

# Recent Chemical Candidates for Tackling Antibiotics-Resistance Crisis Caused by Metallo- $\beta$ -Lactamases

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## Abstract

Antibiotic resistance had been one of the major risks of the healthcare system since early 2000 [1]. After the early 1980s, the number of new antibiotics developed and approved is kept on decreasing while the number of antibiotic resistant strains are on increase due to overuse of antibiotics and international human and animal mobility [2]. When the number of clinically available antibiotics is limited by antibiotic resistance, co-administrating conventional antibiotics with compounds that reverse resistance is an effective approach [3]. In this regard, many  $\beta$ -lactamase inhibitors (BLIs), which reverse resistance against one of the most widely used  $\beta$ -lactam antibiotics [4], are developed. However, until now there is no effective inhibitor for metallo- $\beta$ -lactamases (MBLs), which have a distinct mechanism and structure from serine- $\beta$ -lactamases (SBLs). This is a problem when there is a global prevalence of MBL producing *Enterobacteriaceae* and *P. aeruginosa* strains [5]. In this mini-review article, we will look into some of the most recent (2019-2021) researches regarding MBL inhibitor development to facilitate researches and help find new research objectives. Compounds were sorted into categories according to the criteria of main mechanism of action. Compound's characteristics were summarized with emphasis on the mechanism of action,  $\beta$ -lactam (carbapenem) potentiating activity, and measured/expected cytotoxicity.

**Keywords:** Metallo- $\beta$ -lactamase, MBL,  $\beta$ -lactamase,  $\beta$ -lactamase Inhibitor, BLI, Antibiotics

**Abbreviation:** Metallo- $\beta$ -Lactamase, BLI -  $\beta$ -Lactamase Inhibitor, NDM - New delhi MBL, VIM - Verona integron MBL, IMP - Imipenemase, SBL - Serine- $\beta$ -Lactamase, PBP - Penicillin Binding Protein, OM - Outer membrane, CRE - Carbapenem-Resistant *Enterobacteriaceae*, SPM - São Paulo MBL, GIM - German Imipenemase, CphA - Carbapenem hydrolyzing and first (A) from *Aeromonas hydrophila*, L1 - Labile enzyme, OXA - Active on oxacillin, B1, B2, B3 -  $\beta$ -lactamase of Ambler Class B subclass 1, 2, 3, MPC - Minimum Potentiating Concentration, MIC - Minimum Inhibitory Concentration, MBC - Minimum Bactericidal Concentration, PK, PD - Pharmacokinetics/Dynamics, AMA - Aspergillomarasmine A, PMPCs - 6-Phosphonomethylpyridine-2-carboxylates, DM- N-[2-(dansylamino)ethyl] maleimide, CAMP - Cyclic Antimicrobial Peptide, SLAY - Surface Localization Antimicrobial display, MTT assay - Tetrazolium dye assay. Used to measure cytotoxicity in hRBC in mentioned article, BC - Red blood cell, MMP - matrix metalloproteinases, SICLOPPS - split intein mediated circular ligation of peptides and proteins, PEP4 - Peptidomimetic 4, MCR - mobilized colistin resistance, (Meropenem susceptibility) Breakpoint - By CLSI definition, MIC bigger than 4 mg/L for *Enterobacteriaceae*, and 8 mg/L for *P. aeruginosa* strains. By EUCAST definition, MIC bigger than 8 mg/L for both strains

## Introduction

Carbapenem is a derivative of beta-lactam antibiotics which is favored for their broad-spectrum inhibition capability. The reason why the trend of increasing carbapenem resistance is so daunting is that carbapenem is treated to the patient who has contracted the strains resistant to other types of beta-lactam antibiotics [6]. One of the most commonly encountered carbapenemase producing strains is *Enterobacteriaceae*. *Enterobacteriaceae* is the common

cause of most community and hospital-acquired infection cases [7]. Research documenting enzymes that Carbapenem-Resistant *Enterobacteriaceae* (CRE) produces had shown that the most widely discovered carbapenemase worldwide was New Delhi MBL-1 (NDM-1), an enzyme from MBL subclass 1 [8]. Metallo- $\beta$ -lactamase (MBL) is the subclass of carbapenemase that differed from the serine- $\beta$ -lactamase (SBL) which uses serine as catalytic residue. Unlike

SBL, there is no clinically available inhibitor to MBLs [9]. Combined with the fact that recently elucidated mechanism of bacteria utilizing conjugative plasmids for spreading antibiotic resistance across species [6], metallo- $\beta$ -lactamase poses a real threat to the modern healthcare system. The difficulties of developing effective MBL inhibitors center around the fact that MBL has structurally different subclasses, having zinc ion as the catalytic site which troubles designing suicidal inhibitor and has rather flexible active site loop which makes it difficult to ensuring tight binding of inhibitors. This article's purpose is to document recent attempts to overcome the difficulties and facilitates the development of clinically available MBL inhibitors.

## Main body

### Cyclic Boronate

The early attempt to develop cyclic boronate derivative inhibitors made vaborbactam (RPX7009). Unlike the bicyclic boronates listed below, it was monocyclic and has only moderately inhibited MBLs [10]. However, it did provide a great starting point for looking for unconventional, non-acylating inhibitors [11]. This type of inhibitor work through binding to MBL active site and prevent substrate entry into the active site. In some cases, it follows conformation change by reaction between cyclic boronate and enzyme

bound hydroxide. Though this type of  $\beta$ -lactamase inhibitor (BLI) also inhibits SBLs (Ambler class A, C, and D), this article will mainly discuss inhibition capacity against MBLs [12].

### Taniborobactam [12]

Taniborobactam (VRNX-5133) (Figure 1) has a distinct mechanism to SBL and MBL enzymes. While it inhibits SBL by forming a reversible covalent bond with the boronate chain, it also inhibits MBL by working as a competitive inhibitor. Unlike currently clinically available vaborbactam, taniborobactam can inhibit clinically important MBLs (SPM-1, GIM-1, NDM-1/5/7, and VIM-1/2/4) as well as SBLs. It reacts with the hydroxide anion coordinated by 2 zinc ions of MBL B1 to forms tetrahedral geminal diol formation which mimics the structure of transient tetrahedral intermediate in  $\beta$ -lactam breakdown reaction [13]. It inhibits NDM-1 and VIM-2 utilizing conserved Asn233, 120, and Glu149 residues as well as the interaction between zinc and boronate. However, Taniborobactam still has limits. Though it effectively inhibits most clinically important B1 MBLs, it cannot inhibit clinically important IMP-1, B2, B3 MBLs (CphA and L1). Notably, it is rendered bicyclic as well as tricyclic in crystal structure bound to NDM-1 (PDB ID: 6RMF) [14]. Tricyclic conformation seems to be the result of sp<sup>2</sup> oxygen of the acylamido group reacting with boronate. Its role in binding affinity and target preference is not known.

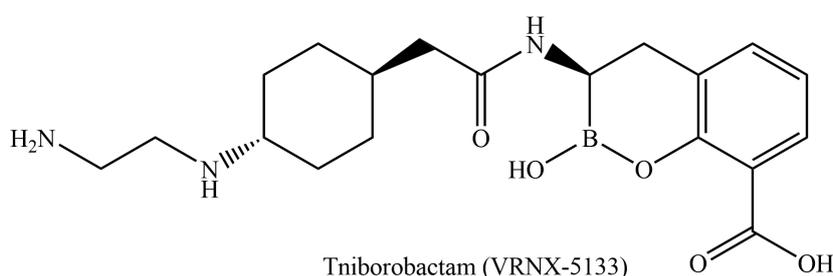


Figure 1: Taniborobactam (VRNX-5133) structure.

### QPX7728 [15]

Although many structures had been tested while developing promising candidates and clinically available BLI, little had been done in respect to substituents on aromatic rings. Hecker and 16 others had experimented on various substitutions in the article about QPX7728 (Compound 35) (Figure 2). They've delved into the effect of substituting largely unmodified regions on the aromatic ring. The most notable founding of this experiment was the potency of the BLI which has thioester group instead of acylamido group. Thioester group had shown overall better minimum potentiating concentration (MPC) when used with other carbapenems against MBLs variants compared to the Taniborobactam. Including the B2, B3, and IMP-1 (B1) which Taniborobactam failed to inhibit efficiently. The other notable improvements from the previous cyclic boronates were the reduced oligomerization inside the aqueous solution and reduced efflux rate by host efflux pump (MexAB-OprM

of *Paeruginosa*). However, even though it had not inhibited human metalloproteases, protective groups and unconventional substituents can have unexpected effects on pharmacokinetics as shown in the case of compound 17B, which had been suspended due to the long-living metabolite in the system.

### Metal Chelators

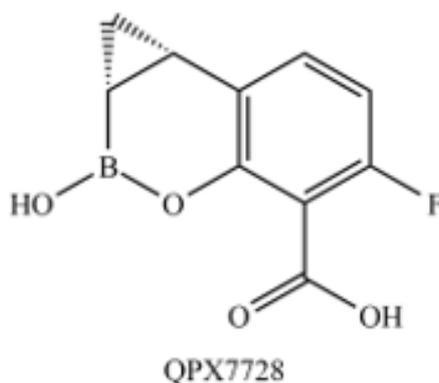
As MBLs activity requires coordinated zinc ions, the idea of using chelators as MBL inhibitors had arisen at the early phase of MBL inhibitor design. Metal chelators inhibit MBL by removing zinc from the active site or through sequestration of zinc ions released from MBL [16]. EDTA was considered as a candidate [17] of this class but consider not suitable for clinical use for its high toxicity [18]. Other metal chelating agents like dipicolinic acid (DPA) are also under clinical survey for further refinement [19]. However, using metal chelator had met with medical challenges as it has various

effects on human metalloproteins like carbonic anhydrase, angiotensin-converting enzyme (ACE), matrix metalloproteinase (MMP), and other proteins with zinc coordinating motif [20]. Hence reducing side effects on human metalloenzymes while having enough chelation activity to deactivate MBL is required.

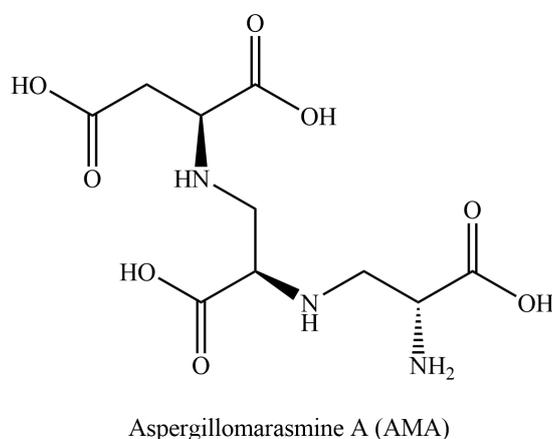
### Aspergillomarasmine A (AMA)[21]

AMA (Figure 3) is a natural fungal product from *Aspergillus* genus [22]. Its chemical isolation was conducted back in 1965, but its capacity as an MBL inhibitor had been discovered in recent years [23,24]. It is still not clear whether AMA makes direct interaction with the enzyme. However, the experiment result indicates that AMA works in a mechanism similar to L-captopril. AMA inhibits NDM-1 in an indirect way which sequesters spontaneously dissociated  $Zn^{2+}$  ions from binding sites with lower affinity (Zinc binding site with Asp 124, Cys 208, and His 250), instead of directly sequestering ions from the enzyme active site. About half the molar equivalent of  $Zn^{2+}$  was release even at the point of total enzyme activity loss. It seems it utilizes the transient state of zinc binding site 2 (ZBS2) [25]. This is backed up by the biotin-maleimide and N-[2-(dansylamino)ethyl] maleimide (DM) assay, which reacts with

exposed cysteine at the zinc binding side 2 of NDM-1. It has shown a similar  $K_d$  (0.20.04 nM) to zinc ion with EGTA (0.6 nM), a wide range chelator. Even though AMA has a relatively low affinity amongst chelators, AMA has narrow selectivity toward Zn, Ni, and Co divalent ions. This allowed 8  $\mu\text{g}/\text{ml}$  of AMA to inhibit NDM-1 in the presence of other metal ions and restores Meropenem sensitivity (2 $\mu\text{g}/\text{ml}$ ) in vitro.  $K_i$  of AMA in zinc depleted medium against NDM-1 was 11 nM, and 7 nM against VIM-2 [23]. However, AMA's activity as BLI is varied upon the concentration of zinc in medium, enzyme stability, and the affinity of the enzyme toward  $Zn^{2+}$ . 10 $\mu\text{M}$  of  $ZnSO_4$  did reverse 90% of NDM-1 inhibition and while  $IC_{50}$  against NDM-1 was between 8  $\mu\text{g}/\text{ml}$  to 16  $\mu\text{g}/\text{ml}$ , more stable and tightly bound IMP-7 shown little change up to 64  $\mu\text{g}/\text{ml}$  of AMA. The narrow selectivity of AMA confers relatively low toxicity against animals (Intravenous  $LD_{50}$  = 160 mg/kg in mice) when compared to EDTA (28.5 mg/kg), a strong zinc chelator ( $K_d = 10^{-9}$  M). However, as this experiment was related to the inhibition of endothelin converting enzyme, which plays an important role in  $\beta\text{ET-1}$ -induced responses such as sudden death, hypertension, and hemoconcentration [18]. Hence it has a limit to compare on par with other BLIs.



**Figure 2:** QPX7728. Bicyclic boronate which had removed conventional acylamido group structure.



**Figure 3:** Aspergillomarasmine A. A fungal product from *Aspergillus* genus. Previously this was tested as an endothelin converting enzyme inhibitor for its metal chelating activity.

## Peptide/Peptidomimetic

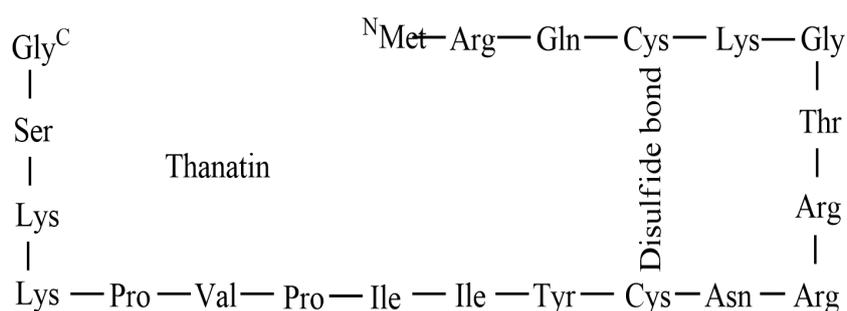
After depleting most of the natural sources for search over the potent leading molecule, research moved into a more novel pool of substances to find potent antimicrobial leading molecules [26]. In 1994,  $\beta$ -lactamase inhibitor protein (BLIP) had been reported [27]. And in 2013, BLIP-II had been reported [28]. While these proteins' inhibition activity is mainly against SBLs, it had preceded the use of peptides as a  $\beta$ -lactamase inhibitor. The biggest pro of this approach is the vast size of possible combinations of amino acids. While the biggest con is the limited mode of administration, acquiring enough stability of peptide until it reaches the target molecule, and acquiring enough selectivity [29].

### NDM-1 inhibiting peptides discovered with SLAY assay [30]

Misha I. Kazi and 12 others from university of Texas used Surface Localization Antimicrobial display (SLAY) to search 1700 random decapeptide sequences for antimicrobial activity. Among the 9 most potentiating peptide adjuvants, P5 (sequence: LRCLMLKFPPI) had shown the highest Minimum Bactericidal Concentration (MBC, determined by 99.90% of the killing of initial inoculums) on tested NDM-1 producing strains with 4 mg/L of meropenem. MBC goes as low as 4  $\mu$ M to 8  $\mu$ M depending on the strain.  $K_i$  value was lowest on P6 (0.11  $\mu$ M, sequence : CLRPSIISRA). Enzyme kinetic assay suggests among the 4 most meropenem-potentiating peptides, 3 had shown competitive inhibition while 1 had shown non-competitive inhibition. Interestingly, except for P4 (sequence: ASVTWLLYAM), all 8 peptide has positive charge like cationic antimicrobial peptide (CAMP), but when treated against NDM-producing *E.coli*, peptide had limited effect on the increase of cell membrane permeability. This indicates that these peptides have a different mechanism of action from the CAMPs, which embed into bacterial membrane and form pores to have bactericidal effect [31]. Cytotoxicity and hemo-

lytic effect of the peptides were tested with MTT assay on human RBC. Among the 4 most potentiating agents, none had shown notable toxicity except for P7 (sequence: WRYQWTILFI), which had shown dose-dependent cytotoxicity against HEK293 at 256  $\mu$ M.

Thanatin (Figure 4) is a pathogen-inducible single-disulfide-bond-containing  $\beta$ -hairpin AMP which was first isolated from the insect *Podisus maculiventris* [33]. Even though our focus is on BLI capacity, thanatin has its own bactericidal capacity. And in sub-MIC dosage, thanatin acts as an adjuvant by displacing zinc ions from the NDM-1 active site by interacting with protein directly. The antimicrobial activity is caused by the removal of divalent cations which bridge the negatively charged phosphate group of lipopolysaccharides (LPS), the staple substance of gram-negative bacterial outer membrane (OM). And it seems there is an alternate mode of action in the above MIC dose by inhibiting LptA and LptD which limits LPS transportation in turn [34,35]. In sub-MIC dose, thanatin can work as BLI through direct interaction with MBL. It is not clear which residue that thanatin interacts with, peptides with a similar mode of action did interact with Trp93 and Asp124 in the case of PEP4 [36] and pterostilbene [37]. Though the article lacks structural data, inductively coupled plasma-mass spectrometry (ICP-MS) confirmed the release of the zinc ions from NDM-1 in vitro setup when treated with thanatin. This was supported with microscale thermophoresis (MST) experiment results.  $K_d$  value of apo NDM-1 to thanatin ( $0.71 \pm 0.06 \mu$ M) was approximately 10 times lower than  $K_d$  of NDM-1 to zinc divalent ions ( $7.36 \pm 0.45 \mu$ M). When its cytotoxicity was tested against human and mice cell lines (human umbilical vein endothelial cells, pulmonary alveolar epithelial cells, and mouse neuron cells) at 200  $\mu$ M, cytotoxicity was far much lower compared to the colistin. Considering that MIC of thanatin was about 0.4 to 0.32  $\mu$ M, much lower toxicity is expected in therapeutic circumstances.



**Figure 4:** Wild type thanatin structure from of *Podisus maculiventris*. 21 residues long peptide with disulfide bond at the middle. (Sequence: GSKKPVPIIYCNRRTGKCQRM).

### Peptidomimetic 4 (PEP4) [36]

PEP4 (Figure 5) is the cyclic analogue of Pentapeptide (Ile-Met-Ile-Ser-Phe) which had been known to inhibit matrix metalloproteinases (MMP), which is structurally similar to NDMs [38]. It has high membrane permeability and high stability in serum due to the cyclization [39]. PEP4 alone did not show any bactericidal effect

and its effect as an adjuvant was restricted to the strains producing MBL, supporting direct inhibition of MBL. The mechanism of action is still being studied, but the Molecular dynamic (MD) simulation based on molecular docking suggests it works via Van der Waals interaction between the Trp93, His122, Gln 123, and Asp 124 residues at the active site of NDM-5 with -10.8 Kcal/mol binding ener-

gy. In in vivo experiment, the meropenem/PEP4 treatment (both 16 mg/kg) increased the survival rate of NDM-producing *E.coli* infected mice from 0% to 70% at 10 days post-infection. When tested in vitro setup, PEP4 had shown  $IC_{50}$  of 5.926  $\mu\text{g/ml}$  against NDM-5. As the original pentapeptide is known to inhibit MMP, which have var-

ious role in human body, cytotoxicity assay was conducted against Vero, CHO and HEK293T mammalian cell lines. The result showed a low level of cytotoxicity at the high concentration (10% at the 128  $\mu\text{g/ml}$ ).

Original structure

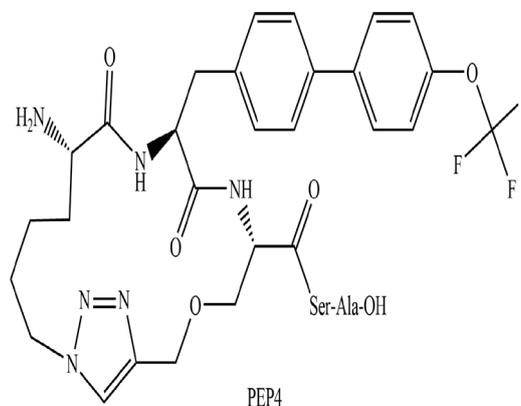


Figure 5: The structure of PEP4.

## Other Type of Compound

### 6-Phosphonomethylpyridine-2-carboxylates (PMPCs) [40]

Even though phosphonate was observed bound to zinc ions in many MBL crystal structures, the utility of phosphonate as a zinc binding group had not been explored thoroughly. Hence Hinchliffe and his team have designed PMPCs to explore the potential of phosphonate as a zinc binding group. PMPC (Figure 6) is consisting of 3 structures, the phosphonate group, pyridine, and carboxylate group. It interacts with B1 and B3 MBLs in a different manner. Against B1, it interacts without displacing any component in the active site. Carboxylate and nitrogen from the pyridine ring interact with  $Zn_2$ , while phosphonate reacts with bound hydroxide (PDB ID: 5EV6). In B3, However, PMPCs displaced both  $Zn_2$  and hydroxide ion, with phosphonate group and pyridine nitrogen and carboxylate making direct interaction with Ser 221, 223 (PDB ID: 1SML). But this starkly different mode of binding is different from what the enzyme kinetic equation suggested. It might be due to the much higher concentration of inhibitors in the crystallization situation. Notably, 3b bound to the L1 was all S-isomer, indicating that adding hydroxyl group in the R-isomer form is highly not recommendable. Even though enzyme kinetics generally indicated it follows competitive inhibition, the inhibition rate was differed by the preincubation time. As the replotted graph of  $[I]$  and  $K_{obs}$  followed linear graph, stepwise dehydration of active site seems to be the cause. Amongst PMPC variants, PMPC 3, Which has a carboxylate group at the C2 and phosphonomethyl group at the C6 of pyridine ring had shown the best potential. Especially 3c, which has a hydrophobic group on the phosphonomethyl group had shown the best potentials against tested B1 MBLs (NDM-1, IMP-1).  $IC_{50}$  range of 0.306~2.91  $\mu\text{M}$  and  $K_i$

range of 0.034~2.91  $\mu\text{M}$ . PMPC3 variant was also effective against tested B3 MBL (L1, observed  $IC_{50}$  1.48~2.05  $\mu\text{M}$ ), but additional hydrophilic/phobic group on the phosphonomethyl group had not shown much difference. However, unlike the in vitro experiment, when tested against clinically isolated strains, PMPC 3a (the one without additional group on phosphonomethyl group) had shown the best potentiating effect as PMPC 3b and 3c failed to reduce *E. coli* UWB75 and *Pseudomonas* spp.'s meropenem MIC below 8 mg/L even with 128 mg/L of inhibitor concentration. PMPC was affected only slightly by the host efflux pump. The potentiating effect was differed only by 2-fold in resistance nodulation division (RND) efflux pump overexpression strain and knockout strain. No noticeable cytotoxicity was observed until the concentration required to lower the meropenem MIC below the breakpoint in tested cell lines (Human epithelial Caco-2, HEPG2, and rat H4IIE).

PEP4 (Figure 5) is the cyclic analogue of Pentapeptide (Ile-Met-Ile-Ser-Phe) which had been known to inhibit matrix metalloproteinases (MMP), which is structurally similar to NDMs [38]. It has high membrane permeability and high stability in serum due to the cyclization [39]. PEP4 alone did not show any bactericidal effect and its effect as an adjuvant was restricted to the strains producing MBL, supporting direct inhibition of MBL. The mechanism of action is still being studied, but the Molecular dynamic (MD) simulation based on molecular docking suggests it works via Van der Waals interaction between the Trp93, His122, Gln 123, and Asp 124 residues at the active site of NDM-5 with -10.8 Kcal/mol binding energy. In in vivo experiment, the meropenem/PEP4 treatment (both 16 mg/kg) increased the survival rate of NDM-producing *E.coli* infected mice from 0% to 70% at 10 days post-infection. When tested in vitro setup, PEP4 had shown  $IC_{50}$  of 5.926  $\mu\text{g/ml}$  against NDM-5. As the original pentapeptide is known to inhibit MMP, which have var-

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## Conclusion

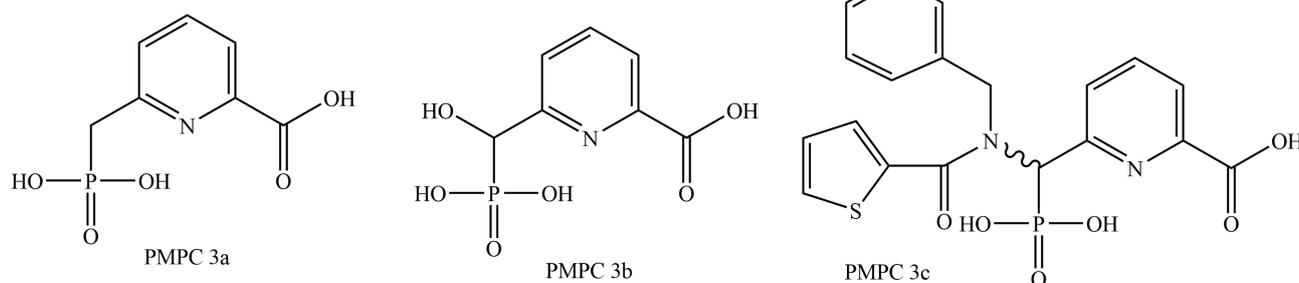
### Importance of Zinc binding site 2 in NDM-1 and VIM-1

The loss of zinc observed from thanatin treated NDM-1 had shown half of the zinc loss per molecule whereas the treatment resulted in almost total inactivation of the enzyme [32]. It might indicate that thanatin treatment affects mainly the weaker binding site of 2 zinc binding site of NDM-1. As this type of impartial effect (either by indirect sequestration or direct displacement) on  $\text{Zn}_2$  coordination had been observed repeatedly, [16,23,40] focusing on Zinc binding site 2 (ZBS2) might be a valid approach. However, I have to admit that there's rapid mutation ongoing on ZBS2, resulting in higher thermostability and zinc binding affinity of ZBS2 [41], and

there is a conflicting report about AMA's mode of action, reporting 1.8 molar ratio of zinc loss per molecule [23].

### Inhibition characteristics of cyclic boronates against MBL

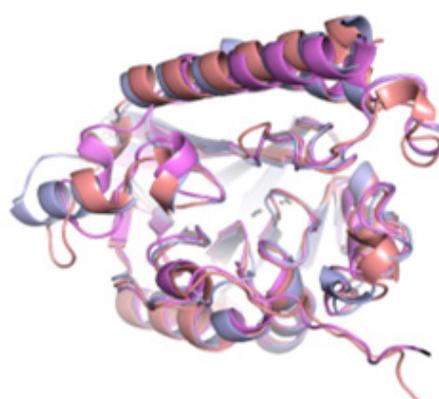
QPX 7728 had shown a better potentiating effect compared to the taniborobactam against the B3 MBLs. Whereas showing a similar level of effect against B1 MBLs [42]. It seems like the substitution of the acylamido group with a much smaller structure have played important role in binding to the 2 different active sites without steric hindrance. As Structural alignment shows acylamido group of taniborobactam cannot fit into the binding site of the B3 MBLs which have a different loop structures around the active site. (Figure 7) And even though most of the attention is gathered around the bicyclic boronate candidates, monocyclic Vaborbactam had shown better performance against the fugitive targets like B2, B3, and IMP-1 variant compared to the Taniborobactam [10]. Leaving the possibility of a binary solution for further refinement in structure.



**Figure 6:** PMPC3 variants. 3a. the basic structure of PMPC with dual interaction with zinc ions. Phosphonate group, the nitrogen of pyridine ring, and sp<sup>2</sup> oxygen of carboxylate group form main interaction with zinc and bridging hydroxide of MBL. 3b. Addition of hydroxyl group on phosphonate. Notable, only S-isomer is rendered L1/3b crystal structure. 3c. The addition of N-benzyl-1-thiopen-2-ylformamido is added to mimic the bulky tail group of carbapenem.



**A.** NDM-1 and L1 with inhibitors



**B.** B3 MBLs (L1, GOB-18, FEZ-1)

**Figure 7:** Comparing the potentiating effect of QPX7728 and Taniborobactam against B1 and B3 MBLs. zinc atoms had move 3.2 Å further from the loop by the alignment process. If taniborobactam did form a similar binding formation as it did with NDM-1, its acyl amido would've collided with the L1's N-terminal residues and loop next to the active site. Even though these Tyr 32, Ile 149, and Phe 145 of the L1 were not conserved, the overall loop structures were well conserved amongst B3 MBL. (A. NDM-1: Blue, L1: Violet, Taniborobactam (VRNX-5133): Yellow, QPX7728: Green) (B. L1: Violet, GOB-18: Salmon red, FEZ-1: Grey) (PDB ID: NDM-1 with Taniborobactam(6RMF), NDM-1 with QPX-7728(6VIM), L1(6U0Y), GOB-18(5K0W), FEZ-1(1K07))

## General side effects by molecular types

Quite a lot of the zinc binding MBL inhibitor candidates were also tested for ACE and MMP inhibitors, including captopril [43], AMA [18], and other metal chelators. Other research indicates that carboxypeptidase and carbonic anhydrase (mammalian metallo-enzymes) [44-46], was also inhibited by MBL inhibitor candidates. Cyclic boronate inhibitors did not show particular toxicity in recent reviews, but clinically approved vaborbactam phase I review indicates a mild level of lethargy [47]. Peptide's off-target bindings are hard to be mentioned in general terms due to the wide range of structure variety, but cationic peptides can have hemolytic side effects by the same pathway as CAMPs.

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## Conflict of interest

No conflict of interest.

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