Small-molecule inhibitors of bacterial metallo-β-lactamases

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Chapter 1

Introduction: β-lactam resistance and β-lactamase inhibitors

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1. Introduction

There is an urgent need to develop new therapeutic options to combat the increasing number of pathogens that have become resistant to β-lactam antibiotics by gaining the ability to express β-lactamase enzymes. The β-lactamases are classified by both structural approaches (Ambler)[1] and functional approaches (Bush-Jacoby-Medeiros).[2] Throughout this chapter, the Ambler classification will be used to describe the β-lactamases. Class A is represented by the classic β-lactamases such as the TEM (named after a patient) and SHV (name derived from sulfhydryl reagent variable) families which inactivate penicillins and narrow-spectrum cephalosporins. Some members of the TEM and SHV families, along with the CTX-M (active against cefotaxime, isolated in Munich) class, are also able to inactivate extended-spectrum β-lactams and are therefore referred to as extended-spectrum β-lactamases (ESBLs). There are also carbapenemases among class A enzymes which include KPC (*K. pneumoniae* carbapenemase), IMI (imipenem-hydrolyzing β-lactamase) and SME (*S. marcescens* enzyme).[3,4] Unlike members of class A/C/D families which hydrolyze β-lactams by action of a serine nucleophile, class B β-lactamases are metalloenzymes that contain zinc ion in their active site. In these so-called metallo-β-lactamases a water molecule, activated via coordination to zinc, serves as a nucleophile and hydrolyzes the β-lactam ring rendering the antibiotic inactive (figure 1). With the exception of monobactams, class B metallo-β-lactamases (MBLs) are able to hydrolyze all classes of β-lactams. The rapidly emerging NDM (New-Delhi metallo-β-lactamase) along with VIM (Verona integron-encoded metallo-β-lactamase) and IMP (imipenemase) are among the most clinically important MBLs which possess carbapenemase activity.[5–8] Class C is represented by CMY (cephamycinase), ACT (AmpC type) and DHA (discovered in Dhahran hospital). Gram-negative bacteria producing this class of enzymes are often resistant to penicillins and some cephalosporins. Class D contains OXA (oxacillinase) family the members of which are able to metabolize penicillins, cephalosporins, and carbapenems. In this regard, the emergence of OXA-producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii* is of particular concern.[9]

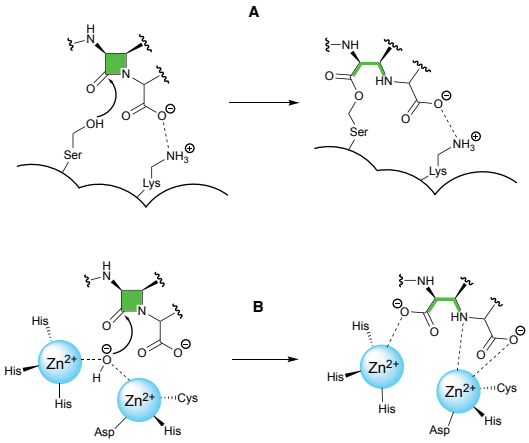


Figure 1. **A**. β-lactam inactivation mediated by serine β-lactamases (Ambler class A, C and D) is facilitated by the attack of a nucleophilic serine. **B**. MBL (class B)-mediated inactivation of β-lactams involves a nucleophilic attack by an activated water molecule coordinated to zinc ions.

For the purpose of clarity, figure 2 provides an overview of the various antibiotics **1**-**13** that have been tested in combination with the β-lactamase inhibitors covered in this chapter. The first generation of β-lactamase inhibitors including clavulanic acid **14**, sulbactam **15**, and tazobactam **16** (figure 3) were granted FDA-approval between 1984 and 1993. They were formulated with penicillins and include amoxicillin **1**-clavulanic acid, ticarcillin **2**-clavulanic acid, ampicillin **3**-sulbactam, and piperacillin **4**-tazobactam combinations.[10] However, the spectrum of activity of these inhibitor/β-lactam combinations covers primarily the β-lactamases of class A (with the exception of KPC). In addition, the emergence of inhibitor-resistant TEM variants with lowered susceptibility to clavulanic acid, sulbactam, and tazobactam has been documented.[11]

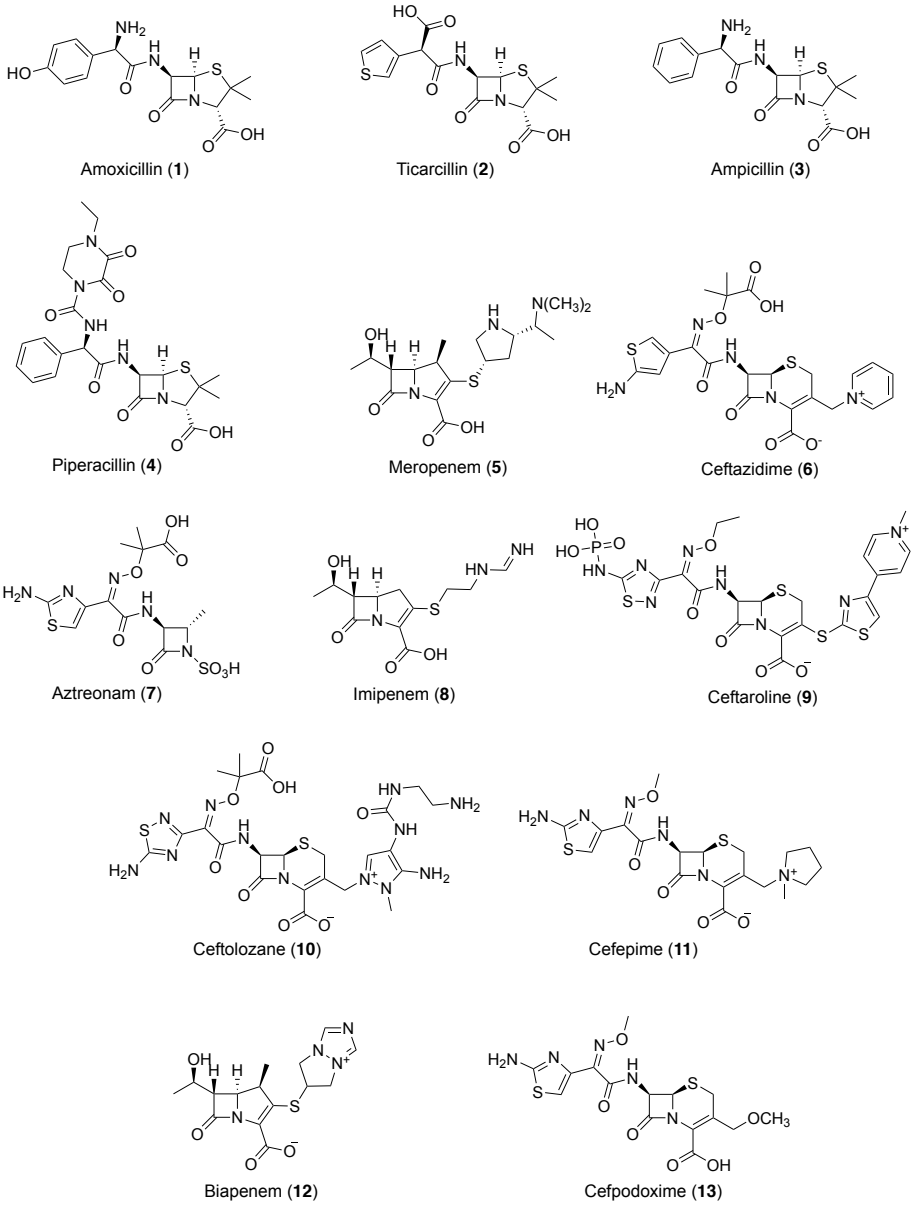
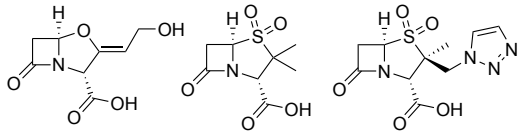


Figure 2. β-lactam antibiotics evaluated in combination with β-lactamase inhibitors.



14 15 16

Figure 3. First generation of β-lactamase inhibitors; clavulanic acid **14**, sulbactam **15** and tazobactam **16**.

In response to the increasing risk of drug-resistant bacterial infections, new generations of β-lactamase inhibitors including avibactam and vaborbactam have been added to our arsenal in recent years. Despite these advances, carbapenem-resistant *Enterobacteriaceae*[12–15] and difficult to treat microorganisms such as *P. aeruginosa* and *A. baumannii* produce a variety of β-lactamases and exhibit other resistance mechanisms that continue to challenge existing antibiotic treatments.[9,16,17] In this chapter we first review the β-lactam/β-lactamase inhibitor (BL/BLI) combinations being marketed or under clinical development. We then continue by an overview of the research articles and patents published on the topic of small-molecule inhibitors of β-lactamases with particular attention paid to progress made in the past decade.

2. Recent FDA-approved BL/BLI combinations

2.1 *Vabomere® (meropenem + vaborbactam)*

Vaborbactam **17** (formerly known as RPX7009, figure 4) is the first FDA-approved β-lactamase inhibitor containing a cyclic boronate pharmacophore.[18–21] The design of vaborbactam is the result of medicinal chemistry efforts to develop a cyclic boronate analog with selectivity towards bacterial β-lactamases over mammalian serine hydrolases. X-ray crystallography studies confirmed that vaborbactam forms a covalent adduct with the catalytic serine residue of CTX-M-15 and AmpC. In addition, vaborbactam inhibited various Class A/C β-lactamases with sub-μM IC_{50} values.[22] The combination of vaborbactam and meropenem **5** was tested against more than 300 *Enterobacteriaceae* clinical isolates, the majority of which carried KPC genes. A fixed vaborbactam concentration of 8 μg/mL potentiated the activity of meropenem **5** by at least 64-fold leading to MIC_{50} and MIC_{90} values of ≤0.06 and 1 μg/mL respectively.[23] A follow-up study on a larger number of non-fastidious gram-negative bacteria collected worldwide confirmed the potent activity of meropenem-vaborbactam against KPC-producing *Enterobacteriaceae* (MIC_{50} and MIC_{90} values of 0.12 and 0.5 μg/mL respectively), however vaborbactam did not reduce the MIC of meropenem **5** against bacterial strains expressing MBLs

(Ambler class B) or OXA-48 (Ambler class D).[24] In a complimentary study, Lomovskaya and co-workers used a panel of engineered *E. coli* strains producing β-lactamases of all four Ambler classes to assess the ability of vaborbactam to potentiate a number of antibiotics.[25] Since most of the strains producing β-lactamases of Ambler class A and C are already susceptible to meropenem **5**, adding ceftazidime **6** and aztreonam **7** to their panel allowed them to fully characterize the inhibition spectrum of combinations with vaborbactam. Their findings reveal a broad spectrum synergistic effect against *E. coli* strains producing β-lactamases of Ambler class A (KPC, SME, NMC, SHV, TEM, CTX) and class C (DHA, MIR, FOX, AmpC-ECL, CMY) when 4 μg/mL vaborbactam is added to meropenem **5**, ceftazidime **6**, or aztreonam **7**. In line with studies employing clinical isolates, vaborbactam did not decrease the MIC of β-lactams against engineered *E. coli* strains producing MBLs including class B (NDM-1, VIM-1) or class D (OXA) enzymes.[25] In addition to strong *in vitro* activity, meropenem-vaborbactam exhibited promising results in clinical trials which indicated the safety, tolerability, and efficacy of the combination.[26,27] In a randomized clinical trial meropenem-vaborbactam along with its comparator drug combination (piperacillin-tazobactam) were evaluated for the treatment of complicated urinary tract infection. Meropenem-vaborbactam was well tolerated by patients and proved to be non-inferior to the comparator therapy.[27] Vaborbactam in combination with meropenem (Vabomere®) was approved by FDA in 2017 for treating complicated urinary tract infections and is marketed by Melinta therapeutics as an injectable solution with each vial containing 1 g of meropenem and 1 g of vaborbactam.[28,29] At present other vaborbactam-antibiotic combinations are under clinical evaluation.

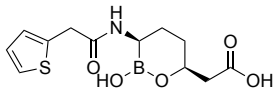


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Figure 4. Vaborbactam **17**.

2.2 *Avycaz® (ceftazidime + avibactam)*

The avibactam/ceftazidime combination marketed as Avycaz was granted FDA-approval in 2015 for the treatment of complicated intra-abdominal infection (cIAI) and complicated urinary tract infection (cUTI). Structurally, avibactam **18** (formerly NXL104, figure 5) is a first-in-class SBL inhibitor with a cyclic urea replacing the β-lactam pharmacophore present in the older generation of β-lactamase inhibitors.[30] Using a variety of biophysical techniques including UV spectroscopy, MS, and NMR, Ehmann and co-workers[31] found that avibactam employs a mechanism based on covalent inhibition of TEM-1 with slow regeneration of the inhibitor. This covalent acylation with reversible deacylation through recyclization is unique to avibactam among β-lactamase inhibitors. When avibactam was tested against a larger panel of β-lactamases including TEM-1, CTX-M-15, KPC-2 (class A), *Enterobacter cloacae* P99 AmpC, *P. aeruginosa* PAO1 AmpC (class C), OXA-10 and OXA-48 (class D), it was confirmed that acylation of enzymes followed by slow release of inhibitor through cyclization could be considered as a general mechanism of inhibition by avibactam.[32] In the case of KPC-2 inhibition however, it was found that recyclization competes with desulfation of avibactam followed by further degradation steps.[32] Studies of avibactam in complex with class A and class C β-lactamases using X-ray crystallography suggest the stability of carbamate bond upon avibactam addition and the substrate-like conformation of the enzyme-bound avibactam as the explanations for the favorability of recyclization over hydrolytic cleavage.[33–35] There are multiple reports on the *in vitro* activity of avibactam combined with cephalosporins, carbapenems, and monobactams against both gram-negative and gram-positive bacterial pathogens. When tested against 126 *P. aeruginosa* clinical isolates, avibactam at 4 μg/mL reduced the MIC_{90} of ceftazidime **6** from 64 to 8 μg/mL, superior to the effect of clavulanic acid **14** and tazobactam which led to no change and two-fold reduction of MIC_{90} respectively. Avibactam also potentiated imipenem **8** with an MIC_{90} reduction of 16 to 2 μg/mL.[36] The combination of avibactam with

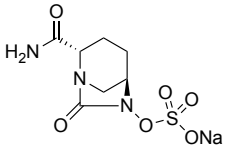

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Figure 5. Avibactam **18**.

ceftaroline **9** inhibited *Enterobacteriaceae* strains containing multiple β-lactamases of class A and C. In addition, avibactam did not appear to adversely affect the activity of ceftaroline **9** against methicillin-resistant *Staphylococcus aureus* (MRSA) strains. The avibactam/ceftaroline **9** combination however showed little activity against *Acinetobacter* spp and *P. aeruginosa* strains containing OXA (class D) enzymes or MBL-producing strains.[37] Another study found the same trend of limited potency of ceftazidime-avibactam combination against *A. baumannii* strains producing PER-1, OXA-51 and OXA-58, while promising activity was observed against *Klebsiella pneumoniae* strains producing CTX-M-15 or OXA-48 and *E. coli* strains producing CTX-M-15.[38] Susceptibility screening of 701 *Enterobacteriaceae* isolates with positive ESBL-phenotype collected from U.S. hospitals showed potent activity of ceftazidime-avibactam as well as tigecycline.[39] Another published screening of 8,640 *Enterobacteriaceae* collected from U.S. medical centers found the similar results with ceftazidime-avibactam, although the combination showed limited activity against *Acinetobacter* spp. isolates and MBL-producers.[40] Avibactam restored the activity of ceftazidime **6** against isolates producing KPC, CTX-M-15-like, CTX-M-14-like and SHV ESBLs and CMY-2-like enzymes ($MIC_{90} \leq 2$ μg/mL in all the cases).[39] Wang and co-workers performed a series of *in vitro* assays with avibactam combined with ceftazidime **6** or aztreonam **7** revealing similar trends.[41] The same study also found that avibactam resensitized *Enterobacteriaceae* isolates producing Ambler class A and C to ceftazidime **6** and aztreonam **7**.[41] Combining avibactam with aztreonam **7** appears to be an appealing strategy to extend the activity to MBL-producers, since aztreonam **7** is a poor substrate for MBLs.[3,42,43] Wang and co-workers found that unlike ceftazidime-avibactam, aztreonam-avibactam did retain potency against the isolates co-producing IMP or NDM.[41] Based on these findings and further *in vitro* susceptibility screenings[44–46] it can be concluded that avibactam greatly potentiates ceftazidime **6** against bacterial pathogens producing class A, C, and some class D β-lactamases and outperforms older generation β-lactamase inhibitors such as clavulanic acid **14** and tazobactam. The ceftazidime-avibactam combination does however exhibit a higher range of MICs against *P. aeruginosa* strains and poor activity against *Acinetobacter* spp and MBL-producer strains.[37,38,40,47] Overproduction of efflux pumps and reduced outer membrane permeability has been suggested to be responsible for ceftazidime-avibactam resistance in *P. aeruginosa* isolates.[48] Ceftazidime-avibactam has also been evaluated in a number of clinical trials for the treatment of complicated urinary tract infections (cUTI) and complicated intra-abdominal infections (cIAI). The published data indicate that overall the combination is well-tolerated by patients and noninferiority to its comparator drugs such as imipenem-cilastatin,

meropenem **5** and doripenem was achieved.[49–52] Avycaz® is manufactured and marketed by Allergan as a powder for injection containing a 4:1 ratio of ceftazidime **6** to avibactam based on dry weight.[53] Clinical trials are ongoing to evaluate the efficacy of avibactam in combination with other β-lactam partners including ceftaroline **9** and aztreonam **7** for a number of other indications (ClinicalTrials.gov identifiers: NCT01624246, NCT01281462, NCT01689207 and NCT03329092).

2.3 *Recarbrio® (imipenem + cilastatin + relebactam)*

As recently summarized by Zhanel and co-workers,[54] the diazabicyclooctane (DBO) analog relebactam **19** (figure 6) has a spectrum of β-lactamase inhibition similar to that of the preeminent DBO-based SBL inhibitor avibactam. Relebactam is active against β-lactamases of Ambler class A including KPC carbapenemase and class C. Again as observed with avibactam, metallo-β-lactamases of class B and OXA-type enzymes of class D are not affected by relebactam.[54] This inhibition spectrum is well reflected in the results of susceptibility screenings using a combination of relebactam and imipenem **8**. Used at 4 μg/mL, relebactam potentiated imipenem **8** against gram-negative clinical isolates.[55] While $MIC_{50/90}$ against *E. coli* strains were retained at 0.25/0.25 μg/mL upon addition of relebactam, the combination was effectively synergistic against *K. pneumoniae*, *Enterobacter* spp., and *P. aeruginosa* isolates with $MIC_{90/50}$ reduced to 0.25/0.25 μg/mL, 0.25/0.5 μg/mL, and 0.5/2 μg/mL respectively. Relebactam also successfully reduced the $MIC_{90/50}$ of KPC-producing *K. pneumoniae* and imipenem-resistant *P. aeruginosa* isolates from 16/>16 μg/mL and 8/>16 μg/mL to 0.25/1 μg/mL and 1/2 μg/mL respectively. However, the combination was not active against *A. baumannii* strains producing OXA-23.[55] Further screenings of gram-negative pathogens collected in U.S. and European hospitals confirmed that *A. baumannii*, along with other organisms that produce MBLs or OXA-type enzymes are likely to present a challenge in the use of imipenem-relebactam.[56,57] The *in vitro* performance of imipenem-relebactam was also evaluated against anaerobic gram-negatives of

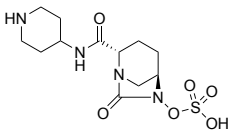

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Figure 6. Relebactam **19**.

Bacteroides group. Among the tested panel of antibiotics, imipenem **8** was found to be most potent with an MIC_{90} of ≤1 μg/mL against all the *Bacteroides* species. However, addition of relebactam did not lead to a further improvement in the activity of imipenem **8**.[58] Similarly, the combination showed excellent activity against gram-positive anaerobes although overall it did not outperform imipenem **8** alone.[59] Phase II studies were conducted in which imipenem-cilastatin plus relebactam or placebo were administered to patients with cIAI[60] and cUTI.[61] Both studies proved non-inferiority of relebactam combination with similar adverse effects profile to the placebo group. Recently, relebactam also completed a Phase III clinical evaluation in combination with imipenem-cilastatin to treat patients with cIAI and cUTI (ClinicalTrials.gov identifier: NCT03293485). Following success in these trials, the combination Recarbrio® (imipenem + cilastatin + relebactam) was granted FDA-approval in 2019 for treating cUTI and cIAI and is currently manufactured and marketed by Merck.

2.4 *Zerbaxa® (ceftolozane + tazobactam)*

Zerbaxa® received FDA approval in 2014 for the treatment of cIAI and cUTI. The drug consists of the novel fifth-generation cephalosporin antibiotic ceftolozane **10** (figure 2) and the established β-lactamase inhibitor tazobactam. Considering that this BL/BLI combination has been the focus of a number of detailed reviews,[62–68] here only the structural features of ceftolozane **10** as well as an overview of the antibacterial spectrum of its combination with tazobactam, including key outcomes of clinical trials, is covered. Ceftolozane **10** was evolved as the result of a medicinal chemistry efforts aimed at developing a cephalosporin with improved potency against AmpC-producing *P. aeruginosa* strains.[69–71] This was achieved by a series of structural modifications of the substituents at C3 and C7 position of the cephalosporin core. On C-7 position, in addition to the thiadiazole ring and oxyimino moiety, which are believed to be responsible for the extended spectrum of anti-gram-negative activity and resistance to some β-lactamases,[72] ceftolozane **10** also contains a dimethylacetic acid moiety which increases affinity to some PBPs, especially PBP3. After evaluating a number of protomolecules, it was eventually established that placement of a pyrazolium ring containing a basic side chain improves permeability, stability against Pseudomonal AmpC, and minimizes off target effects associated with the positively charged moiety.[69,70] To determine to what extent the activity of ceftolozane **10**, then known as FR264205, was affected by major resistance mechanisms of *P. aeruginosa*, it was assayed against variants producing AmpC, overexpressing efflux pumps, and lacking OprD. These studies revealed that ceftolozane **10** showed superior performance to ceftazidime **6** against

all the resistant mutants and its activity was not affected by efflux pump overexpression and OprD loss.[71] The inhibitory activity of tazobactam on the other hand, is highest against class A β-lactamases such as TEM, SHV, CTX-M enzymes.[3] In doing so this inhibitor extends the activity spectrum of ceftolozane **10** against ESBL-producing gram-negative bacteria. Indeed, when tazobactam was combined with ceftolozane **10**, it strongly enhanced the activity of ceftolozane **10** against ESBL-producer and AmpC-hyperproducing gram-negative bacteria in a concentration-dependent manner. Notably, strains producing KPC were not susceptible to the combination.[73] Farrel and co-workers reported the screening results of 7071 *Enterobacteriaceae* strains isolated from U.S. hospitals. Overall, ceftolozane-tazobactam (TOL-TAZ) showed potent activity with an MIC_{90} of 1 μg/mL making it equipotent to cefepime and tigecycline. Also noteworthy was the performance of the ceftolozane-tazobactam combination against *E. coli* isolates with an ESBL phenotype (MIC_{90} = 4 μg/mL) as well as 1971 tested *P. aeruginosa* isolates (MIC_{90} = 2 μg/mL) showing it to be superior to combinations of ceftazidime **6** or piperacillin **4** with tazobactam (MIC_{90} = 32 and >64 μg/mL respectively).[20] These findings were in agreement with the screening results against 2435 *P. aeruginosa* strains isolated from patients in Canadian hospitals.[74] The MIC_{90} of 1 μg/mL for TOL-TAZ was found to be superior to those of colistin (MIC_{90} = 2 μg/mL) and meropenem (MIC_{90} = 8 μg/mL) among the panel of tested antibiotics.[74] Tazobactam also potentiates the activity of ceftolozane **10** against anaerobes. Using a collection of 605 gram-negative and gram-positive anaerobic isolates, Snydman and co-workers observed high activity for TOL-TAZ against *Bacteroides* spp specially *Bacteroides fragilis* (MIC_{90} = 4 μg/mL) and excellent activity against gram-negative anaerobes *Prevotella* spp and *Fusobacterium* spp (MIC_{90} ≤ 0.125 μg/mL).[75] The same study also revealed that ceftolozane-tazobactam has very little activity against *Clostridium* spp. Based on the results described above, TOL-TAZ can be viewed as a new carbapenem-sparing therapeutic option when facing clinically important pathogens such as ESBL-producing *Enterobacteriaceae* and *P. aeruginosa* including AmpC-hyperproducers. However, the antibiotic activity of the combination is expected to be compromised by pathogens expressing highly active carbapenemases and/or MBLs. In this regard a recent phase III clinical trial named ASPECT-cIAI evaluated TOL-TAZ plus metronidazole in patients with complicated intra-abdominal infections (cIAI).[76] The combination showed efficacy against infections with *Enterobacteriacea*e producing CTX-M-type ESBLs and proved to be non-inferior to meropenem **5** as the comparator drug. For the treatment of cUTI including pyelonephritis, another phase III clinical trial known as ASPECT-cUTI was conducted to compare the efficacy of TOL-TAZ with that of levofloxacin. Overall, TOL-TAZ

proved to be non-inferior to levofloxacin and adverse events were moderate.[77] Zerbaxa® is manufactured by Merck as powder for injection comprised of a 2:1 (by weight) mixture of ceftolozane **10** and tazobactam.[78]

3. SBL inhibitors: Recent and ongoing developments

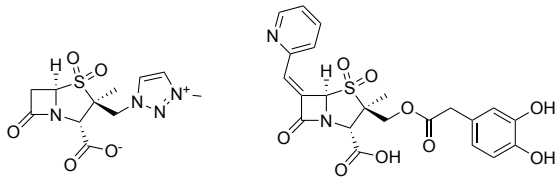
Summarized in table 1 are the drug candidates currently being evaluated in clinical trials spanning the past 10 years. These SBLIs can be structurally classified into β-lactams and non-β-lactams. BLIs with β-lactam structure are represented by the classic inhibitors such as clavulanic acid **14**, sulbactam **15**, and tazobactam. Recently, a structurally similar analog of tazobactam known as AAI101 successfully completed a phase II clinical trial (EudraCT Number in EU clinical trials register: 2016-005161-31). Efforts to discover BLIs among novel scaffolds have also resulted in two important new classes of SBLIs including the diazabicyclooctanes (represented by avibactam) and cyclic boronates (represented by vaborbactam). The following section covers these new SBLIs classes and their current state of clinical development.

3.1. β-lactams

As far as can be gleaned from published reports, AAI101 (**20**, figure 7) is being evaluated in clinical trials as a combination with the fourth-generation cephalosporin cefepime (EudraCT Number in EU clinical trials register: 2016-005161-31). The results of MIC screening using cefepime **11** and various concentrations of AAI101 showed a concentration-dependent

Table 1. BLIs currently in the clinical development stage.

Name/Code	Chemical class	Clinical development phase
Nacubactam	diazabicyclooctane	Phase I in combination with meropenem
Zidebactam	diazabicyclooctane	Phase I in combination with cefepime
ETX2514	diazabicyclooctane	Phase III in combination with sulbactam
Avibactam	diazabicyclooctane	Approved in combination with ceftazidime
		Phase II in combination with ceftaroline fosamil
		Phase I in combination with aztreonam
Vaborbactam	cyclic boronate	Approved in combination with meropenem
		Phase I in combination with biapenem
Taniborbactam	cyclic boronate	Phase III in combination with cefepime
AAI101	penam sulfone	Phase III in combination with cefepime



20 **21**

Figure 7. SBL inhibitor penam sulfones AAI101 (**20**) and LN-1-255 (**21**).

synergistic effect against *K. pneumoniae* and *E. coli* strains with carbapenem-resistance phenotypes.[79] Another study found high activity for the combination particularly against ESBL-producing *Enterobacteriaceae* ($MIC_{50/90}$ = 0.125/0.5 μg/mL).[80]

LN-1-255 (**21**, figure 7) is a penicillin sulfone inhibitor which has been reported to inhibit multiple class of SBLs.[81] Pattanaik and co-workers reported strong inhibition of SHV-1 and SHV-2 (class A) by LN-1-255 and potentiation of ceftazidime **6** against strains producing TEM, SHV, CTX-M and Sme-1 enzymes.[82] Crystallographic data obtained for SHV-1 suggests that LN-1-255 acylates the enzyme followed by rearrangement to a bicyclic indolizine adduct.[82] Also interesting was the potent activity of this inhibitor against multiple enzymes of OXA family and its ability to reduce the MIC of carbapenems against OXA-producing *E. coli*, *K. pneumoniae* and *A. baumannii* strains.[83–85]

3.2. Diazabicyclooctanes

Zidebactam. The acyl hydrazide DBO analog of the DBO family, zidebactam **22** (figure 8) belongs to the newest generation of DBO-based SBLIs with potent PBP inhibitory activity. Although not an inhibitor of class D β-lactamases,[86] zidebactam selectively inhibited *P. aeruginosa* PAO1 PBP2 enzyme. A combination of zidebactam and cefepime **11** effectively inhibited growth of the *P. aeruginosa* PAO1 strain and its knock-outs with defective porins.[87] Also interesting was the increased activity of the combination of zidebactam with selected β-lactams against VIM-1/VIM-2-producing *P. aeruginosa* clones. The most potent activities were observed when the monobactam agent aztreonam **7** was used as β-lactam partner.[87] Likewise, an enzymatic study focusing on *A. baumannii* showed strong and selective inhibition of *A. baumannii* PBP2 by zidebactam, while no inhibition was observed against OXA-23. Interestingly, in antibacterial assays, 8 μg/mL of zidebactam was found to reduce the MIC of cefepime **11** and sulbactam **15** against OXA-23 producing *A. baumannii* to 16 μg/mL (4-fold reduction) and 2 μg/mL (8-fold reduction) respectively. The enhancing effect in this case could

be attributed to the contribution of zidebactam to PBP (and not β-lactamase) inhibition.[88] Zidebactam in combination with cefepime **11** showed excellent *in vitro* inhibition when evaluated against 7876 gram-negative clinical isolate collected worldwide.[89] Overall, the 1:1 combination effectively inhibited *Enterobacteriaceae* isolates with an MIC_{90} of 0.12 μg/mL compared with 16 μg/mL when cefepime **11** was tested alone. The combination also largely enhanced the potency of cefepime **11** by at least 16-fold against clinically important sub-classes including carbapenem-resistant *Enterobacteriaceae*, ESBL phenotype *E. coli*, and ESBL phenotype *Klebsiella* spp. Zidebactam reduced the MIC_{90} of cefepime **11** from 32 to 4 μg/mL against *P. aeruginosa* and from >64 to 32 μg/mL against *Acinetobacter* spp.[89] Another study demonstrated the strong antibacterial activity of a 1:1 mixture of cefepime-zidebactam against a number of *Enterobacteriaceae* expressing various β-lactamases including: CTX-M-15 (MIC_{90} = 1 μg/mL), SHV (MIC_{90} = 0.25 μg/mL), ESBLs (MIC_{90} = 1 μg/mL), plasmid AmpC (MIC_{90} ≤0.06 μg/mL), derepressed AmpC (MIC_{90} = 0.5 μg/mL), KPC (MIC_{90} = 1 μg/mL) and MBLs (MIC_{90} = 8 μg/mL). The inhibitory activity of the same combination had only moderate activity against *P. aeruginosa* and *A. baumannii* isolates.[90] Currently, two phase I clinical trials evaluating the safety, tolerability, and pharmacokitenics of zidebactam have been completed with a third study currently recruiting patients (ClinicalTrials.gov identifiers: NCT02674347, NCT02707107 and NCT02942810).

Nacubactam. Also known as OP0595, nacubactam **23** (figure 8) is an aminoethoxy-substituted analog of avibactam which inhibits class A/C β-lactamase and PBP2. Nitrocefin-based enzyme assays showed inhibition of TEM, CTX-M, KPC-2 (class A), AmpC and CMY-2 (class C) by nacubactam with sub-μM IC_{50} values. This inhibitor showed relatively weak activity against OXA enzymes and none against IMP-1. Similar to zidebactam, nacubactam selectively inhibited PBP2 (IC_{50} = 0.12 μg/mL) and upon incubation with *E. coli*, it induced the formation of spherical cells which is an expected result of PBP2 inhibition.[91] Interestingly, nacubactam has been reported to possess antibacterial activity when tested alone.[92–94] A recent study found that when administered at ≤4 μg/mL, nacubactam inhibited most of the *E. coli*, *Enterobacter* spp., *Citrobacter* spp., and *Klebsiella* spp. strains tested, although it had a poor performance against *Serratia* spp, *P. aeruginosa* and *A. baumannii*.[94] Against those strains with an MIC >4 μg/mL, nacubactam strongly enhanced the activity of aztreonam **7**, cefepime **11**, biapenem **12** and piperacillin **4** in a concentration-dependent manner. In addition, the activity of nacubactam combined with the above-mentioned antibiotics against *Enterobacteriaceae* producing

carbapenemases (KPC, OXA-48 and MBLs) was significant and superior to that of ceftazidime-avibactam. However, nacubactam did not potentiate the same antibiotics when tested against *A. baumannii* strains and MBL-producing *P. aeruginosa*.[94] Since *in vitro* studies of β-lactamase inhibition by nacubactam is complicated due to its inherent antibacterial activity, Livermore and co-workers[95] prepared nacubactam-resistant *Enterobacteriaceae* mutants with elevated MIC values of 8 to >32 μg/mL. When nacubactam was tested against these mutants producing ESBLs, KPC, and OXA enzymes, use of 2 μg/mL of nacubactam, greatly enhanced the activity of piperacillin **4**, cefepime **11**, and aztreonam **7** leading to mean MIC values of <1 μg/mL for these three β-lactamase families. A similar reduction of mean MIC (From 8.43 μg/mL to <1 μg/mL) was observed when nacubactam was combined with meropenem **5** and assayed against KPC-producing mutants. Also interesting was the finding that nacubactam at 1 μg/mL reduced the mean MIC of aztreonam **7** against MBL-producing mutants from 4.68 to 0.072 μg/mL. Taken together the study suggests that the synergy observed by nacubactam is not limited to its PBP2 inhibition but also its inhibition of class A/C β-lactamase. In addition, nacubactam in combination with aztreonam **7** might provide a viable therapeutic option against MBL-producing gram-negative pathogens.[95] To date, two clinical trials evaluating safety, pharmacokinetics, and intrapulmonary lung penetration of nacubactam have been completed (ClinicalTrials.gov identifiers: NCT02134834 and NCT03182504).

ETX2514. Another recently described DBO-based β-lactamase inhibitor known as ETX2514 (**24**, figure 8) has demonstrated a very broad spectrum of activity including inhibition of class A/C/D β-lactamases and PBP2.[96,97] In preparing ETX2514 Durand-Réville and co-workers[96] modified avibactam with the aim of introducing activity against a broader panel of OXA enzymes known to complicate the treatment of resistant *A. baumannii* isolates. Introduction of an endocyclic double bond was implemented to increase chemical reactivity of the ring, and

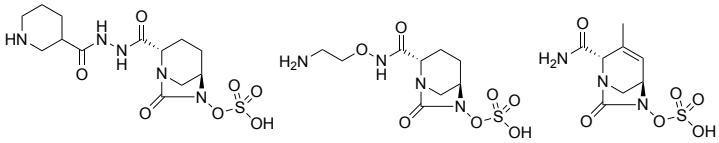


Figure 8. Diazabicyclooctanes in clinical development: zidebactam **22**, nacubactam **23**, ETX2514 **24**.

addition a of methyl group at C-3 (figure 8) led to ETX2514 which displayed a potent inhibitory activity against OXA-24 (IC_{50} = 0.19 μM) along with enhanced biochemical and antibacterial activity. This finding was supported by X-ray crystallography data and molecular modeling of ETX2514 and avibactam which revealed the mode of binding to OXA-24.[96] Another interesting finding was the inhibitory activity of ETX2514 against PBPs with preference to PBP2 of *E. coli* and *A. baumannii*. Use of 4 μg/mL of this inhibitor, decreases the MIC_{90} of imipenem **8** by 8-fold to 2 μg/mL, while its combination with sulbactam **15** most effectively inhibited growth of *A. baumannii* reducing the MIC_{90} of sulbactam **15** from 64 μg/mL to 4 μg/mL. The intrinsic activity of sulbactam **15** against PBP3 plus the dual BL/PBP inhibition by ETX2514 may explain the excellent activity of their combination against *A. baumannii* a challenging nosocomial pathogen that is often multi-drug resistant.[96] A follow-up study showed that similar to avibactam, ETX2514 acylates β-lactamases of class A, C and D.[98] Mass-spectrometry analysis of the resulting enzyme-inhibitor complexes suggested that ETX2514 can recyclize and is released in intact form when incubated with AmpC, CTX-M-15, P99, SHV-5 and TEM-1. On the other hand, interaction with KPC-2, OXA-10, OXA-23, OXA-24 and OXA-48 was accompanied by desulfation and irreversible degradation of the inhibitor. A combination of ETX2514 with imipenem **8** and piperacillin **4** was highly active against isogenic *P. aeruginosa* producing class A, C and D β-lactamases. Compared to avibactam, ETX2514 displayed superior and broader spectrum of activity specially against OXA family of enzymes.[98] Additionally, Iyer and co-workers demonstrated that ETX2514 uses the outer membrane porin $OmpA_{Ab}$ to permeate the *A. baumannii* membrane and synergize with sulbactam **15**.[99] A phase III clinical trial of ETX2514 in combination with sulbactam **15** is currently recruiting participants (ClinicalTrials.gov identifier: NCT03894046).

Review of the recent patent literature reveals a number of other functionalized DBO analogs with SBL inhibitory activity (figure 9). Chang and co-workers reported isoxazoline analogs **25** and specially **26** reduced the MIC of meropenem **5** against *K. pneumoniae* strains producing class A/C/D enzymes by up to 1024-fold.[100] Gu and co-workers reported another group of oxadiazole-substituted analogs **27** as SBL inhibitors.[101] Hydroxamate and hydrazide analogs **28-30** were reported by Maiti and co-workers, as exhibiting potent inhibition of class A and C enzymes with <19 nM IC_{50} values. Of note, compound **30** not only demonstrated high intrinsic antibacterial activity but also when combined with meropenem **5** inhibited *E. coli* and *K. pneumoniae* strains expressing several β-lactamases of class A, B and C.[102] Also noteworthy is

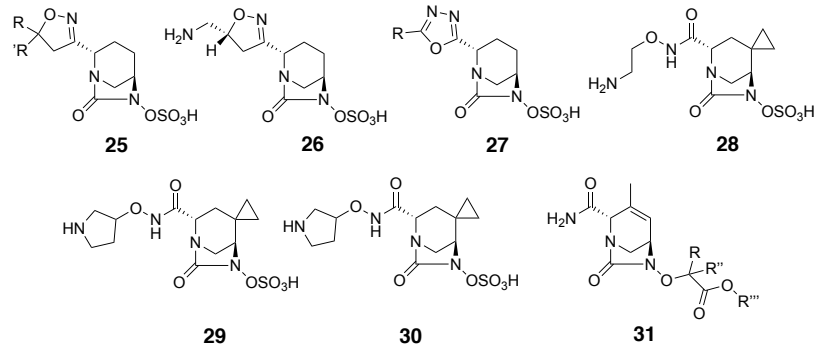


Figure 9. DBO analogs as β-lactamase inhibitors reported in the recent patents.

the report by Comita-prevoir and co-workers of a large library of DBO analogs closely related to ETX2514 wherein the sulfate moiety is replaced by functionalized glycolates.[103] Several compounds with the general structure of **31** demonstrated potent inhibition of TEM-1, AmpC, and OXA-48. These analogs also synergized with cefpodoxime **13** against *Citrobacter freundii*, *E. coli*, and *K. pneumoniae* strains producing multiple β-lactamases of class A, C, and D, and showed *in vivo* efficacy in mouse models of infection.[103]

3.3. Boronates

Boronate-based β-lactamase inhibitors have long been of interest given their resemblance to the tetrahedral intermediate formed upon β-lactam ring attacked by the nucleophilic serine of β-lactamases.[104] For this reason these BLIs are sometimes referred to as boronic acid transition-state inhibitors (BATSIs).[105] Figure 10 shows the chemical structures of a number of such boronates that have been investigated for SBL inhibition including acyclic boronic acids (represented by **32**-**34**)[106–108] or cyclic boronate analogs (represented by **35**, **37** and **38**).[109–111] Of particular note are recent studies aiming at developing cyclic boronates as pan-β-lactamase inhibitors, the rationale being that both MBL- and SBL-mediated hydrolysis of β-lactams involve a tetrahedral transition state that precedes ring opening. Therefore, structures mimicking the transition state have the potential to exert cross-class β-lactamase inhibition (figure 11). Validation of this idea is found in the structural diversity of boronates contained in a number of patent applications claiming both SBL and MBL inhibition. Of note are the acyclic boronic acids represented by **36** which show inhibition of some SBLs and VIM-2 enzyme of class B[112] as well as the cyclic boronates **37**[113,114] and **38**[115–118] (figure 10) which display sub-μM IC_{50}

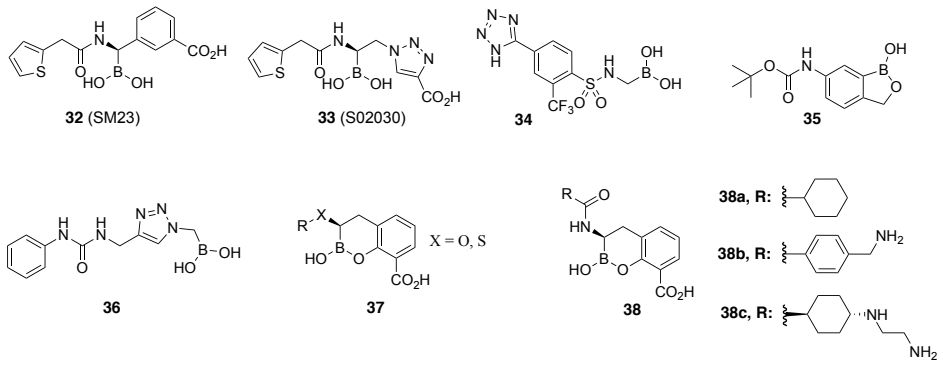


Figure 10. Representative boronic acids as β-lactamase inhibitors

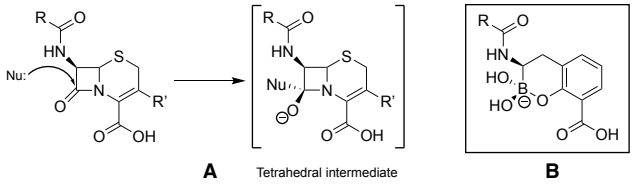


Figure 11. **A**. Tetrahedral intermediate formed by nucleophilic attack of SBLs (Nu: = serine-OH) and MBLs (Nu: is zinc-coordinated OH^-) on β-lactam ring. **B**. Cyclic boronates mimicking the tetrahedral transition state of β-lactam hydrolysis.[110,111]

values for both SBLs and MBLs. By screening a series of cyclic boronates, Brem and co-workers identified a series of SBL-inhibitor analogs with potent activity against MBLs, specifically VIM-2 and NDM-1.[110] Interestingly, **38b** was found to exert potent inhibition of PBP-5. X-ray crystallography studies with **38b** on BcII, VIM-2, OXA-10, and PBP-5 confirmed that the cyclic boronate structure interacts with the crucial β-lactamase residues (and coordinates with Zn^{2+} of MBLs) in the way that mimics the high energy transition state intermediates formed in each case. In addition, **38b** largely enhanced the activity of meropenem **5** towards *Enterobacteriaceae* expressing multiple β-lactamases.[110] A follow-up study confirmed nM range IC_{50}s for the activity of cyclic boronate analogs against TEM-1, CTX-M-15, and AmpC. Compound **38b** exhibited a synergistic relationship with carbapenems against *Enterobacteriaceae* producing multiple β-lactamases including KPC-2, OXA-181 (meropenem only), VIM-1, and VIM-4. However, carbapenemase-producing *P. aeruginosa* and *A. baumannii* strains remained resistant to all combinations.[111] Further structural optimizations with the aim of improving MBL-inhibition and

accumulation in gram-negative bacteria led to the development of taniborbactam (formerly VNRX-5133, **38c**).[119–121] Kinetic experiments using CTX-M-15, KPC-2 and P99 ApmC showed that this compound is a competitive inhibitor of these clinically important β-lactamases.[121] When tested against a large panel of β-lactamases, taniborbactam showed promising biochemical inhibition of enzymes of all 4 Ambler classes. It should, however, be mentioned that taniborbactam did not perform well against the class B enzyme IMP-1 in contrast with its potent inhibition of NDM-1 and VIM-types MBLs.[119] Notably, taniborbactam was found to be a selective β-lactamase inhibitor with no activity against human serine-hydrolases, showed little toxicity toward mammalian cell lines, and its pharmacokinetic parameters are compatible with that of cefepime, a fourth-generation broad-spectrum cephalosporin. In combination with cefepime, taniborbactam significantly reduced the bacterial count in mouse models for lung infection and ascending urinary tract infection.[119] A phase III clinical trial of cefepime/taniborbactam is currently in progress (ClinicalTrials.gov Identifier: NCT03840148).

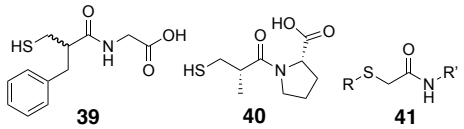
4. Recent advances in the development of MBL inhibitors

Based on their catalytic activities, β-lactamases are classified as serine β-lactamases (SBLs, Ambler class A, C and D) and metallo-β-lactamases (MBLs, Ambler class B). The latter contains zinc ion(s) in the active site which is stabilized by histidine, cysteine and aspartate residues and is also bound to an active water molecule responsible for hydrolyzing β-lactams. MBLs in turn are divided into subclasses B1, B2, and B3. While enzymes of class B1 and B3 contain two zinc ions, B2 functions with only one.[5,122] The most clinically relevant MBLs include NDM, VIM, and IMP enzymes of class B1 which inactivate a broad range of β-lactams but have a low affinity for monobactams.[123] Due to their carbapenemase activity and rapid dissemination, MBLs pose a serious challenge to the antibiotic treatment of infections caused by gram-negative bacteria. The design and development of broad-spectrum MBL inhibitors is challenged by the high active site heterogenicity of the different enzymes of this family.[3,8,124,125] As a result, to date, there are no effective MBL inhibitors currently in clinical use.

Compounds classes with the potential to inhibit MBLs have been the subject of several detailed reviews.[5,123–128] Therefore, the rest of this chapter focuses on new developments in the field of MBL inhibitors over the past decade.

Traditionally, sulfur-containing compounds have been one of the most studied classes of small molecules in the search for MBL-inhibitors. Compounds containing a variety of free thiols, thioethers, thioesters, thioketones, and thioureas have been recently reported to possess inhibitory activity against different class of MBLs.[124] Also of note are thiol-containing drugs that while approved for other indications have shown some capacity to inhibit MBLs. In this regard Klingler and co-workers found that thiorphan (**39**, figure 12), the active metabolite of the antidiarrheal racecadotril, inhibits NDM-1, IMP-7, and VIM-1 with low-μM IC_{50} values and also markedly enhances the activity of imipenem **8** against MBL-producing strains.[129] In addition, captopril **40** an FDA-approved drug used for the treatment of hypertension, has also received some attention for its ability to inhibit NDM-1 (IC_{50} = 7.9 μM).[130] Building upon these findings, efforts have been made to replace the prolyl residue of captopril with various other functional groups,[130–133] as well as modification of the thiolated acyl residue, and/or ring size.[132–134] Brem and co-workers also found the MBL inhibition of D-captopril to be superior to that of its other stereoisomers when evaluated against BCII, IMP-1, VIM-2, NDM-1, and SPM-1.[135] These findings were further supported and could be rationalized by X-ray crystallography studies reported in the same paper.[135]

It has long been known that mercaptoacetic acid and its related structural analogs are among the potent MBL-inhibitors.[124] Recent reports have described the development of aminoacid thioesters of mercaptoacetic acid as inhibitors of L1, an MBL of the B3 class.[136,137] Substituted amide derivatives of mercaptoacetic acid (mercaptoacetamides, **41**) are also prominent in a number of recent studies: Arjomandi and co-workers reported a series of amino acid conjugates of mercaptoacetamide and some longer chain homologs (mercaptpropionamide and mercaptobutyramide) which display IMP-1 inibition.[138] Other studies employed mercaptoacetamide thioethers containing acetate[139] and azolyl ring[140–143] substituents. The diverse library of thiol-containing MBL-inhibitors also include thiomethylbenzoic acids,[144] bisthiazolidines,[145,146] rhodanines and its related thioenolates,[147–150] cysteine-containing oligopeptides,[151,152] mercaptopyridine *N*-oxides,[153,154] phosphonate and tetrazole bioisosteres of



39 **40** **41**

Figure 12. Thiol-containing MBL inhibitors, thiorphan **39**, captopril **40** and substituted mercaptoacetamides **41**.

mercaptoacids[155], and thiones.[156–158] Finally, it should be added that although thiols are among the most potent and broad-spectrum inhibitors of MBLs, their tendency to rapidly oxidize to disulfides poses a serious challenge to further clinical developments. This is important since studies suggest that upon disulfide formation zinc-binding affinity is greatly reduced leading to a loss of MBL-inhibition and *in vitro* synergistic activity.[159,160] Creative chemical modifications to enhance the biological stability of thiol-based inhibitors may be the key to develop such compounds as clinically viable drug candidates.

Picolinic acid derivatives are another well-known class of zinc chelators and act via the same metal-sequestration mechanism as EDTA to inhibit MBLs.[161] In fact pyridine-2,6-dicarboxylic acid also known as dipicolinic acid or DPA (**42**, figure 13) is a commonly used reagent for the phenotypic detection of MBL-producing pathogens.[162–165] By evaluating a series of DPA analogs– represented by compound **43** – Chen and co-workers identified compounds with enhanced NDM-1 inhibition that retained MBL-selectivity over other zinc-dependent metalloenzymes.[166] Compound **43** inhibited NDM-1, VIM-2, and IMP-1 with IC_{50} values of 0.080, 0.21 and 0.24 μM respectively and demonstrated synergistic relationship with imipenem **8** when tested *in vitro* against NDM-1-producing *E. coli* and *K. pneumoniae* isolates. Also of note are the results of various experiments including NMR and equilibrium dialysis suggesting that compound **43** engages in a ternary complex with zinc and NDM-1 unlike its parent compound DPA and EDTA.[166] This was followed up by an isosteric replacement study of DPA (**42**) where the same group found **44** to be a potent inhibitor of NDM-1 (IC_{50} = 0.13 μM). The inhibitory

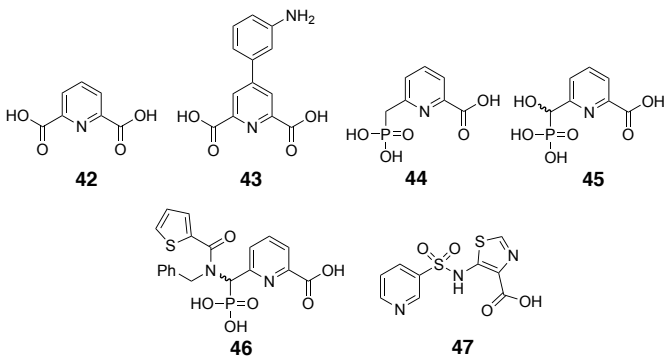


Figure 13. Pyridine derivatives as MBL-inhibitors.

mechanism of **44** studied by membrane dialysis assay and UV/VIS spectroscopy suggested that this compound removes a Zn^{2+} ion from the NDM-1 active site.[167] In a complimentary study, Hinchliffe and co-workers investigated the potential of phosphonate analogs of 2-picolinic acid to inhibit MBLs of B1 and B3 sub-class. They found potent and broad-spectrum inhibition of NDM-1, VIM-2, IMP-1, and L1 by compounds **44**-**46**. Compound **44** reduced the MIC of meropenem **5** down to 8 to <0.125 μg/mL against both recombinant and clinically isolated gram-negative strains producing the earlier mentioned MBLs.[168]

Recently, Antabio Inc. reported the discovery of the sulfonamide small molecule ANT431 (**47**) which was also evolved from 2-picolinic acid.[169] After demonstrating strong inhibition of NDM-1 (K_i = 0.29 μM) and VIM-2 (K_i = 0.19 μM) and the potentiation of meropenem **5** against the BL21 *E. coli* producing the mentioned enzymes, ANT431 was tested against 94 MBL-producing clinical isolates of *Enterobacteriaceae* family. When used at 30 μg/mL, this compound resensitized 72% of the isolates to meropenem **5**. X-ray crystallography studies showed that the thiazole nitrogen as well as carboxylate of ANT431 interact with Zn2 of VIM-2 enzyme.[170] ANT431 also demonstrated *in vivo* efficacy in a mouse model of infection with NDM-1 producing *E. coli* and is currently being considered as a suitable starting point for further lead optimization.[169]

There are multiple reports on the MBL inhibitory activity of dicarboxylic acids.[124] Guided by an X-ray crystallography study of compound **48** (figure 14) in complex with IMP-1, Hiraiwa and co-workers designed and synthesized di-substituted phthalic acids among which the bis(4-hydroxypiperidine) derivative **49** showed strongest inhibition towards IMP-1 (IC_{50} =

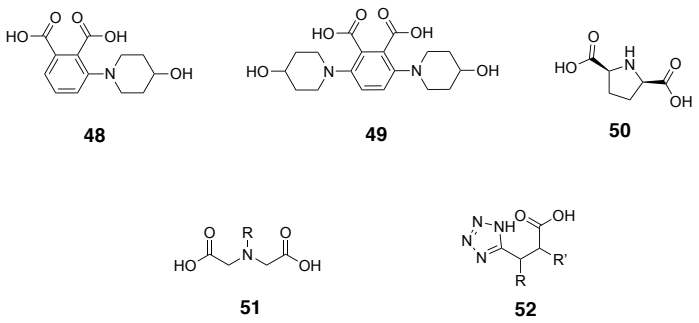


Figure 14. dicarboxylic acid analogs as MBL-inhibitors.

0.270 μM) and reduced the MIC of biapenem **12** against IMP-1 producing *P. aeruginosa* strains by at least 128-fold to ≤0.5 μg/mL.[171] Also recently described as MBL inhibitors are dicarboxylate substituted, five-membered heterocycles with 2,5-pyrrolidinedicarboxylic acid **50** identified as a potent competitive inhibitor of CcrA (K_i = 0.73 μM) and L1 (K_i = 0.69 μM).[172] Notably, compound **50** reduced the MIC of cefazolin against CcrA and L1 producing *E. coli* strains to <1 μg/mL concentrations.[172] As the linear analog of **50**, iminodiacetic acid derivatives (**51**) have been investigated for their potential to inhibit MBLs. Through a series of analog syntheses, it was found that NDM-1 inhibition was best for furyl-substituted analogs, although IC_{50} values were moderate (8.6 μM being the lowest).[173] Tetrazolylpropionic acids such as compound **52** have also been explored as bioisosteres of dicarboxylates and reported to possess potent MBL activity with sub-μM IC_{50} values against NDM-1, IMP-1, and VIM-1.[174]

As described above, Zn^{2+} plays a vital role in the catalytic activity of MBLs and a variety of chelating agents have been shown to inhibit this class of enzymes and resensitize MBL-producing pathogens to β-lactam antibiotics. The MBL-inhibitory activity of aspergillomarasmine A (AMA) – a fungal metabolite with strong zinc chelating ability – was recently reported by King and co-workers.[175] After screening a collection of fungal extracts using a phenotypic assay for synergy with meropenem **5**, they isolated and characterized the active component, AMA (**53**, figure 15) and identified it as an inhibitor of NDM-1 (IC_{50} = 4.0 μM) and VIM-2 (IC_{50} = 9.6 μM). AMA greatly reduced the MIC of meropenem **5** to ≤2 μg/mL against gram-negative strains producing NDM and VIM enzymes and demonstrated promising *in vivo* results in a mouse model of infection with NDM-1 producing *K. pneumoniae*.[175] Soon after this report, multiple chemical[176–179] and chemoenzymatic[180] methodologies were developed to synthesize AMA and its closely related analogs. It was found that the diastereomers of AMA possessed similar activities against NDM-1 and VIM-2.[177] The work by Bergstrom and co-workers[181] shed light upon the action mechanism of AMA as it was shown by isothermal titration calorimetry that AMA strongly binds to Zn^{2+} (K_d = 200 nM). In addition, membrane dialysis and NMR experiments demonstrated that AMA inhibits NDM-1, VIM-2, and IMP-7 by stripping zinc from these enzymes.[181]

The semicarbazide moiety is a well-known metal chelator and has been employed in the search for MBL inhibitors.[182] As an example, compound **54** found in the recent patent literature exhibits strong inhibition of NDM-1 (IC_{50} = 35 nM).[182] Other well established metal-chelators such as 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA, **55**) and 1,4,7,10-

tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA, **56**) and their analogs have also been described as MBL inhibitors with the ability to potentiate carbapenems against gram-negative strains producing NDM, IMP or VIM enzymes.[183,184] Similarly, the well-known zinc binder *N*,*N*,*N*',*N*'-tetrakis-(2-Pyridylmethyl)ethylenediamine (TPEN, **57**) has also been shown to synergize with β-lactam antibiotics to kill strains expressing various MBLs.[185] While such chelating agents have been described as nonhemolytic and nontoxic to mammalian cells *in vitro*, their potential to be advanced to clinical application should be viewed with caution due to their presumed lack of target specificity. To address this problem, Yarlagadda and co-workers covalently linked the zinc binding motif dipicolylamine to vancomycin in an attempt to produce bacterial cell-specific hybrid. Given that vancomycin's inability to effectively kill gram-negative pathogens is generally ascribed to its inability to penetrate the gram-negative outer membrane, it is somewhat surprising that the vancomycin derivative **58** showed activity against strains expressing NDM-1 and restored the activity of meropenem **5** in both *in vitro* and *in vivo* experiments.[186]

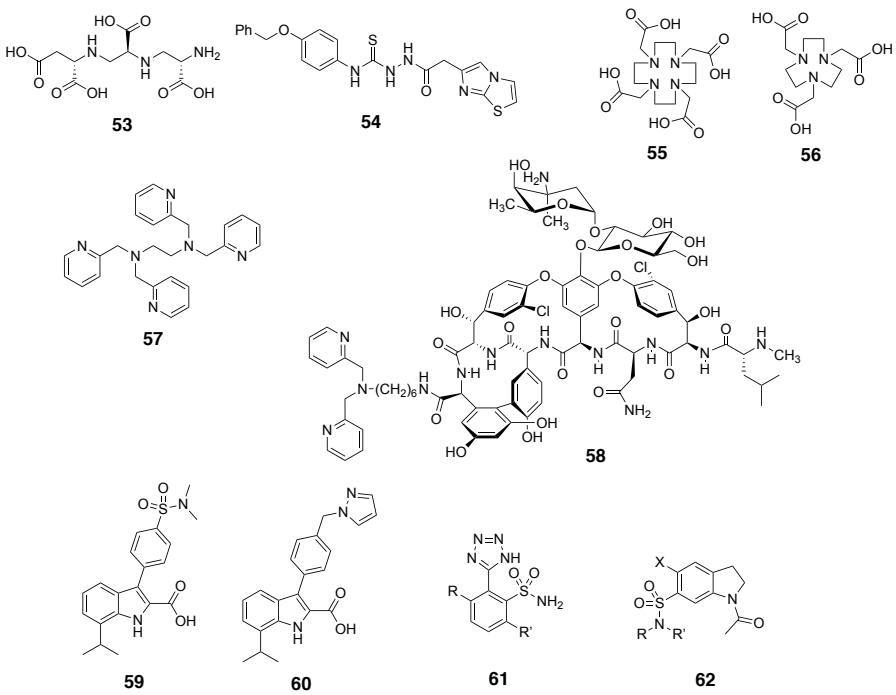


Figure 15. Zinc chelators **52**-**57** and other unique compounds with MBL inhibitory activity.

Another class of MBL inhibitors based on the 3,7-substituted-indole-2-carboxylic acid scaffold was recently reported in the patent literature by Berm and co-workers who screened a large library of analogs for activity against VIM-2, IMP-1, and NDM-1.[187] Several examples were found to possess sub-μM activities among which compounds **59** and **60** were found to be most potent against NDM-1 (IC_{50} values of 0.35 and 0.5 nM respectively). Researchers at Merck have also assessed numerous sulfonamides for MBL inhibitory activity and in a series of patents describe compounds such as 2-tetrazolylbenzenesulfonamides **61**[188,189] as potent inhibitors of IMP-1, VIM-1, and NDM-1. Using a related approach, Fast and co-workers also found indoline-7-sulfonamides such as compound **62** to possess single-digit μM IC_{50} values against NDM-1.[190]

In addition to MBL inhibitors discovered by dedicated screening approaches, a range of other compounds have also been reported to possess anti-MBL activity including: the β-lactam antibiotic cefaclor,[191] 3-formylchromone,[192] ebselen,[193] as well as various hydrazones,[194] phosphonic acids,[195] oxoisoindolines,[196] diphenylpyrroles,[197] and bismuth complexes.[198]

5. Conclusions

In summary, the new generation of SBL inhibitors including avibactam and vaborbactam were significant breakthroughs in that they were developed from non-β-lactam structural backbones. While the activity spectrum of classic β-lactamase inhibitors was limited to non-carbapenemase enzymes of class A and some class C SBLs, avibactam and vaborbactam proved to be potent inhibitors of KPC carbapenemase as well as other class A/C enzymes. Building up the success of this compound class the advanced generation of DBO analogs has provided progress towards achieving broad spectrum SBL inhibitors with activity extending to the clinically important class D OXA enzymes and PBPs. In addition, the advent of cyclic boronate analogs could lead to the first pan-β-lactamase inhibitors due to their structural resemblance to the common transition state formed upon both SBL- and MBL-mediated hydrolysis of β-lactams. For the various other compound classes recently described as MBL inhibitors, challenges including stability in physiological conditions (*i.e.* for thiol-based inhibitors) and site-specificity (as for metal chelators) must first be addressed before their clinical relevance can be properly assessed.

6. Outline

The theme of this thesis is tackling antibiotic resistance through the development of small molecules with the ability to inhibit metallo-β-lactamase enzymes. To this end, we reasoned that molecules that act as zinc-binding ligands could be suitable candidates for preliminary screenings. This effort has been described in **chapter 2**, where a series of commonly-used buffer components previously known to possess metal-binding ability were screened for their inhibitory activity against clinically relevant metallo-β-lactamases. In addition, further analyses on the 2 most potent compounds, including time- and zinc-dependency, zinc-binding affinity using isothermal titration calorimetry (ITC), and synergy assays are described.

The ability of the fungal metabolite AMA to inhibit NDM-1 and VIM-type enzymes was discovered in 2014 after which various groups reported the total synthesis of AMA. Following the success of the Poelarends' group in developing a chemoenzymatic route to synthesize AMA and its related aminocarboxylic acids, we evaluated these new AMA-like compounds for their inhibition of NDM-1, zinc-binding affinity, and ability to rescue meropenem against an NDM-1 producing clinical isolate of *E. coli*. These findings have been described in **chapter 3**.

Soon after the discovery of the MBLs, it was found that a number of thiol-containing molecules exhibit potent and broad-spectrum MBL inhibition. In **chapter 4**, we describe the results of our closer look at the ability of such thiol containing compounds to potentiate meropenem and cefoperazone in cell-based synergy assays. To this end, various concentrations of antibiotic-thiol combinations were tested against a panel of carbapenem-resistant gram-negative bacteria in a checkerboard format. In addition, we evaluated their zinc-binding ability and their chemical stability in culture media.

In **chapter 5** we describe our attempt to address the stability and selectivity problem associated with thiol-based MBL inhibitors using a prodrug approach. The concept applied is based on the hydrolysis pathway of some cephalosporins which leads to fragmentation of the molecule after β-lactam ring opening. This led us to design a prodrug system through which the MBL inhibitor might be released after being hydrolyzed by the MBL enzyme itself. The synthetic route to the novel cephalosporin-thiol conjugates as well as a detailed analysis of their enzyme mediated hydrolysis mechanism studied by ^{1}H-NMR and LCMS are described. The most potent analogs were subjected to further kinetic experiment as well as structure-activity relationship studies.

Chapter 6 describes our kinetic experiments on the bacterial lysate containing a newly identified class A carbapenemase. Previous DNA-sequencing and antimicrobial susceptibility assays performed at the Wageningen Bioveterinary Research centre hinted towards the preference of this new carbapenemase for carbapenems over third-generation cephalosporins. Our kintetic assays provided support for this pattern of substrate preference as well as the sensitivity of this enzyme to clavulanic acid.

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