Diversity and proliferation of metallo-β-lactamases: a clarion call for clinically effective metallo-β-lactamase inhibitors

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Running head: Finding effective metallo-β-lactamase inhibitors

Abstract

The worldwide proliferation of life-threatening metallo-β-lactamase (MBLs)-producing Gram-negative bacteria is a serious concern to public health. MBLs are compromising the therapeutic efficacies of β-lactams, particularly carbapenems, which are last-resort antibiotics indicated for various multidrug-resistant bacterial infections. Inhibiting enzymes mediating antibiotic resistance in bacteria is one of the major promising means in overcoming bacterial resistance. Compounds having potential MBLs-inhibitory activity have been reported, but none are currently under clinical trials. The need for developing safe and efficient MBL inhibitors (MBLIs) is obvious, particularly with the continuous spread of MBLs worldwide. In this review, the emergence and escalation of MBLs in Gram-negative bacteria are dicussed. The relationship between different class B β-lactamases identified up to 2017 are represented by a phylogenetic tree and summarized. On the other hand, approved and/or clinical-phase serine β-lactamase inhibitors are recapitulated to reflect the successful advances made in developing class A ϐ-lactamase inhibitors. Reported MBLIs, their inhibitory properties and purported mode of inhibition are herein delineated. Insights into MBLs' structural variations and the challenges involved in developing potent MBLIs are also elucidated and discussed. Currently, natural products and MBL-resistant ϐ-lactam analogues are the most promising agents that can become clinically efficient MBLIs. A deeper comprehension of the mechanism of action and activity spectrum of the various MBLs and their inhibitors will serve as a bedrock for further investigations that can result in clinically useful MBLIs to curb this global menace.

Keywords: β-lactamase; Metallo-β-lactamase; Metallo-β-lactamase Inhibitors; Gram-negative bacteria; antibiotic resistance; β-lactam antibiotics.
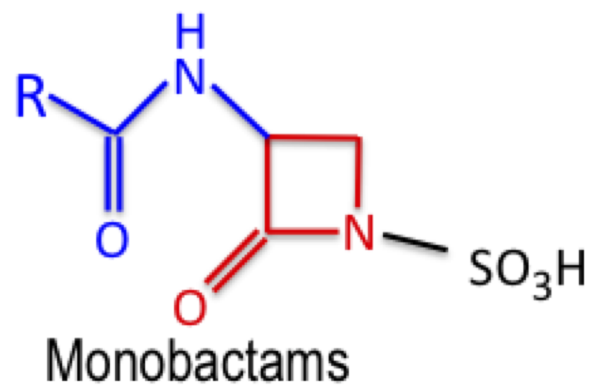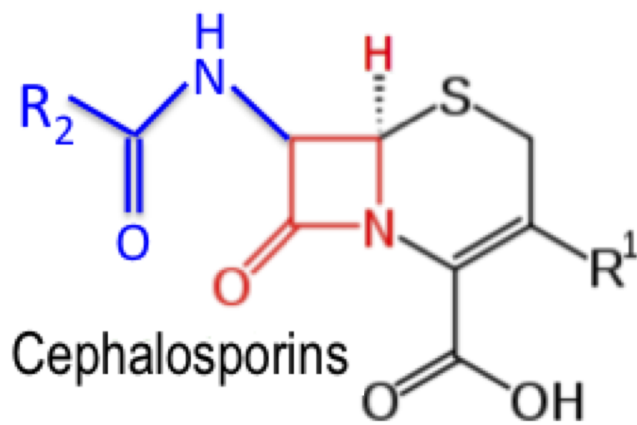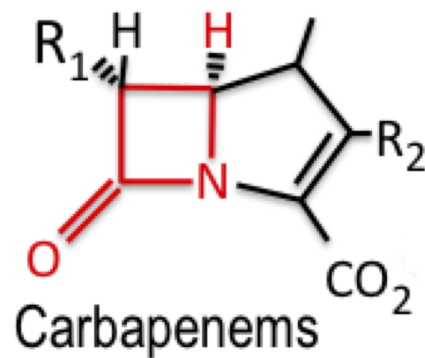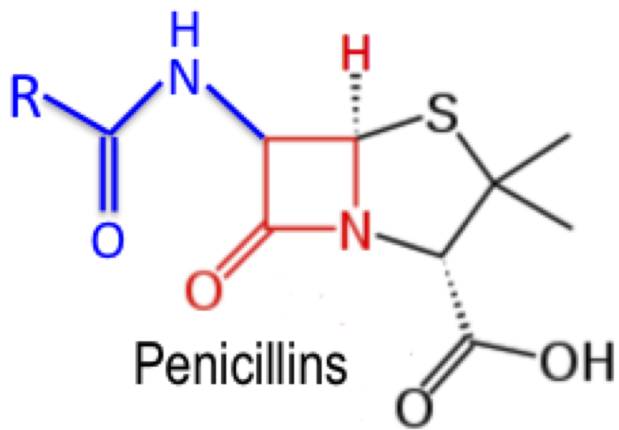
Introduction

The alarming spread of antimicrobial resistance (AMR) presents a major challenge to public health worldwide [121,135]. The dissemination of AMR is spearheaded by increasing world trade, rising human

and animal populations, economic factors, changing climatic conditions and air travel, which are breaking down geographical borders between countries and continents, exposing humans and animals to diverse kinds of infections [81]. The immense clinical benefits obtained from antimicrobials in the management of infectious diseases were eroded by the emergence of AMR in pathogens, a few years after their introduction and adoption into clinical medicine [26,134]. As new antibiotics have been discovered and introduced into clinical use, a similar resistance cycle has ensued: resistance to cephalosporins due to the expression of extended spectrum β-lactamases (ESBLs); carbapenem resistance due to carbapenemases; resistance to colistin due to plasmid-mediated *mcr-1* gene and chromosomal mutations; tigecycline resistance due to chromosomal mutations [122,123].

Gram-negative bacteria develop resistance to β-lactams through different mechanisms [111], including the production of enzymes called β-lactamases that hydrolyze the β-lactam ring. Resistance can also be developed through modification(s) of the normal penicillin-binding proteins (PBPs), reduced porin expression leading to impermeability of the outer membrane and active antibiotics expulsion from the bacteria through efflux pump systems. The presence of one or more of these mechanisms in microorganisms can lead to β-lactam resistance [97,98,111,152].

Bacterial β-lactamases are members of an enzyme family capable of impairing the efficacy of β-lactam antibiotics such as penicillins, cephalosporins, monobactams, and carbapenems (Figure 1)[14,58] by hydrolysing their β-lactam rings [136]. There are two globally accepted classification schemes for β-lactamases. The first scheme is based on the amino-acid sequence of the enzyme and comprises four classes i.e., class A, B, C and D. The second scheme is based on the enzyme's functionality or substrate and includes three major groups: Group 1 or cephalosporinases (Class C), Group 2 or serine β-lactamases (Classes A and D), and Group 3 or metallo-β-lactamases (Class B). Each of these groups are further sub-divided into several different sub-groups [20,137]. Classes A, C and D β-lactamases possess the amino acid serine at their active site and are thus known as serine β-lactamases (SBLs) while Ambler class B β-lactamases contain one or two zinc ions at their active site, and are thus called

Figure 1: Structures of selected β-lactam antibiotics. In a clockwise order, are the structures of penicillin, cephalosporin, monobactam and carbapenem. Images were adapted from references 14,58

metallo-β-lactamases (MBLs).

The production of class B β-lactamases in bacteria, together with other resistance mechanisms, has narrowed down treatment options for infections caused by these pathogens [21]. One approach for treating β-lactamase-producing bacterial infections is by combining existing β-lactam antibiotics with β-lactamase inhibitors [22]. β-lactam-β-lactamase inhibitor combinations resulted in the restoration of β-lactam antibiotics' activity against β-lactamase-producing bacteria. Such combination drugs currently approved by the FDA and available for clinical treatment [22] do not include Ambler class B (metallo-β-lactamase) inhibitors (MBLIs). There is thus far no MBLI in clinical use, which indicates that the presence of this class of β-lactamases in resistant bacteria needs much attention. Class B or MBLs include plasmid-encoded enzymes such as Imipenemase enzyme (IMP), Verona integron-encoded metallo-β-lactamase (VIM), Sao Paulo metallo-β-lactamase (SPM), German imipenemase (GIM), Seoul imipenemase (SIM), New-Delhi Metallo-β-lactamase (NDM), and Dutch imipenemase (DIM), which are increasingly being detected in Gram-negative bacteria with considerable clinical impact [5].

Invariably, the increasing worldwide prevalence of MBLs is a major threat to global healthcare, affecting both community and hospital settings [39,55,74,133]. Mortality rates of up to 50% have been reported for carbapenem-resistant Enterobacteriaceae (CRE), including MBL-positive MBL infections [37,77]. Over the last decade, the prevalence of these drug-resistant bacterial infections has been increasing, affecting over a million patients worldwide. This has led to prolonged hospitalization, longer terms of disability, increased healthcare-associated costs, higher mortalities etc. [39,129,185]. The World Health Organization (WHO) recently listed CRE pathogens as "critical priority pathogens" for which novel and efficient antibiotics are required urgently [189].

The absence of clinical MBLIs for MBL-mediated drug-resistant infections, particularly involving CREs, makes this class of β-lactamases especially important in infectious diseases. Hence, this review aims to map out advances made so far in finding MBLIs that can fight MBL producers and point out

challenges involved in designing clinically useful MBLIs.

Metallo-β-lactamases (MBLs)

MBLs confer resistance to all β-lactams except monobactams by using their active-site zinc ions to activate a nucleophilic water molecule, which opens the β-lactam ring (through hydrolysis) and render it ineffective [182]. For many decades, MBLs were considered to be clinically irrelevant enzymes that were chromosomally encoded in non-pathogenic organisms [85,95,143,179]. This situation has changed with the increasing spread of NDM-, VIM- and IMP-mediated AMR in Gram-negative pathogens, including Enterobacteriaceae. The above-mentioned enzymes are carried on chromosomes or plasmids and located on mobile genetic cassettes inserted into integrons (IMP & VIM) and/or bracketed by composite transposons (NDM) [30,87]. Transposons are DNA segments capable of moving to different positions in the genome of a single cell while Integrons are key elements that can shuttle genes between integrons on plasmids, therefore allowing the plasmids to transfer genetic material to different bacteria [11]. These mechanisms mediate the spread of MBL-mediated resistance worldwide. A typical example is the dissemination of NDM-1 MBL, first isolated from a Swedish patient transferred from India [193] in 2008, worldwide in diverse Enterobacteriaceae species. And the incidence rate continues to grow [193].

MBLs are divided into three (3) subclasses namely, B1, B2 and B3 (Table 1), based on differences in their primary zinc coordination shell and their amino acid sequence. Sequence identity between subclass B1 and B2 enzymes ranges from 14 to 24% while sequence identity between subclass B3 and both subclasses B1 and B2 enzymes ranges from 2% to 14% [73]. Subclass B1 possesses a binuclear active site, within which either one or two zinc ions can exist. B1 binds one zinc ion (Zn1) with three Histidine residues (H116, H118, H196) and a second zinc ion (Zn2) with three different residues, including a Cysteine (D120, C221, H263) in particular. Subclass B1 is a clinically relevant and notorious MBL, comprising the largest number of MBLs located on plasmids. Thus, it is more likely to spread to other organisms to cause β-lactam-resistant clinical infections [43].

Table 1: Historical timeline and characteristics of Metallo-β-lactamases discovered up to December 2017

| MBL subclasses (B1, B2, & B3)	Year detected	Species first detected in	Number of Variants	Country of origin	β-lactam antibiotics hydrolysis profile	Genetic location	Accession number	Reference(s)
B1								
BcII	1966	*B. cereus*	7	Uruguay	Broad spectrum β-lactams	Chromosome	M11189	(30, 31, 87, 88)
CcrA or cfiA	1990	*B. fragilis*	25	England	Broad spectrum β-lactams	Chromosome	AB087225	(89, 90)
IMP	1994	*S. marcescens*	67	Japan	Broad spectrum β-lactams	Plasmid/Chromosome	HM036079	(91, 92)
BlaB	1998	*E. meningoseptica*	15	-	Broad spectrum β-lactams	Chromosome	AF189298	(93–95)
VIM	1999	*P. aeruginosa*	54	Italy	Broad spectrum β-lactams	Plasmid/Chromosome	GU724868	(92, 96)
IND	1999	*C. indologenes*	16	France	Broad spectrum β-lactams	Plasmid/Chromosome	EF394436	(97)
EBR	2002	*E. brevis*	1	France	Narrow spectrum Cephalosporins,	Chromosome	AF416700	(98)
SPM	2002	*P. aeruginosa*	1	Brazil	Broad spectrum β-lactams	Plasmid/Chromosome	GU831565	(99, 100)
Bla	2003	*B. anthracis*	2	-	Broad spectrum β-lactam	Chromosome	Q93T42	(69)
GIM	2004	*P. aeruginosa*	2	Germany	Broad spectrum β-lactam	Plasmid	JF414726	(101)
SIM	2005	*A. baumannii*	2	Korea	Broad array β-lactams, narrow-carbapenem	Plasmid/Chromosome	GQ288397	(102)
SLB	2005	*S. livingstonensis,*	1	Islands	Broad spectrum	Chromosome	AY590118	(103)
SFB	2005	*S. frigidimarina*	1	Islands	Narrow spectrum	Chromosome	AY590119	(103)
PEDO-3	2015	*P. kyungheensis*	1	United Kingdom	Broad spectrum β-lactams	Chromosome	NG_049959	(104)
JOHN	2003	*F. johnsoniae*	1	-	Broad spectrum β-lactams	Chromosome	AY02846	(105)
CGB	2002	*C. gleum*	1	-	Broad spectrum β-lactams	Chromosome	EF672680	(106)
MUS	2002	*M. odoratimimus*	2	-	Broad spectrum, exception of aztreonam	Chromosome	AF441286	(107, 108)
TUS	2002	*M. odoratus*	1	-	Large spectrum, exception of aztreonam	Chromosome	AF441287	(107)
KHM	2008	*C. freundii*	1	Japan	Broad spectrum β-lactam	Plasmid	AB364006	(109)
DIM	2010	*P. stutzeri*	1	Netherlands	Broad spectrum spared Aztreonam	Plasmid	GU323019	(110)
NDM	2008	*K. pneumonia, E. coli*	18	India	Broad spectrum β-lactams	Plasmid/Chromosome	JQ080305	(111–114)
HMB	2017	*P. aeruginosa*	1	Germany	Broad spectrum β-lactam	Chromosome	NG_052225	(115)
FIM	2012	*P. aeruginosa*	1	Italy	Broad spectrum β-lactams	Chromosome	JX570731	(116)

Subclass B2 MBLs have a Zn1 binding site with one altered residue (N116, H118, H196), but retain a similar Zn2 site (D120, C221, H263); it selectively hydrolyses carbapenems and possess the fewest members compared with the others. Subclass B3 MBLs have a varied Zn1 binding site (H/Q116, H118, H196) and a distinctive Zn2 binding site that lacks a Cysteine residue (D120, H121, H263) [108]. These structural variations in known and emerging MBLs offers a greater challenge towards discovering potent MBLIs that can inhibit all MBLs. The historical timeline and characteristics of MBLs discovered up to 2017, retraced from different databases such as the Comprehensive Antibiotic Resistance Database (CARD) [71] and β-Lactamase Database (BLDB), are summarized in Table 1 [113].

MBLs were detected from diverse species of organisms as shown in Table 1. However, subclass B2 was mostly detected from *Aeromonas spp.* Interestingly, most MBLs were initially identified in Europe (France, Germany, Denmark, UK, Italy etc.). Africa had the least discovered number of these MBLs, not because of their absence in the continent but rather a lack of proper awareness, skilled personnel, resistance diagnosis and surveillance system. Class B3 also contained the enzyme Adelaide Imipenemase (AIM) that is plasmid-borne (Table 1).

Phylogenetic Analysis and Metadata of MBLs

All the reported MBLs together with their available metadata are illustrated on a phylogenetic tree to demonstrate the associated characteristics of the different sub-classes: B1, B2 and B3. Protein sequences of all reported MBLs (Table 1) were downloaded from GenBank using their accession numbers. They were then respectively aligned using default parameters/settings of classical sequence analysis with the CLC Genomics Workbench (version 10.1.1) software to generate an aligned file. The created aligned file was used to draw the maximum likelihood phylogenetic tree to infer the evolutionary relationship using optimized parameters as follows: construction method: UPGMA; nucleotide substitution model: Jukes cantor; protein substitution model: WAG; transition/transversion ratio: 2; estimate substitution rate: Yes; number of substitution rate: 4; perform bootstraps analysis: Yes; replicates: [1000]. Metadata associated with the aligned sequences (Table 1) was imported to provide a
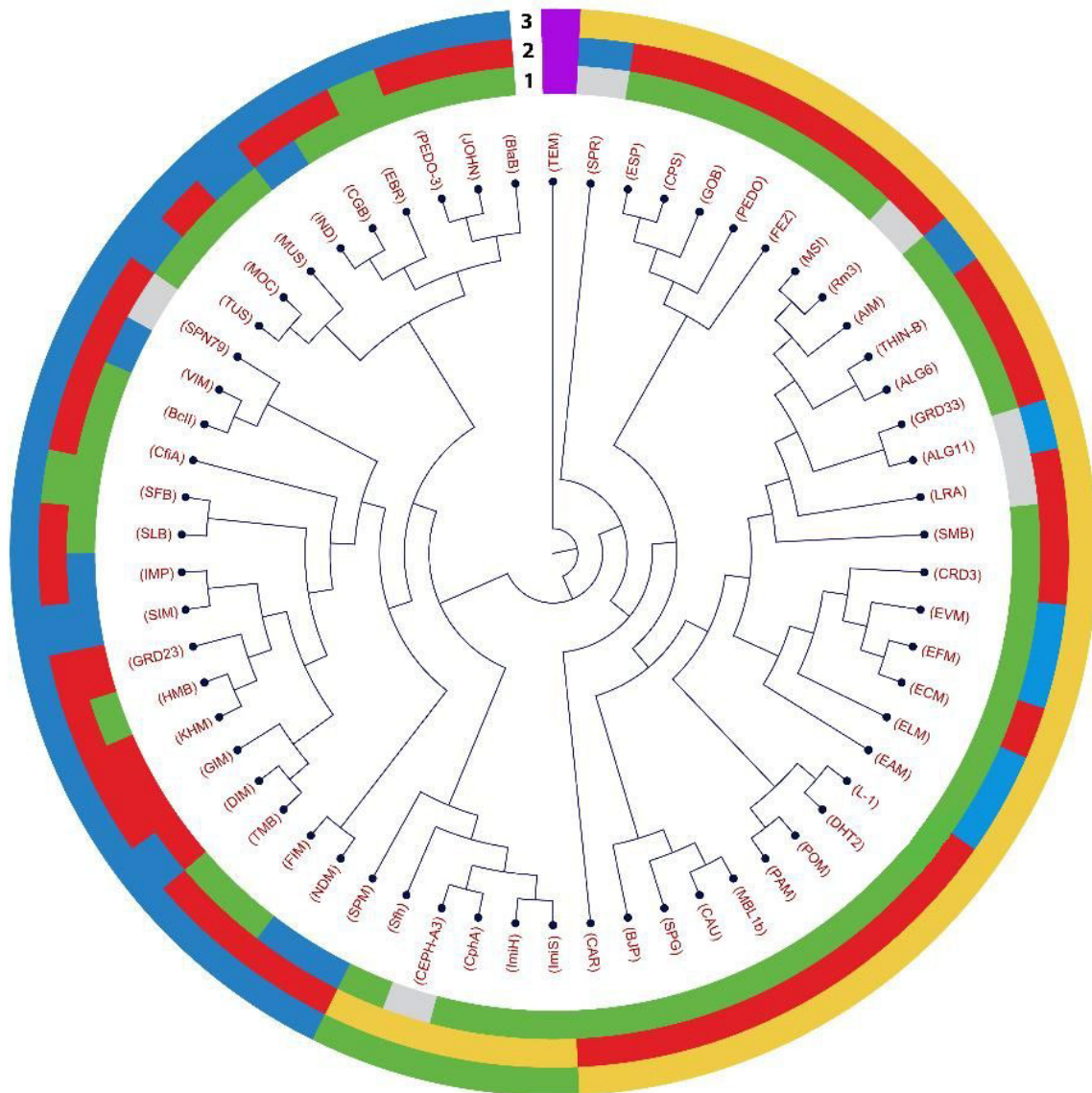
Figure 2: A graphical view of the phylogenetic and metadata of metallo-β-lactamases. The TEM gene of Ambler class A β-lactamases was rooted and used as the out-group (reference) in the tree. The patterns on the circular phylogram are: 1) Genetic location (inner layer), 2) β-lactam antibiotics hydrolysis profile (middle layer), and 3) Metallo-β-lactamase (outer layer).

comprehensive analysis of the generated phylogenetic tree. TEM-1 (Ambler class A β-lactamase) was used as the outgroup (reference) to root the tree to enable easy configuration of the phylogenetic distance between the MBL enzymes on the branches. The circular version of the tree is shown in Figure 2.

The phylogenetic analysis depicted a clear distinction between sub-classes B1 and B3 as reported in the literature [56,57], where they were more phylogenetically related to each other compared to B2 (Figure 2). Metadata analysis provided a deeper insight into the associated characteristics and distinctions between all the sub-classes. Specifically, sub-classes B1 and B3 MBLs act on a broad spectrum of β-lactam antibiotics while class B2 MBLs have a narrower β-lactam spectrum, affecting only carbapenems. This diversity further complicates the management of CREs in the clinical setting making it a huge challenge for a single inhibitor to act efficiently on all MBLs sub-groups. The metadata also indicated that B2 and B3 were mostly chromosomal while B1 was both plasmid- and chromosomally-mediated. Even though SPM was phylogenetically clustered with sub-class B2, the metadata differentiated them into a distinct sub-group as illustrated in Figure 2, reiterating the importance of visualizing phylogenetic structures in relation to their metadata [3].

Novel MBLs recently discovered through genomics and metagenomics

The advent of modern techniques such as next/whole-genome sequencing as a diagnostic tool in clinical microbiology has led to the discovery of novel genes and diverse mechanisms of drug resistance that could have been otherwise impossible to find using conventional diagnostic methods. Berglund and collaborators in 2017[12] assumed that there is an existence of a large number of unexplored reservoir of uncharacterized MBLs. Hence, with the aid of new computational methods based on hidden Markov's model, they identified novel MBL genes of subclass B1, from bacterial genomes, plasmids and metagenomic data. The findings predicted the existence of 76 novel genes of subclass B1. Based on the evolutionary origin of the genes, a phylogenetic analysis revealed that the

subclass B1 could be differentiated into five groups (subclass B1-1 to B1-5). However, these recently discovered novel MBLs are not listed in Table 1 due to the lack of proper nomenclature and very scant information or metadata (i.e. no information on country, bacteria isolated from and genetic location).

These current findings by Berglund *et al.* (2017), thus echoes the diversity and proliferation of MBLs as well as their possible threat to clinically available antibiotic options, cautioning scientist to be prepared for the challenges that may occur in the future regarding the control and treatment of these life-threating pathogens. Therefore, there is an urgent need to find efficient and effective MBLIs to overcome this global scourge.

Metallo-β-Lactamase Inhibitors (MBLIs)

β-lactam/β-lactamase inhibitor/adjuvant combinations have been developed and are in clinical use for the treatment of infections caused by SBLs, while others are in clinical trials [22] (Tables 2 and 3). These molecules fail to inhibit MBLs, thus urgently necessitating the development of inhibitors that target bacterial metallo-enzymes.

In areas with increasing proliferation of MBL-positive CREs, clavulanic acid, sulbactam, tazobactam, and avibactam, which are all serine-based β-lactamase inhibitors in clinical use (Table 2), do not provide much benefit [22]. One approach to overcome the effects of MBLs is by designing specific inhibitors that can be co-administered with β-lactam antibiotics. Studies have been executed in this direction where a number of potent MBLIs have been identified, although none of these inhibitors has thus far reached clinical trials [104]. The general challenge in identifying and developing broad-spectrum MBLIs stems from the structural and mechanistic differences within the three sub-classes of MBLs. Reported MBLI groups include thiol and thioester derivatives [166], tetrazoles and hydroxamates [170,184] sulfonic acid derivative s[41], β-lactam analogues derivatives[23], pyrroles-based inhibitors [107], pyridine dicarboxylates[67], peptides[17] natural products[127], nucleic acid[153], and metal chelators [154] .(Table 4).

Table 2: Approved β-lactam β-lactamase inhibitor combinations

β-lactam β-lactamase inhibitor combinations	Year introduced into clinical use	Route of administration	Dosage regimen	References
Amoxicillin-clavulanate	1981	Oral	20-40 mg/kg/day	(147)
Ticarcillin-clavulanate	1985	Intravenous	80 mg/kg every 6-8 hours	(148)
Ampicillin-sulbactam	1997	Intravenous	100-200 mg/kg/day	(148)
Cefoperazone-sulbactam	-	Intravenous	50-100 mg/kg/day	(149)
Piperacillin-tazobactam	1993	Intravenous	150-400 mg/kg/day	(150)
Ceftolozane-tazobactam	2014	Intravenous	1 g/0.5 g every 8 hours	(151)
Ceftazidime-avibactam	2015	Intravenous	2.5 g infusion in 2 hours every 8 hours	(152)

Table 3: β-lactam β-lactamase inhibitor combinations in clinical phases

β-lactam β-lactamase inhibitor combinations	Clinical phase	Expected activity	Potential indications	References
Ceftaroline-avibactam	Phase 2	Active against Gram-negative ESKAPE and CDC pathogens	Bacterial infections	(153)
Aztreonam-avibactam	Phase 2	Active against Gram-negative ESKAPE and CDC pathogens	Complicated intra-abdominal infections	(154)
Imipenem-cilastatin-Relebactam (MK-7655)	Phase 3	Active against Gram-negative ESKAPE and CDC pathogens	cUTIs*, cIAIs**, hospital-acquired and ventilator-associated bacterial pneumonia	(155)
Meropenem-vaborbactam (RPX-7009) also known as carbavance	New Drug Application (NDA) Submitted	Active against Gram-negative ESKAPE and CDC pathogens	cUTIs, hospital-acquired and ventilator-associated bacterial pneumonia, febrile neutropenia, bacteremia, infections caused by carbapenem-resistant Enterobacteriaceae	(156, 157)
Biapenem-vaborbactam (RPX-7009)	Phase 1	Active against Gram-negative ESKAPE and CDC pathogens	Anaerobic bacterial infection	(158)
ETX2514SUL	Phase1	Active against Gram-negative ESKAPE pathogens	Infection caused by A. baumannii	(159)
Cefepime-zidebactam	Phase 1	Active against Gram-negative ESKAPE pathogens, Possible activity against CDC pathogens	cUTIs, hospital-acquired and ventilator-associated bacterial pneumonia	(160)

*cUTIs: complicated urinary tract infections,
**cIAIs: complicated intra-abdominal infections,

Table 4: Types and Characteristics of Known Metallo-β-lactamase Inhibitors

Metallo-β-lactamase inhibitor	Source	Ki (μM)			IC$_{50}$ (μM)			Type of inhibition	References
		B1	B2	B3	B1	B2	B3		
Carboxylates, tetrazoles and hydroxamates									
Maleic acids	Synthetic	0.41-120	-	-	2.5-12.6	-	-	Competitive inhibitor	(65, 66)
Succinic acid	Synthetic	3.3	-	-	0.0027-490	-	15-300	Irreversible	(161–163)
Phthalic acid	Synthetic	-	-	-	0.2-243	-	-	-	(67, 68, 164)
N-acyl hydrazones	Synthetic	-	-	-	1.02-23.9	-	-	-	(165)
2-substituted-4,5-dihydrothiazole-4-carboxylic acid	Synthetic	3.3-5.1	-	-	4.9-77	-	-	-	(71)
3-Mercapto-1,2,4-triazoles and N-acylated thiosemicarbazides	Synthetic	11-75	-	-	-	-	-	Mixed inhibition	(166)
N-Heterocyclic dicarboxylic acid derivatives	Synthetic	0.64-5.9	3.5-7.1	0.69-1.9	-	-	-	Mixed inhibition	(167)
Biphenyl tetrazoles	Synthetic	0.59-1.6	-	-	0.3-860	-	-	Competitive inhibitor	(47, 168)
Hydroxamates (amino-acid derived)	Synthetic	-	-	6.1-17.9	-	-	-	Competitive inhibitor	(48, 169)
Phenazines									
SB 212021	Streptomyces	-	-	-	37	-	19	Reversible	(170)
SB212305	Streptomyces	-	-	-	75	-	1	Reversible	(170)
Trifluoromethyl-ketones and alcohols	Synthetic	30-1000	6-217	1.5- 5000	-	-	-	Irreversible for sub-class B2	(171)
Trifluoromethyl-ketone 5b (3S)	Synthetic	500	6	15	-	-	-	Irreversible for sub-class B2	
Trifluoromethyl-ketone 5'a (3R)	Synthetic	700	11	3	-	-	-	Irreversible for sub-class B2	
Trifluoromethyl-ketone 5a (3S)	Synthetic	300	44	1.5	-	-	-	Irreversible for sub-class B2	
Trifluoromethyl-alcohol 4'b (2R, 3R); (2S, 3R)	Synthetic	30	20	>5000	-	-	-	Irreversible for sub-class B2	
Trifluoromethyl-alcohol 4b (2R, 3S); (2S, 3S)	Synthetic	1000	19	>5000	-	-	-	Irreversible for sub-class B2	
Trifluoromethyl-alcohol 4'a (2R, 2R); (2S, 3R)	Synthetic	700	217	35	-	-	-	Irreversible for sub-class B2	
β-lactam analogues									
1β-methylcarbapenem	Synthetic	0.0037-0.83	-	1	<0.1- >10	-	-	Reversible	(77)
Penicillin derived inhibitors	Synthetic	-	-	-	1.4- >200	-	0.1->200	-	(50)
Thioxo-cephalosporin derivatives	Synthetic	29-720	-	-	-	-	-	Competitive inhibitors	(79, 172)
Cyclobutanone analogues of β-lactams	Synthetic	-	-	-	122-1000	-	-	Reversible	(81)
β-lactam substrates	Synthetic	2300	-	-	-	-	-	Irreversible inhibitors	(173, 174)
Peptides									
Cys-Val-His-Ser-Pro-Asn-Arg-Glu-Cys	Synthetic	-	-	16/9	-	-	-	Mixed inhibition	(175)

Metallo-β-lactamase inhibitor	Source	K_i (µM)			IC_{50} (µM)			Type of inhibition	References
		B1	B2	B3	B1	B2	B3		
Homo-cysteinyl peptide	Synthetic	-	-	0.0021-1.03	-	-	-	Reversible competitive	(176)
Cysteinyl peptide	Synthetic	3.0-1000	-	0.88-3.67	-	-	-	Reversible	(53)
Pyridine Dicarboxylates									
2-picolinic acid	Synthetic	54-95	5.7	29-62	-	-	-	Competitive inhibitors	(52)
Pyridine-2, 4-dicarboxylic acid	Synthetic	78-98	4.5	65-78	-	-	-	Competitive inhibitors	(52)
Pyridine monothiocarboxylic acid analogues	Natural products	-	-	-	0.14-250	-	0.6-340	Reversible	(84)
Natural products									
Flavonoids: galangin, quercetin	From plant	-	-	18.5-185	-	-	-	Irreversible inhibition	(74)
Tricyclic natural products: SB238569, SB236050, SB 236049	Chaetomium fanicola	17-88	3.4-15	-	0.7-256	2-29	>1000	Competitive inhibitors	(54)
Polyketides	Penicillium sp.	-	-	-	87.9-94.9	-	-	-	(75)
Triazoles and N-acylated thiosemicarbazides									
Sulphonyl-triazole	Synthetic	0.41-1.4	-	-	3.3- >56	-	-	Competitive inhibitors	(177)
NH-1,2,3-Triazole inhibitors	Synthetic	0.01-0.18	-	-	0.07-21	-	-	Competitive and Mixed inhibitors	(178)
4-methyl-5- (trifluoromethyl)-4H-1,2,4-Triazole-3-Thiol	Synthetic	970	-	-	-	-	-	Competitive inhibition	(179)
3-mercapto-1, 2, 4-triazoles and N-acylated thiosemicarbazides	Synthetic	11-75	-	-	-	-	-	Mixed inhibition	(166)
Pyrroles Derivatives									
Pyrroles based inhibitorors	Synthetic	12-33	-	-	-	-	-	Competitive inhibition	(51)
Tetrahydropyrimidine-2-thione and pyrrole derivatives	Synthetic	19-235	-	-	-	-	-	Competitive inhibitors	(73)
Nucleic Acids									
ssDNAs	DNA	0.00031-0.0092	-	-	0.15-0.75	-	-	Reversible and non-competitive inhibition	(55, 180)
dsDNAs	DNA	0.11-0.103	-	-	0.011-0.021	-	-	Reversible	(55)
DNA nanoribbon	DNA	-	-	-	3.32-40	11.76	5.66	Non-intercalative binding	(181)
Sulfonic Acid Derivatives									
Bulcegin A	Microorganisms	230	-	2.5	-	-	-	Competitive inhibitor	(182)
4-Morpholinoethanesulfonic acid	Synthesis	23000	-	-	-	-	-	Competitive inhibitor	(49)
N-arylsulfonyl hydrazones	Synthesis	0.7-6.6	-	-	1.6-150	-	-	Reversible competitive	(183)
Metal chelators									
Ca EDTA	Synthesis	-	-	-	27.9	-	-	Non-competitive	(71)

Metallo-β-lactamase inhibitor	Source	Ki (µM)			IC$_{50}$ (µM)			Type of inhibition	References
		B1	B2	B3	B1	B2	B3		
Aspergilomarasamine A	Aspergillus versicolor	-	-	-	4-10.8	-	-	Irreversible	(76, 184)
NOTA, DOTA, TPEN, DPA, NODAGA	Synthetic	-	-	-	-	-	-	-	
Dipicolinic acid	Synthetic	-	5.7	-	0.14-250	-	0.6-340	Competitive inhibitors	(52, 84)
0-phenanthroline	Synthetic	-	-	-	-	55	-	Reversible	
Thioester and thiol derivatives									
Thiodepsipeptides	Synthetic	-	-	-	0.25-240	-	-		(57)
Thioesters and thiols	Synthetic	0.01- 4	-	-	0.00041-740	-	-	Reversible competitive	(57, 185, 186)
Mercaptoacetic acid derivatives	Synthetic	3.9	-	-	28-645	0.55-30	2 - 186	Irreversible inhibitors	(59, 187)
Mercaptophenylacetic acid derivatives	Synthetic	0.02-1500	144	0.27-0.56	22-479	-	1.95 - 8	Reversible competitive	(188)
Mercaptocarboxylate	Synthetic	-	-	-	1.1-16.4	-	-	-	(189)
Mercaptophosphonate derivatives	Synthetic	1-16	0.25-24	0.4-40	-	-	-	Competitive inhibitors	(190)
1β-methylcarbapenamens	Synthetic	0.0037-0.83	-	1	>0.1-9	-	-		(77)
D-captropil	Synthetic	0.5-700	2.7-72	-	0.072-261.8	-	-	Competitive inhibitors	(62, 72, 191)
L-captopril	Synthetic	1.5-65	19-950	-	4.4-157.4	-	-	Competitive inhibitors	(62, 72, 192)
(R)-Thiomandelic acid	Synthetic	0.029-0.8	144	0.27-0.56	-	-	-	Reversible	(60, 193)
Various charged and neutral thiols	Synthetic	-	-	-	1.3-200	-	-	Slow-binding Reversible	(194)
Dansyl derived thiols	Synthetic	0.14-1.3	-	-	0.7-6.3	-	-	-	(195)
Penicillin derived thiols	Synthetic	-	-	-	1.4-106.2	-	0.10-72.3	-	(50, 78)
Bisthiazolidines derivatives	Synthetic	2.9-84	0.26-29	10-41	-	-	-	Competitive inhibitors	(196)

(-) no data found

-lactamase (outer layer).

Thioester, thiol and captopril derivatives

Thiols and thiol-carboxylates are widely reported to be MBLIs [51,166]. In 1997, Goto et al. reported that thiol esters, 2-mercaptopropionic acid and mercaptoacetic acid, are among the numerous thiol-molecules that strongly and competitively inhibited IMP-1, a sub-class B1 MBL [50]. Payne et al. (1997) also demonstrated that a series of mercaptoacetic acid thiol ester derivatives inactivate the catalytic activity of members of class B MBLs such as BcII, CFiA, L-1, and CphA [125]. Mass spectrometry analysis revealed that thiol esters inhibit MBLs by forming a disulfide bond with the active site of the enzymes [125].

Thiomandelic acid and derivatives were also found to be broad-spectrum MBLIs [109]. During the establishment of a library of thiomandelic acid analogues, Mollard and coworkers (2001) realized that thiols with a carboxylic moiety improved the efficacy of the compounds while the thiol group remained essential for the inhibitory effect. This was revealed by the substitution of thiol group with a bromo, hydroxyl, or amidoxime function, and it was found that the thiomandelic acid analogues failed to inhibit the activity of BcII MBL [109]. The racemic-thiomandelic acid exhibited greater inhibitory activity with a Ki value of 0.09 µM than the isomer-thiomandelic acid with Ki of 1.28 µM against BcII enzyme. MBL members of sub-class B1 were the most largely inhibited by thiomandelic acid and thiol-containing compounds; however, they were also effective against sub-class B2 and B3 [94,109,166].

Captopril derivatives, L- and D- captopril, were investigated for their inhibitory activity against MBL by Heinz et al. (2003) [60]. Both enantiomeric inhibitors demonstrated competitive inhibition against sub-class B1 and B2. Potential inhibitory activity of this group of MBLIs was observed against most class B β-lactamases; however, L-captopril exhibited weak inhibition toward NDM-1 with a half-inhibitory concentration of 202 µM than D-captopril, which had an IC_{50} of 8 µM [18]. These captopril derivatives act by binding to the two zinc ions at the active side of the MBL enzymes, thus displacing the hydroxyl group that normally binds the two metal ions[18]. Furthermore, Bai et al. (2015) synthesized analogues of D- and L-cysteine and reported that some cysteine or homocysteine derivatives could efficiently

inactivate the NDM-1 enzyme. The design of these analogues were inspired by the potent inhibition of MBLs by captopril and derivatives [31]. The most potent analogue exhibited an IC_{50} of 1 µM, which was far more potent than the D-diastereoisomer captopril (IC_{50} = 8 µM).

Dicarboxylate, carboxylic acid and hydroxamate derivatives

ME1071, a maleic acid derivative discovered by Meiji Seika Kaisha Ltd. (Tokyo, Japan) as a novel and specific MBLI, was evaluated in vitro in 2010 by Yoshikazu Ishii et al. (2010) to determine its ability to potentiate the activity of biapenem and ceftazidime against IMP-1 and VIM-2 MBL-producing *P. aeruginosa* [70]. *In vivo* efficacy of ME1071 was also carried out in a murine model mimicking ventilator-associated pneumonia involving an MBL-producing *P. aeruginosa*. A dose of 100mg/kg of biapenem alone or in combination with ME1071 was administered to infected mice, and the survival rates and bacterial burden in the lungs were evaluated. Treatment of the infected mice with biapenem and ME1071 significantly resulted in longer survival and relatively lower bacterial burden in the lungs than biapenem alone. These findings showed ME1071 as a potent and effective MBLI for treating ventilator-associated pneumonia infections [190].

Dicarboxylic acid group inhibitors, such as 3-amino and 3-alkoxy derivatives, 3-(4-hydroxypiperidin-1-yl) phthalic acid and 3,6-bis(4-hydroxypiperidin-1-yl) showed inhibitory potency against diverse MBL-producing bacteria, particularly those in sub-class B1 [63,64]. Studies have also shown the potential ability of hydroxamic acid to inactivate the catalytically essential zinc ions of the metallo-protease matrix linking with the peptide backbone, making them potential MBLIs [102]. In 2016, Kim and collaborators [75] demonstrated that compounds with hydroxamic acid moieties exhibit reversible, competitive inhibitory activity against Bla2 metallo-β-lactamase. This study showed that both sides of the dihydroxamic acids play an important role in the binding affinity, which explains the failure of the monohydroxamic-containing molecule to inhibit Bla2. Therefore, compounds containing dihydroxamic acid moieties may be promising MBLIs [75]. The strong binding affinity of the complexes IMP-1 and 2-benzylthiazole-4-carboxylic acid was demonstrated by Pinhong Chen et al. in 2012, where the IC_{50} was found to be 38

μM, thus making this molecule a potential MBLI compound [28]. Therefore, analogues were synthesized based on the structure of that molecule to improve the inhibitory activity against MBLs. The aromatic thiazole ring containing 2-benzyl exhibited greater inhibition property (IC_{50}= 35 μM) than the one with phenyl group (IC_{50} > 200 μM); on the other hand, the partially saturated 4, 5-dihydrothiazole ring with a 2-benzyl group was inactive. The analogue (R)-2-phenyl-4, 5-dihydrothiazole-4 carboxylic acid, was the best compound of the group that inhibited IMP-1 with an IC_{50} value of 5.5 μM and was even more active than 2-benzylthiazole-4-carboxylic acid [28].

Cyclic boronates are broad-spectrum β-lactamase inhibitors, which act against both serine- and zinc-based β-lactamases, as well as target penicillin-binding proteins (PBP) [19]. This group of inhibitors was found to be active against sub-class B1 MBLs, providing an avenue for the development of dual-action inhibitors targeting both serine- and zinc-based β-lactamases, in addition to possessing antimicrobial activity by inhibiting PBPs.

Pyrrole-based inhibitors

Synthesized pyrrole derivatives were investigated for their inhibitory properties against acquired IMP-1 MBL in *P. aeruginosa* and *K. pneumoniae*. Among the assayed compounds, six exhibited good inhibitory effect with Ki values ranging from 10 to 30 μM [107]. Hussein et al. (2012) also synthesized two sets of tetrahydropyrimidine-2-thione and pyrrole derivatives, and their ability to inhibit MBLs were examined against IMP-1; the recorded inhibition constant varied from 20 to 80 μM [68]. The interactions of this group of inhibitors with the MBLs are not yet well-established, due in part to the absence of crystal structures of the enzyme-pyrrole derivatives. Modelling and docking studies of the most potent compound in the pyrrole derivatives' series indicated that they bind to the two zinc ions through the thiol anion, with sulfur-metal distances of 2.2 Å for zinc (I) and 2 Å for zinc (II) [68,107]. More data is required to elucidate the benefit of this group of inhibitors as potential MBLIs for clinical use.

Natural products

Natural products or metabolites play an important role in antimicrobial discovery. Diverse compounds from natural products exhibited good inhibitory activity against MBLs. A series of tricyclic products, SB238569, SB236050 and SB236049 from *Chaetomium funicola* showed an inhibitory effect against CfiA, IMP-1 and BcII enzymes, with SB236049 as the lead compound exhibiting an IC_{50} of ≤2 µM [127]. These tricyclic molecules showed minimal or no inhibitory activity toward the angiotensin-converting enzyme (ACE), which is a mammalian metallo-enzyme, thus predicting their specificity for class B MBLs. The flavonoids galangin and quercetin from *Stenotrophomonas maltophilia* irreversibly inhibited the L-1 MBL from *S. maltophilia* [33]. Polyketides and derivatives from *Penicillium spp.* also showed activity against the clinically relevant sub-class B1 enzyme, NDM-1 [44]. Aspergillomarasmine A (AMA), a natural product and metal chelating agent produced by a fungus, recently identified by King et al. (2014), has shown an ability to inhibit the activities of the class B1 enzymes, VIM-2 and NDM-1 [78]. The property of these natural products to avoid sequestration of human metallo-enzymes would make them safer adjuvants. Nevertheless, systemic application of natural products as antimicrobial agents has been limited by toxicity.

β-Lactams stable against hydrolysis by MBLs

β-lactam analogues that are stable against MBL hydrolysis have been identified. Nagano and co-workers in 1999 tested a variety of carbapenem analogues (1β-methylcarbapenem conjugates) against MBLs [114], and showed that 1β-methylcarbapenems containing dithiocarbamate, benzothienythio, or pyrrolidinylthio moieties at the C-2 position exhibited resistance to hydrolysis by series of MBL enzymes. The most promising compound among this group was J-110,441, a molecule possessing a benzothienylthio moiety at the C-2 position of 1beta-methylcarbapenem, which inhibited IPM-1, Ccra, L-1, and BcII MBLs with Ki values of 0.004, 0.2, 1, and 0.8 µM respectively. Interestingly J-110,441 also inhibited other classes of beta-lactamases (Class A and C), thus possessing a broad spectrum anti-MBL activity [114]. Similarly, penicillin derivatives have been as well reported as potent ϐ-lactamase inhibitors targeting both serine and zinc based ϐ-lactamases [6,23] while thioxo derivatives of

cephalosporins were found to be inhibitors of BcII MBLs [173]. In order to develop simultaneous inactivation of serine and zinc-based ß-lactamases, series of cephalosporin analogues, reverse hydroxamates and oximes were prepared and tested against MBLs to evaluate their inhibitory activity. The reverse hydroxamates were found to inhibit the GIM-1 MBL [45] whereas 6-alkylidene-2-substituted penam sulfones, and cyclobutanone derivatives of β-lactams were also reported as inhibitors of Bla2 and IMP-1 respectively [6,72]. β-lactam analogues inhibitors are likely to be broad spectrum, extending their activity to both classes of β-lactamases (serine- and zinc-based β-lactamases).

Metal Chelators

The use of zinc-chelating agents is an approach for inhibiting MBLs as MBLs require one or two zinc ions in their active site to hydrolyze β-lactams [36]. The utilization of zinc-chelating agents will interfere with the functionality of the zinc ions by sequestering them from the active site of MBLs, thus reducing the MBLs' activity [154].

Several metal chelators with a strong affinity for zinc ions have shown inhibitory effects against different classes of MBLs. Docquier et al. (2003) demonstrated that VIM-2 was susceptible to inactivation by metal chelators, indicating that the zinc ions of this enzyme were probably loosely bound and thus subject to easy sequestration by zinc-chelating agents [36]. Among the numerous metal-chelating agents, ethylene diamine tetra-acetic acid (EDTA) has been widely reported as a potential MBLI. It exhibited an IC_{50} of 27.9 µM for the acquired IMP-1 enzymes [28]. In addition, this molecule possesses essential antimicrobial properties such as potentiation of the activity of other antibiotics through the disruption of the outer membrane of Gram-negative bacteria, as well as the neutralization of enzymes and toxins produced by organisms. Due to its toxicity, EDTA was not considered suitable for therapeutic use [196]. In 2010, Nobumasa Aoki et al. evaluated the efficacy of calcium-EDTA (Ca-EDTA) as an MBLI, which was found to be less toxic than EDTA for *in-vivo* assays using murine models. They discovered that imipenem's activity was restored in the presence of Ca-EDTA in all the tested *P. aeruginosa* isolates

producing IMP and VIM enzymes, but failed to restore its activity in all the non-MBL-producing strains [196]. An *in-vivo* study demonstrated significant reduction in bacterial colony forming units (CFU) in the lung of infected mice when treated in combination with imipenem and Ca-EDTA. These findings suggest that Ca-EDTA could be used clinically due to its lesser toxic effect compared to EDTA [196].

Dipicolinic acid is another metal-chelating agent that demonstrated good inhibitory effect against members of the three MBL classes namely, CcrA, CphA and L1 [139]. Pyridine 2,4-dicarboxylic acid is also one of the numerous zinc-chelating agents that inhibited CphA [67]. A natural fungal product, AMA, which is structurally similar to EDTA, was identified as a potent inhibitor of NDM-1 and VIM-2 in 2014 [78]. Irreversible inhibition was observed with AMA after its removal by gel filtration; nevertheless, the activity of the sub-class B1 enzyme was restored by adding an excess $ZnSO_4$, suggesting that AMA acts by metal sequestration. The observed survival rates of infected mice with a lethal dose of *K. pneumoniae* strains producing NDM-1 was 95%, demonstrating that AMA prevented the hydrolysis of the β-lactam antibiotic *in vivo* [78]. This potent MBLI also showed less toxicity compared to EDTA. In 2015, Somboro et al. evaluated two cyclic chelators, 1,4,7-triazacyclononane-triacetic acid (NOTA) and 1,4,7,10-tetraazacyclododecane-tetraacetic acid (DOTA) as potent MBLIs, with NOTA being the most promising agent [159]. In 2016, Azumah et al. also investigated two acyclic zinc chelators, N,N,N',N'-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) and di-(2-picolyl)amine (DPA), as well as peptide derivatives of 1,4,7- triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODAGA) as MBLIs [4]. These cyclic and acyclic metal chelators restored the activities of imipenem and meropenem against carbapenem-resistant bacteria producing NDM, IMP and VIM enzymes, by presumably binding to the zinc ions of the enzymes [4,159].

The greatest challenge to the clinical use of metal chelators is that they also inhibit human metallo-enzymes such as matrix metallo-proteinase, carbonic anhydrase and carboxypeptidases necessitating further investigations into their systemic effect on living tissues.

Conclusion and future perspectives

This review elucidates the diversity, and alarming dissemination of MBLs causing carbapenem resistance. It is known that the discovery of efficient MBLIs is difficult, in part, due to the flexible active sites of the multiple enzymes and the challenges associated with targeting metallo-enzymes. Therefore, the hunting of MBLIs should ideally be specific to bacterial metallo-enzymes and circumvent other zinc-containing enzymes in humans. Inhibitors should either mimic the structure of the enzymes' substrates or allosterically inhibit the activity of this enzyme. The other challenge is that the performance of these inhibitors varied from one subclass to another. Worse still, structural variations are known to occur within the same subclass, rendering the discovery and development of MBLIs more challenging. Particularly, it will be a great feat to identify compounds that inhibit plasmid-encoded subclass B1 enzymes, which includes the most widespread and clinically important MBLs.

Emphasis needs to be placed on designing and developing MBLIs to extend their inhibitory spectrum to the three classes of MBLs, restore the efficacy of available β-lactam antibiotics and improve their pharmacological properties. Moreover, genomic data generated with current methods such as next/whole genome sequencing, coupled with phylogenetics and metadata are currently one of the tools that give valuable insight into the identification and characterization of MBLs. They also help elucidate the global spread or epidemiology of these MBLs. These modern methods also have a quick turnaround time compared to traditional ones and should be explored to find lasting solutions to this menace. In-depth analysis and optimization of MBLIs, employing a multidisciplinary research approach involving advanced computational simulations, biochemical, microbiological, bio-informatics and animal studies to develop novel metallo-β-lactamase inhibitors are needed to precipitate the development of efficient clinical MBLI adjuvants.

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