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MINIREVIEW

Diversity and Proliferation of Metallo-β-Lactamases: a Clarion Call for Clinically Effective Metallo-β-Lactamase Inhibitors

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ABSTRACT The worldwide proliferation of life-threatening metallo-β-lactamase (MBL)-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. Inhibition of enzymes mediating antibiotic resistance in bacteria is one of the major promising means for overcoming bacterial resistance. Compounds having potential MBL-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 discussed. The relationships between different class B β-lactamases identified up to 2017 are represented by a phylogenetic tree and summarized. In addition, 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 their purported modes of inhibition are delineated. Insights into structural variations of MBLs 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 mechanisms of action and activity spectra 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

The alarming spread of antimicrobial resistance (AMR) presents a major challenge to public health worldwide (1, 2). 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 (3). 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 (4, 5). 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 the plasmid-mediated *mcr-1* gene and chromosomal mutations, and tigecycline resistance due to chromosomal mutations (6, 7).

Gram-negative bacteria develop resistance to β-lactams through different mechanisms (8), including the production of enzymes called β-lactamases that hydrolyze the

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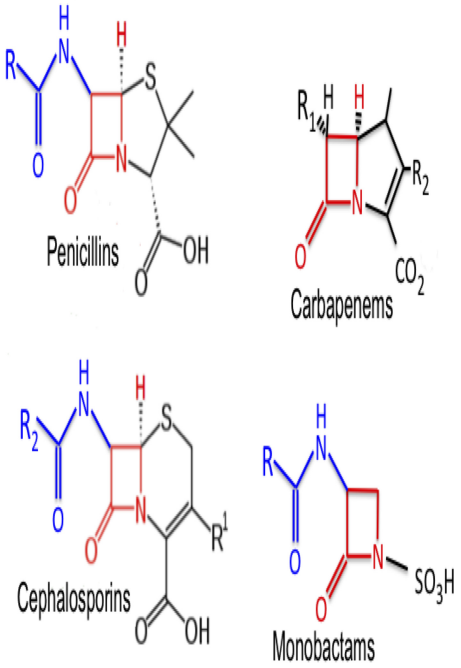

FIG 1 Structures of selected β-lactam antibiotics (penicillins, cephalosporins, monobactams, and carbapenems). Images were adapted from references 12 and 13.

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 antibiotic 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 (8–11).

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 (Fig. 1) (12, 13) by hydrolyzing their β-lactam rings (14). 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., classes A, B, C, and D. The second scheme is based on the enzyme's functionality or substrate and includes three major groups: group 1, cephalosporinases (class C); group 2, serine β-lactamases (SBLs) (classes A and D); and group 3, metallo-β-lactamases (class B). Each of these groups is further subdivided into several different subgroups (15, 16). Class 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 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 (17). One approach for treating β-lactamase-producing bacterial infections is by combining existing β-lactam antibiotics with β-lactamase inhibitors (18). β-lactam/β-lactamase inhibitor combinations resulted in the restoration of the activity of β-lactam antibiotics against β-lactamase-producing bacteria. Such combination drugs that are currently approved by the FDA and available for clinical treatment (18) 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 β-lactamases (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

being detected in Gram-negative bacteria increasingly and with considerable clinical impact (19).

Invariably, the increasing worldwide prevalence of MBLs is a major threat to global health care, affecting both community and hospital settings (20–23). Mortality rates of up to 50% have been reported for carbapenem-resistant *Enterobacteriaceae* (CRE), including MBL-positive infections (24, 25). 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 health care-associated costs, higher mortalities, etc. (23, 26, 27). The World Health Organization (WHO) recently listed CRE pathogens as "critical priority pathogens" for which novel and efficient antibiotics are required urgently (28).

The absence of clinical MBLIs for MBL-mediated drug-resistant infections, particularly those 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 to point out challenges involved in designing clinically useful MBLIs.

METALLO-β-LACTAMASES

Metallo-β-lactamases (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 renders it ineffective (29). For many decades, MBLs were considered to be clinically irrelevant enzymes that were chromosomally encoded in nonpathogenic organisms (30–33). 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 and VIM) and/or bracketed by composite transposons (NDM) (34, 35). 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 (36). These mechanisms mediate the spread of MBL-mediated resistance worldwide. A typical example is the dissemination of the NDM-1 MBL, first isolated from a Swedish patient transferred from India (37) in 2008, worldwide in diverse *Enterobacteriaceae* species. The incidence rate continues to grow (37).

MBLs are divided into three 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 subclass B1 and B2 enzymes ranges from 2% to 14% (38). 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, and H196) and a second zinc ion (Zn2) with three different residues, including a cysteine (D120, C221, and H263) in particular. Subclass B1 is a clinically relevant and notorious MBL subclass, 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 (39).

Subclass B2 MBLs have a Zn1 binding site with one altered residue (N116, H118, or H196), but retain a similar Zn2 site (D120, C221, H263); it selectively hydrolyzes carbapenems and possess the fewest members compared with the others. Subclass B3 MBLs have a varied Zn1 binding site (H/Q116, H118, or H196) and a distinctive Zn2 binding site that lacks a cysteine residue (D120, H121, or H263) (40). These structural variations in known and emerging MBLs offer a greater challenge to 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) (41) and β-Lactamase Database (BLDB), are summarized in Table 1 (42).

MBLs were detected from diverse species of organisms, as shown in Table 1.

TABLE 1 Historical timeline and characteristics of metallo-β-lactamases discovered up to December 2017

MBL subclass and enzyme	Yr detected	Species in which first detected	No. of variants	Country(ies) of origin	β-Lactam antibiotic hydrolysis profile	Genetic location(s)	Accession no.	Reference(s)
B1								
BcII	1966	*Bacillus cereus*	7	Uruguay	Broad spectrum β-lactams	Chromosome	M11189	32, 33, 89, 90
CcrA or CfiA	1990	*Bacteroides fragilis*	25	England	Broad-spectrum β-lactams	Chromosome	AB087225	91, 92
IMP	1994	*Serratia marcescens*	67	Japan	Broad-spectrum β-lactams	Plasmid, chromosome	HM036079	93, 94
BlaB	1998	*Elizabethkingia meningoseptica*	15	—[a]	Broad-spectrum β-lactams	Chromosome	AF189298	95, 96, 97
VIM	1999	*Pseudomonas aeruginosa*	54	Italy	Broad-spectrum β-lactams	Plasmid, chromosome	GU724868	94, 98
IND	1999	*Chryseobacterium indologenes*	16	France	Broad-spectrum β-lactams	Plasmid, chromosome	EF394436	99
EBR	2002	*Empedobacter brevis*	1	France	Narrow-spectrum cephalosporins	Chromosome	AF416700	100
SPM	2002	*P. aeruginosa*	1	Brazil	Broad-spectrum β-lactams	Plasmid, chromosome	GU831565	101, 102
Bla	2003	*Bacillus anthracis*	2	—	Broad-spectrum β-lactams	Chromosome	X62244	71
GIM	2004	*P. aeruginosa*	2	Germany	Broad-spectrum β-lactams	Plasmid	JF414726	103
SIM	2005	*Acinetobacter baumannii*	2	Korea	Broad-array β-lactams, narrow carbapenem	Plasmid, chromosome	GQ288397	104
SLB	2005	*Shewanella livingstonensis*	1	Islands	Broad spectrum	Chromosome	AY590118	105
SFB	2005	*Shewanella frigidimarina*	1	Islands	Narrow spectrum	Chromosome	AY590119	105
PEDO-3	2015	*Pedobacter kyungheensis*	1	United Kingdom	Broad-spectrum β-lactams	Chromosome	NG_049959	106
JOHN	2003	*Flavobacterium johnsoniae*	1	—	Broad-spectrum β-lactams	Chromosome	AY02846	107
CGB	2002	*Chryseobacterium gleum*	1	—	Broad-spectrum β-lactams	Chromosome	EF672680	108
MUS	2002	*Myroides odoratimimus*	2	—	Broad spectrum, exception of aztreonam	Chromosome	AF441286	109, 110
TUS	2002	*Myroides odoratus*	1	—	Large spectrum, exception of aztreonam	Chromosome	AF441287	109
KHM	2008	*Citrobacter freundii*	1	Japan	Broad-spectrum β-lactam	Plasmid	AB364006	111
DIM	2010	*Pseudomonas stutzeri*	1	Netherlands	Broad spectrum, exception of aztreonam	Plasmid	GU323019	112
NDM	2008	*Klebsiella pneumonia, Escherichia coli*	18	India	Broad-spectrum β-lactams	Plasmid, chromosome	JQ080305	37, 113, 114, 115
HMB	2017	*P. aeruginosa*	1	Germany	Broad-spectrum β-lactams	Chromosome	NG_052225	116
FIM	2012	*P. aeruginosa*	1	Italy	Broad-spectrum β-lactams	Chromosome	JX570731	117
MOC	—	*M. odoratus*	1	—	Broad-spectrum β-lactams	Chromosome	KX371616	Unpublished
TMB	2012	*Achromobacter* sp.	2	Libya	Broad-spectrum β-lactams	Chromosome	FR771847	118, 119
GRD23	2016	*Proteobacteria*	1	Denmark	Broad-spectrum β-lactams	Plasmid	KU167043	120
SPN79	2016	*Proteobacteria*	1	Spain	Broad-spectrum β-lactams	Unknown	KU167036	120
VarG	2017	*Vibrio cholerae*	1	—	Large spectrum β-lactams	Chromosome	AAF94716	121
B2								
CphA	1990	*Aeromonas hydrophila*	17	Italy	Narrow spectrum, more strictly carbapenems	Chromosome	AY261378	63, 122, 123
ImiS	1996	*Aeromonas veronii*	1	United Kingdom	Narrow spectrum, more strictly carbapenems	Chromosome	Y10415	124, 125
ImiH	2003	*A. hydrophila*	1	Australia	Narrow spectrum, more strictly carbapenems	Chromosome	AJ548797	126
CEPH-A3	—	*A. veronii*	1	—	Narrow spectrum, more strictly carbapenems	—	AY112998	Unpublished
Sfh	2003	*Serratia fonticola*	1	Portugal	Narrow spectrum, more strictly carbapenems	Chromosome	AF197943	127
B3								
L-1	1982	*Stenotrophomonas maltophilia*	19	—	Broad-spectrum β-lactams	Chromosome	AJ272109	128, 129, 130
FEZ	2000	*Legionella gormanii*	1	—	Broad-spectrum β-lactams	Chromosome	Y17896	131
GOB	2000	*E. meningoseptica*	19	France	Broad-spectrum β-lactams	Chromosome	AF090141	97, 132
THIN-B	2001	*Janthinobacterium lividum*	1	Italy	Broad-spectrum β-lactams	Chromosome	AJ250876	133
MBL1b	2001	*Caulobacter crescentus*	1	—	Broad-spectrum β-lactams	Chromosome	AJ315850	134
CAU	2002	*Caulobacter vibrioides*	2	—	Broad-spectrum β-lactams	Chromosome	AJ308331	135
BJP	2006	*Bradyrhizobium japonicum*	1	—	Broad-spectrum β-lactams	Chromosome	NP_772870	136
AIM	2012	*P. aeruginosa*	1	Australia	Large spectrum, including aztreonam	Plasmid	AM998375	137, 138
CAR	2008	*Erwinia carotovora*	1	—	Broad-spectrum β-lactams	Chromosome	NC_004547	139
POM	2011	*Pseudomonas otitidis*	1	—	Broad-spectrum β-lactams	Chromosome	EU315252	140, 141
PAM	2013	*Pseudomonas alcaligenes*	1	Japan	Broad-spectrum β-lactams	Chromosome	AB858498	142
PEDO	2015	*Pedobacter roseu, Pedobacter borealis*	2	Denmark, Norway	Broad-spectrum β-lactams	Chromosome	NG_049957	106
CPS	2015	*Chryseobacterium piscium*	1	United Kingdom	Broad-spectrum β-lactams	Chromosome	NG_048587	106, 143
ESP	2015	*Eristalis tenax*	2	United Kingdom	Broad-spectrum β-lactams	Chromosome	NG_049088	106
MSI	2015	*Massilia oculi*	1	Spain	Broad-spectrum β-lactams	Chromosome	NG_049323	106
SPG	2015	*Sphingomonas* sp.	1	Norway	Broad-spectrum β-lactams	Chromosome	NG_050139	106
Rm3	2016	From soil sample	1	United Kingdom	Broad-spectrum β-lactams	—	KF485393	144
LRA	2009	*J. lividum*	8	United States (Alaska)	Broad-spectrum β-lactams	—	EU408347	145
SMB	2011	*S. marcescens*	1	Japan	Broad-spectrum β-lactams	Chromosome	AB636283	146
GRD33	2016	*Gemmatimona*	1	Denmark	Broad-spectrum β-lactams	Chromosome	KU167042	120
CRD3	2016	*Erythrobacter*	1	Denmark	Broad-spectrum β-lactams	Chromosome	KU167037	120
DHT2	2016	*S. maltophilia*	1	Germany	Broad-spectrum β-lactams	Chromosome	KU167035	120
ALG6	2016	*J. lividum*	1	Algeria	Broad-spectrum β-lactams	Chromosome	KU167038	120
ALG11	2016	*Burkholderia*	1	Algeria	Broad-spectrum β-lactams	—	KU167039	120

(Continued on next page)

TABLE 1 (Continued)

MBL subclass and enzyme	Yr detected	Species in which first detected	No. of variants	Country(ies) of origin	β-Lactam antibiotic hydrolysis profile	Genetic location(s)	Accession no.	Reference(s)
EAM	2012	*Erythrobacter aquimaris*	1	France	Broad-spectrum β-lactams, specific for carbapenem	Chromosome	JN558591	147
ECM	2012	*Erythrobacter citreus*	1	France	Broad spectrum, specific for carbapenem	Chromosome	JN558590	147
EFM	2012	*Erythrobacter flavus*	1	France	Broad spectrum, specific for carbapenem	Chromosome	JN558587	147
ELM	2012	*Erythrobacter longus*	1	France	Broad spectrum, specific for carbapenem	Chromosome	JN558589	147
EVM	2012	*Erythrobacter vulgaris*	1	France	Broad spectrum, specific for carbapenem	Chromosome	JN558588	147
SPR	2014	*Serratia proteamaculans*	1	United States	Broad spectrum, specific for carbapenem	—	CP000826	Unpublished

[a]—, unpublished.

However, subclass B2 was detected mostly from *Aeromonas* spp. Interestingly, most MBLs were initially identified in Europe (France, Germany, Denmark, the United Kingdom, Italy, etc.). Africa had the least discovered number of these MBLs, not because of their absence in the continent but rather because of a lack of proper awareness, skilled personnel, resistance diagnosis, and surveillance systems. Class B3 also contained the enzyme Adelaide imipenemase (AIM), which 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 subclasses (B1, B2, and B3). Protein sequences of all reported MBLs (Table 1) were downloaded from GenBank using their accession numbers. They were then aligned using the 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, unweighted pair group method using average linkages (UPGMA); nucleotide substitution model, Jukes-Cantor; protein substitution model, WAG (198); transition/transversion ratio, 2; estimate substitution rate, yes; number of substitution rate, 4; perform bootstrap analysis, yes; replicates, 1,000. Metadata associated with the aligned sequences (Table 1) were imported to provide a comprehensive analysis of the generated phylogenetic tree. TEM-1 (Ambler class A β-lactamase) was used as the outgroup 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 Fig. 2.

The phylogenetic analysis depicted a clear distinction between subclasses B1 and B3, as reported in the literature (43, 44), where they were more phylogenetically related to each other than to B2 (Fig. 2). Metadata analysis provided a deeper insight into the associated characteristics and distinctions between all the subclasses. Specifically, subclass 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 CRE infections in the clinical setting, making it a huge challenge for a single inhibitor to act efficiently on all MBL subgroups. 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 subclass B2, the metadata differentiated it into a distinct subgroup, as illustrated in Fig. 2, reiterating the importance of visualizing phylogenetic structures in relation to their metadata (45).

NOVEL MBLs RECENTLY DISCOVERED THROUGH GENOMICS AND METAGENOMICS

The advent of modern techniques such as next-generation and whole-genome sequencing as diagnostic tools in clinical microbiology has led to the discovery of novel genes and diverse mechanisms of drug resistance that could otherwise have been

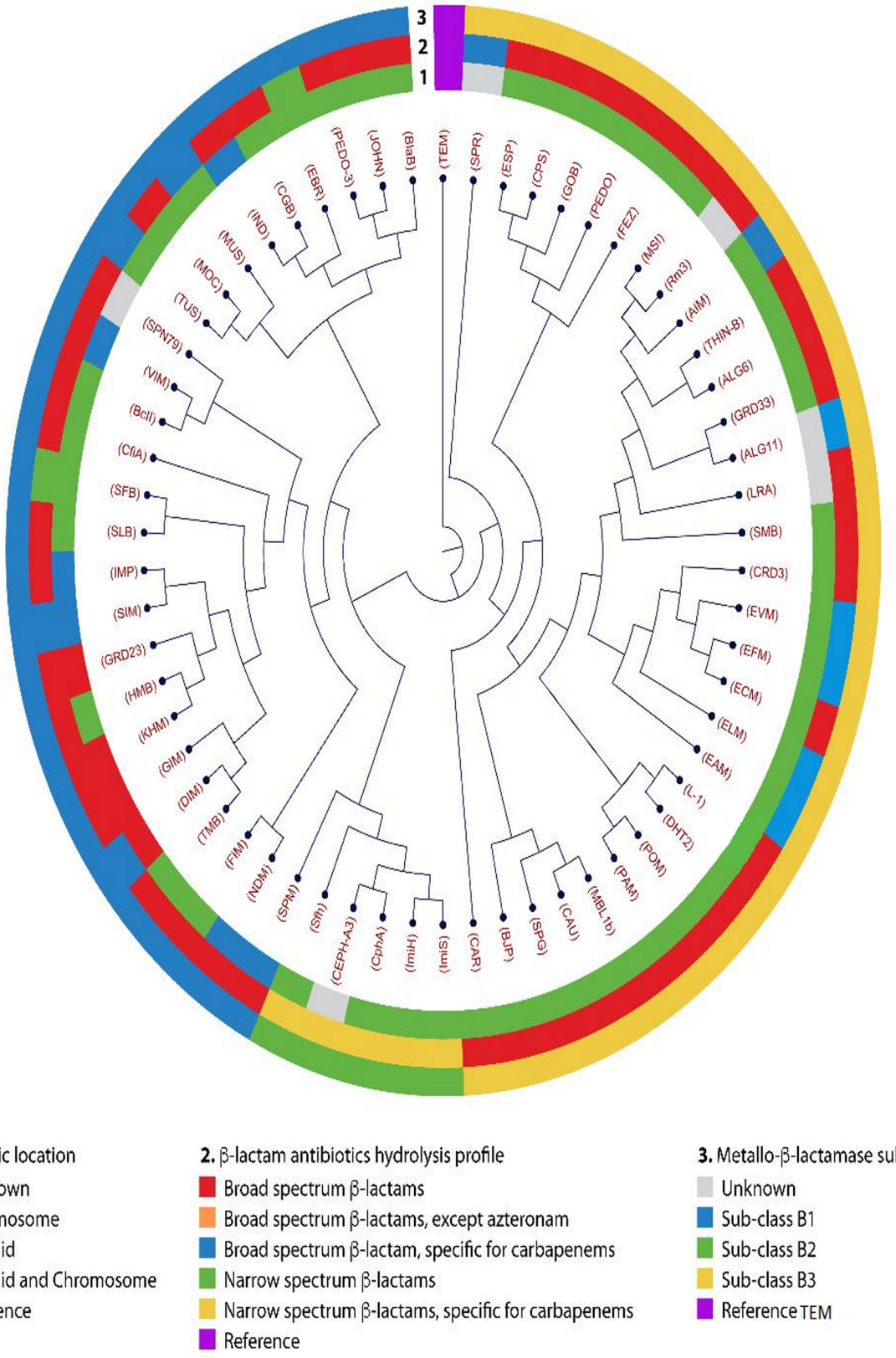

FIG 2 Graphical view of the phylogenetic and metadata of metallo-β-lactamases. The TEM-1 of Ambler class A β-lactamases was rooted and used as the outgroup in the tree. The patterns on the circular phylogram are as follows: 1, genetic location (inner layer); 2, β-lactam antibiotic hydrolysis profile (middle layer); and 3, metallo-β-lactamase (outer layer).

TABLE 2 Approved β-lactam/β-lactamase inhibitor combinations

β-Lactam/β-lactamase inhibitor combination	Yr introduced into clinical use	Route of administration	Dosage regimen	Reference
Amoxicillin-clavulanate	1981	Oral	20–40 mg/kg/day	148
Ticarcillin-clavulanate	1985	Intravenous	80 mg/kg every 6–8 h	149
Ampicillin-sulbactam	1997	Intravenous	100–200 mg/kg/day	149
Cefoperazone-sulbactam		Intravenous	50–100 mg/kg/day	150
Piperacillin-tazobactam	1993	Intravenous	150–400 mg/kg/day	151
Ceftolozane-tazobactam	2014	Intravenous	1 g/0.5 g every 8 h	152
Ceftazidime-avibactam	2015	Intravenous	2.5-g infusion in 2 h every 8 h	153

impossible to find using conventional diagnostic methods. Berglund and collaborators in 2017 (46) assumed that there is a large number of unexplored reservoirs of uncharacterized MBLs. Hence, with the aid of new computational methods based on hidden Markov models, 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 (subclasses 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 from which the MBL was isolated, and genetic location).

These current findings by Berglund et al. (46) thus echo the diversity and proliferation of MBLs as well as their possible threat to clinically available antibiotic options, cautioning scientists 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

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 (18) (Tables 2 and 3). These molecules fail to inhibit MBLs, thus urgently necessitating the development of inhibitors that target bacterial metalloenzymes.

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 (18). One approach to overcome the effects of MBLs is by designing specific inhibitors that can be coadministered 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 (47). The general challenge in identifying and developing broad-spectrum MBLIs stems from the structural and mechanistic differences within the three subclasses of MBLs. Reported MBLI groups include thiol and thioester derivatives (48), tetrazoles and hydroxamates (49, 50) sulfonic acid derivative s (51), β-lactam analogues derivatives (52), pyrroles-based inhibitors (53), pyridine dicarboxylates (54), peptides (55) natural products (56), nucleic acid (57), and metal chelators (58) (Table 4).

THIOESTER, THIOL, AND CAPTOPRIL DERIVATIVES

Thiols and thiolcarboxylates are widely reported to be MBLIs (48, 59). In 1997, Goto et al. reported that the thiol esters 2-mercaptopropionic acid and mercaptoacetic acid are among the numerous thiol molecules that strongly and competitively inhibited IMP-1, a subclass B1 MBL (60). Payne et al. also demonstrated that a series of mercaptoacetic acid thiol ester derivatives inactivate the catalytic activity of class B MBLs such as BcII, CFiA, L-1, and CphA (61). Mass spectrometry analysis revealed that thiol esters inhibit MBLs by forming a disulfide bond with the active site of the enzymes (61).

Thiomandelic acid and derivatives were also found to be broad-spectrum MBLIs (62). During the establishment of a library of thiomandelic acid analogues, Mollard and

TABLE 3 Clinical phases for β-lactam/β-lactamase inhibitor combinations

β-Lactam/β-lactamase inhibitor combination	Clinical phase	Expected activity	Potential indications[a]	Reference(s)
Ceftaroline-avibactam	2	Active against Gram-negative ESKAPE and CDC pathogens	Bacterial infections	154
Aztreonam-avibactam	2	Active against Gram-negative ESKAPE and CDC pathogens	Complicated intra-abdominal infections	155
Imipenem-cilastatin-relebactam (MK-7655)	3	Active against Gram-negative ESKAPE and CDC pathogens	cUTIs, cIAIs, hospital-acquired and ventilator-associated bacterial pneumonia	156
Meropenem-vaborbactam (RPX-7009) (also known as carbavance)	New drug application 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*	157, 158
Biapenem-vaborbactam (RPX-7009)	1	Active against Gram-negative ESKAPE and CDC pathogens	Anaerobic bacterial infection	159
ETX2514SUL		Active against Gram-negative ESKAPE pathogens	Infection caused by *A. baumannii*	160
Cefepime-zidebactam	1	Active against Gram-negative ESKAPE pathogens, possible activity against CDC pathogens	cUTIs, hospital-acquired and ventilator-associated bacterial pneumonia	161

[a]cUTIs, complicated urinary tract infections; cIAIs, complicated intra-abdominal infections.

coworkers 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 the thiol group with a bromo, hydroxyl, or amidoxime function, and it was found that the thiomandelic acid analogues failed to inhibit the activity of the BcII MBL (62). The racemic thiomandelic acid exhibited greater inhibitory activity, with a K_i of 0.09 M, than the isomer thiomandelic acid, with K_i of 1.28 M, against the BcII enzyme. MBL members of subclass B1 were the most largely inhibited by thiomandelic acid and thiol-containing compounds; however, they were also effective against subclass B2 and B3 (48, 62, 63).

The captopril derivatives L- and D-captopril were investigated for their inhibitory activity against MBL by Heinz et al. (64). Both enantiomeric inhibitors demonstrated competitive inhibition against subclasses B1 and B2. Potential inhibitory activity of this group of MBLIs was observed against most class B β-lactamases; however, L-captopril exhibited weaker inhibition toward NDM-1, with a 50% inhibitory concentration (IC_{50}) of 202 M, than D-captopril, which had an IC_{50} of 8 M (65). These captopril derivatives act by binding to the two zinc ions at the active site of the MBL enzymes, thus displacing the hydroxyl group that normally binds the two metal ions (65). Furthermore, Bai et al. 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 was inspired by the potent inhibition of MBLs by captopril and derivatives (66). The most potent analogue exhibited an IC_{50} of 1 M, which was far more potent than the D-diastereoisomer captopril (IC_{50} = 8 M).

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 Ishii et al. to determine its ability to potentiate the activity of biapenem and ceftazidime against IMP-1 and VIM-2 MBL-producing *Pseudomonas aeruginosa* strains (67). The *in vivo* efficacy of ME1071 was also determined in a murine model mimicking ventilator-associated

TABLE 4 Types and characteristics of known metallo-β-lactamase inhibitors

Metallo-β-lactamase inhibitor(s)	Source	K_i (μM)			IC_{50} (μM)			Type of inhibition	Reference(s)
		B1	B2	B3	B1	B2	B3		
Carboxylates, tetrazoles, and hydroxamates									
Maleic acids	Synthetic	0.4–120	—[a]	—	2.5–13	—	—	Competitive	67, 68
Succinic acid	Synthetic	3.3	—	—	0.0027–490	—	15–300	Irreversible	162, 163, 164
Phthalic acid	Synthetic	—	—	—	0.2–243	—	—	—	69, 70, 165
N-Acylhydrazones	Synthetic	—	—	—	1.02–24	—	—	—	166
2-Substituted 4,5-dihydrothiazole-4-carboxylic acid	Synthetic	3.3–5.1	—	—	5–77	—	—	—	73
3-Mercapto-1,2,4-triazoles and N-acylated thiosemicarbazides	Synthetic	11–75	—	—	—	—	—	Mixed	167
N-Heterocyclic dicarboxylic acid derivatives	Synthetic	0.6–6	3.5–7.1	0.7–2	—	—	—	Mixed	168
Biphenyl tetrazoles	Synthetic	0.6–1.6	—	—	0.3–860	—	—	Competitive	49, 169
Hydroxamates (amino acid derived)	Synthetic	—	—	6.1–18	—	—	—	Competitive	50, 170
Phenazines									
SB 212021	Streptomyces	—	—	—	37	—	19	Reversible	171
SB212305	Streptomyces	—	—	—	75	—	1	Reversible	171
Trifluoromethyl ketones and alcohols	Synthetic	30–1,000	6–217	1.5–5,000	—	—	—	Irreversible for subclass B2	172
Trifluoromethyl ketone 5b (3*S*)	Synthetic	500	6	15	—	—	—	Irreversible for subclass B2	172
Trifluoromethyl ketone 5′a (3*R*)	Synthetic	700	11	3	—	—	—	Irreversible for subclass B2	172
Trifluoromethyl ketone 5a (3*S*)	Synthetic	300	44	1.5	—	—	—	Irreversible for subclass B2	172
Trifluoromethyl alcohol 4′b (2*R*, 3*R*), (2*S*, 3*R*)	Synthetic	30	20	>5,000	—	—	—	Irreversible for subclass B2	172
Trifluoromethyl alcohol 4b (2*R*, 3*S*), (2*S*, 3*S*)	Synthetic	1,000	19	>5,000	—	—	—	Irreversible for subclass B2	172
Trifluoromethyl alcohol 4′a (2*R*, 2*R*), (2*S*, 3*R*)	Synthetic	700	217	35	—	—	—	Irreversible for subclass B2	172
β-Lactam analogues									
1β-Methylcarbapenem	Synthetic	0.0037–0.8	—	1	<0.1–>10	—	—	Reversible	79
Penicillin-derived inhibitors	Synthetic	—	—	—	1.4–>200	—	0.1–>200	—	52
Thioxo-cephalosporin derivatives	Synthetic	29–720	—	—	—	—	—	Competitive	81, 173
Cyclobutanone analogues of β-lactams	Synthetic	—	—	—	122–1,000	—	—	Reversible	83
β-Lactam substrates	Synthetic	2,300	—	—	—	—	—	Irreversible	174, 175
Peptides									
Cys-Val-His-Ser-Pro-Asn-Arg-Glu-Cys	Synthetic	—	—	16/9	—	—	—	Mixed	176
Homocysteinyl peptide	Synthetic	—	—	0.0021–1	—	—	—	Reversible competitive	177
Cysteinyl peptide	Synthetic	3.0–1,000	—	0.9–3.7	—	—	—	Reversible	55
Pyridine dicarboxylates									
2-Picolinic acid	Synthetic	54–95	5.7	29–62	—	—	—	Competitive	54
Pyridine-2,4-dicarboxylic acid	Synthetic	78–98	4.5	65–78	—	—	—	Competitive	54
Pyridine monothiocarboxylic acid analogues	Natural products	—	—	—	0.14–250	—	0.6–340	Reversible	86
Natural products									
Flavonoids: galangin, quercetin	Plants	—	—	18.5–185	—	—	—	Irreversible	76
Tricyclic natural products: SB238569, SB236050, SB 236049	*Chaetomium fanicola*	17–88	3.4–15	—	0.7–256	2–29	>1,000	Competitive	56
Polyketides	*Penicillium* sp.	—	—	—	88–95	—	—	—	77

(Continued on next page)

TABLE 4 (Continued)

Metallo-β-lactamase inhibitor(s)	Source	K_i (μM)			IC_{50} (μM)			Type of inhibition	Reference(s)
		B1	B2	B3	B1	B2	B3		
Triazoles and N-acylated thiosemicarbazides									
Sulfonyltriazole	Synthetic	0.41–1.4	—	—	3.3–>56	—	—	Competitive	178
NH-1,2,3-triazole inhibitors	Synthetic	0.01–0.2	—	—	0.07–21	—	—	Competitive and mixed	179
4-Methyl-5-(trifluoromethyl)-4H-1,2,4-triazole-3-thiol	Synthetic	970	—	—	—	—	—	Competitive	180
3-Mercapto-1,2,4-triazoles and N-acylated thiosemicarbazides	Synthetic	11–75	—	—	—	—	—	Mixed	167
Pyrrole derivatives									
Pyrrole-based inhibitors	Synthetic	12–33	—	—	—	—	—	Competitive	53
Tetrahydropyrimidine-2-thione and pyrrole derivatives	Synthetic	19–235	—	—	—	—	—	Competitive	75
Nucleic acids									
Single-stranded DNAs	DNA	0.00031–0.0092	—	—	0.15–0.8	—	—	Reversible and noncompetitive	57, 181
Double-stranded DNAs	DNA	0.1–0.103	—	—	0.01–0.02	—	—	Reversible	57
DNA nanoribbon	DNA	—	—	—	3.3–40	11.7	5.7	Nonintercalative binding	182
Sulfonic acid derivatives									
Bulcegin A	Microorganisms	230	—	2.5	—	—	—	Competitive	183
4-Morpholinoethanesulfonic acid	Synthesis	23,000	—	—	—	—	—	Competitive	51
N-Arylsulfonyl hydrazones	Synthesis	0.7–6.6	—	—	1.6–150	—	—	Reversible competitive	184
Metal chelators									
Ca-EDTA	Synthesis	—	—	—	28	—	—	Noncompetitive	73
Aspergilomarasamine A	*Aspergillus versicolor*	—	—	—	4–11	—	—	Irreversible	78, 185
NOTA, DOTA, TPEN, DPA, NODAGA	Synthetic	—	—	—	—	—	—	—	
Dipicolinic acid	Synthetic	—	5.7	—	0.14–250	—	0.6–340	Competitive	54, 86
o-Phenanthroline	Synthetic	—	—	—	—	55	—	Reversible	
Thioester and thiol derivatives									
Thiodepsipeptides	Synthetic	—	—	—	0.25–240	—	—		59
Thioesters and thiols	Synthetic	0.01–4	—	—	0.0004–740	—	—	Reversible competitive	59, 186, 187
Mercaptoacetic acid derivatives	Synthetic	4	—	—	28–645	0.55–30	2–186	Irreversible	61, 188
Mercaptophenylacetic acid derivatives	Synthetic	0.02–1,500	144	0.3–0.6	22–479	—	1.95–8	Reversible competitive	189
Mercaptocarboxylate	Synthetic	—	—	—	1.1–16.4	—	—	—	190
Mercaptophosphonate derivatives	Synthetic	1–16	0.3–24	0.4–40	—	—	—	Competitive	191
1β-Methylcarbapenamens	Synthetic	0.004–0.8	—	1	>0.1–9	—	—		79
D-Captropil	Synthetic	0.5–700	2.7–72	—	0.072–261.8	—	—	Competitive	64, 74, 192
L-Captopril	Synthetic	1.5–65	19–950	—	4.4–157.4	—	—	Competitive	64, 74, 193
(*R*)-Thiomandelic acid	Synthetic	0.03–0.8	144	0.3–0.6	—	—	—	Reversible	62, 194
Various charged and neutral thiols	Synthetic	—	—	—	1.3–200	—	—	Slow-binding reversible	195
Dansyl-derived thiols	Synthetic	0.14–1.3	—	—	0.7–6.3	—	—	—	196
Penicillin-derived thiols	Synthetic	—	—	—	1.4–106		0.1–72	—	52, 80
Bisthiazolidines derivatives	Synthetic	3–84	0.3–29	1,041	—	—	—	Competitive	197

[a]—, no data found.

pneumonia involving an MBL-producing *P. aeruginosa* strain. A dose of 100 mg/kg of biapenem alone or in combination with ME1071 was administered to infected mice, and the survival rates and bacterial burdens in the lungs were evaluated. Treatment of the infected mice with biapenem and ME1071 significantly resulted in longer survival and relatively lower bacterial burdens in the lungs than treatment with biapenem alone. These findings showed ME1071 as a potent and effective MBLI for treating ventilator-associated pneumonia infections (68).

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 subclass B1 (69, 70). Studies have also shown the potential ability of hydroxamic acid to inactivate the catalytically essential zinc ions of the metalloprotease matrix linking with the peptide backbone, making them potential MBLIs (71). In 2016, Kim and collaborators (72) demonstrated that compounds with hydroxamic acid moieties exhibit reversible, competitive inhibitory activity against the 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 acid-containing molecule to inhibit Bla2. Therefore, compounds containing dihydroxamic acid moieties may be promising MBLIs (72). The strong binding affinity of the complexes IMP-1 and 2-benzylthiazole-4-carboxylic acid was demonstrated by Chen et al. in 2012, where the IC_{50} was found to be 38 M, thus making this molecule a potential MBLI compound (73). 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 (IC_{50} = 35 M) than the one with a phenyl group (IC_{50} > 200 M); 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, inhibiting IMP-1 with an IC_{50} of 5.5 M, and was even more active than 2-benzylthiazole-4-carboxylic acid (73).

Cyclic boronates are broad-spectrum β-lactamase inhibitors which act against both serine- and zinc-based β-lactamases and target penicillin binding proteins (PBP) (74). This group of inhibitors was found to be active against subclass 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 *Klebsiella pneumoniae*. Among the assayed compounds, six exhibited a good inhibitory effect, with K_i values ranging from 10 to 30 M (53). Hussein et al. 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 M (75). 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. Modeling and docking studies of the most potent compound in the pyrrole derivative 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) (53, 75). More data are 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 M (56). These tricyclic molecules showed minimal or no inhibitory activity toward the angiotensin-converting

enzyme (ACE), which is a mammalian metalloenzyme, 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* (76). Polyketides and derivatives from *Penicillium* spp. also showed activity against the clinically relevant subclass B1 enzyme NDM-1 (77). Aspergillomarasmine A (AMA), a natural product and metal-chelating agent produced by a fungus, recently identified by King et al., has shown an ability to inhibit the activities of the class B1 enzymes VIM-2 and NDM-1 (78). The ability of these natural products to avoid sequestration of human metalloenzymes 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 coworkers in 1999 tested a variety of carbapenem analogues (1-methylcarbapenem conjugates) against MBLs (79) and showed that 1-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 1-beta-methylcarbapenem, which inhibited the IPM-1, Ccra, L-1, and BcII MBLs with K_i values of 0.004, 0.2, 1, and 0.8 M, respectively. Interestingly J-110,441 also inhibited other classes of beta-lactamases (classes A and C) and thus possessed a broad-spectrum anti-MBL activity (79). Similarly, penicillin derivatives have also been reported as potent β-lactamase inhibitors targeting both serine- and zinc-based β-lactamases (52, 80), while thioxo derivatives of cephalosporins were found to be inhibitors of BcII MBLs (81). In order to develop simultaneous inactivation of serine- and zinc-based β-lactamases, series of cephalosporin analogues, reverse hydroxamates, and oximes were prepared and tested against MBLs to evaluate their inhibitory activities. The reverse hydroxamates were found to inhibit the GIM-1 MBL (82), whereas 6-alkylidene-2-substituted penam sulfones and cyclobutanone derivatives of β-lactams were also reported as inhibitors of Bla2 and IMP-1, respectively (80, 83). β-Lactam analogue 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 (84). 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 activity of the MBLs (58).

Several metal chelators with a strong affinity for zinc ions have shown inhibitory effects against different classes of MBLs. Docquier et al. 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 (84). Among the numerous metal-chelating agents, EDTA has been widely reported as a potential MBLI. It exhibited an IC_{50} of 27.9 M for the acquired IMP-1 enzymes (73). 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 (85). In 2013, Yoshizumi et al. evaluated the efficacy of calcium-EDTA (Ca-EDTA) as an MBLI, which was found to be less toxic than EDTA in *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 that activity failed to restored in all the non-MBL-producing strains (85). An *in vivo* study demonstrated significant reductions in bacterial CFU in the lungs of infected mice when treated with a combination of imipenem and Ca-EDTA. These

findings suggest that Ca-EDTA could be used clinically due to having a lesser toxic effect than EDTA (85).

Dipicolinic acid is another metal-chelating agent that demonstrated good inhibitory effects against members of the three MBL classes, namely, CcrA, CphA, and L1 (86). Pyridine 2,4-dicarboxylic acid is also one of the numerous zinc-chelating agents that inhibited CphA (54). 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 subclass B1 enzyme was restored by adding an excess of $ZnSO_4$, suggesting that AMA acts by metal sequestration. The observed survival rates of mice infected with lethal doses of *K. pneumoniae* strains producing NDM-1 were 95%, demonstrating that AMA prevented the hydrolysis of the β-lactam antibiotic *in vivo* (78). This potent MBLI also showed less toxicity than EDTA. In 2015, Somboro et al. evaluated two cyclic chelators, 1,4,7-triazacyclononane-triacetic acid (NOTA) and 1,4,7,10-tetra-azacyclododecane-tetra-acetic acid (DOTA), as potent MBLIs, with NOTA being the most promising agent (87). 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 (88). These cyclic and acyclic metal chelators restored the activities of imipenem and meropenem against carbapenem-resistant bacteria producing NDM, IMP, and VIM enzymes, presumably by binding to the zinc ions of the enzymes (87, 88).

The greatest challenge to the clinical use of metal chelators is that they also inhibit human metalloenzymes such as matrix metalloproteinase, 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 metalloenzymes. Therefore, the hunt for MBLIs should ideally be specific to bacterial metalloenzymes and circumvent other zinc-containing enzymes in humans. Inhibitors should either mimic the structures of the enzymes' substrates or allosterically inhibit the activity of the 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 include 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-generation and 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 and biochemical, microbiological, bioinformatic, 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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