carbapenem resistance genes.

BACKGROUND

β –Lactamases and bacterial resistance –

The bacterial resistance against β-lactam antibiotics poses a continuous challenge, thus it is highly relevant and important to develop general and more specific inhibitors conferring to the antibiotic resistance. The β-lactam antibiotics hold a beta-lactam ring as its key feature. The β-lactam antibiotics works by inhibition of bacterial peptidyltransferase by forming a stable acyl-enzyme intermediate after a nucleophilic attack governed by a serine residue in the active site of peptidyltransferase, where the attack results in a cleavage of the β-lactam ring. Peptidyltransferase is critical for the peptidoglycan biosynthesis of bacterial cell wall. Similar to peptidyltransferase, most beta-lactamases (not MBLs) contain a serine residue within its active site, which performs a nucleophilic attack on the amide bond and cleaves the β-lactam ring. These types of enzymes are called serine β-lactamases (SBLs), and on the basis of sequence and structural homology, they have been suggested to be grouped into classes A, C and D by Ambler. β-lactamases that employ one or two active-site Zn(II) ions to catalyze the cleavage of β-lactams are called metallo-β-lactamases and belong to class B2. Based on amino acid sequence identity and structural features, MBLs can be further classified into three subclasses: B1, B2 and B33. Similar for them all is that they have an αβ/βα-fold4, where the two β-sheets are in between the two α-helices. This is called the metallo-β-lactamase fold5. The active site with one or two Zn(II) ions is located at the edge of the two β-sheets, and the positions of the Zn(II) ions are essential for substrate binding and hydrolysis.

All three subclasses of MBLs have a binuclear active site, which requires one or two zinc ions for full activity. For some binuclear B1 and B3 MBLs, two Zn(II) ions are not necessary for activity. The second Zn(II) ion in the Zn_2 binding site serves to stabilize the built-up negative charge on the anionic N atom during hydrolysis of the peptide bond, thereby increasing the activation free energy for nucleophilic attack. As a result, the binuclear enzyme has an overall better catalytic efficiency than the mononuclear variant. Subclass B2 enzymes are catalytically active with one Zn(II) ion binding to the Zn_2 binding site. MBLs hydrolyze all β-lactam antibiotics, except for the monobactam aztreonam.

Reductive amination-

Reductive amination15 of aldehydes/ketones is a useful reaction in organic chemistry and is an important tool in the synthesis of amines. The aldehyde/ketone condensates with an amine and generates an imine followed by reduction to furnish the desired amines. The reaction mechanism for reductive amination is shown in Figure 1.

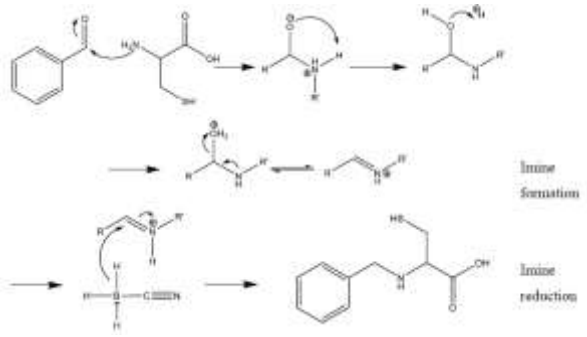

Figure 1 Reaction mechanism of reductive amination with NaBH3CN as the reducing agent.

The reaction mechanism for reductive amination involves an initial formation of an intermediate hydroxylamine followed by elimination of water which furnishes the imine. The reaction is reversible and the imine formation is the slow step. Subsequent

reduction of the imine with a suitable reducing agent produces the amine.

Among the various reduction methods at hand are catalytic hydrogenation, reduction with amine borane complexes or by using metallo-hydride reducing agents such as sodium cyanoborohydride, sodium triacetoxyborohydride, sodium- or zinc borohydride. Among the latter, sodium cyanoborhydride and sodium triacetoxyborhydride are the most common because of their versatility and will be described later. The success of the reaction depends on the choice of the reducing agent, as it might have to reduce imines selectively over ketones/aldehydes under the same reaction conditions. The rate of the reduction of iminium ions is in addition, much faster than that for aldehydes or ketones, but requires catalytic amount of acid. As a result, reductive amination can be carried out as a one-pot procedure by introducing the reducing agent into a mixture of the amine and carbonyl compound at the start of the reaction.

Sulfonation of amino acids-

The most common way of preparing sulfonamide derivatives of unprotected amino acids is by nucleophile acyl substitution at suitable sulfonyl chloride in aqueous solution in the presence of a base. Schröder et al (2001) have proposed a general procedure for preparing Nsulfonylated amino acids involves addition of the sulfonyl chloride in a solution of the amino acid in aqueous base before heating the mixture with vigorous stirring. This procedure works for most of the natural amino acids but they tend to favor different bases for optimal yields. NaOH, DIEA, K_2CO_3, and Na_2CO_3 are the most frequently used bases.

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). Osmotic stability is preserved by a rigid cell wall comprised of alternating *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) units.

These glycosidic units are linked by transglycosidases. A pentapeptide 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 transpeptidases. 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–Dalanine of the NAM pentapeptide, and PBPs "mistakenly" use the β-lactam as a "building block" during cell wall synthesis. This results in acylation of the PBP, which renders the enzyme unable to catalyze further transpeptidation reactions. 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.

RESISTANCE TO β-LACTAM ANTIBIOTICS

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

(i) Production of β-lactamase 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*. Through natural transformation and recombination with DNA from other organisms, *Neisseria* spp. and *Streptococcus* spp. have acquired highly resistant, lowaffinity PBPs. 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*. 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 PBP2β). 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, β-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 nonfermenter *Pseudomonas aeruginosa*. Resistance to imipenem and meropenem has also been associated with the loss of the CarO OMP in clinical isolates of multidrug-resistant *Acinetobacter baumannii*. Point mutations or insertion sequences in porin-encoding genes can produce proteins with decreased function and thus lower permeability to β-lactams. 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. 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. 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).

Ambler class	Bush-Jacoby-Medeiros class	Preferred substrate	Inhibited by clavulanate	Representative enzyme(s)
A (serine penicillinases)	2a	Penicillin	+	PC1 from *S. aureus*
	2b	Penicillin, narrow-spectrum cephalosporin	+	TEM-1, TEM-2, SHV-1
	2br	Penicillin, narrow-spectrum and extended-spectrum cephalosporin	+	SHV-2 to SHV-6, TEM-3 to TEM-26, CTX-Ms
	2br	Penicillin	–	TEM-30, SHV-72
	2c	Penicillin, carbenicillin	+	PSE-1
	2e	Extended-spectrum cephalosporin	+	FEC-1, CepA
	2f	Penicillin, cephalosporins, carbapenem	±	KPC-2, SME-1, NMC-A
B (metallo-β-lactamases)	3	Most β-lactams, including carbapenems	–	IMP-1, VIM-1, CcrA, and BcII (B1); CphA (B2); L1(B3)
C (cephalosporinases)	1	Cephalosporins	–	AmpC, CMY-2, ACT-1
D (oxacillinases)	2d	Penicillin, cloxacillin	±	OXA-1, OXA-10
Not classified	4			

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

CONCLUSION

Several novel chemical classes of MBL inhibitors have been reported recently and there is now a rich source of structural information on the chemical details of the enzyme-inhibitor interaction. While none of these classes constitute the 'ideal MBL inhibitor', the challenge for the pharmaceutical industry will be to continue to explore the chemical classes presented here for sufficient broad spectrum activity against the most important clinical pathogens expressing MBLs. Careful monitoring of clinical isolates for the presence of MBLs, within the context of other important resistance mechanisms, will provide the ultimate test of whether development of an MBL inhibitor will add value to the current antibiotic armamentarium.

MBLs have emerged as a major threat to global health. They inactivate an increasing number of commonly used antibiotics and spread easily among various pathogens on mobile genetic elements. Crystal structures for several MBLs have been determined and an extensive amount of information about their biochemical properties has been accumulated. Some potent *in vitro* inhibitors of MBLs have also been detected. However, to date none of the available MBL inhibitors are of clinical use. The search for universal and clinically applicable MBL antagonists is still very much at the beginning.

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Corresponding Author

Rustam Singh Tomar*

Research Scholar, Mewar University, Chittorgarh, Rajasthan

rustamsinghtomar7@gmail.com