Addendum

Summary

If left unmitigated, antibiotic resistance will continue its path to become a global catastrophe in the 21st century. β-Lactams are the most prescribed group of antibiotics making modern medicine and many surgical interventions possible. However, infections, especially those caused by gram-negative pathogens are becoming increasingly difficult to treat largely due to the presence of β-lactamases. A number of innovative drugs have been developed over the past decades to rescue the efficacy of β-lactams while treating β-lactamase-producing bacterial infections. However, these agents only inhibit serine-type β-lactamases (SBLs, Ambler class A, C, and D). This renders β-lactams, including the last-resort carbapenems, clinically ineffective against the infections caused by the bacteria that express the rapidly emerging metallo-β-lactamases (MBLs, Ambler class B). There is an unmet and urgent need to add inhibitors that target MBLs to our antibiotic arsenal.

The β-lactamase inhibitors approved by the FDA or being evaluated in clinical trials have been reviewed in **chapter 1**. While the earlier generations of SBL inhibitors such as clavulanic acid, sulbactam, and tazobactam were all β-lactam derivatives themselves, discovery of avibactam (a diazabicyclooctane) and vaborbactam (a cyclic boronate) broadened the chemical space available to develop potent and broad-spectrum β-lactamase inhibitors. Interestingly, multiple studies have shown that certain cyclic boronates can mimic the intermediates formed when β-lactams are attacked by both SBLs and MBLs. This provides a valuable opportunity in the pursuit of a "pan-spectrum" β-lactamase inhibitor. Taniborbactam, which is currently in a phase III clinical trial is a cyclic boronate optimized to inhibit the clinically important β-lactamases of all 4 Ambler classes (figure 1). MBLs are a diverse group of zinc metallo-enzymes capable of hydrolyzing penicillins, cephalosporins, and also carbapenems. The majority of the reported MBL-inhibitors either act by zinc-sequestration or binding the active site zinc thereby

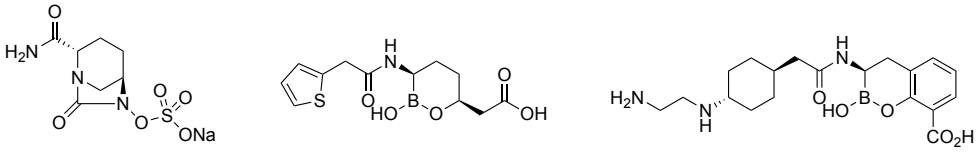


Figure 1. Structures of approved SBL inhibitors avibactam, and vaborbactam, and the SBL-MBL inhibitor taniborbactam.

forming a ternary complex. Since zinc is essential for the hydrolytic activity of MBLs, many chelating agents have been applied for the phenotypic screening and biochemical characterization of β-lactam resistant bacterial isolates. Other inhibitors containing a zinc-binding moiety include thiols, diacids, and picolinic acid derivatives (figure 2). The following 4 chapters describe our efforts to identify new compounds with ability to *a*. inhibit clinically relevant MBLs of the NDM, VIM, and IMP families, and *b*. restore the activity of β-lactams in cell-based phenotypic assays. This was followed up by our attempts to use a prodrug approach to improve the druggability of the MBL-inhibitors.

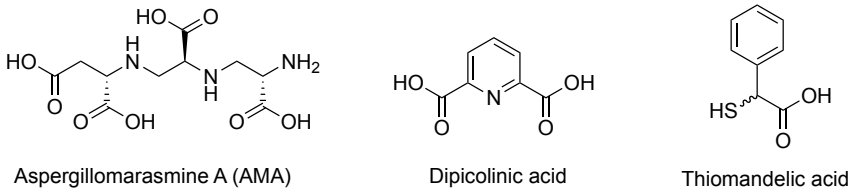
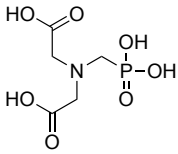
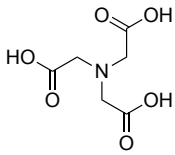


Figure 2. Zinc-chelators, picolinic acid derivatives, and thiols represented by AMA, dipicolinic acid, and thiomandelic acid respectively, all reported as MBL inhibitors.

In **chapter 2**, we describe our study leading to the identification of *N*-(phosphonomethyl)iminodiacetic acid (PMIDA) and nitrilotriacetic acid (NTA) as potent inhibitors of NDM-1 (figure 3). Building on literature reports in which commonly used buffering agents were reported to possess MBL inhibitory activity, we screened a larger group of buffering agents known to have metal-binding affinity. Among the tested compounds, PMIDA and NTA exhibited the highest potency against purified NDM-1 and VIM-2, with moderate to weak activity against IMP-28. The results of Zn-dependency studies and isothermal titration calorimetry (ITC) assays shed light on the inhibitory mechanism of the most potent compounds, showing that they act primarily by chelating the zinc ions crucial for the catalytic activity of the MBLs. Our data do not support the possibility of the inhibitors forming a complex with the metallo-enzyme itself. Phenotypic screenings revealed the strong synergistic relationship between PMIDA/NTA and meropenem when tested against a large panel of carbapenem-resistant gram-negative clinical isolates. We suggest that such readily available small molecules can serve as biochemical tool compounds for enzymatic and phenotypic studies of MBLs, as well as leads for further optimization on the path towards clinical development.

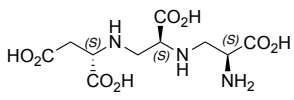




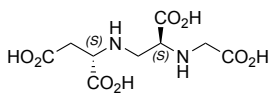
	PMIDA	NTA
IC_{50} (μM) against NDM-1	0.91	1.3
Zn dissociation constant (K_d, nM)	56	121

Figure 3. PMIDA and NTA are potent NDM-1 inhibitors and strong zinc-binders.

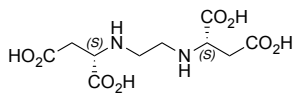
Chapter 3, describes additional efforts to identify MBL inhibitors by screening a series of small-molecule aminocarboxylic acids related to the secondary metabolites produced by *Aspergillus* spp. including aspergillomarasmine A (AMA) and aspergillomarasmine B (AMB), as well as the chelating agent ethylenediamine-*N*,*N*'-disuccinic acid (EDDS, figure 4). The various analogs synthesized in Prof. Gerrit Poelarends' group were first tested for their inhibition of the NDM-1 enzyme. The promising IC_{50} values of some of the analogs prompted us to evaluate their Zn-binding affinity using ITC. There was a clear correlation between Zn-binding affinity and inhibitory activity of the aminocarboxylic acids. Notably, the 2 methylene units between the aspartate fragments of EDDS were found to be the optimal length for maximum zinc-binding and NDM-1 inhibition. Interestingly, some of the potent inhibitors identified did not exhibit synergy with meropenem when tested against an NDM-1 producing *E. coli* isolate, most probably due to sub-optimal cellular accumulation. On the other hand, a methyl substituted EDDS, as well as a methylene homolog of AMB, where among the most potent synergizers rescuing meropenem at 100 μM.



AMA



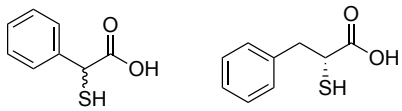
AMB



(*S*,*S*)-EDDS

Figure 4. Previously reported aminocarboxylic acids as potent NDM-1 inhibitors.

In **chapter 4**, another group of small-molecule MBL inhibitors (*i.e.* thiols) were evaluated. Focusing on a series of previously reported thiol-containing MBL inhibitors, we reported the first checkerboard synergy assays and zinc-binding affinity studies, and assessed the chemical stability of these compounds. In the synergy experiments, thiomandelic acid and 2-mercapto-3-phenylpropionic acid (compounds **3** and **4** respectively in chapter 4, figure 5) largely reduced the MIC of meropenem and cefoperazone when tested against a panel of MBL-producing gram-negative clinical isolates. The lack of synergy against KPC- and OXA-producing isolate indicates that the activity of the thiols is MBL-specific. Despite their strong synergistic activity and zinc-binding affinity, we found that thiols **3** and **4** suffer from poor stability in the assay medium. With a half-life of *ca*. 5 hours, they oxidize to their corresponding disulfides which in turn exhibit moderate to no synergistic activity.



3 **4**

Figure 5. Thiomandelic acid (**3**) and 2-mercapto-3-phenylpropionic acid (**4**).

Chapter 5, describes the synthesis and bioactivity evaluation of a series of cephalosporin-thiol conjugates which were designed to act as prodrugs of the thiols described in chapter 4. Following our findings on the poor stability and specificity of these thiols and given the well-known hydrolysis mechanism of cephalosporins, we designed and synthesized a small group of cephalosporin thiol conjugates (compounds **5**-**7** in chapter 5, figure 6). The IC_{50} assays against IMP-1, IMP-28, NDM-1 and VIM-2 revealed the potent activity of compounds **6** and **7** with selectivity towards IMP enzymes. The same trend was observed in synergy assays against MBL-producing gram-negative bacterial isolates where **6** and **7** largely reduced the MIC of meropenem against the IMP-producing bacteria. Despite the promising activity data, mechanistic studies using ^{1}H-NMR and LC-MS indicated that exposure of the cephalosporin conjugates to IMP-28 does not lead to the expected release of the small-molecule thiols. This observation prompted us to determine the mode of action of the cephalosporin conjugates. In doing so, a structure-activity relationship analysis of compound **6** was performed by synthesizing and testing a series of structural variants. The bioactivity data obtained pointed towards the contribution of both phenyl and carboxylate residues of compound **6** to its potency. Secondly, we

determined the kinetic parameters of the MBL-mediated hydrolysis of the synthesized cephalosporins. The calculated catalytic efficiency values suggest slow substrate turn-over as the inhibitory mechanism of compounds **6** and **7**. Finally, the bioactivity evaluation of the partially hydrolyzed products of **6** and **7** (*i.e.* **6H** and **7H**) revealed that IMP enzymes can be inhibited by both substrates **6** and **7** as well as their hydrolysis products **6H** and **7H**.

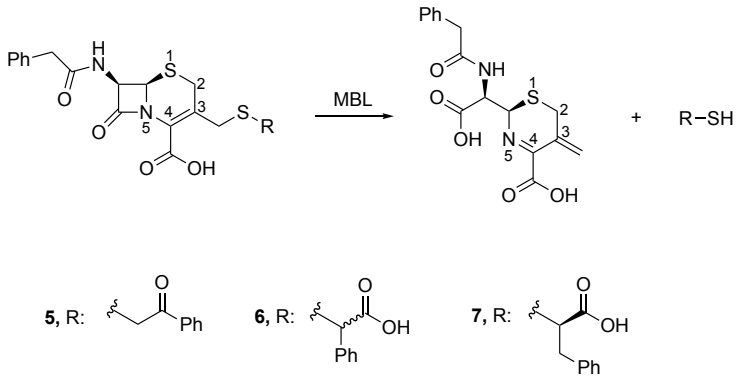


Figure 6. The cephalosporin conjugates hypothesized to release the thiol MBL-inhibitors after MBL-mediated hydrolysis.

Chapter 6 was the result of a collaborative project with Dr. Mike Brouwers from Wageningen Bioveterinary Research to evaluate the enzymatic activity of a newly discovered class A carbapenemase. The enzyme, named FLC-1 (FRI-like carbapenemase-1), was originally identified in *E. cloacae* isolated through the screening of food products imported to The Netherlands. After transformation of *E. coli* with the FLC-1 plasmid and arabinose-induced over-expression, the protein fraction of the bacterial culture was prepared and used for the determination of kinetic parameters. The FLC-1 fraction demonstrated preference for hydrolyzing the tested carbapenems over third-generation cephalosporins as indicated by higher catalytic efficiency values. This preference was mirrored by the MIC data previously determined where the transformed *E. coli* showed higher resistance to carbapenems vs. third generation cephalosporins. We also found that FLC-1 is inhibited by clavulanic acid with an IC_{50} value of 1.97 μM.