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Navigating the complexities of drug development for metallo-β-lactamase inhibitors

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The rising antibiotic resistance rates, especially among carbapenem-resistant Enterobacterales with metallo-β-lactamases (MBLs), highlight the urgent need for effective MBL inhibitors (MBLIs). Navigating the complexities of drug development for MBLIs requires addressing the significant challenges that have hindered its progress. Despite numerous efforts in pre-clinical development, the lack of standardized approaches has led to disparities, stalling the translation of potential MBLIs from research into clinical use. Alarmingly, there is only one metallo-β-lactamase inhibitory candidate in the pre-registration phase of development. This review highlights the need for a global consensus on key aspects of MBLI development, including standardized *in vitro* testing, refined animal models, harmonized toxicity assessments, consistent pharmacokinetic data, and uniform *in silico* methods. It also proposes solutions to these challenges, aiming to bridge the gap between research and clinical application.

1. Introduction

Antimicrobial resistance (AMR) has garnered significant attention in recent years, mainly due to the inefficacy of carbapenem antibiotics and the proliferation of multidrug-resistant pathogens.[1] These pathogens are primarily of Gram-negative origin and carry genes responsible for expressing β-lactamases.[2] These enzymes hydrolyze the amide bond in β-lactams, neutralizing the antibiotics and preventing them from interacting with bacterial penicillin-binding proteins (PBPs).[3] Given the current global antimicrobial crisis and the World Health Organization's (WHO) call for more antibacterial interventions,[4–6] currently, only about 60 antibacterial agents are in phase 1–3 clinical trials, with >50%[7] targeting critical priority pathogens such as Carbapenem resistant – *Enterobacterales*, *Acinetobacter baumanii*, and *Pseudomonas aeruginosa*.[8] Restoring the activity of existing antibiotics through the development of β-lactamase inhibitors is crucial in addressing the growing threat of AMR. This approach extends the lifespan of our current antibiotic arsenal, reduces the reliance on discovering new drugs, and helps curb the spread of resistant bacteria, ultimately safeguarding global public health, in the fight against AMR.[9]

While many serine β-lactamase inhibitors (SBLIs) are effective in clinical practice, there is still a need for an approved metallo β-lactamase inhibitor (MBLI), to reach clinical dispensation (Fig. 1).[10] Broad-spectrum cyclic boronate β-lactamase inhibitors (BLIs) taniborbactam (1)[11–13] – previously known as VNRX-5133 and xeruborbactam (2)[14–17] – previously known as QPX7728 (Fig. 2),[18] are promising candidates that will provide patients with better treatment outcomes in a shorter timeframe.

Cyclic boronates elicit their inhibitory activity by masking as analogues to the primary tetrahedral intermediate, which is shared by both serine β-lactamases (SBLs) and metallo β-lactamases (MBLs) during bicyclic β-lactam hydrolysis.[19] They may also inhibit several PBPs upon interaction.[20] Compound **1**, in combination with cefepime (administered intravenously),[11–13] has completed phase 3 clinical trials and is currently in the pre-registration phase of development.[21] This compound inhibits Ambler class A, C, and D enzymes, as well as class B New-Delhi metallo-β-lactamase (NDM) and Verona integron-encoded metallo-β-lactamase (VIM) through reversible, competitive enzyme inhibition.[13] Meanwhile, **2**,[14–17] in phase 1 clinical trials, is administered with

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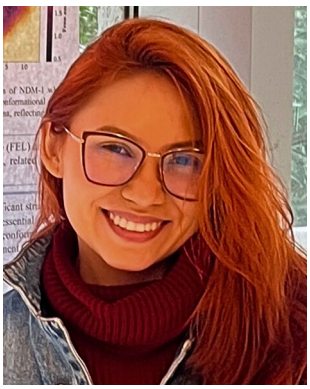
Nakita Reddy

Dr. Nakita Reddy (UKZN/SAMRC) and Dr. Christiaan A. (Arno) Gouws (UP/NuMeRI) are postdoctoral researchers tackling critical healthcare challenges through innovative pharmaceutical research. Nakita's work targets antimicrobial resistance by evaluating novel drug candidates and exploring resistance mechanisms in WHO priority pathogens. Arno focuses on radiopharmaceutical development for PET imaging, designing tracers for infection and cancer diagnostics. Both obtained their PhDs from the Catalysis and Peptide Research Unit at University of KwaZulu-Natal in 2023 and 2024, respectively, and are passionate about translating cutting-edge science into impactful health solutions across Sub-Saharan Africa.



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Dr Awelani Mutshembele is a Specialist Scientist at the South African Medical Research Council, in the Office of AIDS and TB. She leads the South African arm of the NIHR Global Health Research Programme and contributes to BRICS STI and AMR research initiatives. As a lead scientist in the East Africa AMR-STOP programme, she investigates antibiotic resistance and virulence genes in E. coli and K. pneumoniae across six African countries. She also supports One Health AMR surveillance in the Lake Victoria Basin. Awelani's expertise includes molecular surveillance, AI for AMR prediction, and strengthening health systems through capacity development and community engagement.

Per I. Arvidsson

Prof. Per I. Arvidsson is the founding Director of the national Swedish Drug Discovery & Development Platform at the Science for Life Laboratory (SciLifeLab). SciLifeLab DDD supports academic researchers with drug discovery infrastructure and expertise for small molecules, biologics, oligonucleotides, and new modalities. Since 2014, this academic infrastructure has evaluated >500 academic ideas and matured prioritized programs into 18 commercial exits, of which 5 programs have reached clinical trials. Before recruitment by SciLifeLab & Karolinska Institutet in 2013, Prof. Arvidsson held various roles at AstraZeneca as well as academic appointments at Uppsala University and the University of KwaZulu-Natal.

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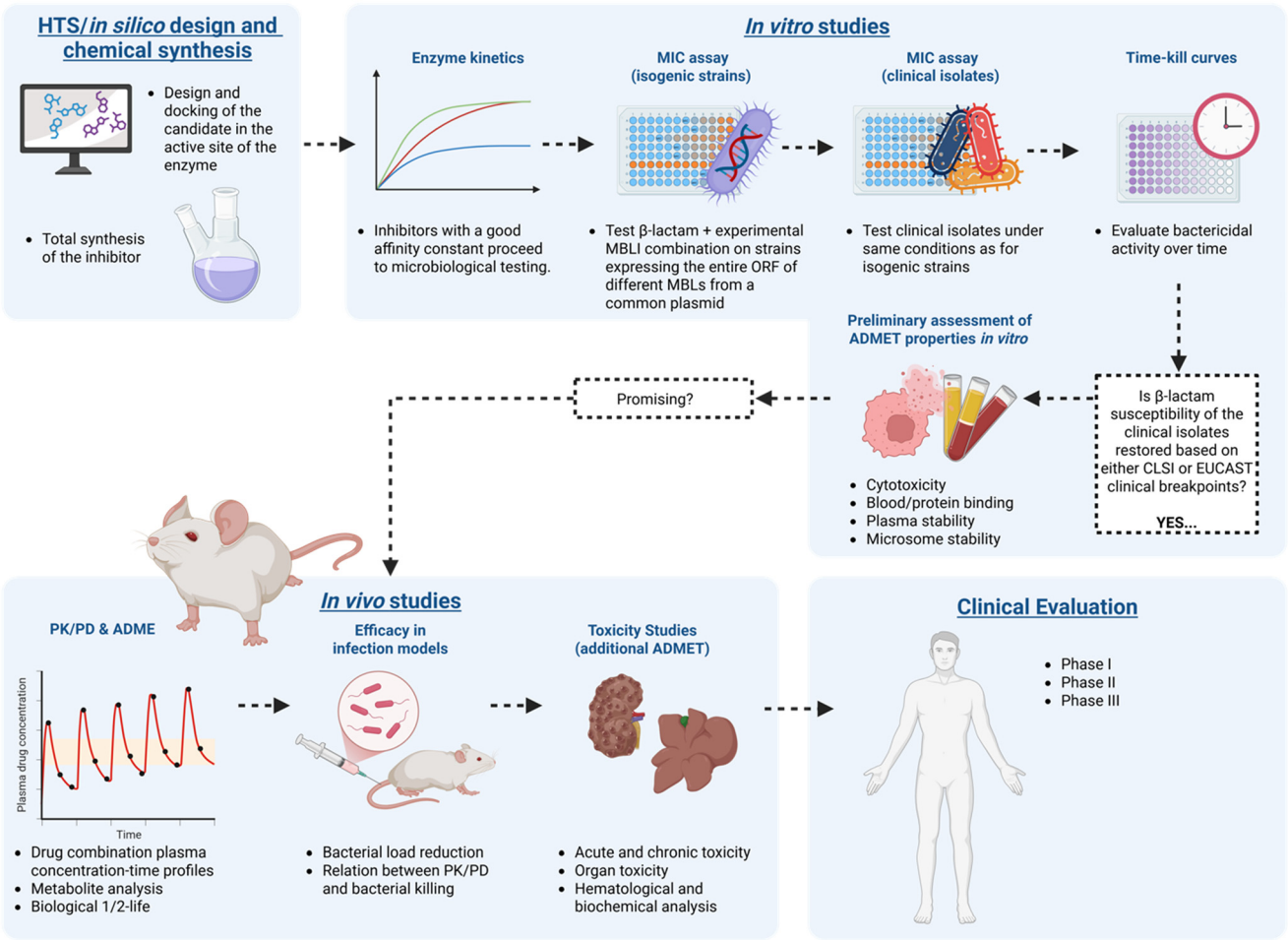

Fig. 1 Schematic representation of the MBLI discovery workflow towards an investigational new drug. Created with **https://Biorender.com**.

meropenem intravenously or ceftibuten orally and targets a broader spectrum of β-lactamases, including the MBL imipenemase (IMP).[14]

In contrast to the rigorous regulatory testing and evaluation required for an Investigational New Drug (IND) proposal there is a noticeable lack of uniformity in the pre-clinical evaluation practices when reporting potential BLIs/MBLIs in the literature (Fig. 1). While some researchers perform in-depth evaluations with *in vitro* and *in vivo* assays, biochemical analyses, and computational methods, many challenges arise in comparing the results across the various research groups, underscoring the need for greater consistency in this field.

This review advocates for the implementation of standard protocols to help establish consensus guidance that can facilitate

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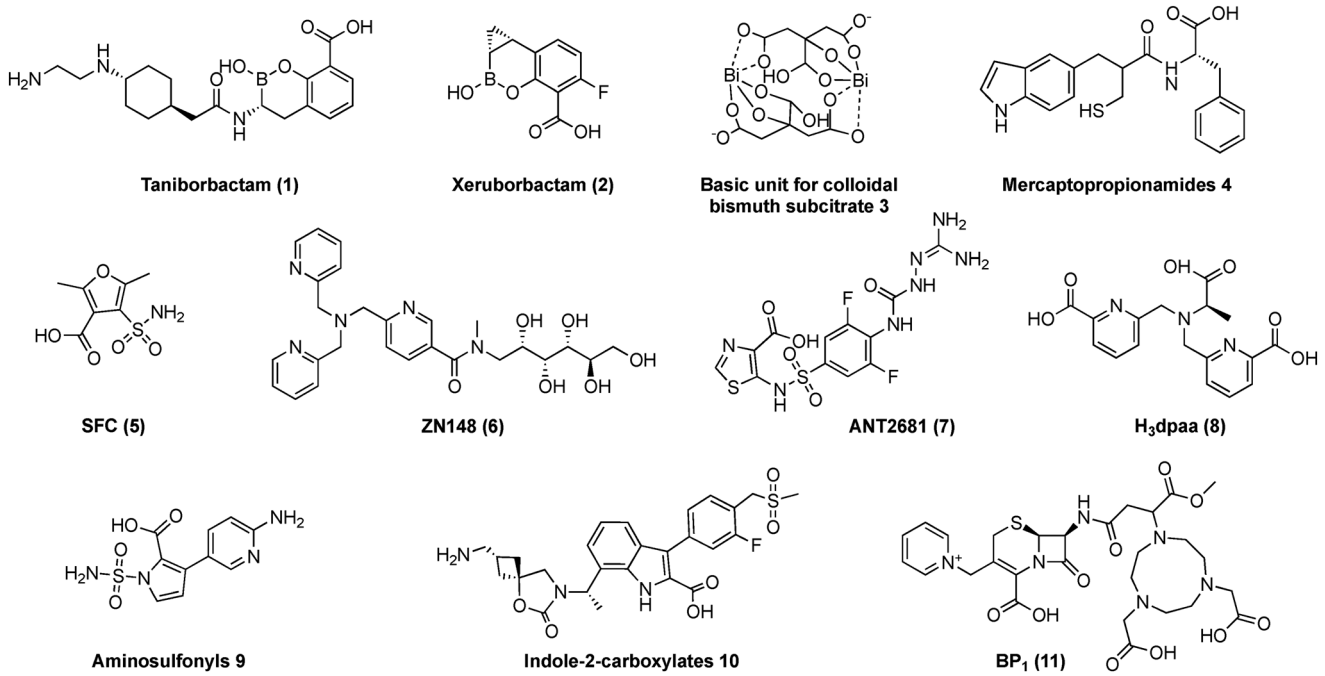

Fig. 2 Representative MBLIs selected, that underwent comprehensive pre-clinical testing from 2018 to 2023 (**1** and **2** have progressed to the clinical development stage, with **1** undergoing pre-registration).

comparison, reproducibility and optimize the pre-clinical development of highly sought-after MBL inhibitors. The text is organized according to the hypothetical MBLI discovery workflow outlined in Fig. 1. It highlights shortcomings in these areas by utilizing the selected MBLIs depicted in Fig. 2 that have undergone thorough *in vitro–in vivo–in silico* examination using similar experiments with differing methodologies and outcomes. The authors acknowledge that many other representative MBLIs have been reported. However, this article does not aim to provide a comprehensive review but rather to offer a critical perspective with these examples.

2. Computational methods

2.1 High-throughput screening and *in silico* observations

High-throughput screening (HTS) is a popular drug discovery tool used to identify starting compounds with biological potential.[22] HTS entails assaying and screening numerous biological effectors and modulators against a specific selected target when minimal information about the target exists.[23] Screening assays are evaluated against a vast array of DNA, chemistry, and protein libraries, at a fast-paced rate, which can exceed thousands of compounds per day, thus accelerating the drug discovery process as "hit compounds" are identified.[24] HTS comprises several steps including target recognition, compound management, reagent preparation, assay development, and screening.[25] Assays used include; biochemical (enzyme-based), ligand-based or cell-based assays;[22] with detection techniques comprising of; fluorescence resonance energy transfer (FRET), and colorimetric/photometric absorbance, among others.[26]

HTS however, is disadvantaged by the practical limitations of not having the bioactive compound in existence, with synthesis-on-demand libraries, requiring a weeks/months that require significant material and experimental resources.[27] Computational approaches have emerged as valuable tools that complement or, in some cases, serve as alternatives to traditional HTS, fragment-based screening (FBS), and newer platforms like DNA-encoded chemical libraries (DELs).[27] Therefore, in the context of designing MBL inhibitors, *in silico* methods are an alternative to HTS. It is a crucial early step in drug discovery, enabling researchers to identify promising candidates before synthesis. Computational approaches such as molecular docking, virtual screening, molecular dynamics and artificial intelligence-driven drug discovery (discussed later in section 8) help predict how potential inhibitors interact with metallo-β-lactamases at the atomic level, guiding the optimization of binding affinity and specificity. Although computational methods offer a solution to improve HTS, it may not replace it,[27] instead, it could be used in conjunction with HTS, to deliver a more robust outcome, as a drug discovery tool.

2.2 Computational methods used to identify MBL–MBLI interactions at a molecular level

The challenges in understanding MBL enzymes begin with their classification (Table 1). Particularly from a computational perspective, the difficulty in adequately describing the inhibition mode (Table 1) and the reaction mechanism induced by inhibitors stems from the challenging depiction of the MBL metal centers (Fig. 3). Although classical and

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Table 1 Chemical classification and inhibition modes for compounds **1–11**

Compound	Chemical class[28]	Inhibition mode
1	Boronic acid	Masks as analogues to the primary tetrahedral intermediate, common to SBLs and MBLs. **1** also formed bicyclic and tricyclic structures with NDM-1, during crystallography analyses[11–13,28]
2	Boronic acid	Similar to **1**, the cyclopropyl of **2** is positioned to occupy the hydrophobic pocket near the zinc ions of NDM-1 and VIM-2 (ref. 14–17 and 28)
3	Colloidal bismuth	NDM-1 was inhibited irreversibly by one Bi(III) displacing two zinc ions at the MBL active site[29]
4	Thiols	Mimic substrate intermediates responsible for β-lactam ring opening. Through IMP-1 structural observations, thiol displaces the hydrolytic water molecule, responsible for bridging the two metal ions, carboxylate groups then form electrostatic hydrogen bonds with anchor residues, $Lys224_{IMP-1}$ (ref. 28 and 30)
5	Sulfonamides	The carboxylate group of sulfamoyl heteroarylcarboxylic acid binds to the MBL active site by masking as a β-lactam substrate. The sulfamoyl group coordinates to the two zinc ions[28,31]
6	Nitrogenous aromatic carboxylic acid	Tris-picolylamine meglumine derived acyclic chelator that mimics as a β-lactam substrate[32]
7	Nitrogenous aromatic carboxylic acid	Masks as a β-lactam substrate. In VIM-2 the carboxylate and pyridine nitrogen bind to the second zinc ion, displacing two "non-bridging" water molecules present in the native structure[28,33–35]
8	Nitrogenous aromatic carboxylic acid	Acylic dipicolinic acid pentadentate-chelating N–O ligands act by mimicking pharmacological substrates[36]
9	Sulfonamides	The di-zinc bridging water molecule is displaced by the *N*-sulfamoyl group, whilst the carboxylate group facilitates ligation to the second zinc ion[28,37]
10	Nitrogenous aromatic carboxylic acid	The C7 alkyl groups and the indole NH forms a stable retained di-zinc ion-bridging hydroxide complex. Whilst the carboxylate ligates to Zn_2, to mimic the β-lactam substrate. Indole-2-carboxylates engage with various motifs during binding[28,38]
11	Nitrogenous aromatic carboxylic acid	Masks as a β-lactam substrate, β-lactam derived cyclic amino chelator[39]

quantum methods aim to describe the correct geometry and energetically coherent values relative to experimental data, this understanding still has several gaps to fill.

As previously delineated in the scientific literature, it is widely accepted that MBL enzymes are reliant on Zn^{2+} ions for the execution of their biological activities.[40,41] Therefore, excluding these ions from simulations is an impractical approach for faithfully representing the system from a computational perspective. There is no doubt that the most challenging aspect in the computational understanding of MBL enzymes lies in the accurate description of Zn^{2+} ions using the classical and quantum mechanical methods.[42] Additionally, the nature of the inhibition reaction of MBL enzymes must be considered, as inhibitors can be classified as non-covalent and covalent,[28] further contributing to the computational challenge. Various computational studies employing molecular docking, hybrid quantum mechanics/molecular mechanics (QM/MM) simulations, density functional theory (DFT), and Molecular Dynamics (MD) are used to shed light on the intricate interactions and mechanisms involved in MBL inhibition.

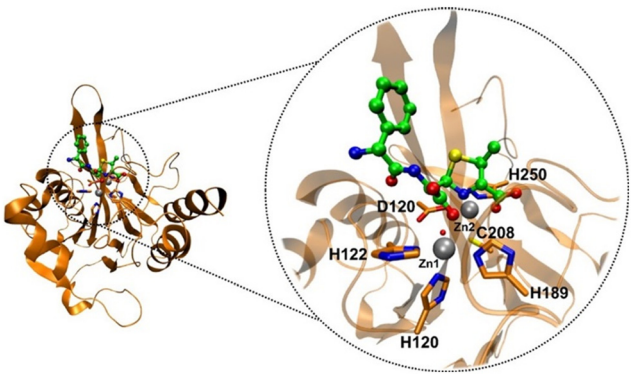

Fig. 3 Detailed 3D structure of NDM-1 in complex with hydrolyzed ampicillin (PDB code 5ZGE), showcasing the zinc-binding residues and hydrolyzed ampicillin in a ball-and-stick representation. The carbon atoms within the active residues are highlighted in orange, while those in the hydrolyzed ampicillin are depicted in green. Additionally, zinc ions are illustrated as grey spheres and hydroxide ions are represented by red spheres, providing a clear visualization of the system's complexity.

Particularly, concerning the compounds **1–11** highlighted in this study, **7**[33–35] and **11**[39] underwent computational analyses through molecular docking techniques,[34,39] while **1**[11–13] was assessed using classical molecular dynamics techniques, due to its theoretical and computational nature, only classical structural features were considered in the analyses.[43] While thermodynamic parameters, such as free energy, play a crucial role and are derived from these classical simulations, it is critical to recognize that the energies associated with docked bindings offer only a basic approximation. To obtain more precise binding energies of the MBLI interactions, conducting QM/MM, Our Own N-layered Integrated molecular Orbital and molecular Mechanics (ONIOM) calculations or implementing QM/MM molecular dynamics simulations is essential.[44,45]

In the literature, it is possible to find some studies that provide computational insights for the description of MBLI systems: Olsen *et al.* (2004)[46] conducted a detailed analysis of *Bacillus cereus* MBL interaction with benzylpenicillin, highlighting the influence of metal ions on the enzyme's mechanism and revealing distinct binding modes. Wang and

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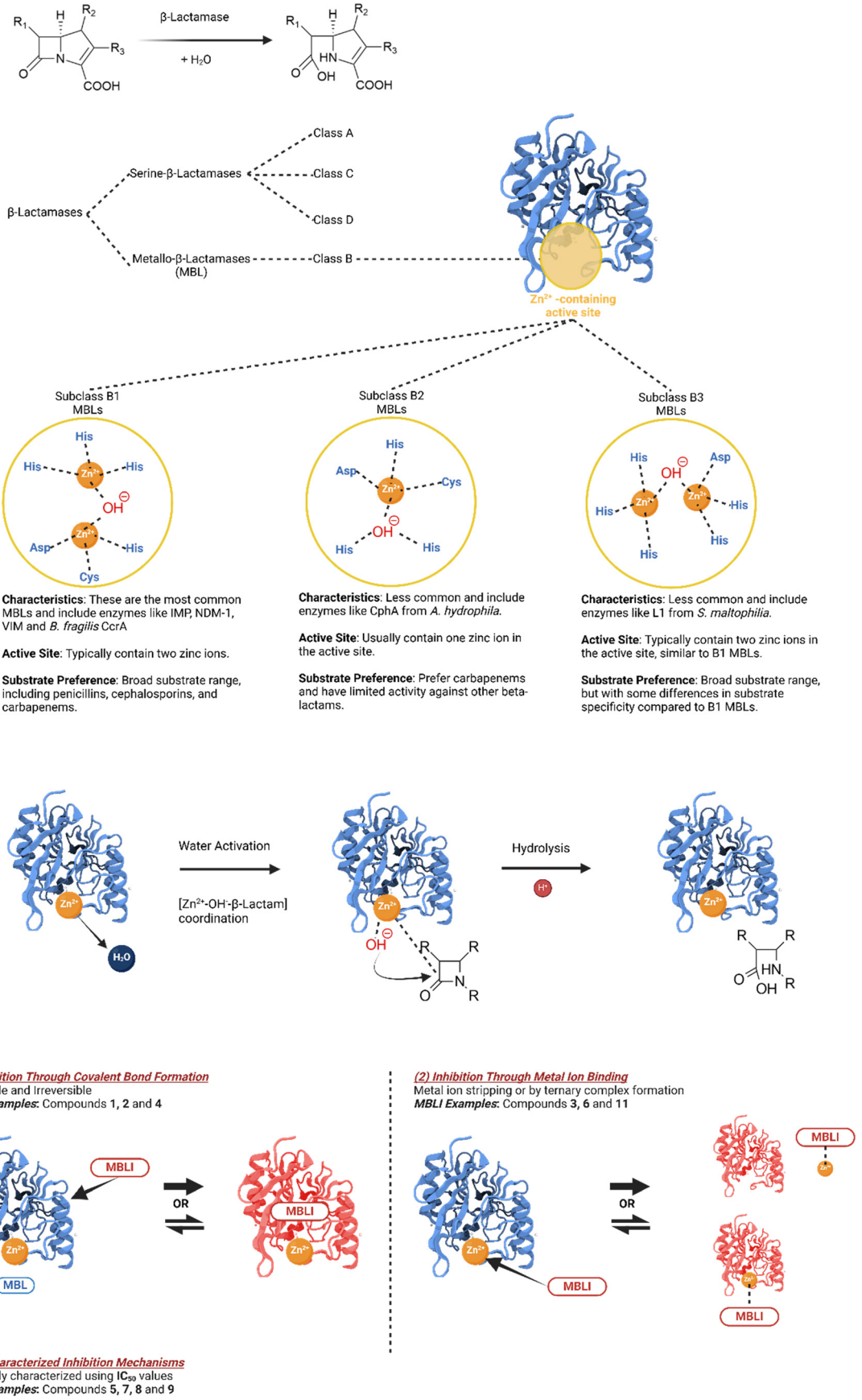

Fig. 4 The different inhibition mechanisms of metallo-β-lactamase inhibitors. Abbreviations: MBL (metallo-β-lactamase), MBLI (metallo-β-lactamase inhibitor). Created with **https://Biorender.com**.

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Guo (2007)[47] employed DFT and hybrid QM/MM simulations to elucidate the mode of action of inhibitors of the MBL IMP-1 from *P. aeruginosa*, emphasizing the crucial role of direct binding to the metal. Zhu *et al.* (2013)[48] and Zheng and Xu (2013)[49] investigated the hydrolysis mechanism of NDM-1 through MD and ONIOM methods, emphasizing the importance of ionized residues and water molecules in the catalytic process. Chen *et al.* (2014)[50] explored the impact of mutations in NDM-1, while Levina *et al.* (2020)[51] analyzed cephalosporin-L1 MBL complexes, employing potential energy surface (PES) calculations and QM/MM methods. More recently, Gervasoni *et al.* (2022)[52] addressed the choice of QM methods, emphasizing the limitations of empirical models and advocating for higher-level calculations like DFT/MM. Medina and Jana (2022)[53] investigated the catalytic mechanism of meropenem hydrolysis by VIM-1, identifying key residues and proposing mutants for inhibiting MBL activity. These studies emphasize the computational challenges of studying covalent and non-covalent inhibitors, stressing the importance of accurately describing the metal center in simulations.

3. Inhibition of enzyme activity

Another integral component of *in vitro* evaluation is the incorporation of enzyme analysis of an MBLI. The inhibitory candidate must be able to bind to the MBL at a suitable concentration, generally in the micro/nanomolar range, to be regarded as an efficient inhibitor. Other important factors include the mechanisms of inhibition expressed by the MBLI (Fig. 4). It is important to note that metal-binding agents function by binding to and extracting metal ions from enzymes, thereby disrupting enzyme activity without forming a permanent interaction. In contrast, covalent inhibitors establish a chemical bond with the enzyme, which can be either reversible (temporary) or irreversible (permanent), directly modifying the enzyme's function. Covalent irreversible inhibitors have the advantage of; prolonged action, target acquisition, and the ability to compete with high substrate concentrations.[54] Successful covalent inhibition has been observed with many SBLIs.[55] The MBLIs **3**,[29] **6**,[32] and **11**[39] are classified as metal binders, which typically operate through an unclear irreversible binding mechanism, which may increase the enzyme's susceptibility to irreversible chemical modification.

A more comprehensive understanding of the mode of action of enzymatic inhibitors involves recognizing the presence of non-covalent and covalent states throughout the inhibition process. Consequently, relying solely on a straightforward examination of K_i (inhibition constant of the inhibitor) or IC_{50} (half the maximum inhibitory concentration) values is insufficient for determining the optimal MBLI potency, as the nature of inhibition should be considered.

Besides the covalent inhibitors' challenge, the traditional view in drug discovery is that irreversible inhibition is undesirable due to fears of off-target effects and toxicity. The reliance on equilibrium measures like IC_{50} values, which may underestimate the potency of inhibitors for slow-onset inhibitors, highlights the limitations of these metrics. Instead, kinetic parameters, particularly second-order parameters like k_{inact}/K_i, offer a more accurate assessment by incorporating both inactivation rates and pseudo-affinity constants.[56] Although it is the gold standard, this value is typically derived from *in vitro* assays, which can limit efficiency when studying enzyme families with multiple homologs, like PBPs. Moreover, enthalpy/entropy compensation warrants careful consideration for covalent inhibitors, as irreversible inhibitors often incur a more significant entropic penalty than their reversible counterparts.[57] These points provide a clearer picture of the covalent inhibitors' effectiveness, sidestepping the pitfalls of equilibrium-based evaluations and affirming their therapeutic potential.

The IC_{50} of irreversible inhibitors is indeed time-dependent due to the formation of covalent bonds, unequivocally making K_i a more precise parameter for comparing binding affinities. In contrast, reversible inhibitors consistently exhibit time-independent IC_{50} values, with K_i directly reflecting their equilibrium binding affinity.[58] Using an IC_{50} that approximates the K_i (K_{iapp}) will depend on the assay conditions (insufficient enzyme or weak substrate), which vary among research laboratories. Except for **10**,[38] all other compounds shown in Table 2 were evaluated for either the IC_{50} (**1**,[11–13] **3**,[29] **9**,[37] **10**[38]) or K_i (**2**,[14–17] **4**,[30] **5**,[31] **6**,[32] **7**,[33–35] **8**[36]) or both (**11**).[39] In particular, the inhibition values for compound **10** (ref. 38) are in the nanomolar range. Then, its activity values are expressed as pIC_{50} ($-\log IC_{50}$) and are reported to be a better indicator of enzyme inhibition for MBLs.[38] MBLIs that function as metal chelators are known to be non-specific and may be limited in therapeutic use if they bind to other essential human enzymes that contain zinc.[39]

Metal binding inhibitors such as **3**, **6** and **11** can overcome MBL resistance since they bind to the active site of MBLs, which is their defining feature and the most conserved part of the class B1 family.[59] As highlighted previously, metal-binding inhibitors function by a metal ion stripping mechanism or ternary complex formation.[54] In metal ion stripping, MBL active site metal ions are actively removed by inhibitors or sequestered when metal ions leave the active site.[54] Ternary complex formation occurs when the inhibitor binds to the metal ions and surrounding protein residues, thereby blocking access to the antibiotics to be degraded by the MBL.[54]

Uncharacterized inhibitor mechanisms (compounds **5**,[31] **7**,[33–35] **8**,[36] **9**[37]) are solely identified through their potency, where the IC_{50} is determined and active site binding is ignored.[60] This approach is unfavored since it does not consider the structure–activity relationship of MBL *versus* MBLI. In addition, a true IC_{50} cannot be determined for covalent inhibitors since it depends on time, the binding and kinetic components of inhibition.[61] Although the IC_{50} is not an absolute constant, it remains a useful

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Table 2 Pre-clinical inhibitory potential and binding specificity of the selected MBLIs, **3**–**11**, (**1** and **2** have progressed to the clinical development stage, with **1** undergoing pre-registration)

Compound	Inhibitory parameter assessed and binding mode	MBL: inhibition concentration (μM)	Mammalian metalloproteinase: inhibition concentration (μM)
1 (ref. 11–13)	IC_{50}, covalent reversible	NDM-1: 0.19 VIM-2: 0.026 IMP-1: 39.8	MMP – 1, 2, 3, 9: 100
2 (ref. 14–17)	K_i, covalent reversible	NDM-1: 0.032 VIM-1: 0.008 IMP-1: 0.22	ECE-1: 431 ACE-2: >1000 MMP-1: >1000 MMP-9: >1000
3 (ref. 29)	IC_{50}, metal ion binder and meta displacement mechanism	NDM-1: 2.81 VIM-2: 3.55 IMP-4: 0.7	Not determined
4 (ref. 30)	IC_{50}, covalent irreversible	NDM-1: 0.12	Not determined
5 (ref. 31)	K_i, uncharacterized	NDM-1: 9.82 VIM-2: 2.81 IMP-1: 0.22	ACE: >1000
6 (ref. 32)	K_i, metal ion binder and unclear irreversible inhibition	NDM-1: 310 VIM-2: 2.24	Gly-2: >500
7 (ref. 33–35)	K_i, uncharacterized	NDM-1: 0.04 VIM-1: 0.63 IMP-1: 3.81	ACE: 133 Gly-2: >200 MMP-2: 173 MMP-9: >200
8 (ref. 36)	K_i, uncharacterized	NDM-1: 1.70 VIM-2: 5.83 IMP-1: 1.54	Not determined
9 (ref. 37)	IC_{50}, uncharacterized	NDM-1: 0.83 VIM-2: 32.4 IMP-1: 0.43	MMP-1, 2, 3, 7, 8, 9, 12, 13, 14: % inhibition using 100 μM of **9**: −0.1–22.2[a]
10 (ref. 38)	[b]IC_{50} (pIC_{50}), non-covalent	NDM-1: <0.06 nM (>10.2) VIM-1: 0.4 nM (9.4) VIM-2: 0.16 nM (9.8) IMP-1: 10 nM (8.0)	Not determined
11 (ref. 39)	IC_{50} K_i, metal ion binder and unclear irreversible inhibition	253.3 (NDM) and 45.8 (VIM-2) 97.4 (NDM) and 24.8 (VIM-2)	Gly-2: >500

ACE = angiotensin-converting enzyme, ECE = endothelin converting enzyme, Gly-2 = glyoxylase II, MMP = matrix metalloproteases. [a] <25% inhibition of important human MMPs indicating good selectivity towards bacterial MBLs. [b] IC_{50} measured in nanomolar units, pIC_{50} is unitless.

and 'quick' parameter for comparing compounds under consistent experimental conditions, regardless of the inhibition mechanism. We believe that determining the exact mode of inhibition and the corresponding kinetic constants (*e.g.*, K_i, k_2/K, or k_{off}) is essential for a more mechanistic understanding of inhibitor behaviour. Regarding EC_{50}, although not commonly reported for MBLIs, we consider this parameter relevant during the cellular validation phase of development. Once a candidate demonstrates potent enzyme inhibition *in vitro*, its efficacy can be evaluated in whole-cell assays where the EC_{50} reflects the concentration required to restore β-lactam activity in the presence of the inhibitor. Thus, for covalent (reversible and irreversible) MBLI, one must consider the contribution of non-covalent interactions that favour the removal of Zn, justifying the high values of K_i, since the entire inhibition process needs to be considered, advocating that higher K_i values do not equate to compound inferiority.

MBL concentrations are dependent on whether the enzyme is purchased (**4**,[30] **11**[39]) or expressed (**2**,[14–17] **3**,[29] **5**,[31] **6**,[32] **8**,[36] **9**,[37] **10**[38]) and in what quantity. At the same time, activity is dependent on the sensitivity of each batch of enzyme, which can be influenced by temperature and freeze–thaw cycles.[62] Freeze–thaw cycles of enzyme aliquots are not recommended. Still, they are practised in many labs due to the high cost of purchasing enzymes or the poor yield and technical difficulties in expressing and purifying MBLs of interest.[63] Although these are standard biochemistry principles, they are often taken for granted by laboratory personnel in fast-paced environments or overlooked in labs that do not primarily focus on enzyme studies. The Assay Guidance Manual[64] provides experimental information regarding enzyme assays and should be consulted when designing enzyme studies. For research labs lacking a dedicated enzymologist or the experience to troubleshoot assays, commercially available enzyme assay kits offer a promising solution to address this challenge and expedite the MBLI development process. These kits could include MBLs, MBLI controls, substrates, buffers, and optimized protocols, which aid in validating results. This standardization allows for results to be compared across compounds evaluated using the same assay kit. Furthermore, on a molecular level, heterologous MBL sequences (the reason why **1**[11–13] inhibits NDM and VIM but not IMP

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B1 MBLs), shallow active sites, the presence of catalytic residues, and similarity to human metalloenzymes often impede the development of an MBLI.[65]

4. Inadequate predictive *in vitro* assays

Pre-clinical research necessitates robust *in vitro* assays to detect the presence of MBLs, evaluate the potency of BLIs and their capacity to restore the activity of β-lactam antibiotics.

4.1 MBL detection using various diagnostic assays

IMP-1, the first MBL discovered in Japan in 1990 from *P. aeruginosa* and *Serratia marcescens*,[66] currently comprises approximately 98 variants.[67,68] A few years later, in 1997, VIM was reported in *P. aeruginosa*,[69] with about 85 variants identified.[67,68] NDM was subsequently reported in 2008,[70] with 65 variants currently known.[67,68] However, data collected by the SENTRY antimicrobial surveillance program in India during 2006–2007 revealed that 38.5% of CRE isolates harbored $bla_{\text{NDM-1}}$,[71] indicating its circulation before its discovery in 2008. NDM also shares a 32.4% amino acid similarity with VIM-1/VIM-2,[72] underscoring the critical need for precise detection methods and the essential requirement for accurate diagnostic methods that can differentiate among MBLs.

Another concern arising from antimicrobial susceptibility testing is the existence of two recognized guides for categorizing the generated minimal inhibitory concentration (MIC). These guidelines are from the Clinical and Laboratory Standards Institute (CLSI)[73] and the European Committee for Antimicrobial Susceptibility Testing (EUCAST).[74] Utilizing either of these guidelines in pre-clinical labs, will have an altering effect on the MIC categorization of the β-lactam that is used in combination with the MBLI, since most MBLIs work by restoring the efficacy of the β-lactam. Harmonizing these two guidelines will effectively reduce possible discrepancies in the MIC categorization.[75] Speculation of an initiated agreement between the relevant stakeholders from EUCAST and CLSI has been mentioned, however, there is no conclusive evidence on this matter. Therefore, in the interim, the authors suggest providing an MIC interpretation using both guidelines.

Diagnostic tests vary in usage across countries due to factors such as accessibility, infrastructure, cost, and the expertise of laboratory personnel.[72] Consequently, the specific protocols and techniques influence the uniformity of antimicrobial susceptibility testing (AST). The WHO does not support molecular methods, for the precise detection of carbapenemases.[76] Instead, phenotypic detection is endorsed, through characterization of the MIC, which is reported by AST. This is currently regarded as the gold standard by WHO,[76] with time-kill assays preferred over broth microdilution assays. Although there are flaws in the MIC and classification of antimicrobial susceptibility, it is still widely used and relied upon, particularly in many developing countries where access to specialized equipment is limited, and cost is a significant factor. Regrettably, it is in these countries where the burden of resistance is highest, and the need for surveillance and adequate reporting on prevalence and controlled antimicrobial usage is critical.[72,77]

4.2 Antimicrobial susceptibility testing and the associated shortcomings

Challenges in AST using the broth microdilution method have revealed significant discrepancies in the MIC of bacteria harboring MBLs. Adjusting the AST media to mimic *in vivo* conditions accurately should be explored when establishing predictive *in situ* resistance. For example, in contrast to *in vitro* resistance assessed by AST, many case studies have reported positive outcomes in patients infected with MBL-producing bacteria.[78] This can occur from using a 2–3 antibiotic combination of β-lactams or non-β-lactams and a carbapenem, to target various PBPs. In *Escherichia coli*, meropenem targets PBP2,[79] whilst cefepime/ceftazidime targets PBP3,[80] using a combination of both antimicrobials, has shown clinical significance during treatment.[78] Enhancers that bind to PBP2, such as Zidebactam, have also been used similarly. Zidebactam, in combination with PBP3-based β-lactams (cefepime), have successfully targeted and inhibited MBL-producing bacteria.[81] However, bacteria are constantly evolving through resistance mechanisms (explained further in section 7), with NDM-1 and NDM-5 harbouring *E. coli* isolates, PBP-3 modifications have been observed, conferring resistance to the aztreonam-avibactam combination treatment.[80]

MBL activity largely depends on the presence of the metal zinc, with *in vivo* homeostasis studies indicating that intracellular and plasma zinc exists in a bound state, with no observations of free zinc being present.[82] However, the free zinc concentrations used in Mueller–Hinton broth (MHB) for microdilution AST are reportedly supraphysiological.[83] A study by Asempa *et al.*[84] found that all MBL-harboring pathogens producing NDM, IMP, and VIM MBLs were resistant to meropenem at concentrations of 16 to >64 mg L^{-1} when using cation-adjusted Mueller–Hinton broth (CAMHB). However, these MBL-producing pathogens were susceptible to meropenem (<0.06–0.5 mg L^{-1}) when tested with chelex or EDTA-treated MHB. In mice infected with MBL-pathogens in the thigh and lung, respectively, a 2.24 and 3 $\log_{10}$ decrease in the colony-forming units (CFU) was observed when utilizing meropenem monotherapy.[84] Thus, the precise resistance level may be overestimated with *in vitro* tests compared to the resistance observed within *in vivo* models.

According to Bilinskaya *et al.*,[85] another concerning factor in the AST of MBL-harboring pathogens is the complications of unspecified and variable zinc content among the different broth manufacturers and within lot batches of the same brand. This has led to an eight-fold difference in meropenem MIC among the commercial lots and has impacted the susceptibility categorization of MBL-producing bacteria.[85] The utilization of zinc-limited media, by adding EDTA/chelex

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to the prepared broth, offers a more thorough approach to MIC classification for β-lactamase-producing bacteria in the absence of molecular testing. However, there are still some practical challenges in quantifying free zinc and EDTA-bound zinc before AST, as inductively coupled plasma mass spectrometry (ICP-MS) – the current method of choice, was unable to differentiate between these two types of zinc available to the pathogen.[85–87] As a result, the efficacy of MBLIs under study will be compromised due to the extent of the EDTA/Chelex interference on susceptibility breakpoints. So far, there is no consensus on EDTA concentrations in such procedures, and the issues raised have not yet been settled.

A communication from Rennie[88] states that manufacturers necessitate zinc measurement to attain low zinc ion concentrations for carbapenem susceptibility testing. However, this letter does not suggest an acceptable reference range for the zinc ion concentration used in the CAMHB. Instead, it stipulates that at low levels, the zinc ion concentration will indefinitely vary by more than 0.1 or 0.2 mg L^{-1} and criticizes the use of EDTA to deplete zinc ions, as other cations could be negatively impacted. In response to Rennie's letter, Asempa and Nicolau,[89] stated unawareness of any such requirement and argued on what is regarded as "low zinc concentrations". During the study, unsuccessful communication attempts with the respective MHB manufacturers prevented data validation, with each representative citing proprietary reasons. This observation implied potential zinc sequestration and/or supplementation in manufacturing. Asempa and Nicolau advocate for open access to these methods and the dissemination of zinc target requirements in CLSI standards and broth container labels. This is recommended to guide quality control efforts and investigational studies utilizing zinc ions to achieve performance standards.

Aligning to these concerns, the quantity of zinc in the broth directly affects MICs, which may be exaggerated for MBLIs such as **3**,[29] **4**,[30] **6**,[32] and **11**[39] that function by an unclear irreversible binding mechanism that will hamper the MBLI's full potency. Many laboratories do not have access to ICP-MS instrumentation used to quantify the zinc content pre- and post-addition of EDTA/Chelex in the broth. Therefore, it is unknown if **3**,[29] **4**,[30] **6**,[32] and **11**[39] partially bind to the zinc in the broth before encountering the MBL pathogen. Given the present challenges with the varying zinc content of CAMHB, as well as the mitigation of zinc-ion interference by the addition of EDTA, it is recommended that the effect on other cations, such as iron, be also considered. The inhibitor **6** exhibits irreversible inhibition, and it is essential to note that the mechanism behind this process is not yet fully understood.[32] Nonetheless, multiple studies have suggested that chelating agents enhance the susceptibility of active-site amino acids to chemical modifications, such as the oxidation of Cys.[90–92]

Compounds **1**–**11** (Fig. 2) have all undergone AST using the broth microdilution technique in combination with meropenem against one or more MBLs. The meropenem MIC can vary depending on the amount of inhibitory compound used. The general reporting of MIC is done in mg L^{-1} units; however, **6**[39] (Fig. 2) was reported in micromolar units, which considers the molar mass of the compound, which researchers do not always disclose. This presents an extra step before direct comparisons to other MBLIs can be made. It is also important to note that not all compounds were assessed for synergy with the partnering β-lactam. Compounds **3**,[29] **5**,[31] **8**,[36] and **11**[39] were the only MBLIs to incorporate the checkerboard assay with the FICI criteria for synergy assessment. The checkerboard assay involves studying a range of MBLI concentrations, and based on the produced activity, the researcher can select an MBLI-antibiotic combination, with the lowest concentration that produces effective inhibition. Utilizing a very high concentration of the MBLI may prove to be toxic, and detrimental to further biological evaluation. Increasing or decreasing the MBLI concentration directly impacts the antibiotic's MIC and the relationship shared between the two compounds. Hence this AST method is an excellent screening assay for MBLI/antibiotic compatibility.

The EUCAST interpretative criteria were used for compounds **1**,[11–13] **3**,[29] **4**,[30] **6**,[32] and the CLSI guidelines were used for **2**,[14–17] **5**,[31] **8**,[36] **9**,[37] whilst **7**,[33–35] **10**,[38] and **11**[39] utilized both guidelines (Table 3). Testing guidelines should list an approved number of isogenic MBL strains that are widely accessible and well-characterized for MIC testing. This will facilitate cross-comparisons of MBLIs, as MICs are species-specific and can be influenced by the presence of other β-lactamases (in clinical strains) when tested with the selected antibacterial agent.

5. Limited toxicity and pharmacokinetic data

5.1 Toxicity and drug safety

Many MBLIs have undergone toxicity testing and pharmacokinetic analysis in pre-clinical research. However, there is no consensus on how it is conducted, which cell line(s) are used for cytotoxicity, the protocol used to determine cytotoxicity, and the pharmacological parameters evaluated. Frequently, the choice of the cell line for cytotoxicity studies or the selection of software and equipment for pharmacokinetics assessment is dictated by the resources available within the laboratory. This underscores the critical importance of adequate infrastructure and accessibility in certain countries. For instance, some of the most commonly used cell lines for evaluating the cytotoxicity of a candidate MBLI (Table 4) are HeLa cells (the first human cancer cells used),[11,31] human embryonic kidney cells,[30] human hepatoma,[32,34,37–39] and red blood cells.[36] Human hepatoma cells (HepG2) were the most common cell line used among **6**,[32] **7**,[33–35] **9**,[37] **10**,[38] **11**.[39] However, **1**[11–13] and **4**[30] were assessed against three cell lines, which is important to identify possible discrepancies; in these cases, there were none. The frequently utilized laboratory tests include hemolysis, the 3(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

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Table 3 MICs, assays, and the interpretative criteria used to study the pre-clinical activity of MBLIs **3**–**11**, (**1** and **2** have progressed to the clinical development stage, with **1** undergoing pre-registration)

Compound	Clinical/reference/engineered strains	MBLI MIC(MBC): mg L^{-1}	Meropenem MIC(MBC): mg L^{-1}	Interpretive criteria and assay(s) used
1	*Klebsiella pneumoniae* VIM-4	4	0.5	EUCAST broth microdilution method
(ref. 11–13)	*Pseudomonas aeruginosa* VIM-2	4	0.06	and time-kill assay
	K. pneumoniae VIM-27 (CDC-0040)	4	4	
	Escherichia coli NDM (CDC-0452/CDC-0055)	4	0.125–2	
	P. aeruginosa VIM (CDC-0457)	4	0.125	
	K. pneumoniae NDM CDC-0049	(4)	[a](4)	
2	*K. pneumoniae* IMP-26 (KP1160)	2.5	1	CSLI broth microdilution method
(ref. 14–17)	*K. pneumoniae* NDM-1 (KP1081)	0.6	[b]1	
	K. pneumoniae VIM-1 (KP1054)	0.16	[b]1	
3 (ref. 29)	*E. coli* NDM-1	32(64)	2(24)	EUCAST broth microdilution method
	K. pneumoniae NDM-1	32	4	(checkerboard- with FICI calculation),
	Citrobacter freundii NDM-1	32	0.5	and the time-kill assay
	E. coli BL21(DE3, pET-28a-VIM-2)	32	0.5	
	E. coli BL21(DE3, pET-28a-IMP-4)	32	4	
4 (ref. 30)	*E. coli* NDM-1	16	8	EUCAST broth microdilution method
	K. pneumoniae NDM-1	32	32	with FICI calculation
	Sample ID 2470	32	64	
	Sample ID2472			
5 (ref. 31)	*E. coli* IMP-1 NUBL-24	(10)	(1)	CSLI broth microdilution method
	P. aeruginosa			(checkerboard – with FICI calculation),
	PAO1/pME-IMP-1	50	16	and the time-kill assay
	PAO1/pME-NDM-1	10	4	
	PAO1/pME-VIM-2	50	16	
	E. coli			
	DH5α/pBC-IMP-1	8	0.031	
	DH5α/pBC-NDM-1	32	≤0.25	
	BL21(DE3)/pET-SPM-1	8	0.063	
6 (ref. 32)	*K. pneumoniae* NDM-1 (K66-45)	50 μM(50 μM)	0.125(4)	EUCAST broth microdilution method
	P. aeruginosa VIM-2 (K34-7)	50 μM	0.125	and the time-kill assay
	E. coli NDM-1/5	50 μM	0.06–2	
	E. coli IMP	50 μM	≤0.125	
7	*K. pneumoniae* NDM	8	<0.06–32	CLSI + EUCAST broth microdilution method
(ref. 33–35)	*Enterobacter cloacae* NDM	8	<0.06–8	
	E. coli NDM	8	<0.06–4	
8 (ref. 36)	*E. coli*-DH5a/pUC-NDM-1	16	<0.03	CLSI agar disc diffusion, broth microdilution
	E. coli NDM-1/3/5	16	<0.03–0.5	method (checkerboard- with FICI calculation),
	E. coli IMP-4	16(16)	<0.03–0.25(0.25)	and the time-kill assay
	E. cloacae NDM-1/5	16	<0.03–1	
	K. pneumoniae IMP-4	16	0.125–2	
	K. pneumoniae NDM-1	16(16)	<0.03–0.125(0.25)	
	P. aeruginosa VIM-1	16(16)	0.25(1)	
9 (ref. 37)	*K. pneumoniae* NDM-1	4	0.25–4	CLSI broth microdilution method and the
	E. coli NDM-1:			time-kill assay
	BAA-2452	4(4)	0.03(0.12)	
	BAA-2469	4	0.06	
	E. coli IMP	4	0.03	
	K. pneumoniae VIM-1	4	0.06	
10 (ref. 38)	*Acinetobacter baumanii* NDM (76885-C)	8	2	CLSI + EUCAST broth microdilution method
	A. baumanii NDM-1 (76030-E-G, CH3504, S7–29)	8	2–8	(checkerboard), and the agar dilution assay
	E. coli NDM (55 N/B53)	8	0.25	
	K. pneumoniae NDM (86259, 48F, I39, IR18, 76664-G)	8	0.125–0.5	
	C. freundii NDM-1 (84646-E-B, 85524-E-Pi, 85558-E-Pi, 85569-E-Pi)	8	0.125–0.5	
11 (ref. 39)	*E. coli* NDM-1/4	16	0.25	CLSI + EUCAST broth microdilution method
	E. cloacae NDM-1	16	0.5	(checkerboard- with FICI calculation) and the
	K. pneumoniae NDM	16(32)	0.125	time-kill assay
	E. coli IMP-1	8	0.5(2)	
	E. cloacae VIM-1	32	0.03	
			0.5	

Bacterial inhibition was achieved by co-administering meropenem with an MBLI (**1**–**11**). CLSI = Clinical Laboratory Standards Institute, EUCAST = European Committee on Antimicrobial Susceptibility Testing, FICI = fractional inhibitory concentration index, MBC = minimum bactericidal concentration, shown in brackets and obtained from the time-kill assay. [a] Cefepime is used instead of meropenem. [b] Biapenem is used instead of meropenem. Note compound **11** is a β-lactam-derived MBLI and possesses antimicrobial activity when used at concentrations >64 mg L^{-1}.

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Table 4 Pre-clinical evaluation of the safety and tolerability of **3**–**11** utilizing cell lines and/or animal studies (**1** and **2** have progressed to the clinical development stage, with **1** undergoing pre-registration)

Compound	Cell line/animal used	Assay conducted	Result
1 (ref. 11–13)	HeLa, MRC-5, 3T3	Cell viability – MTT	IC_{50} = 256 μM
	Human primary renal proximal tubule cells	Toxicity assessments	Non-toxic up to 1000 μg mL^{-1}
2 (ref. 14–17)	Rodents	Acute toxicity	No change in tissue histology or clinical chemistry up to 300 mg kg^{-1}
3 (ref. 29)	N/A	N/A	Low toxicity, repurposed drug that is in clinical use
4 (ref. 30)	HUVEC, LO2, HEK293 cells	CCK-8 assay, LDH release assay	IC_{50} > 200 μM
	ICR mice	Acute toxicity	LD_{50} = 350.1 mg kg^{-1}
5 (ref. 11–31)	HeLa cells	CellTiter 96 AQueous one solution cell proliferation assay	IC_{50} > 100 μM
	CD1 mice	Acute toxicity	LD_{50} = 246 mg kg^{-1}, i.v. LD_{50} > 1000 mg kg^{-1}, i.p.
6 (ref. 32)	HepG2 cells	AlamarBlue cell viability assay	IC_{50} > 100 μM
	Balb/c mice	Acute toxicity	Up to 252 mg kg^{-1}, s.c.
7 (ref. 33–35)	HepG2 cells	Cell viability	IC_{50} > 100 μM
8 (ref. 36)	Red blood cells	Hemolysis	HC_{50} > 1024 μg mL^{-1}, <7% hemolysis
	Balb/c mice	Acute toxicity	Nontoxic up to 50 mg kg^{-1}, i.p.
9 (ref. 37)	HepG2 cells	CellTiter-GloV viability assay	CC_{50} > 256 mg L^{-1}
10 (ref. 38)	HepG2 cells	Cell viability	IC_{50} > 64 μM
	NMRI mice	Acute toxicity	Up to 300 mg kg^{-1}, s.c.
11 (ref. 39)	HepG2 cells	LDH and MTT assays	IC_{50} > 1000 μg mL^{-1}

N/A = not applicable, HepG2 = hepatoblastoma, HeLa = cervical cancer, MRC-5 = human fetal lung fibroblast, 3T3 = mouse fibroblast, HUVEC = umbilical vein endothelial cells, LO2 = HeLa derivative, and HEK293 = human embryonic kidney. CCK-8 = cell counting kit assay, LDH = lactate dehydrogenase assay, MTT = cell metabolic activity, alamarBlue = nontoxic alternative to MTT assay. IC_{50} = half the maximum inhibitory concentration, LD_{50} = half the maximal lethal dose, HC_{50} = the concentration needed to cause 50% hemolysis of human red blood cells, CC_{50} = 50% cytotoxic concentration.

colorimetric dye-reduction assay, the lactate dehydrogenase assay,[39] and the Cell Titer-Blue assay[31,37,93] to measure the effects on cell viability with and without the MBLI. This indicates that various assays and commercially available assay kits are available to the researcher; hence, the results generated will be validated for the specific assay used and cannot be extrapolated to other cell lines or assay procedures. Hence, it emphasizes the significance of a standardized test, or an assay kit designed to incorporate the commonly used assays and cell lines, that are accessible, cost-effective and allows for cross-comparisons among MBLIs in toxicity assessment.

5.2 Pharmacokinetics and the absorption, distribution, metabolism, and excretion (ADME) parameters

The standard pharmacodynamic (PD) index for β-lactam antibiotics is T > MIC, which represents the time that the drug concentration exceeds the MIC.[94] For β-lactamase inhibitors, maintaining a threshold concentration is often essential for efficacy. Pharmacokinetics (PK) plays a crucial role in drug discovery research, as compounds that may not be ideal therapeutics based on *in vitro* models can exhibit good absorption and distribution *in vivo*. This becomes even more important with the co-administration of a β-lactamase inhibitor and β-lactam antibiotic (combination therapy) since the inhibitor can restore the efficacy of the β-lactam. The efficacy of this combination therapy relies heavily on the PK and PD properties of both agents. PK parameters typically assessed are the peak drug concentration(C_{max}), the time taken to reach peak concentration (T_{max}), the terminal half-life ($T_{1/2}$), the area under the curve (AUC), and clearance (CL) of the drug.[95]

T_{max} is a critical PK parameter indicating the time it takes for each drug to reach its highest concentration in the bloodstream. For combination therapy to be effective, the T_{max} of the β-lactamase inhibitor should ideally align with that of the β-lactam antibiotic. This synchronization ensures that both agents are present at therapeutic levels simultaneously, maximizing their synergistic potential against bacteria. The MIC, a fundamental PD parameter, defines the lowest concentration of an antibiotic required to inhibit the visible growth of a microorganism. Achieving and maintaining drug concentrations above the MIC for a sufficient dosing/treatment interval (T > MIC) is vital to the antimicrobial efficacy of β-lactam antibiotics.

Optimizing therapeutic outcomes involves correlating T_{max} and MIC values. This can be done by adjusting the dosing schedule to incorporate frequent doses of the β-lactamase inhibitor, or by using continuous infusions of the β-lactam/BLI (in a larger animal model), to maintain T > MIC. This alignment allows both agents to exert their maximal inhibitory effects simultaneously, thereby enhancing the overall success of the treatment.

The PK/PD profile of multiple β-lactams in clinical practice has been examined extensively; however, insufficient

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Table 5 A summary of the pre-clinical *in vivo* study design and outcomes for compounds **1**–**11** (noting that **1** and **2** were tested on non-MBL strains)

Compound	Animal model used	Bacteria used for infection	Administration route of drug	Dosage (mg kg^{-1})	$\log_{10}$ decrease in CFU
1 (ref. 11–13)	Murine, lung	*K. pneumoniae* CTX-M-14	s.c.	16	>4
	Murine, ascending urinary tract	*E. coli* CTX-M-15	s.c.	8	>2
2 (ref. 14–17)	Neutropenic murine, thigh	*K. pneumoniae* KP1244	i.p.	50	>2
3 (ref. 29)	Murine, peritonitis	*E. coli* NDM-HK	i.p.	10	ND[a]
4 (ref. 30)	Murine, acute toxicity	N/A	i.p. and i.v.	50–400	N/A
5 (ref. 31)	Murine, systemic	*E. coli* IMP-1	i.p.	100	ND[a]
6 (ref. 32)	Neutropenic murine, peritonitis	*K. pneumoniae* NDM-1	s.c.	10	>3
7 (ref. 33–35)	Neutropenic murine, thigh	Multiple NDM *Enterobacterales*	s.c.	160	3
8 (ref. 36)	Murine, sepsis	*K. pneumoniae* NDM-1	i.p.	10	≥3 (liver and kidney), ≥2 (spleen)
9 (ref. 37)	Murine, thigh	*E. coli* NDM-1	s.c.	100	>3 (vehicle), >1 (meropenem monotherapy)
10 (ref. 38)[b]	Murine, peritonitis	*E. coli* ATCC 25922 ISAba 125 bla_{NDM-7}	i.v.	10 or 30	>5
	Murine, thigh		i.v.	10 or 30	2
11 (ref. 39)	Neutropenic murine, thigh	*K. pneumoniae* NDM	i.p.	100	>3

s.c. = subcutaneous, i.p. = intraperitoneal, i.v. = intravenously, NA = not applicable, ND = not determined. [a] Denotes that the survival rate of mice was assessed instead of a $\log_{10}$ decrease in the bacterial CFU. A survival rate of 50% was achieved for **3**, 87.5% (i.p. 400 mg kg^{-1} and i.v. 300 mg kg^{-1}) or 100% (i.p. 300 mg kg^{-1} and i.v. 200 mg kg^{-1}) for **4**, and 100% for **5**. [b] Multiple *in vivo* studies done on **10**, varying the dose, pathogen/strain and animal model infection site *i.e.* utilization of either peritonitis/septicemia infection model or thigh infection model.

information exists for the PK/PD profile of MBLIs.[96] For the past 20 years, dosing regimens have been optimized using time-dependent PK/PD,[97] which has been enforced on MBLIs, although data are scarce.[96] Thus far, PK data has only been made available for MBLIs (broad-spectrum BLIs) **1**[11–13,98] and **2**,[14–17,99] which have reached clinical trials.

There are very few studies, such as those by Yan *et al.*,[100] that conduct acute toxicity testing of MBLIs in animals. However, from the eleven compounds in Table 5; **2**,[14–17] **4**,[30] **5**,[31] **6**,[39] **8**,[36] and **10**[38] incorporated this test in the evaluation process. This test is important to determine if there are any side effects of the candidate MBLI from multiple doses or a single dose monitored over time. It can also be a cost-efficient way to prevent further development of the MBLI if found to be toxic *in vivo*.

To guarantee the integrity of the generated bioanalytical data, the method validation providing the PK and ADME parameters of the MBLIs needs to conform to acceptance standards as part of the evaluation criteria. For example, in regulatory studies, the guidelines outlined by the European Medicines Agency (EMA) documents[101] are utilized in facilities accredited for good laboratory practices (GLP). In contrast, non-regulatory studies do not adhere to such stringent practices as it is not a requirement to advance the research. However, implementing a single guideline for researchers conducting non-regulatory studies will help promote accurate results and eliminate discrepancies among candidate MBLIs.

6. Restricted animal models with variable pre-clinical study designs

Animal models are important tools used in pre-clinical research to bridge the gap between *in vitro* efficacy and clinical trials of MBLI candidates.[102,103] They are utilized to assess the MIC generated by phenotypic tests, establish dosing regimens, and determine pharmacokinetic and pharmacodynamic (PK/PD) parameters.[102,104] Fig. 3 summarizes an evaluation report of *in vivo* efficacies published between the years 2000 and 2020.[105]

6.1 Types of animal models used in MBLI efficacy studies

The thigh murine infection model was most frequently used in the evaluation report, with only a single study utilizing a rabbit model (Fig. 5). The neutropenic model (61%) was preferred over the immunocompetent model (39%)[105] because it allowed for the infection to be established successfully without interference from the host's immune system and promoted the undisturbed evaluation of the antimicrobial agent against the target pathogen.[106] The neutropenic murine thigh infection model was also a popular animal model utilized among compounds **2**,[14–17] **7**,[33–35] **9**,[37] **11**[39] in Table 3. Although the remaining compounds experimented with using different models, all incorporated mice as the animal of choice to study. The use of mouse models as the standard choice for efficacy studies can provide researchers with a foundational basis for designing their MBLI studies.

6.2 Pathogen selection and inoculation

Apart from five studies[107–111] in the evaluation report (Fig. 5), all animal trials established an *Enterobacterale* NDM-1 infection model with either clinical *Klebsiella pneumoniae* or *Escherichia coli*. However, such studies should not be limited to NDM-1 since the global prevalence of MBLs also includes VIM and IMP in significant numbers.[112]

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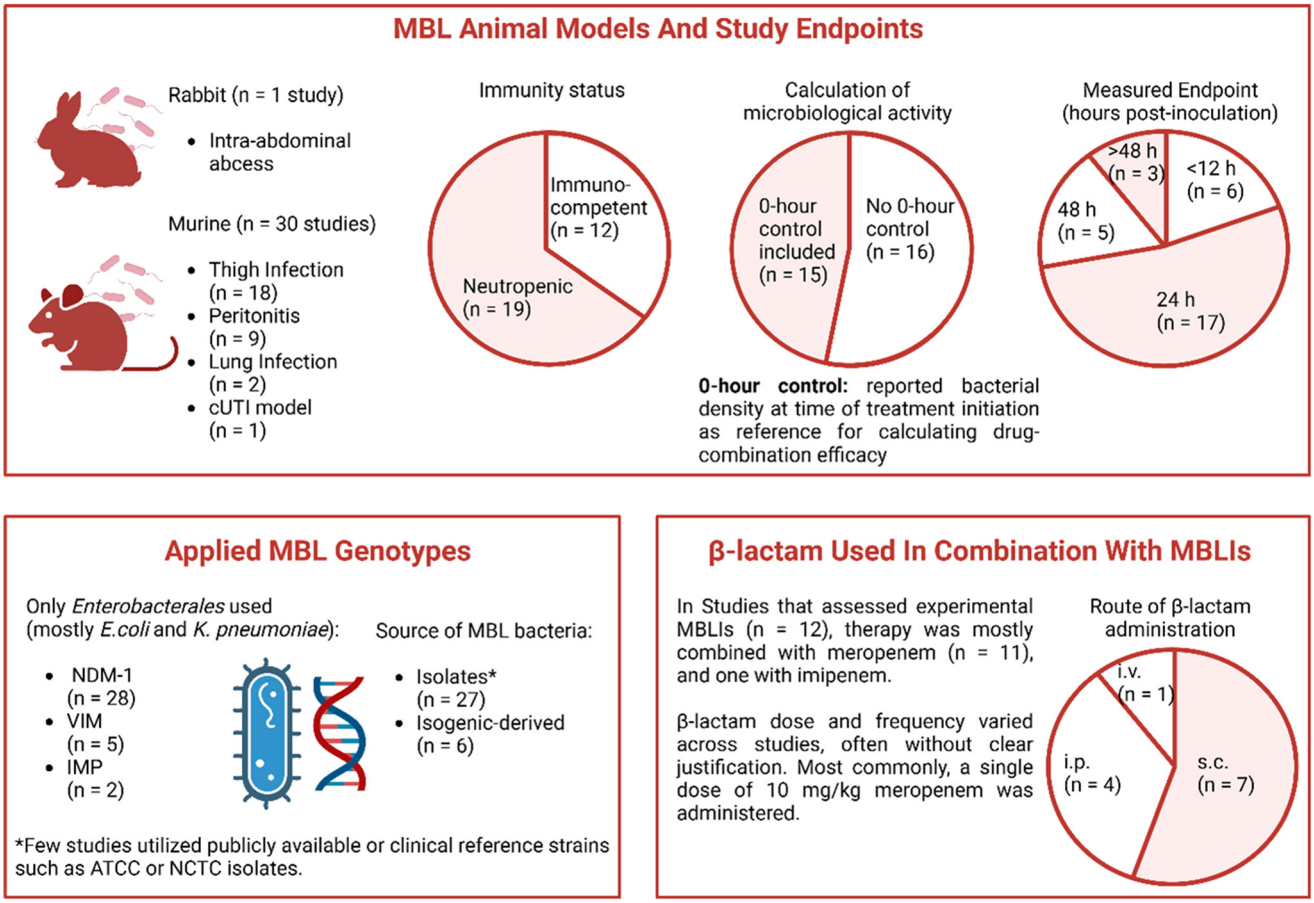

Fig. 5 Animal model variabilities amongst 31 studies (from 27 publications) were used to study β-lactam and MBLI efficacy against MBL-harbouring bacterial genotypes between 2000–2020. Of these, 12 animal studies assessed the efficacy of novel experimental MBLIs. Image adapted from data provided by Asempa T. E. *et al.* (2021).[105] Created with **https://Biorender.com**.

Moreover, the evolution of MBL variants and their respective zinc sensitivities suggests that a wide collection of genotypes should be investigated when determining MBL efficacy *in vivo*.[85,113,114]

An implementation that will improve the CFU data extracted from animal trials is the use of a standardized inoculum density, such as the 0.5 McFarland standard. Since a higher bacterial inoculum density will influence the treatment outcomes of the drug in neutropenic animal models, infecting with fewer bacteria may not lead to infection in immunocompetent hosts. Therefore, when incorporating the McFarland standard into the study design, researchers will need to plate an aliquot of the bacterial suspension. This will be done to determine the exact number of bacteria used to infect the animal.

Many of the reviewed studies in the evaluation report (Fig. 5) did not include CFU counts from 0 hours post-inoculation, such as an imipenem/ertapenem study using an engineered *E. coli* NDM-1 strain,[115] and it was excluded, thereby raising concerns over the bacteriostatic effects produced and the effects of *in vitro–in vivo* inoculum density.[116] Another concern with engineered MBL strains is the failure to establish infection in a mouse model, hindering the evaluation process of the MBLI.[100] Therefore, genetically well-characterized bacterial strains harboring multiple MBLs should be deposited into commercial biobanks and made accessible to researchers from diverse ethnic and social backgrounds.

6.3 Antibiotic administration and dosage

Selecting the appropriate β-lactam antibiotic and dosage to co-administer with an MBLI is another concern in the design of animal models. Meropenem is the most frequently utilized β-lactam, often administered at a dose of 10 mg per kg body weight and provides stability when co-administered with a developing inhibitor (Fig. 5). This is further evidenced by all inhibitory compounds in Fig. 2 (**1–11**), utilizing meropenem as the partnering β-lactam antibiotic. However, the clearance rate requires administration every two hours, and this poses a technical challenge for its use in mouse models. While studies have compensated for this rapid clearance,[37,39] injecting the animal repeatedly over the advised 24 hour study period is impractical.[117]

Although monitoring an animal for >24 hours would provide in-depth efficacy data, such scrupulous studies are

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almost impossible to design. As they require larger research labs, more lab personnel, and are governed by animal welfare and ethical restrictions. Another concern reported by Moya *et al.*[118] were failed murine infection models amongst four NDM-harboring isolates designed using a humanized meropenem regimen. These findings indicate that meropenem may not be the ideal β-lactam antibiotic for pre-clinical animal study assessment and necessitates the inclusion of a meropenem monotherapy treatment arm.

When designing an animal model, many administration routes can be used depending on the preference of the injector, the infection site, and the PK/PD properties of the drug. The most common administration routes include intraperitoneal (i.p.), sub-cutaneous (s.c.), and intravenous (i.v.).[105] In Table 5, most of the compounds employed either the i.p. (**2**,[14–17] **3**,[29] **5**,[31] **8**,[36] **11**[39]) route or the s.c. route (**1**,[11–13] **6**,[32] **7**,[33–35] **9**[37]), with only **10**[38] employing the i.v. route and **4**[30] utilizing both i.v. and i.p. routes. The concern is that the results generated depend on the administration route used. Studies by Ooi *et al.*,[37] and Everett *et al.*,[119] demonstrated that utilizing i.v. and s.c. administration routes with identical meropenem dosing regimens produced varying degrees of efficacy in an *E. coli* NDM infection model.

These findings were in accordance with **4**,[30] as an identical dose led to one mouse dying using the i.p. route and seven mice dying with the i.v. route, thus highlighting inconsistencies based on the choice of administration route used. The choice between i.v. and i.p. routes carries significant implications for exposure, pharmacokinetics (including potential drug–drug interactions), and acute toxicity. It is acknowledged that the exposure profile and PK parameters can vary between these routes, with acute toxicity, particularly concerning effects on the heart, being a critical consideration.[120,121] This suggests that acute toxicity, such as cardiac effects leading to fatalities in mice, may result from high maximum plasma concentration associated with i.v. administration. However, it is proposed that such toxicity could potentially be avoided with i.p. administration. Despite this, the recommendation is not an outright preference for i.p. administration, as i.v. administration is typically the chosen route in clinical settings. We would like to emphasize a nuanced approach: rather than deciding solely based on the route that appears to yield a more promising result, researchers of non-GLP studies should delve deeper into understanding the specific factors driving toxicity for each compound.

6.4 Determination of MBLI efficacy in animal studies

Another trend observed is that there is an *in vitro–in vivo* discordance among the generated efficacies (explained previously in 4.2, in greater detail). In an animal model, bacteria could also fail to express or lose MBL-harbored plasmids.[122,123] Therefore, a β-lactam monotherapy treatment arm should be included in animal trials to account for this finding of pharmacodynamic susceptibility.[124,125] While animal models provide a great amount of information regarding the therapeutic efficacy of the drug and guide approval applications by regulatory organizations,[126,127] until the shortcomings mentioned above are addressed, it is advisable to exercise prudence in drawing *in vivo*-related conclusions when evaluating MBLI.

7. Challenges in overcoming resistance mechanisms

Bacteria may possess intrinsic resistance to β-lactams expressed by chromosomal genes, or they may acquire resistance from mobile genetic elements such as plasmids.[128] Acquired resistance is associated with producing multi-drug resistant bacterial strains, as other antibiotic resistance determinants are also carried on plasmids and may code for resistance to other classes of antimicrobials.[129,130] For instance, the bla_{NDM} gene also carries resistance to aminoglycosides since they are linked to ArmA and RmtB methyltransferase genes. While bla_{IMP} and bla_{VIM} genes are generally found on integrons that also have aac(6′) genes that code for acetyltransferase expression, which offers resistance to amikacin and tobramycin.[131] Antimicrobial agents targeting the bacterial cell wall have a higher chance of reaching their target than antibiotics targeting the cytoplasm.[1] Therefore, β-lactams are a popular treatment choice among clinicians.

Nonetheless, there are many challenges to consider when examining β-lactam resistance, unlike other soluble MBLs in the periplasm, NDM is a lipoprotein in the outer membrane vesicles.[132] Recent observations indicate that outer membrane vesicles protect the MBL and facilitate the transfer of the bla_{NDM-1} gene in carbapenem-resistant *K. pneumoniae* to other strains of *K. pneumoniae*.[133] Other reports of NDM-1 expression convey that a fitness cost is not exerted on the bacteria in *P. aeruginosa*, *E. coli*, or *A. baumannii*. Expression of MBLs such as VIM-2 and Sãu Paulo metallo-β-lactamase 1 (SPM-1), are well tolerated in *P. aeruginosa*, but poorly tolerated when the same genes encoding for VIM-2 and SPM-1 are expressed in *E. coli* or *A. baumannii*. This indicates that MBL expression and compatibility may be species-specific.[134]

Since 2009, NDM-1 has undergone rapid evolution, as evidenced by the many existing variants.[135,136] NDM-4, first detected in 2012,[137] is one such variant that has gained importance. NDM-4 differs from NDM-1 by a single amino acid substitution (M154L), which increases the hydrolysis of carbapenems and cephalosporins,[138] leading to increased pathogenicity in bacteria harboring the bla_{NDM-4} gene. Almost half of all NDM variants have the M154L amino acid substitution, among other amino acid substitutions.[68] Consequently, the MBL inhibitor design process should consider targeting the M154L-containing gene in addition to other mechanisms of resistance.

Although similar activity is produced *in vitro*, NDM-1 is more prevalent than IMP-1.[2] This is because a strongly conserved promoter (bla_{NDM-1} gene occurs downstream to the ISAba125 insertion sequence) facilitates higher NDM-1 expression.[139] Additionally, the gene can frequently switch between the

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bacterial host genome and plasmid, as evidenced in many species.[140] While IMP-1 is downstream-regulated, it has a variable promoter with differing strengths, leading to weaker resistance.[139] For example, in *E. coli*, the binding of a protein upstream from the bla_{IMP-6} gene led to transcriptional repression of the gene, resulting in susceptibility to imipenem and meropenem,[141] and therefore did not require the use of an MBLI. Upon inspection of the MICs of **1–11**, clear observations can be noted of NDM-expressing bacterial strains undergoing more frequent assays, as compared to VIM or IMP harborants. This is often done because NDM variants possess higher MICs, contributing to the pathogens' virulence and, thus, treatment regimen. This further emphasizes the need for a better understanding of the various resistance mechanisms at play on a cellular level.

Gram-negative bacteria are intrinsically more resistant due to an outer membrane that functions as a permeability barrier to antimicrobial substances targeting the bacterial cell's periplasm and inner plasma membrane.[142] Therefore, they mainly constitute the list of WHO's priority pathogens.[143] The production of multidrug efflux pumps that can expel different antimicrobials out of the bacterial cell should not be ignored during the process of MBLI inhibitor synthesis. When efflux pumps are overexpressed in MBL-harboring pathogens, a significant amount of resistance is conferred to typically effective antibiotics.[144] This can pose an enormous setback in MBLI development.

Other mechanisms of escaping the potent effects of antimicrobials include persistence and tolerance, which the scientific community has significantly underestimated.[145] Bacterial cells that are "persisters" are dormant bacteria that can withstand the effects of antimicrobials without affecting the MIC and the host's immune response and are responsible for recurrent infections. Uropathogenic *E. coli* and some *P. aeruginosa* variants are persisters,[145] and they also express MBLs. Consequently, when assessing MBLI potency with antimicrobial susceptibility assays, it is crucial to also incorporate diagnostic methods like molecular assays to confirm the results.

The sharp incline in MBL resistance rates, specifically with NDM, is primarily attributed to poor hygiene practices,[146] subpar medical facilities, over-prescription of antimicrobials,[147] underlying health conditions, bacterial resistance strategies,[148] WHO priority pathogens,[7,149] asymptomatic carriage,[139] and environmental exposures and reservoirs.[146] Although measures are in place to address these confounders, implementing strategies on a national scale with involved stakeholders may help alleviate resistance, persistence, and tolerance to antimicrobial chemotherapy, ultimately promoting the better development of MBL inhibitory compounds.

8. Artificial intelligence influence over drug discovery in AMR

Artificial intelligence (AI), is a field of computer science that mimics human intelligence,[150] offers innovative approaches to accelerate scientific discoveries. This is common in medicinal chemistry, where AI has enabled the discovery of new drugs and expedited the overall process of drug development and clinical investigation. AI has become a central component of interdisciplinary efforts aimed at addressing the AMR crisis[151–153] as the scale of biological data grows, various AI-driven methods have emerged to analyse it. AI technology enables computers to learn and improve automatically without explicit programming and to build and predict models using available data. The methodological domain of AI primarily involves reasoning, knowledge representation, solution search, and machine learning (ML).[154]

Deep learning (DL), a subset of ML, involves neural networks that mimic the brain's structure to recognise and differentiate between patterns of language, imagery, and various biological data types.[155,156] DL-related algorithms have advanced rapidly in recent years to include several typical algorithms; convolutional neural networks, recurrent neural networks, deep reinforcement learning, and generative adversarial networks (GANs).[157] These are unsupervised learning algorithms that have been extensively applied in various fields of drug discovery.[158–160]

Recently, Ding *et al.* demonstrated that AI can enhance the accuracy and efficiency of BL detection.[161] Traditional methods often struggle due to environmental factors such as variations in temperature and pH, which can lead to unreliable results. AI-assisted systems, including smartphone-based AI clouds, can automatically correct these errors, intelligently analyze data, and provide real-time outcomes. Integrating AI with fluorogenic probes and microfluidic devices facilitates the detection of rapid, low-cost, and highly sensitive BLs. This advancement is essential for the early identification of resistant bacteria, which aids in infection control and improves antibiotic treatment options. This method could also be applied to MBLs.

AI technology, therefore should not be ignored in drug discovery for combating AMR.[162–166] For instance, data-driven methods can predict new antibiotic compounds, while image-based methods can aid in identifying resistant bacteria.[167] AI-assisted compound library screening or the novel design of compound structures can help rapidly identify the most promising antimicrobial compounds. Additionally, AI can leverage known data, such as genomic information, to predict potential resistance sites and related enzymatic functions, laying the groundwork for the design of better antibiotics.[168] Moreover, AI has facilitated target identification and dynamic modeling, the design and synthesis of peptides, the evaluation of structure–activity and structure-toxicity relationships, and drug repurposing.[169] In this context, an ML model has recently identified a novel NDM-1 inhibitor,[170] which significantly reduced the MIC of Meropenem against a panel of *E. coli* and *K. pneumoniae* clinical isolates expressing NDM-1, highlighting its potential in combating antibiotic resistance.

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9. Outlook and conclusion

AMR poses a significant threat to global public health, with projections suggesting it could cause 10 million deaths annually by 2050, primarily due to infections affecting the respiratory and gastrointestinal tracts.[171] The development of MBLIs to restore the activity of existing antibiotics is a critical advancement in the fight against AMR. As drug-resistant bacteria continue to render many antibiotics ineffective, these inhibitors target enzymes that degrade β-lactam antibiotics. By revitalizing the efficacy of these essential drugs, MBLIs can significantly reduce the need for new antibiotic development, mitigate the spread of resistant infections, and safeguard public health. This approach provides an immediate and cost-effective strategy for saving lives and preserving the effectiveness of our antibiotic arsenal. However, there are still relatively few compounds that have shown significant promise, highlighting the need for further development of MBLIs. Among recent advancements, compound **1** is progressing toward clinical registration as a treatment targeting MBL-expressing bacteria, though resistance to some NDM variants underscores the ongoing challenges in this field.[172] Adjuvant **2** shows considerable promise as an ultra-broad-spectrum inhibitor of serine SBLs and MBLs, effectively evading resistance mechanisms such as efflux pump expression and porin structural modifications.[28]

A critical aspect of advancing MBLI development lies in standardizing methodologies to facilitate comparison and reproducibility. Reliable assays for evaluating inhibitors' efficacy and specificity are necessary to expedite progress. A unified MIC breakpoint guideline could address discrepancies in assessing antibiotic efficacy. Moreover, zinc content quantification in testing media should become standard practice, with widely available protocols to ensure consistency. Incorporating EDTA/Chelex protocols and emphasizing enzyme inhibition potency through K_i values, IC_{50} during early enzymatic screening, and EC_{50} during cellular efficacy testing in the AST guidelines would enhance optimisation. For covalent (reversible and irreversible) MBL inhibitors, the full inhibition mechanism, including non-covalent interactions that promote zinc removal must be considered, supporting the view that higher K_i values do not necessarily indicate reduced compound efficacy. The development of low-cost MBLI enzyme inhibition kits, standardized protocols, and globally accessible materials would not only foster data comparability but also enable the integration of machine-learning models in inhibitor design.[173]

Pre-clinical research must also focus on addressing bacterial resistance mechanisms at multiple levels. Ethical and reproducible animal models are essential for assessing efficacy while mimicking β-lactamase-mediated resistance. Comprehensive pharmacokinetic data, toxicology studies, and optimized dosing strategies, including C_{max} and $T >$ MIC parameters, are critical to ensuring the clinical success of combination therapies. Cellular-level research and multifaceted approaches to bacterial phenotypes, β-lactamase activity, and B1 MBL sequence homology are also imperative to inform inhibitor development.

Emerging computational techniques, supported by artificial intelligence, offer significant potential in early-stage MBLI research. Methods such as ligand-based pharmacophore modeling, structure-based virtual screening, and molecular docking can streamline the initial stages of *in silico* assessment.[174–176] Whether employing classical or quantum methods, researchers must carefully account for factors such as water molecule coordination in the Zn^{2+} ion active site to refine inhibition strategies.

To achieve meaningful progress in the fight against AMR, global collaboration is essential. Standardized approaches in MBLI development, supported by robust financial investments from organizations such as the Wellcome Trust, Gates Foundation, and CarbX, could enable a "Moonshot" initiative against superbugs. This vision would involve distributing standardized kits, establishing centralized data repositories, and implementing clear pre-clinical frameworks for decision-making. By aligning resources, uniform research methodologies, and stakeholder priorities, MBLIs can move closer to developing effective therapeutic strategies to combat resistant pathogens and help mitigate the AMR crisis.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

Author contributions

NR was responsible for data curation, and formal analysis, wrote the initial draft, and revised the manuscript accordingly. AMB and JRAS were responsible for some data curation, visualization aspects, funding acquisition and writing – review & editing. AM was responsible for writing – reviewing & editing and the funding acquisition. CAG was responsible for the visualization aspects and for writing – review & editing. HGK and PIA provided resources, funding acquisition and writing – review & editing. TN and TG conceptualized the idea, provided resources, funding acquisition, project administration, writing – review & editing and supervision of the manuscript.

Conflicts of interest

There are none to declare.

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