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(Article begins on next page)

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An update on β-lactamase inhibitor discovery and development.

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ABSTRACT

Antibiotic resistance, and the emergence of pan-resistant clinical isolates, seriously threatens our capability to treat bacterial diseases, including potentially deadly hospital-acquired infections. This growing issue certainly requires multiple adequate responses, including the improvement of both diagnosis methods and use of antibacterial agents, and obviously the development of novel antibacterial drugs, especially active against Gram-negative pathogens, which represent an urgent medical need.

Considering the clinical relevance of both β-lactam antibiotics and β-lactamase-mediated resistance, the discovery and development of combinations including a β-lactamase inhibitor seems to be particularly attractive, despite being extremely challenging due to the enormous diversity, both structurally and mechanistically, of the potential β-lactamase targets. This review will cover the evolution of currently available β-lactamase inhibitors along with the most recent research leading to new β-lactamase inhibitors of potential clinical interest or already in the stage of clinical development.

1. Introduction – The relevance of β-lactamases as drug targets

Antibacterial resistance has now reached alarming levels, with major bacterial pathogens (including Gram-negative organisms such as *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*) quickly evolving towards pandrug resistance phenotypes (Monaco et al., 2014; Rossolini et al., 2014). Without an adequate response to this medical issue, the burden and mortality associated to infectious diseases, and especially hospital-acquired bacterial infections, is consequently expected to significantly increase in the near future (O'Niel, 2014; World Health Organization, 2014).

β-lactam antibiotics, the first natural antibacterial compounds to be successfully developed, and subsequently modified, by the pharmaceutical industry, still represent an outstanding class of antibiotics, thanks to both their excellent antibacterial activity and selectivity, accounting for over 65% of injectable antibiotics in the clinical setting in the U.S.A. (Bush and Bradford, 2016). However, and like any other antibacterial drug used in the clinical setting so far, their use is soon or later followed by the emergence of resistant strains which, following the acquisition of resistance determinants, can rapidly spread on a global scale (the rapid and global diffusion of NDM-1-producing *K. pneumoniae* isolates is a striking example) (Dortet et al., 2014a; Khan et al., 2017).

Mechanisms of resistance to β-lactams include the production of efflux pumps, the modification or reduced production of outer membrane porins (in Gram-negative bacteria), alterations of Penicillin-Binding Proteins (the molecular target of β-lactams) and the production of an enzyme able to inactivate the antibiotic (a β-lactamase) (Frère et al., 1991).

β-lactamase production represents the most relevant mechanism of resistance in Gram-negative pathogens. For that reason, the pharmaceutical industry successfully employed two strategies to overcome β-lactamase-mediated resistance to β-lactams: (a) the optimization of β-lactamase-stable antibiotics (such as the expanded-spectrum cephalosporins and carbapenems that are resistant to hydrolysis by narrow-spectrum β-lactamases or extended-spectrum β-lactamases, respectively) and (b) the development of selective β-lactamase inhibitors to be co-administered with a β-lactam antibiotic. In

that perspective, the discovery of a *Streptomyces clavuligerus* secondary metabolite, clavulanic acid (Scheme 1, **a**), represented a significant milestone in the field of antibacterial discovery. This β-lactam molecule is able to specifically inhibit most of the β-lactamases circulating in the 80's, while showing a modest (if any) antibacterial activity, and allowed for the development of the first β-lactam-β-lactamase inhibitor combination, Augmentin (amoxycillin/clavulanate). This drug encountered a huge commercial success and was followed by the introduction of other combinations with tazobactam (Scheme 1, **b**) and sulbactam (Scheme 1, **c**).

The fast evolution of β-lactamases, with an exponentially-increasing number of variants showing an impressive diversification of functional properties, including the ability to hydrolyze the carbapenems and/or being insensitive to available β-lactam-based β-lactamase inhibitors surely represent an important factor in the evolution of relevant Gram-negative pathogens towards multi-drug resistance (MDR) and extensively-drug resistance (XDR) phenotypes (Bush, 2013). For that reason, colistin, a polymyxin antibiotic is progressively replacing carbapenems as last-resort agents to treat complicated infections caused by carbapenem-resistant XDR pathogens (including carbapenem-resistant isolates of *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*), despite presenting some potential safety issues and requiring a very tightly controlled administration regimen (Biswas et al., 2012).

The rise of antibiotic resistance in the last two decades and the growing need for new antibacterial drugs contributed to a significant regain of interest towards β-lactam/β-lactamase inhibitor combinations. Still considered among the most reliable strategies to deliver antibacterial drugs able to address the development of resistance, a significant number of new combinations entered the stage of clinical development since 2010 (Table 1). One of the first successes deriving from these efforts led to the approval, in March 2015, of the first non-β-lactam β-lactamase inhibitor, avibactam (Scheme 1, **d**), available in combination with ceftazidime (4:1 ratio) for the treatment of complicated urinary tract infections. Following avibactam, the first member of the diazabicyclooctonane class of β-lactamase inhibitors, other structurally-related β-lactamase inhibitors also reached the stage of clinical development, including relebactam (MK-7655) (Scheme 1, **e**), developed by Merck in combination with imipenem-

cilastatin), nacubactam (FPI-1459, RG6080, OP0595) (Scheme 1, **f**) and EXT2514 (Scheme1, **g**). The two latter compounds interestingly show significant inhibition of both β-lactamases and penicillin-binding proteins (Morinaka et al., 2015; Shapiro et al., 2016). Finally, an additional class of non-β-lactam β-lactamase inhibitors, boronic acids (Scheme 1, **h**), successfully reached the final stages of clinical development (Carbavance [RPX7009/meropenem]).

In this review, the major advances in β-lactamase inhibitor discovery and development will be presented, with a focus on the structural basis for inhibition of clinically relevant β-lactamases and the emerging mechanisms of resistance to available next-generation β-lactam/β-lactamase inhibitor combinations.

2. "Classical" β-lactamase inhibitors – oxapenam and penam sulfones – and their evolution

Clavulanate, tazobactam and sulbactam, the three β-lactam BLIs commercially available, behave as "mechanism-based" β-lactamase inactivators. They all irreversibly form a covalent adduct with the catalytic serine of serine-β-lactamases, which further evolves via slow hydrolysis or fragmentation of the inhibitor molecule. Interestingly, the fragmentation of the inhibitor can lead to the formation of inactivated β-lactamase adduct whose half-life is even longer than the initially formed acyl-enzyme (Kuzin et al., 2001; Matagne et al., 1993). The relatively complex mechanism of interaction between these molecules and relevant β-lactamases has been extensively studied (Helfand et al., 2003; Monnaie and Frere, 1993).

These β-lactam BLIs overall show a similar spectrum of activity, being essentially better inhibitors of class A β-lactamases than of other serine-β-lactamases (molecular classes C and D). They do not expectedly inhibit class B β-lactamases, or metallo-β-lactamases, considering the different chemistry underlying catalysis in these enzymes. Metallo-β-lactamases can actually hydrolyze these compounds with a consistent catalytic efficiency (k_{cat}/K_M, 1.6 – 7.2 × 10^4 $M^{-1}\cdot s^{-1}$) (Docquier et al., 2003; Prosperi-Meys et al., 1999).

After they became available, clinical isolates showing resistance to β-lactam/BLI combinations emerged in the clinical setting. These isolates either acquired a β-lactamase natively poorly susceptible to inhibition by BLIs or accumulated mutations in the β-lactamase-encoding genes, which would determine a so-called "inhibitor-resistant" variant. This last strategy was well investigated in TEM- and SHV-type enzymes, in which various specific substitutions were responsible for the alteration of the properties of the enzyme. Interestingly, these substitutions could be located either in or close to the conserved motifs of serine-β-lactamases, such as Met69 or Arg244 or located, more surprisingly, rather far away from the active site (Bush and Jacoby, 2010; Rossolini and Docquier, 2006).

There is still considerable interest in similar β-lactamase inhibitors, and the progressive addition of chemical substituents at positions 2 and 6 of penam (Scheme 1, i) and penem sulfones led to molecules

with interesting properties, especially regarding the potential inhibition of class D β-lactamases which are commonly less susceptible to available conventional inhibitors. Alkylidene penam sulfones, including LN-1-255 (Scheme 1, **i**), were shown to be able to inhibit a wide range of β-lactamases, including important carbapenemases such as OXA-24 and OXA-48 (Bou et al., 2010; Drawz et al., 2010b; Vallejo et al., 2016).

In addition, the development of novel β-lactam/β-lactamase combinations were also driven by the optimization of β-lactams with interesting properties. Ceftolozane-tazobactam is an example of combination between a new cephalosporin in which the addition of tazobactam is beneficial and grant protection from several common clinically-relevant enzymes. As a result, the combination shows a strong antibacterial activity on Gram-negative pathogens, and especially against drug-resistant *Pseudomonas aeruginosa* (Bush, 2012; Scott, 2016).

Following a similar idea, and although these compounds are not always intended to be used in combination with a β-lactam inhibitor, other modified β-lactam molecules have been investigated and contain a "siderophore" (iron-chelating) moiety (Page, 2013). This approach allowed to obtain antibiotics characterized by efficient permeation in the bacterial cell by luring the iron uptake system. These molecular "Trojan horses" are usually characterized with a high antibacterial activity on Gram-negative organisms considered difficult due to the intrinsically low permeability of their outer membrane, especially *Pseudomonas* and *Acinetobacter* (Moynié et al., 2017).

BAL19764, a dihydroxypyridone monosulbactam, was tested in combination with a bridged monobactam BAL29880 and clavulanate (Page et al., 2011). The clever rationale behind using modified monobactams was their stability to hydrolysis by metallo-β-lactamases and their inhibition of class C enzymes. Consequently, this triple combination (known as BAL30376) showed activity on carbapenem-resistant metallo-β-lactamase-producing organisms. Further modification of the molecule yielded its dihydroxypyridone monosulfactam analogue, BAL30072 (Higgins et al., 2012). This compound showed significant activity on a wide range of Gram-negative pathogens producing different kinds of β-lactamases (Hofer et al., 2013; Mushtaq et al., 2013). It also showed a synergistic activity with the carbapenem

meropenem (Hornsey et al., 2013). Although BAL30072 entered phase 1 trials in 2011 and 2014, alone or in combination with meropenem, and in 2015 as an inhaled formulation for the treatment of cystic fibrosis-related infections, its future development seems to be hindered by hepatotoxicity issues (Paech et al., 2017).

This strategy was further exploited in a recent study, in which several substituents were added on the BAL30072 molecule, most notably an isopropyl substituent on the carbon atom between the pyridine and the oxyimino group, yielding a compound with significant activity (MICs, <0.03-1 μg/ml) on several MDR pathogens, including KPC-producing *Enterobacteriaceae*, OXA-23-producing *A. baumannii* and MBL-producing *P. aeruginosa*, favorable pharmacological properties and *in vivo* efficacy in a murine systemic infection model (Tan et al., 2017).

Finally, cefiderocol (S-649266) represents another siderophore mimic-containing β-lactam which also appears to be relatively stable to hydrolysis by clinically-relevant β-lactamases (Ito-Horiyama et al., 2016; Ito et al., 2016b; Kohira et al., 2016). From a structural standpoint, it is an oxyimino-cephalosporin in which the oxyimino group substituent (2-carboxypropyl) is that found in ceftazidime, while the C3 substituent is similar to that found in cefepime (1-methylpyrrolidinium) but includes a catechol (siderophore) moiety. It shows activity on *P. aeruginosa*, *A. baumannii* and carbapenem-resistant *Enterobacteriaceae* producing various types of carbapenemases (metallo-β-lactamase, KPC, NMC-1, OXA-48) (Dobias et al., 2017; Falagas et al., 2017; Ito et al., 2016a).

3. Diazabicyclo-octanones, a versatile scaffold of reversible β-lactamase inhibitors

In response to the increasing prevalence of MDR, XDR or PDR (pandrug resistant) clinical isolates, the need for novel β-lactam/β-lactamase inhibitor combinations that would overcome the weaknesses of classical BLIs and/or last-resort β-lactam agents (such as carbapenems) has been growing in the last two decades. Based on the available knowledge regarding β-lactamase mechanism of action, scientists at Sanofi-Aventis designed a small-sized molecular scaffold that would represent a structural mimicry of β-lactams, although it would be structurally different from a β-lactam, and thus would have the potential to escape the known mechanism of resistance of β-lactam BLIs (Bonnefoy et al., 2004). This molecular scaffold, the diazabicyclo-octanones (DABCO, Scheme 1, **d-g**), allowed to identify avibactam as a potent inhibitor of β-lactamases, with an inhibition spectrum that was considerably broadened when compared to that of clavulanate. Indeed, avibactam efficiently inhibit most class A β-lactamases, including the inhibitor-refractory KPC-type carbapenemases, both the chromosome- and plasmid-encoded class C enzymes and some class D β-lactamases, including the OXA-48 carbapenemase (Bonnefoy et al., 2004; Ehmann et al., 2012; Livermore et al., 2008; Mushtaq et al., 2010; Stachyra et al., 2009).

The crystal structures of different β-lactamase-avibactam covalent adducts provided a structural basis for the broad spectrum of inhibition of the inhibitor (Lahiri et al., 2013; Lahiri et al., 2015). The avibactam molecule bound to the catalytic serine, in which the oxygen carbonyl is located in the oxyanion hole, creates a network of interactions that is very similar in both class A and class C enzymes (Fig. 1A) and closely resembles that of acylated β-lactam substrates. The sulfonate moiety interacts with residues of the conserved KTG motif, while the carboxamide side chain is stabilized by interactions with Asn/Gln residues essentially found in the SxN conserved motif and in the omega loop. The presence of two nitrogen atoms in the inhibitor allows these atoms to interact with catalytically relevant elements of β-lactamases. The N6 atom interacts with conserved residues Ser130 (class A) or Tyr150 (class C). Furthermore, and in class A enzymes, the N1 atom is H-bonded to the deacylation water molecule, in turn bound to the Glu166 which was found to be protonated in the ultra-high resolution structure of the CTX-M-15 adduct (Lahiri et al., 2013). Consequently, this water molecule is not properly oriented for

the attack to the carbonyl carbon and thus unable to proceed towards deacylation (which requires a deprotonated Glu166 to activate and orient the water molecule for the nucleophilic attack on the carbonyl carbon of the substrate/inhibitor).

A similar network of interaction is partially found in class D enzymes, although the overall more hydrophobic nature of the residues defining the active site pocket prevents the carboxamide side chain to be stabilized by polar interactions (Fig. 1B). Furthermore, it has been shown that the binding of avibactam in the OXA-48 carbapenemase induced the shift of some residues, including Ile102, Val120 and Thr213. This difference might explain the overall lower "acylation" rates of avibactam for class D enzyme, although the enzyme-inhibitor adduct showed a similar, if not lower, deacylation rate (Ehmann et al., 2013). The active site carboxylated Lys70 in class D enzymes was also modified upon avibactam binding, as the structures of avibactam-OXA-48 adducts clearly showed a decarboxylated lysine and the presence of a linear CO_2 molecule. Although it is unclear whether lysine decarboxylation happens during or after the formation of the covalent bond between avibactam and the catalytic serine, that situation not only corresponds to a catalytically unproductive state of the enzyme (poorly relevant for avibactam recyclization, see below) but also induces changes in charge distribution in the active site. This likely explains the lower deacylation rates observed with class D enzymes ($6.3 \times 10^{-6} – 1.2 \times 10^{-5}$ s^{-1}), when compared to that of class A or class C β-lactamases (3×10^{-4} $s^{-1} – 2 \times 10^{-3}$ s^{-1}) (Lahiri et al., 2015)

Noteworthy, the mechanism of inhibition of avibactam is significantly different from that of clavulanate or penam sulfones, in that avibactam acts, with most β-lactamases, as a reversible inhibitor, while being a covalent inhibitor (Ehmann et al., 2012). The latter resides in the specific conformation adopted by the serine-bound avibactam in the β-lactamase active site, which shows significant differences with β-lactam substrates or inhibitors (Fig. 2). In the avibactam molecule, the presence of a piperidine ring located "above", rather than besides, the scissile C7-N6 bond is keeping the inhibitor in a relatively strained conformation. As a consequence, and despite the hydrolytic deacylation water molecule was present in most X-ray structures of the complexes, the N6 atom of avibactam is a better nucleophile than the

hydrolytic water, resulting in the (slow) formation of the original molecule and the release of the free enzyme (Ehmann et al., 2013; Lahiri et al., 2013, 2015).

A notable exception to this reversible covalent mechanism of inhibition is observed with the clinically-relevant carbapenemase KPC-2, which shows the ability to undergo inhibitor transformation through a rather complex mechanism, following inhibitor desulfatation and involving the formation of an imine intermediate and, ultimately, the release of 5-oxopiperidine-2-carboxamide (**5**) (Ehmann et al., 2013).

Before the ceftazidime-avibactam combination became available, the potential mechanism underlying the acquisition of inhibitor resistance has been extensively studied in laboratory variants of several clinically-relevant enzymes, including class C β-lactamases (AmpC) and the acquired carbapenemase KPC-2 (Lahiri et al., 2014; Lahiri et al., 2015; Livermore et al., 2015; Papp-Wallace et al., 2015).

Papp-Wallace and colleagues investigated the properties of several KPC-2 laboratory variants for their ability to confer resistance to the combination ceftazidime-avibactam, identifying several substitutions that could lead to a significant reduction of sensitivity of the enzyme towards avibactam inactivation. The S130G KPC-2 variant, in which an catalytically-important residue is modified (Ser130 is a conserved amino acid of serine-β-lactamases), turned out to have a greatly reduced "acylation" rate (rate of formation of enzyme -inhibitor covalent complex) and was able to confer a very high level of resistance to ampicillin, even in the presence of 4 μg/ml avibactam. Interestingly, mass spectrometry data revealed that this variant was unable to hydrolyze or modify the inhibitor at a significant level. Thus, resistance to the combination mainly relies in this case in the lack of reactivity of the enzyme towards the intact inhibitor. Furthermore, it should be noted that KPC-2 exhibits a rather poor hydrolytic activity on ceftazidime, thus benefiting the antibacterial activity of the ceftazidime-avibactam on KPC-2 producers.

More recent reports identified additional substitutions in KPC-3-related variants produced by clinical isolates showing phenotypic resistance to ceftazidime-avibactam that were selected during antibiotic treatment (Shields et al., 2017a). Considering that KPC-3 is a largely spread enzyme and that it shows a higher activity on ceftazidime than KPC-2, the development of resistance to ceftazidime-avibactam

potentially mediated by single mutations is extremely worrying. These KPC-3 variants had one or two substitutions located either in the omega loop (Asp179Tyr) or shortly after the conserved KTG motif (V240G, T243M/A) (Fig. 3) and determined a ≥4-fold decrease of susceptibility to the combination in *E. coli* isogenic strains. Interestingly, such substitutions also apparently impacted on either the production level and/or the functional properties of the variants, with an apparent decrease of its hydrolytic activity on carbapenems, some cephalosporins or aztreonam (Compain and Arthur, 2017; Papp-Wallace et al., 2015; Shields et al., 2017a). This is in agreement with the fact that some, but not all, *K. pneumoniae* clinical isolates with the aforementioned mutations were actually susceptible to meropenem. Although carbapenems might thus represent a viable alternative for the treatment of infections caused by these peculiar KPC-producing *Klebsiella pneumoniae* isolates, it might be anticipated that the accumulation of additional mutations might restore carbapenem resistance in these isolates, either via modification of the KPC enzyme or involving other mechanism (decreased outer membrane permeability) (Shields et al., 2017b).

Avibactam most certainly illustrates how successful the DABCO scaffold could be to generate potent β-lactamase inhibitor, or even more generally, penicillin-recognizing enzymes with a nucleophilic serine, which could lead to the interesting idea of developing DABCO compounds endowed with antibacterial activity due to efficient PBP inhibition (avibactam indeed has a moderate antibacterial on *Enterobacteriaceae*, see below). It is therefore not surprising that several other compounds from this class are currently under investigation. Relebactam (MK-7699, Merck) (**5**), showing a piperidiniun substitutent on the carboxamide side chain, is currently being developed in combination with imipenem-cilastatin (Blizzard et al., 2014). The inhibitor shows properties very similar to that of avibactam except a lower activity on the *Klebsiella* OXA-48 carbapenemase. Being developed in combination with a carbapenem, rather with a oxyimino-cephalosporin, it however shows a slightly different profile of antibacterial activity, especially with OXA-type-producing *Enterobacteriaceae* (Bush and Bradford, 2016).

Nacubactam (RG6080, FPI1459, OP0595) (**6**), another DABCO showing a substituent on the carboxamide side chain (in this case, a 2-aminoethoxy group), interestingly shows a potent inhibition of

both β-lactamases (IC_{50} values as low as 10 nM) and PBP2 (IC_{50}, 370 nM) (Livermore et al., 2015b; Morinaka et al., 2015). Thus, nacubactam has an better intrinsic antibacterial activity (MIC, 1-4 μg/ml with *Enterobacteriaceae* and non-ESBL-producing *K. pneumoniae*) than avibactam (MIC, 8-32 μg/ml) (Livermore et al., 2015a, 2015b). Due to the dual inhibition of both serine-β-lactamases and PBP2, nacubactam shows a remarkable synergy with various β-lactams, translating in a so-called "enhancer" effect. The MIC of piperacillin, a PBP3 inhibitor, is decreased from 1 μg/ml to ≤0.008 μg/ml in the presence of 4 μg/ml nacubactam. Even more interestingly, combinations with piperacillin, cefepime, aztreonam or meropenem yielded MIC values in the susceptibility range with concentrations of RG6080 as low as 1-2 μg/ml with strains producing either an ESBL, an AmpC-type β-lactamase or a KPC carbapenemase. Although RG6080-resistant mutants could be rather easily selected (mutation frequency, $\approx 10^{-7}$), it has been shown that both the β-lactam potentiation and the enhancer effects were maintained in these strains (Doumith et al., 2016).

Relebactam and nacubactam both differ from avibactam by the presence of a bulky substituent on the carboxamide nitrogen. X-ray crystallography studies conducted with class A and class C β-lactamases showed the mode of binding of the inhibitor was essentially unaltered, when compared to that of avibactam, as the substituent was pointing outwards the active site and did not further interact with other relevant residues (Fig. 3), except the terminal amino group of nacubactam side chain, interacting with the Gln145 (already engaged in a H-bond with inhibitor carboxamide carbonyl oxygen) (Blizzard et al., 2014; Morinaka et al., 2015).

Another strategy, besides the diversitifcation of the carboxamide substitutent, regarded the modification of the piperidine ring of the inhibitor and led to the discovery of the more recent DABCO inhibitor, EXT2514 (Scheme 1, **g**). This compound also shows PBP2 inhibition while keeping a potent inhibitory activity towards β-lactamases, with an intrinsic antibacterial activity against *Enterobacteriaceae* (MIC_{90}, 1-8 μg/ml, *Escherichia coli* and *Klebsiella pneumoniae* isolates) (Durand-Réville et al., 2017; Shapiro et al., 2016, 2017). EXT2514 was rationally designed to improve its reactivity towards β-lactamases and its diffusion through the outer membrane of Gram-negatives, without affecting its stability. When compared to

relebactam or nacubactam, EXT2514 shows a more rapid and effective inhibition of all tested serine-β-lactamases, with a large improvement on class D β-lactamases, including the *Acinetobacter* OXA-24 carbapenemase. Exploiting the moderate intrinsic activity of sulbactam on *Acinetobacter* spp., the combination sulbactam/EXT2514 showed a good *in vitro* activity on multidrug-resistant *Acinetobacter baumannii* (MIC_{90}, 4 μg/ml; 191 clinical isolates with resistance to carbapenems, ceftazidime, aztreonam and sulbactam).

4. Metallo-β-lactamase inhibitors: advances and challenges

Metallo-β-lactamases (MBLs) are zinc enzymes constituting the class B of β-lactamases. They are characterized by having a broad substrate specificity being able to hydrolyze all β-lactam antibiotics with the exception of monobactams like aztreonam (Walsh et al., 2005). However, monobactams are inactivated by serine-β-lactamases that are usually co-expressed with MBLs, extending the resistance capability of bacteria. Moreover, MBLs are insensitive to most of the clinically available β-lactamase inhibitors and hence represent one of the main bacterial resistance factors. For the above reason the search of new and potent MBLs inhibitors is of paramount importance and efforts by many laboratories around the world are addressed to this goal.

The difficulty in finding a 'universal' inhibitor of MBLs arises from the structural and mechanistic differences spread among the 3 subclasses of MBLs recognized up to now and that have been classified on the basis of structural similarity about 12 years ago (Garau et al., 2004). This classification is still valid as more MBLs have been detected and added to the three subclasses. Recent reviews are available on this subject (Meini et al., 2015; Mojica et al., 2016) and we limit ourselves to just outline the three subclasses. Despite large sequence divergence, all MBLs share a common αββα fold (Figure 4A). This fold is widely present in Nature, from bacteria to humans, and it is characteristic of a superfamily of dinuclear metallo-enzymes catalyzing a variety of chemical reactions and acting on different substrates (for specialized reviews see (Carine Bebrone, 2007; Pettinati et al., 2016, and references therein). The MBLs metal binding site is located in between the two facing β-sheets and the three MBLs subclasses differ in the metal binding residues and in secondary structure features surrounding the metal binding site that are involved in substrate/inhibitor interactions. These significant differences have been readily identified since the appearance of the first crystal structures of MBLs from all subclasses and have been already properly described in a review by Bebrone (Bebrone, 2007)

All MBLs possess two potential zinc-binding sites and share a small number of conserved motifs bearing some of the residues that coordinate the zinc ion(s). Crystal structures of all subclasses of MBL have been reported containing either one or two zinc ions depending on subtle modifications of the H-

bonding network involving active site residues or, possibly, oxidative modifications of Cys residues and experimental conditions. This has made very difficult to obtain a consistent, general description of the exact role played by the two zinc ions in these enzymes. Although there are examples of mono-zinc B1 enzymes displaying some activity (Murphy et al., 2006), it is generally accepted that subclasses B1 and B3 MBLs are di-zinc enzymes where the binding of the second zinc ion bring some enhancement of the catalytic activity (Palzkill, 2013; Karsisiotis et al., 2014; Meini et al., 2015). On the contrary, subclass MBLs B2 enzymes are inhibited in a non competitive manner by the binding of the second zinc ion and hence these enzymes are active only in the mono-zinc form (Bebrone et al., 2009).

Subclass B1 collects enzymes that bind one Zn^{2+} ion (Zn1) by three conserved histidine residues (His116, His118, His196), a water/hydroxide molecule bridging it to the second Zn^{2+} ion (Zn2) completes its tetrahedral coordination sphere. Zn2 is coordinated by one aspartate, one cysteine and one histidine residue (Asp120, Cys221, His263), the bridging water/hydroxide and an apical water molecule in a slightly distorted trigonal bipyramidal geometry as reported in Figure 4B. Residue labels follow the numbering suggested by Garau et al., 2004. It should be noted that subclass B1 enzymes count among the most clinically-relevant acquired MBLs, including variants of the IMP-, VIM- and NDM-subtypes. Furthermore, clinical strains producing these major subtypes have been detected virtually worldwide (Dortet et al., 2014b; Nordmann et al., 2011).

In subclass B2 an asparagine substitutes His116, while the functional Zn2 site remains unaltered and it is the one where catalysis occurs (Figure 4C). A water molecule completes the tetrahedral coordination geometry of Zn2.

Finally, subclass B3 is characterized by a 116 position hosting either histidine or glutamine (His/Gln116, His118, His196) and a peculiar Zn2 ligand set constituted by an aspartate and two histidines (Asp120, His121, Hid263; see Figure 4D). The two zinc sites maintain the same geometry observed in B1 enzymes.

Several crystal structures of MBLs complexes with inhibitors of hydrolyzed substrates have been reported and deposited within the Protein Data Bank (PDB). Table 1 SI reports the PDB codes of crystal

structures of representative native and inhibited MBLs. These structures provide a large amount of valuable information to deepen our understanding of MBLs mechanism of action and inhibition that has been exploited to design and synthesize new molecules able to inhibit these enzymes. Comprehensive reviews on this subject are available (Palzkill, 2013; Fast and Sutton, 2013; Meini et al., 2015; Mojica et al., 2016). An useful way to rationalize MBLs inhibitors mode of action is to group them depending on their chemical determinants able to interfere with the MBLs active site as done by Fast and Sutton (Fast and Sutton, 2013). We will follow this line of reasoning for the present review by limiting our analysis to the most recent acquisitions about MBLs inhibitors.

4.1 Zinc binding inhibitors

Following the introduction of N-heterocyclic dicarboxylic acid derivatives and diaryl-substituted azolylthioacetamides as possible broad-spectrum MBLs inhibitors (Feng et al., 2012; Zhang et al., 2014), Yang and co-workers exploited the azolylthioacetamide scaffold (**a**, Scheme 2) for the discovery of new inhibitors and found molecules able to increase to some extent the antibacterial effect of antibiotics, reported as MIC values, on *E. coli* BL21(DE3) cells expressing CcrA (B1) and ImiS (B2)(Yang et al., 2015). However, the tested azolylthioacetamide compounds had Kis in the low μM range only for the ImS enzyme (Yang et al., 2015). More recently, Zhai and co-workers, by following this line of research, have reported a new family NDM-1 inhibitors, based on the triazolylthioacetamide scaffold (Zhai et al., 2016). These most potent inhibitors have the basic structure reported in Scheme 2 **a**, where substitutions on ring 3 seem not to have effect on their potency, while substitutions on ring 1 appear to diminish the inhibitor activity. The most potent inhibitors have IC_{50} values below 1 μM and display mixed-type inhibition of NDM-1 hydrolysis of cefazolin, with K_i around 500 nM. The crystals structures of NDM-1 complexes with those inhibitors are not available, but docking studies propose that the triazole ring binds in between the two Zn^{2+} ions (Zhai et al., 2016), replacing the bridging water/hydroxide.

L-captopril ((2S)-1-[(2S)-2-methyl-3-sulfanylpropanoyl]pyrrolidine-2-carboxylic acid; **b**, Scheme 2), a potent angiotensin converting enzyme (ACE) inhibitor, is a widely used drug for the treatment of

hypertension and contains thiol and carboxylate groups able to establish coordination bonds with metal ions. These chemical determinants are responsible for the quite strong inhibitory effect of L- and D-captopril (Scheme 2, **c**) on MBLs of all subclasses (BcII, NDM-1, CphA, L1, FEZ-1) that has been established since a quite long time (Garcia-Saez et al., 2003; Nauton et al., 2008; King et al., 2012; Brem et al., 2016b). Upon realizing that several other drugs, already present in the pharmacopoeia, contain free thiols or carboxylate donor groups, Klingler and co-workers have established a promising platform for the discovery and development of new MBLs inhibitors (Franca-M Klingler et al., 2015). Their approach has the advantage of using already approved drugs that can be possibly repositioned for antibacterial therapy and succeeded in finding, besides captopril, three molecules having IC50 in the low μM range for NDM-1, IMP-7 and VIM-1 and interesting biological activity in restoring imipenem susceptibility of clinical isolates or *E. coli* transformants carrying such MBLs. These molecules (Scheme 2) are thiorphan (3-oxo-5-phenyl-5'-sulfanylleucine; **d**), dimercaprol (2,3-disulfanyl-1-propanol; **e**) and tiopronin (N-(2-sulfanylpropanoyl)glycine; **f**) that show low μM IC50 for inhibition of clinically relevant NDM, VIM and IMP enzymes and were demonstrated able to restore the activity of imipenem against clinical isolates expressing these enzymes. The crystal structure of tiopronin to NDM-1 (PDB: 5A5Z) confirms the binding of the molecule to the enzyme.

More recently, Brem *et al.* (Brem et al., 2016b) have analyzed the modes of binding of D- and L-isomers of captopril (2S,2R and 2S,2S, respectively) as revealed by high resolution crystal structures of the complexes with the B1 subclass MBLs BcII, IMP-1, VIM-2. The structures have been compared with the existing crystal structures of BlaB–D-captopril (Garcia-Saez et al., 2003), NDM-1–L-captopril (King et al., 2012), L1–D-captopril (Nauton et al., 2008), FEZ-1–D-captopril (García-Sáez et al., 2003) and CphA–D-captopril (Liénard et al., 2008). In all cases, except for FEZ-1 and the B2 enzyme CphA, the structures show that, captopril stereoisomers always bind the enzymes by the sulfur atom that replaces the bridging water/hydroxide of the dizinc cluster (see Fig. 5). Inhibition data show that D-captopril is always the most potent inhibitor with respect to the other stereoisomers, comprising *epi*-L- and *epi*-D-captopril (2R,2S and 2R,2R, respectively). The structural rationale consists in the observation that the D-

isomer carboxylate group is establishing charge-assisted hydrogen bonds with basic residues (Lys224/Arg228 for IMP-1, BcII, VIM-2, or Lys176 for BlaB). In the B3 enzyme L1 the carboxylate group of D-captopril is H-bonded to a Ser225 (Nauton et al., 2008) that is the structural substituent of Lys224. On the contrary, the L-isomer constrains the carboxylate group to point away from such residues losing the above interactions in all cases. However, it should be pointed out that L-captopril can establish similar interactions within the active sites of other MBLs as exemplified by the IMP-1 (Brem et al., 2016b) B1 and SMB-1 (Wachino et al., 2016) B3 enzymes where the carboxylate group still binds Lys224 and Lys 252, respectively.

In addition, the work of Brem et al. (Brem et al., 2016b) shows the ability of D-captopril to potentiate the effects of meropenem against pathogens expressing different MBLs.

In 2016, a paper from Arjomandi et al. proposed (Arjomandi et al., 2016) new thiol derivatives of tyrosine (Scheme 2, **g**) that constitute the development of the original compound proposed by Lienard et al. (Liénard et al., 2008) as IMP-1 inhibitors . These compounds have inhibition constants similar to the original compound.

The inhibitory properties of other mercaptoacetic acid thioester amino acid derivatives (Scheme 2, **h**) towards NDM-1, ImiS-1 and L1 have been reported (Liu et al., 2015) indicating that this class of compounds are strong inhibitors of L1 with inhibition constants in the low μM range. Weaker inhibition is observed for ImiS, NDM-1. Docking studies suggest that the binding of these compounds to L1 Zn^{2+} ions occurs by the mercaptoacetic carboxylate group assisted by hydrogen bond to Ser221.

A further attempt to find new routes for MBLs inhibition has been delineated in a paper by the Spencer and Shofield groups (Brem et al., 2014) where a molecule with a thiazolidine core (N-(4-methylpiperazin-1-yl)-2-[(5Z)-4-oxo- 2-thioxo-5-(2,3,6-trichlorobenzylidene)-1,3- thiazolidin-3-yl]acetamide, Scheme 2, **i**) acts as a precursor of (2Z)-2-sulfanyl-3-(2,3,6-trichlorophenyl)2-propenoic acid (Scheme 2, **j**), a thioenolate inhibitor generated by the hydrolysis of the parent molecule. The crystal structure (PDB: 4PVO) shows the inhibitor chelating VIM-2 Zn2 by its thiolate sulfur and by the carboxylate groups

formed upon hydrolysis of the thiazolidine ring. The inhibitor sulfur is further bound to Zn1. The same occurs in the complex with BcII MBL (PDB: 4TYT).

Soon after this paper, a novel scaffold based on thiazolidines appeared in the literature. This consists of bisthiazolidines (BTZ) that present analogies with the β-lactams. Two papers report the crystal structure of the VIM-2 (Mojica et al., 2015) and NDM-1 (González et al., 2015) complexes (PDB:4UA4 and 4U4L, respectively) with the L-isomer of a bisthiazoline inhibitor (L-CS319: 3R,5R,7aS)-5-(Sulfanylmethyl)tetrahydro[1,3]thiazolo[4,3-b][1,3]thiazole-3-carboxylic acid; Scheme 2, **k**). Both structures show the inhibitor bound to the dizinc center by the only thiol/thiolate group bridging the two Zn^{2+} ions. The intact bisthiazolidine rings make interactions with the surrounding hydrophobic residues while the carboxylate group is H-bonded to Arg228 in case of VIM-2, or involved in water-mediated H-bonds with Lys224 in case of NDM-1. The thiol mode of binding is that already observed for other thiolate inhibitors like the captopril complexes described above.

A following study (Hinchliffe et al., 2016) completes the analysis of the binding modes of the different isomers of two bisthiazolidines (L- and D-CS319 and L- and D-VC26, see schemes 2, **k-n**) towards the B1 enzymes BcII and IMP-1, the B2 enzyme Sfh-I and the B3 enzyme L1. The crystal structures of the complexes with this series of inhibitors (see Table 2 SI for the listing of PDB entries) show the versatility of the bisthiazolidine isomers scaffold in binding the diverse subclasses of enzymes by varying the interactions of the thiolate and carboxylate groups with the Zn^{2+} ions and the surrounding active site residues. L-CS319 binds B1 enzymes by making the same type of interactions already described for VIM-2 and NDM-1 (Figure 6A). The binding of L-VC26, which differs from L-CS139 by bearing a gem-dimethyl group, parallels that of L-CS319. The D-CS319 isomer binds B1 enzymes BcII and IMP-1 adopting the same poses. In both cases, D-CS319 forms the usual thiolate bridge between the two metal ions with the carboxylate group coordinating Zn2 and H-bonding Lys224. Interestingly, the carboxylate binding to Zn2 displaces Asp120 from its coordination sphere as shown in Figure 6B. The remaining of the molecule, namely the bisthiazolidine rings, is involved in hydrophobic interactions with residues Trp59 (BcII) or Val31 (IMP-1). A completely different binding mode of L-CS139 is shown by the

monozinc B2 enzyme Sfh-I. Here is one carboxylate group that binds zinc and not the thiol, while the second carboxylate oxygen is H-bonded to His196 and Asn233 (Figure 6C). Finally, all bisthiazolidine isomers (D-CS139, L-CS139 and D-VC26) bind the B3 enzyme L1 by the thiolate sulfur bridging the dizinc center with the carboxylate group H-bonded to Ser225 only for the L-isomer (Figure 6D), while H-bonded only to water molecules in both D-isomers.

In spite of the different binding modes, the inhibition constants towards all tested β-lactamases (K_i in the units - tens μM range) and the ability to potentiate β-lactam activity against pathogens producing metallo β-lactamases are all similar. The promising aspect of the thiadiazolidine scaffold is the ability to inhibit metallo β-lactamases of all subclasses. However, from the structural analysis it appears that the cross-class inhibition is essentially due to the zinc binding capability of all compounds through the thiolate sulfur (only D-CS319 chelates Zn^{2+} by sulfur and carboxylate groups). Consequently, increasing the affinity by introducing groups able to establish H-bonds and/or hydrophobic interactions with the surrounding residues, if successful, will probably increase binding at the expenses of generality due to the scarcity of common amino-acid motifs in MBL active sites.

Fragment-based screening approaches also proved useful for the discovery of compounds able to inhibit clinically relevant MBLs, such as VIM-2, IMP-1 and NDM-1. Vella and coworkers successfully identified fragments showing submicromolar inhibition of IMP-1 (Vella et al., 2011).

A fragment screening performed by surface plasmon resonance (SPR) provided different compounds with inhibition constants against VIM-2 varying in the mM to μM range (Christopeit and Leiros, 2016). The crystal structure (PDB: 5ACX) of one of them, 2-(4-fluorobenzoyl)benzoic acid, shows binding to Zn2 by the carboxylate group of the molecule. Zn2 is present in the enzyme despite the oxidation of Cys221.

More recently, Chen and co-workers report the identification of a new type of broad-spectrum MBL inhibitor, effective against VIM-2, NDM-1 and IMP-1 (Chen et al., 2017). The inhibitor is a derivative of dipicolinic acid (DPA) identified by a fragment-based approach directed to find metal-directed inhibitors.

Chemical modification of DPA lead to a compound (4-(3-aminophenyl)pyridine-2,6-dicarboxylic acid) showing a different inhibition mechanism towards NDM-1 with respect to the parent compound. While DPA is a "zinc stripper", that compound acts as a metal-binding competitive inhibitor, showing significant inhibition of clinically-relevant MBLs together with a 10-20-fold potentiation of imipenem when tested on clinical isolates of MBL-producing carbapenem-resistant *Enterobacteriaceae*.

Other compounds containing carboxylate groups able to inhibit MBLs with a sufficient selectivity were indeed previously described. The maleic acid derivative ME1071 was shown to potentiate the activity of carbapenems and cephalosporins on MBL-producing clinical isolates but also to improve survival in a ventilator-associated pneumonia murine model with an MBL-producing *P. aeruginosa* strain (Ishii et al., 2010; Livermore et al., 2013; Yamada et al., 2013).

Further carboxylate bearing inhibitors were synthesized by Hiraiwa and co-workers (Hiraiwa et al., 2014). The crystal structure of 3-(4-hydroxypiperidine-1-yl)phthalic acid (Scheme 2, **o**) bound to both IMP-1 Zn1 and Zn2 through the carboxylate groups and H-bonded to Ser119 by the piperidine ring para-hydroxyl group was used as the starting point for further development. New derivatives of this scaffold were obtained, displaying K_i below 1 μM and having synergistic effect with the carbapenem biapenem against clinical isolates of *P. aeruginosa* strains producers of IMP-1.

Furthermore, computational methods were also used to identify new MBL inhibitors. Early attempts successfully delivered compounds showing inhibition of metallo-β-lactamases with K_i values in the range 7-15 μM, although these compounds were unfortunately tested on enzymes with a limited clinical relevance, such as *Bacillus cereus* BcII and *Stenotrophomonas maltophilia* L1 (Olsen et al., 2006). Fragment-based *in silico* screening methods were also adopted, although it only identified fragments with a narrow-spectrum of inhibition (Klingler et al., 2015). The implementation of high-throughput docking methods together with the availability of large ligand databases allowed the identification of additional compounds, which showed inhibition of clinically-relevant MBLs and a synergistic activity with β-lactams on MBL-producing laboratory strains (Brindisi et al., 2016).

Finally, 1,2,4-triazole-3-thione derivatives have been recently investigated for their ability to inhibit clinically-relevant metallo-β-lactamases (Sevaille et al., 2017). The mode of binding of initial compounds (IIIA and IIIB) was investigated in the L1 enzyme and showed unexpected differences between the two compounds (Nauton et al., 2008; Sevaille et al., 2017). This subsequently allowed a new chemical series, based on IIIB analogues, to be synthesized. Among this series, some compounds showed a broad-spectrum inhibition of relevant enzymes, including NDM-1, IMP-1 and VIM-2.

4.2 Zinc-chelating inhibitors

An obvious way to inhibit metallo β-lactamases (as well as any metallo-enzyme) is to use strong chelating agents as, for example, EDTA to sequester and extract zinc from MBLs. Although effective, such compounds strongly suffer from being aspecific, and thus potentially associated with cytotoxicity issues.. However, Ca-EDTA was shown to significantly improve the efficacy of imipenem in a murine pneumonia model, in which the animals were infected with an MBL-producing strain of *P. aeruginosa*, not only by inhibiting the MBL but also by limiting the tissue-damaging activity of the metallo-proteases produced by the pathogen (Aoki et al., 2010). The potential clinical application of Ca-EDTA was further demonstrated in a murine sepsis model of infection (Yoshizumi et al., 2013).

The phytotoxin aspergillomarasmine A (AMA), a product of pathogenic fungi, was reported to efficiently inactivate clinically-relevant metallo-β-lactamases like VIM-2 and NDM-1 (King et al., 2014). Aspergillomarasmine A is a derivative of L-aspartic acid and contains four carboxylates groups (Scheme 2, **p-q**). This molecule was already known since 1965 (Haenni et al., 1965) and tested as inhibitor of angiotensin converting enzyme (ACE) (Mikami and Suzuki, 1983). AMA inhibits VIM-2 and NDM-1 (IC50 in the low μM range) by sequestering both Zn^{2+} ions, thanks to the chelating properties of the four carboxylate groups and the three nitrogen groups (King et al., 2014), but the structure of the Zn^{2+}-AMA complex is not available and the geometry and coordination number of zinc bound to AMA are not known. The original stereochemistry for AMA proposed was 2''R, 2'R, 2S for the three stereocenters

present in the molecule, corresponding to: N-[(2R)-2-{[(2R)-2-Amino-2-carboxyethyl]amino}-2-carboxyethyl]-L-aspartic acid. This assignment has been now corrected upon achieving the total synthesis of the inhibitor (Koteva et al., 2016, Liao et al., 2016) that has now been identified as the 2"S, 2'S, 2S isomer (Scheme 2, **q**) which is the actual MBL inactivator. AMA was effective in potentiating the activity of carbapenems on MBL-producing strains and in an *in vivo* murine model of infection (with an NDM-1-producing *K. pneumoniae* strain).

The increasing interest towards such kind of inhibitors is illustrated by the identification of chemical entities that significantly differ from EDTA or AMA, while endowed with similar Zn-chelating properties, and thus potentially MBL inactivation. Falconer and co-workers explored a series of spiro-indoline-thiadiazole compounds for their potential to inhibit MBLs (Falconer et al., 2015). Starting from chelators originally identified as interfering with iron hometostasis in *E. coli*, analogue SIT-5Z emerged as a moderately potent inhibitor of NDM-1, although its activity on other MBLs (VIM-2 and IMP-7) was lower, if not absent. Interestingly however, this compound, in combination with meropenem, significantly reduced the bacterial load in both the liver and spleen of mice infected by an NDM-1-producing *K. pneumoniae* strain (peritoneal infection).

Additional Zn^{2+}-chelating agents, including the macrocycles 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA, Scheme 2, **r**) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA, Scheme 2, **s**) were later described and shown to restore meropenem and imipenem activity in different MBL-producing bacterial strains (Somboro et al., 2015) with MICs lower than those reported for AMA.

4.3 MBLs covalent inhibitors

An unusual type of inhibitor is the selenium-bearing compound ebselen (2-phenyl-1,2-benzoselenazol-3-one, Scheme 2, **t**) that has been shown to efficiently inactivate NDM-1 and restore meropenem activity on NDM-1 expressing *E. coli* cells (Chiou et al., 2015). It appears that the activity of this molecule is due

to the opening of the selenazol ring and formation of a covalent Se-S bond with the zinc binding Cys221 residue.

Along this line of research also 4-oxo-4H-1-benzopyran-3-carboxaldehyde (3-formylchromone, Scheme 2, **u**) has been shown to be a covalent inhibitor NDM-1 by binding to the active site Lys224 (Christopeit et al., 2016). the occurrence of covalent inhibitors of metallo-enzymes is not new as exemplified by the inhibition of urease by β-mercaptoethanol and 1,4 benzoquinone (Benini et al., 1998, Mazzei et al., 2016) and by fumagillin and ovicillin inhibition of methionine aminopeptidase (Sin et al., 1997, Griffith et al., 1997).

Finally, it should also be noted that some β-lactams might undergo additional chemistry after being hydrolyzed by MBLs, progressing towards a covalent binding to the enzyme, and thus its inactivation. This has been investigated in depth with moxalactam (Zervosen et al., 2001), whose leaving group released upon substrate hydrolysis, is able to combine with the Cys221 active site residue, although this was observed with the subclass B2 enzyme CphA, which is most among the most clinically-relevant MBLs. More recently, a similar behavior was observed between NDM-1 and cefaclor, an early cephalosporin. Interestingly, enzyme inactivation occurred by chemical modification of the active lysine Lys224, rather than with the cysteine residue, thus representing a new mechanism of covalent adduct formation in MBLs (Thomas et al., 2014). The hope is that, like serine β-lactamases covalent inhibitors, covalent MBL inhibitors would reach the stage of clinical development.

5. Boronic acids – towards the next-generation pan-β-lactamase inhibitors?

β-lactamases of all classes share a common aspect of their catalytic mechanism that consist in the occurrence of one or two tetrahedral intermediates and transition states along the reaction pathway (Drawz and Bonomo, 2010, Meini et al., 2015). Therefore, compounds able to mimic such steps are good candidates to become strong inhibitors of the enzymes. On the other hand, tetrahedral intermediates occur in many enzymes and metallo-enzymes catalyzed reactions involving C-C, C-N or C-O bonds (as well as others), making specificity a relevant issue when such approach is undertaken. Indeed, similarly to serine proteases, β-lactamases catalyze the hydrolysis of an amide bond

The versatile chemistry of boron has been exploited since many years in pharmaceutical drug design (Nielsen, 1991, Ban and Nakamura, 2015). Trigonal boron(III) compounds behave as Lewis acids and are prone to react with nucleophiles resulting in tetrahedral covalent adducts that resist hydrolysis by enzymes. B-O bonds in tetrahedral compounds are somewhat longer than C-O single bonds (by about 0.06 Å) and hence represent a quite good mimic for a tetrahedral transition state characterized by stretched C-O bonds. This is the rationale behind the inhibitory activity of boronic acid compounds that are found active against different families of enzymes like proteases of all kinds (Smoum et al., 2012; Lei et al., 2016), histone deacetylases (Hideshima et al., 2011) and whole proteasome (Ban and Nakamura, 2015). The properties of boron compounds as inhibitors of a collection of target enzymes, relevant for drug design and development has been recently and extensively reviewed (Smoum et al., 2012).

The activity of boronic acid compounds as inhibitors of serine β-lactamases has been firstly reported more than three decades years ago (Kiener and Waley, 1978, Beesley et al., 1983). Since then a variety of boronic acid inhibitors have been synthesized and tested for their inhibitory activity against β-lactamases of all classes. In 1996 Strynadka and co-workers, reported a rationally designed, potent boronic inhibitor of class A TEM-1 enzyme (Scheme 3, **a**) (Strynadka et al., 1996) inspired by the crystal structure of TEM-1 from *E. coli* (Strynadka et al., 1992). These compounds were then refined resulting in the strong inhibitors **b** and **c** (Scheme 3) (Ness et al., 2000). Work in this direction has been continued by Schoichet and others resulting in numerous new compounds achieving low nM inhibition constants towards class

C β-lactamases (Weston et al., 1998, Morandi et al., 2003, Morandi et al., 2008, Ferrari et al., 2011, Tondi et al., 2014). Fragment based drug design eventually lead to sulfonamide boronic acids having up to 50 pM molar Ki (Scheme 3, **d**) and able to triple the survival of mice infected with AmpC–overproducing *E. coli* (Scheme 3, **e**) when administered together with cefotaxime with respect to the antibiotic alone (Eidam et al., 2012).

Boronates and cyclic derivatives of antibiotics like ceftazidime and cefoperazone have been shown to be strong inhibitors of class A and C β-lactamases (Drawz et al., 2010a; Ke et al., 2011; Winkler et al., 2013). Also β-lactam analogue-boronate inhibitors, where the carboxamide group characteristic of penicillins and cephalosporins has been replaced by sulfonamide, resulted to be strong AmpC inhibitors (~ 20 nM Kis) (Eidam et al., 2010; Tondi et al., 2010).

Recently, Hecker and co-workers considered the possibility to achieve higher selectivity towards β-lactamases, without losing affinity by screening cyclic boronate compounds (Hecker et al., 2015). Indeed, human serine hydrolases have, in general, sterically constrained active sites, better suited to host linear compounds, while β-lactamases have wider active sites able to bind cyclic molecules mimicking tetrahedral transition states. This idea lead to compound **f** (RPX7009; Scheme 3), a strong wide-spectrum inhibitor (Ki 50-100 nM) towards relevant serine β-lactamases (KPC, CTX-M, SHV, CMY) able to significantly increase MICs of cefepim and carbapenems towards a series of pathogen clinical isolates expressing A, C and D β-lactamases. RPX7009 appears also to be provided of high selectivity not being able to inhibit mammalian serine proteases and to potentiate the effect of biapenem and meropenem activity against a carbapenem-resistant strain of K. pneumoniae in the neutropenic mouse lung infection model (Hecker et al., 2015). All these properties, together with favorable toxicology and pharmacokinetics have led to successful completion of phase 1 trials for RPX7009 alone (under the trademark Vaborbactam; Griffith et al., 2016) and in combination either with biapenem (ClinicalTrials.gov Identifier: NCT01772836) or meropenem (trademark Carbavance; ClinicalTrials.gov Identifiers: NCT02020434, NCT02073812 and NCT01897779). These allowed this compound to enter a clinical phase 3 trial in combination with meropenem (trademark Carbavance; Hecker et al., 2015).

Figure 7 A-C shows the comparison of the binding mode of compound **a** to TEM-1 and of compound **f** to class A (CTX-M-15) and class C (AmpC) β-lactamases. The binding modes adopted by the inhibitors **a** and **f** in the two enzymes of different classes are similar. Besides the covalent bond formed by boron with the catalytic serine side chain oxygen, compound **a** and **f** carboxylates are H-bonded to the KTG motif and to neighboring residues, while the carbonyl oxygen receives H-bonds from the S(T)A(D)N motifs. The thiophene ring present in **f** is involved only in loose contacts with the cavity.

The ability of boric acid, borate anions, boronic acids to act as inhibitors of mononuclear and dinuclear metallo-enzyme inhibition has been exploited either for mechanistic studies and for development of new drugs as exemplified by the cases of *Sporosarcina pasteurii* urease (a dinuclear nickel enzyme; Benini *et al*., 2004) and *Schistosoma mansoni* or human arginase (dinuclear manganese enzymes; Van Zandt et al., 2013; Hai et al., 2014).

The further modification of the cyclic boronate vaborbactam (RPX7009), scientists at Rempex Pharmaceuticals (then the Medicines Company) were able to introduce chemical modifications in the inhibitor leading to a substantial improvement of their inhibitory activity on metallo-β-lactamases (Hecker et al., 2015).

A first optimization of the inhibitor was achieved with the introduction of an aminothiadiazole side chain in RPX7262 (Scheme 3, **h**) which kept nanomolar potency against serine carbapenemase and a promising inhibition of the NDM-1 metallo-β-lactamase (K_i, 7.4 μM). However, the most significant increase in the potency of inhibition towards metallo-β-lactamases was achieved with a more drastic modification of the molecule, in which the amide group was substituted by a sulphur atom, followed by a similar thiadiazole moiety. This compound (RPX-7282; Scheme 3, **i**) showed pan-spectrum inhibition of β-lactamases, with a potent and similar activity against both serine- and metallo-carbapenemases (K_i, 0.01-0.03 μM; Hecker et al., 2015). RPX-7282 showed a good synergistic activity with carbapenems, and were notably able to restore carbapenem susceptibility with VIM-2- or NDM-1-poducing strains at inhibitor concentrations as low as 0.3 μg/ml (Lomovoskaya et al., 2015).

Schofield et al. have followed this path by testing a variety of boronic acids, some of which were already known SBL or PBP inhibitors, in search of inhibitors of MBL enzymes like NDM-1 (Brem et al., 2016a). They characterized cyclic boronates that interestingly displayed inhibitory activity against SBL, MBL and PBP enzymes. Among them compound **j** (Scheme 3; dubbed as compound 2 in ref. 29), a compound actually already described in the patent literature (Burns et al., 2010), has the highest inhibition towards members of all three enzyme classes and has the ability to restore meropenem activity against Gram-negative bacterial strains carrying both SBL and MBL genes (Brem et al., 2016a). The crystal structures of **j** bound to the MBLs BcII and VIM-2 (subclass B1) reveal a similar binding mode for the compound that binds the two active site Zn^{2+} ions as exemplified in Figure 8. Two of the boron-bound oxygen atoms asymmetrically chelate Zn1, while the third boron-bound oxygen atom and one carboxylate oxygen of **j**, bind Zn2. The two zinc ions adopt trigonal bipyramidal coordination geometry. The binding of **j** to BcII and VIM-2 mimics the high-energy tetrahedral oxianion intermediate proposed to occur in β-lactam hydrolysis by B1 MBLs enzymes (Aitha et al., 2016; Brem et al., 2016a).

6. Conclusion

There is an increased interest in the development of new β-lactam/β-lactamase inhibitor combinations. Besides representing a validated strategy to overcome resistance in the major Gram-negative pathogens, and thus restore the efficacy of older but still potentially very reliable β-lactams (such as oxyimino-cephalosporins or carbapenems), the approval of Avycaz (ceftazidime-avibactam) also shows that new chemical entities (i.e. not β-lactams) could not only lead to an *in vitro* inhibition of β-lactamases (this has been known for a long time with boronic acids), but also lead to compounds amenable to a successful clinical development. The recent discovery of pan-β-lactamase boronic acid inhibitors further highlights the validity of such new chemical entities and their ability to inhibit structurally and mechanistically different enzymes (serine- and metallo-β-lactamases), while apparently being perfectly selective and thus devoid of any toxicity-related issues. Thus, a natural step of development of both DABCO and boronic acids is therefore represented by their optimization into potent inhibitors of penicillin-binding proteins, which would confer these molecules a direct antibacterial activity, as exemplified in this review. The perspective of the availability of clinically-useful inhibitors of bacterial PBPs that would not be β-lactams could be definitely interesting, as it could represent a valid strategy to overcome at least β-lactamase-mediated resistance and could open a new (and hopefully successful) era of antibacterial chemotherapy.

TABLES

Table 1. β-lactam-β-lactamase inhibitor combinations that reached the stage of clinical development since 2010.

Combination	Status
Ceftolozane-tazobactam (2:1)	Approved in 2014 in the U.S.A. and in 2015 in Europe
Ceftazidime-avibactam (4:1)	Approved in 2015 in the U.S.A. and in 2016 in Europe
Ceftaroline-avibactam	Various phase 1 trials completed 2011-2017
Aztreonam-avibactam	Phase 1 studies initiated in 2012 (completed in 2016) and 2015.
Meropenem-Vaborbactam (Carbavance)	Phase 3 initiated in 2014
Imipenem-cliastatin-relebactam (2:2:1)	Phase 3 initiated in 2015.
Nacubactam[a]	Phase 1 concluded in 2014.
Sulbactam-ETX2514	Phase 1 initiated in 2016.
VNRX-5133[a]	Phase 1 initiated in 2016.

[a] β-lactamase inhibitor, partner β-lactam currently unspecified

Table 1SI. Crystal structures of representative MBLs of all subclasses and of their complexes with inhibitors or hydrolyzed substrates.

MBL subclass	Name	Crystal structures of native (variants)/metal substituted (no-active site ligands) of representative MBLs	Crystal structures of inhibited MBLs
B1	BcII	2BC2 (mono-Zn) 2BFK (R121C variant, pH 7.0) 2BFL (R121C variant, pH 5.0) 2BFZ (R121C variant, pH 4.5, C221 oxidized) 2BG2 (R121C variant, pH 4.5, C221 reduced) 2M5C (mono-Zn) 2UYX(D120S variant) 3I13(di-Zn pH 5.8) 4C09(di-Zn,atomic res.) 4NQ4 (di-Zn pH 7.0) 5FQA (diferric),	4C1C (D-captopril), 4C1H(L-captopril), 4NQ5 (L-CS319),4NQ6 (D-CS319) 4NQ7 (D-VC26) 4TYT(ML302F) 5FQB (boronic acid derivative),
	CCrA(CfiA)	1ZNB, 2BMI,	1A8T (L-159,061), 1KR3 (SB-236050), 1HLK (tricyclic inhibitor)
	BlaB		1M2X (D-captopril)
	SPM-1	2FHX 4BP0 (closed form),	
	NDMs	3RKJ,3RKK,3SBL (NDM-1) 3SFP (NDM-1 mono-Zn) 3ZR9 (NDM-1), 3SPU(apoNDM-1) 4TZE (NDM-5)	3Q6X (NDM-1 ampicillin), 4EXS (NDM-1 l-captopril), 4EY2 (NDM-1 methicillin), 4EYL (NDM1 benzylpenicillin), 4EYB (NDM1 meropenem), 4EYB (NDM1 oxacillin), 4HL2(NDM1 ampicillin), 4HKY (NDM1 faropenem), 4U4L (NDM1-L-CS319), 4RL0 (NDM1 cephalosporin), 5A5Z (NDM-1 tiopronin)
	IMP-1	5EV6(diZn)	1DD6 (mercapto), 1DDK (acetate), 1DD6 (mercaptocarboxylate), 1JJE(succinic acid derivative), 1JJT (succinic acid derivative) 1VGN (propan-thiolate), 3WXC (3-aminophalate), 4C1F,4C1G (L,D captopril) 5EV8 (D-CS319), 5EWA (L-VC26)
	VIMs	2Y87(VIM-7), 1KO2 (VIM-2 mono-Zn), di-Zn), 5ACU (VIM-2 native), 5ACV (VIM-2 oxidized)	2YZ3 (VIM-2mercaptocarboxylate), 4BZ3(formiate), 4PVO (ML302, ML203F, rhodanine), 4C1D (L-captopril), 4C1E (D-captopril), 4UA4 (bisthiazolidine), 5ACW (VIM-2 triazole), 5ACX (fluoro-phenyl-cerbonyl-benzoic acid)
B2	CphA,	3T9M (apo, C221D variant) 3F9O(CphA di-Zn), 1X8G(CphA mono-Zn),	1X8I(biapenem), 2QDS(d-captopril), 2GKL(pyridincarboxylate), 3IOF(CphA mut phosphonate), 3IOG(CphA mut+phosphonate)
	Sfh-I	3SD9	3Q6V(GOL), 5EW0 (L-CS319)

	ImiS,		
B3	L1	1SML (di-Zn), 2H6A (mono-Zn), 2FU6 (apo), 2FM6 (di-Zn),	2FU8 (D-captopril), 2FU9 (mp2), 2GFJ (pyrazole-dicarboxylate), 2GFK(furan-dicarboxylate inhibitor), 2HB9. 5DPX (1,2,4-triazole-3-thione) 2QDT(mercaptoalanine derivative), 5EVD(D-VC26), 5EVB(D-CS319), 5EVK(L-CS319)
	GOB-1,	5K0W(GOB-18),	
	FEZ-1,	1K07	1JT1(captopril)
	BJP-1	2GMN, 3LVZ	3M8T (benzenesulfonamide)
	SMB-1	3VPE (glycerol)	3VQZ (mercaptoacetic acid), 5AXO (meropenem), 5AXR (2-mercaptoethanesulfonate, 5AYA (L-captopril, 5B15 (doripenem), 5B1U (imipenem)
	AIM-1	4AWY	
	CAU-1, THIN-B, Mbl1b		

700 Table 2SI. Crystal structures of metallo-β-lactamase-bisthiazolidine complexes.

	D-CS319	**L-CS319**	**D-VC26**	**L-VC26**
IMP-1	5EV8 (Hinchliffe et al., 2016)			5EWA (Hinchliffe et al., 2016)
BcII	4NQ6 (Hinchliffe et al., 2016)	4NQ5[a]	4NQ7[a]	
VIM-2		4UA4(Mojica et al., 2015)		
NDM-1		4U4L(González et al., 2015)		
Shf-I		5EW0 (Hinchliffe et al., 2016)		
L1	5EVB (Hinchliffe et al., 2016)	5EVK (Hinchliffe et al., 2016)	5EVD (Hinchliffe et al., 2016)	
[a]Released in PDB only				

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SCHEME AND FIGURE LEGENDS

Scheme 1. Chemical structures of β-lactamase inhibitors, showing their expanding chemical diversity. Besides the β-lactam compounds (clavulanic acid, tazobactam and sulbactam, **a-c**), new chemical entities showing an expanded spectrum of inhibition successfully entered the stage of clinical development: diazabicyclooctanone compounds (avibactam, relebactam and nacubactam, **d-f**) and boronic acids (RPX7009, **h**). Chemical structure of the alkylidene penam sulfone LN-1-255 (**i**), showing the presence of unusual chemical substituents at positions 2 and 6.

Scheme 2. Chemical structures of metallo-β-lactamase inhibitors of different classes. Zinc binding inhibitors (**a-o**). Zinc chelating inhibitors (**p-s**). Covalent (Cys bond) inhibitors (**t-u**).

Scheme 3. Chemical structures boronic-acid-based β-lactamase inhibitors (**a-j**).

Figure 1. Interaction of avibactam with β-lactamases of class A, C (panel A) and D (panel B). The inhibitor (balls and sticks representation) is covalently bound to the enzyme catalytic serine and interacts with many conserved functionally-relevant residues, providing a structural basis for both its broad-spectrum of inhibition and its reversible mechanism. A) superimposition of the class A extended-spectrum β-lactamase CTX-M-15 (green) and the *Pseudomonas aeruginosa* class C AmpC (orange) inhibited by avibactam. B) Avibactam bound to the class D carbapenemase OXA-48 (magenta); the position of active site residues in the native enzyme (white, PDB code, 3HBR) is also shown.

Figure 2. The three-dimensional structure of KPC-type carbapenemases, showing the position of residues involved in the resistance to ceftazidime-avibactam combination (red, naturally occurring substitutions; blue, substitutions identified in laboratory variants only).

Figure 3. Superimposition of the three-dimensional structure of the *Pseudomonas aeruginosa* class C β-lactamase AmpC inhibited by avibactam (pink), relebactam (cyan) and nacubactam (green). All inhibitors showed a similar binding mode with the enzyme and only differ by the nature of the carboxamide side chain, which points outwards the enzyme activie site.

Figure 4. A) The MBL fold. The active site location is shown by representing active site residues as sticks. B) Subclass B1 active site and zinc coordination sphere. C) Subclass B2 active site and zinc coordination sphere. D) Subclass B3 active site and zinc coordination sphere. The coordination bonds to the Zn^{2+} ions (grey spheres) are represented as thin black lines.

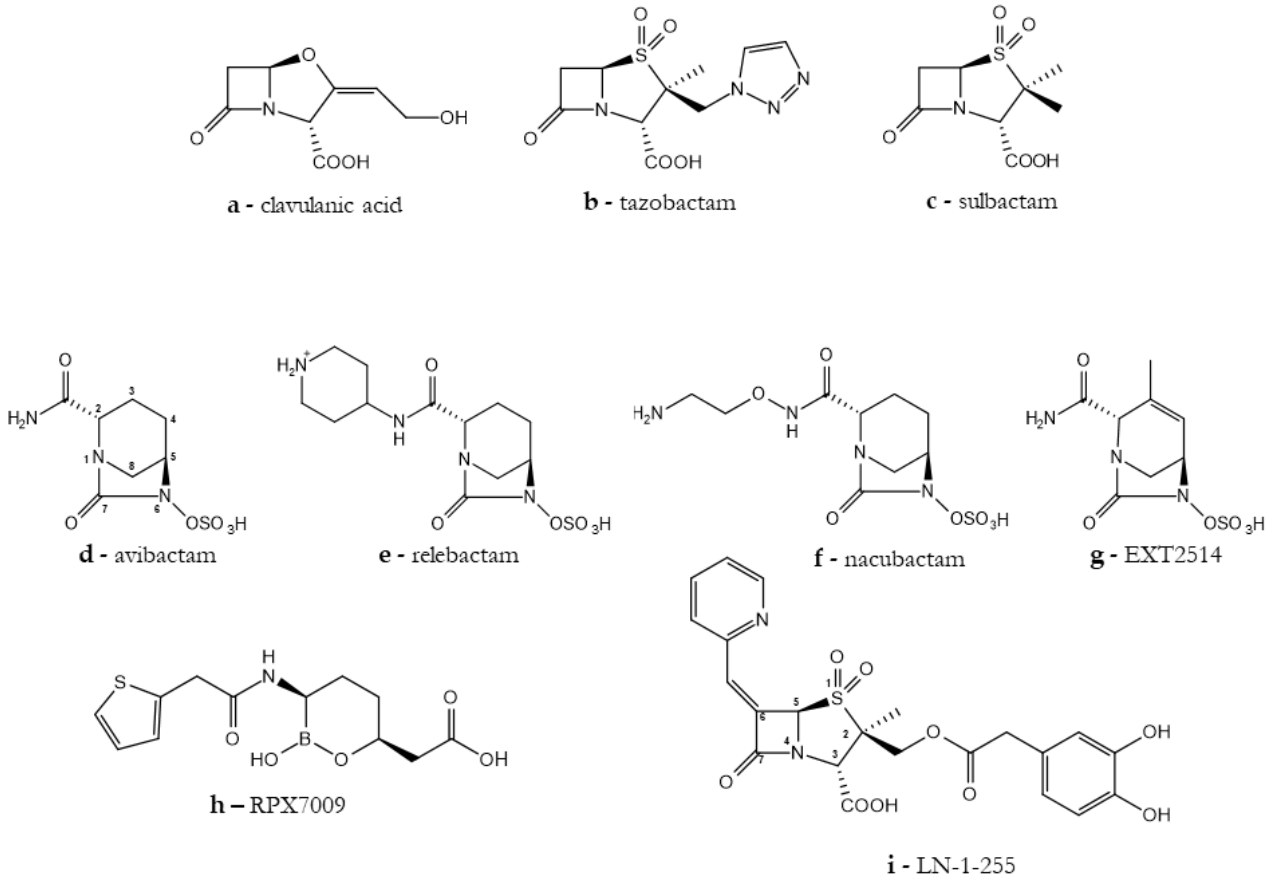
Figure 5. Comparison the binding modes of D-captopril with one representative MBL from each subclass. D-captopril binding to B1 (gold carbon atoms), to B2 (ice blue carbon atoms) and B3 (coral carbon atoms) enzymes. Notice the different binding by carboxylate to Zn2 in B2 subclass. The Zn^{2+} ions are represented as spheres colored following the subclass scheme above. The remaining atoms are colored following the standard color code (nitrogen: blue; oxygen: red; sulfur: yellow).

Figure 6. A) Binding of L-CS319 to representative B1 MBL (BcII). B) Binding of D-CS319 to BcII. Notice the carboxylate binding to Zn2 and the displacement of Asp120 from Zn2 coordination sphere. C) Binding of L-CS319 to a representative B2 enzyme (Sfh-I). Here, is the inhibitor carboxylate group that coordinates the Zn^{2+} ion. D) Binding of L-CS319 to a representative B3 enzyme (L1). The

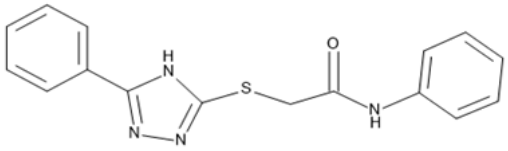
coordination bonds to the Zn^{2+} ions (grey spheres) are represented as thin black lines. The inhibitor carbon atoms are represented as green spheres, while the remaining atoms are colored following the standard color code.

Figure 7. A) Binding of compound **a** (Scheme 3) to TEM-1. B) Binding of compound **f** (Scheme 3) to CTX-M-15. C) Binding of compound **f** (Scheme 3) to AmpC. The inhibitor carbon atoms are represented as green spheres, while the remaining atoms are colored following the standard color code.

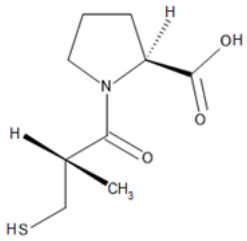
Figure 8. Binding of compound **j** (Scheme 3) to BcII. The coordination bonds to the Zn^{2+} ions (grey spheres) are represented as thin black lines. The boron atom is colored gray. The inhibitor carbon atoms are represented as green spheres, while the remaining atoms are colored following the standard color code.


a - clavulanic acid
b - tazobactam
c - sulbactam
d - avibactam
e - relebactam
f - nacubactam
g - EXT2514
h – RPX7009
i - LN-1-255

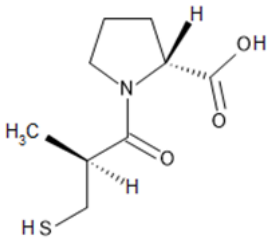
Scheme 1.



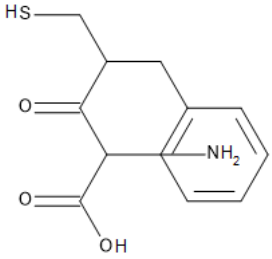
a - Example of diaryl-substituted triazolylthioacetamide scaffold



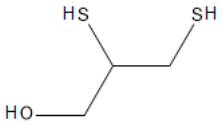
b - L-captopril



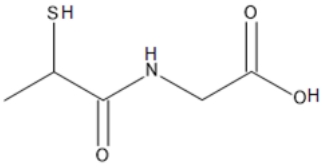
c – D-captopril



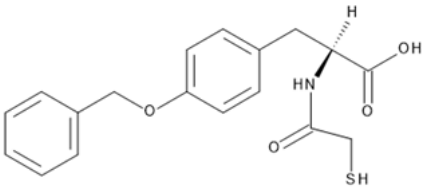
d – thiorphan



e – D,L-dimercaprol

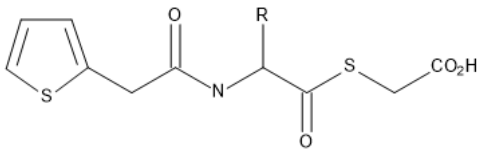


f – tiopronin

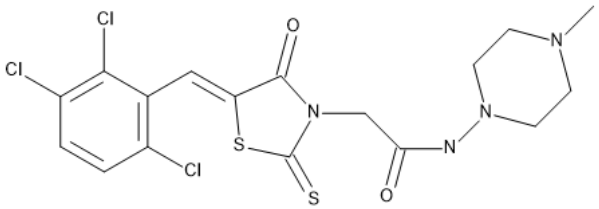


g – Tyrosin-thiol derivative (13a)

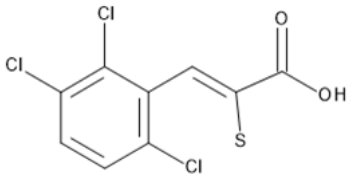
Scheme 2.



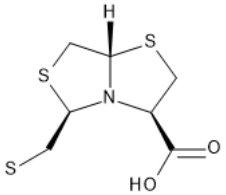
h – Scaffold of acetic acid thioester amino acid derivative



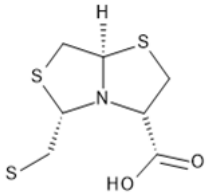
i - (N-(4-methylpiperazin-1-yl)-2-[(5Z)-4-oxo- 2-thioxo-5-(2,3,6-trichlorobenzylidene)-1,3- thiazolidin-3-yl]acetamide



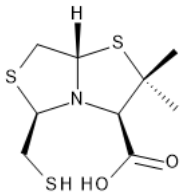
j - (2Z)-2-sulfanyl-3-(2,3,6-trichlorophenyl)2-propenoic acid



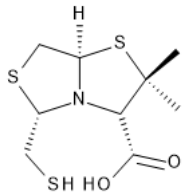
k - L-CS319



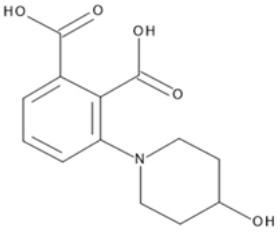
l - D-CS319



m – L-VC26

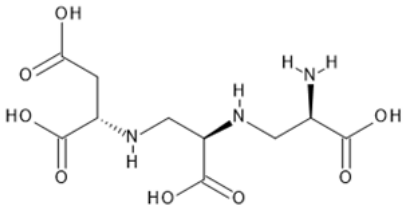


n – D-VC26

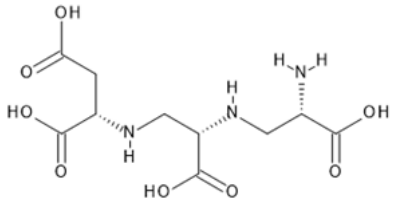


o - 3-(4-Hydroxy-1-piperidinyl)phthalic acid

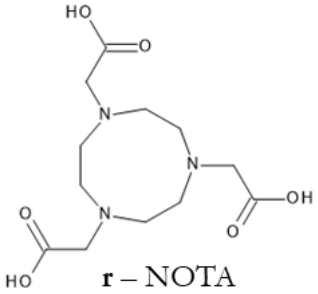
Scheme 2. cntd



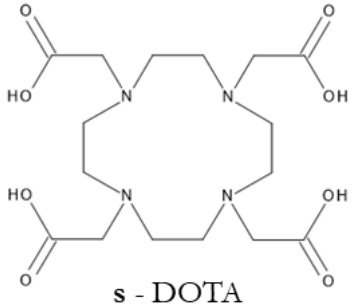
p - 2"R, 2'R, 2S-Aspergillomarasmine



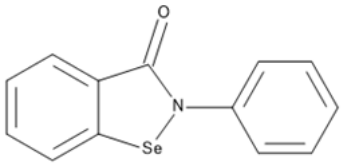
q- 2"S, 2'S, 2S-Aspergillomarasmine



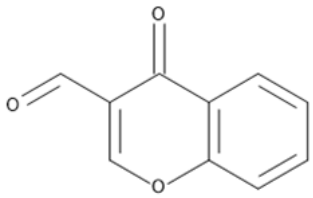
r – NOTA



s - DOTA



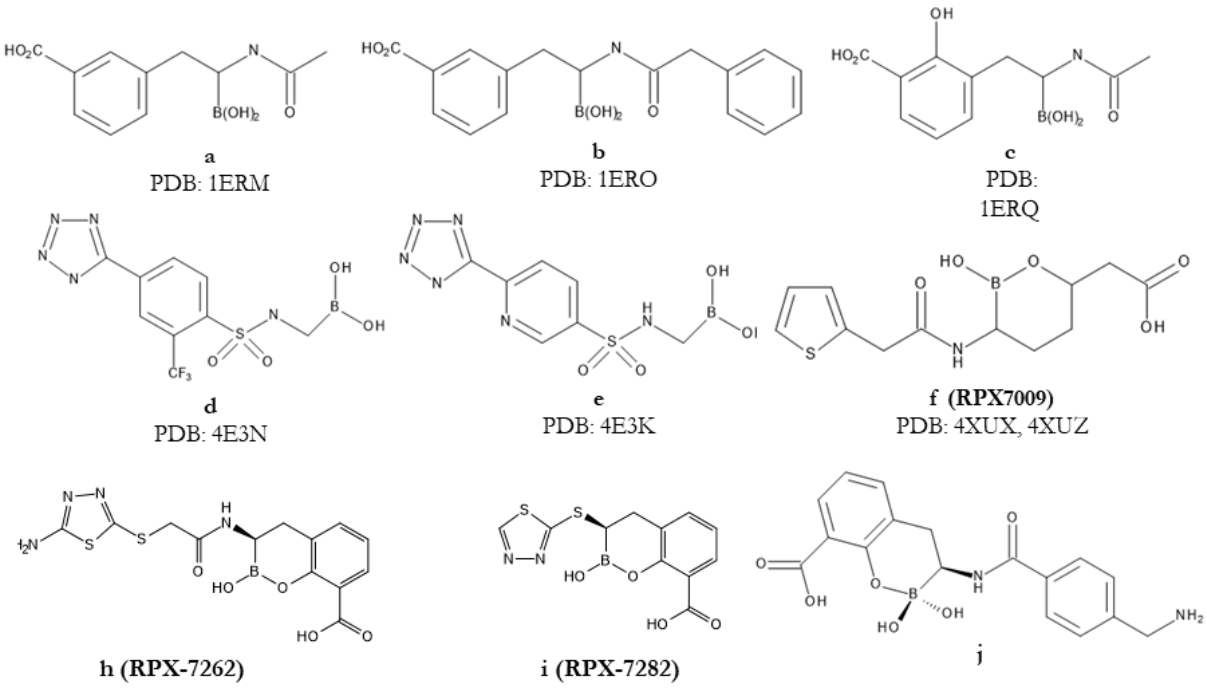
t – ebselen



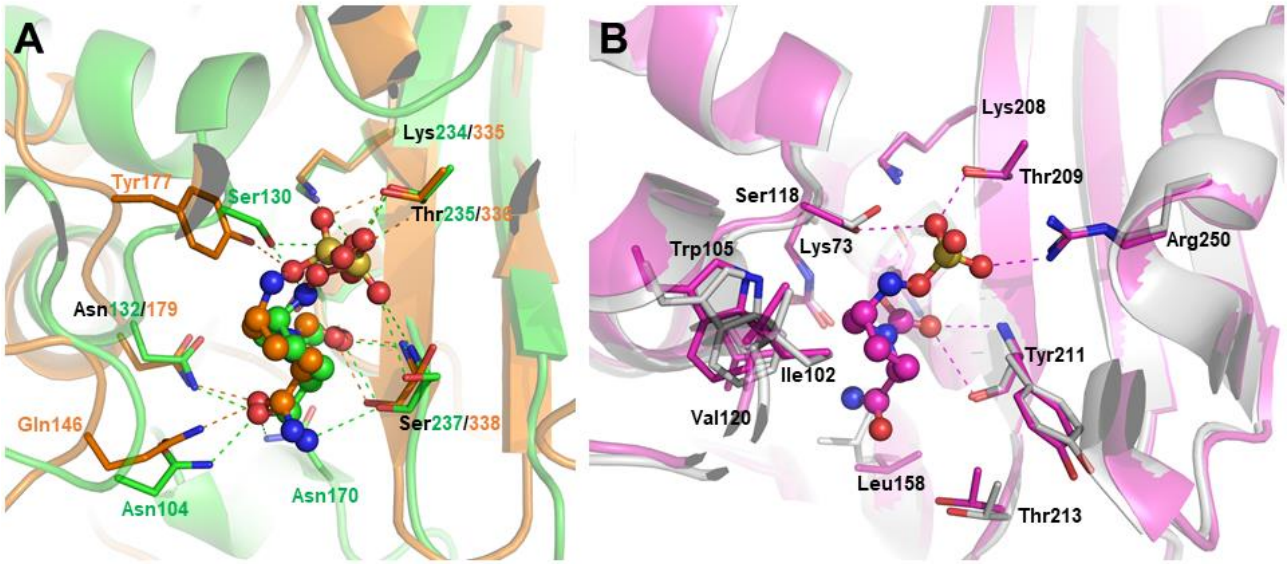
u – 3-formylchromone

1245

Scheme 2. cntd


a
PDB: 1ERM
b
PDB: 1ERO
c
PDB: 1ERQ
d
PDB: 4E3N
e
PDB: 4E3K
f (RPX7009)
PDB: 4XUX, 4XUZ
h (RPX-7262)
i (RPX-7282)
j

Scheme 3.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Ser130
Thr235/336
Asn132/179
Gln146
Ser237/338
Asn104
Asn170
B
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Thr209
Ser118
Trp105
Lys73
Arg250
Tyr211
Ile102
Val120
Leu158
Thr213

Figure 1. A, B

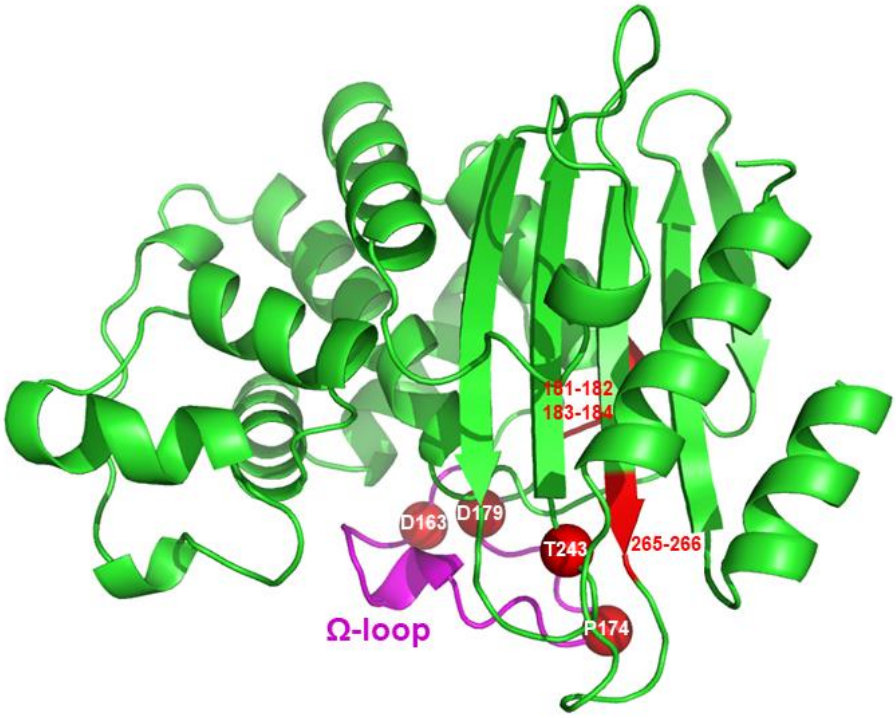

Figure 2.

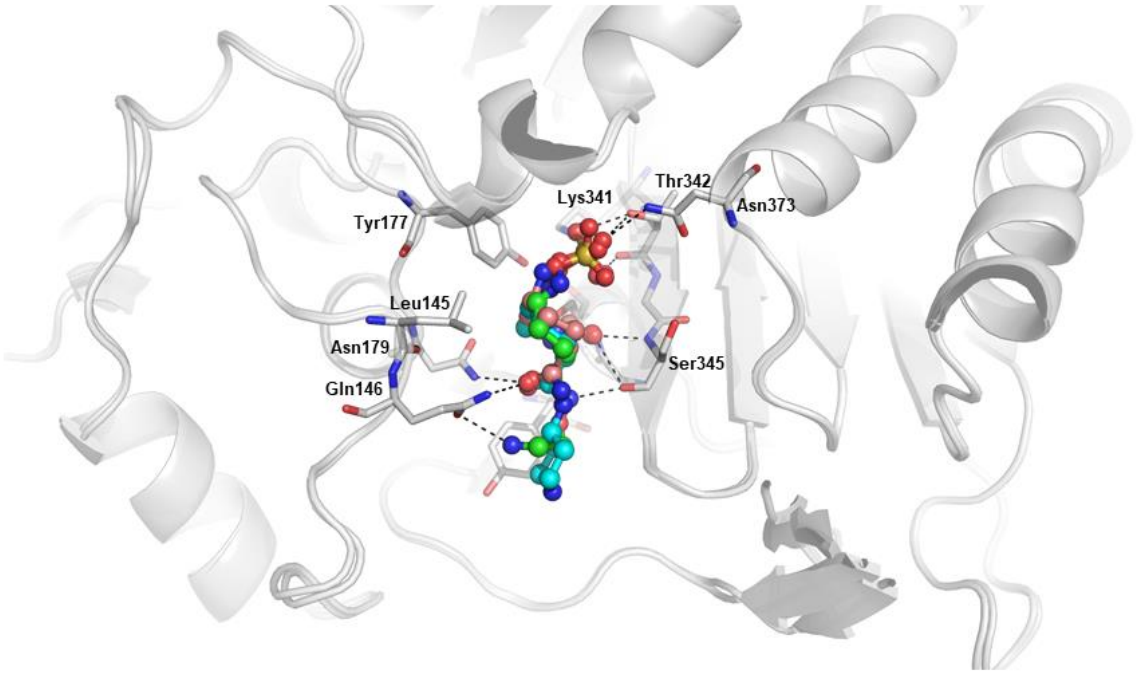

Figure 3.

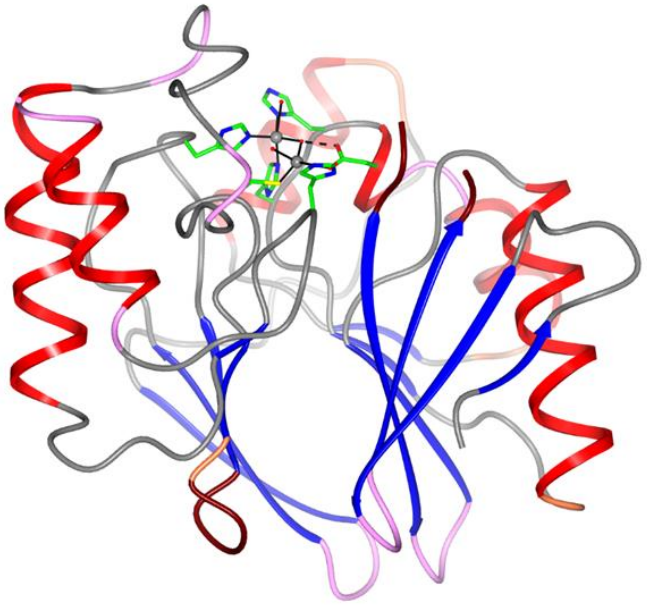

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Figure 4A.

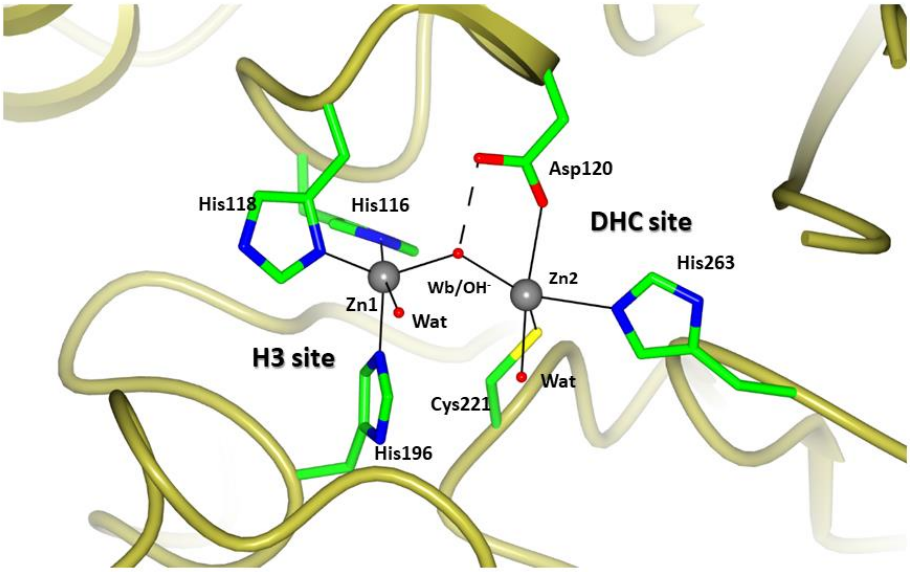


Figure 4B.

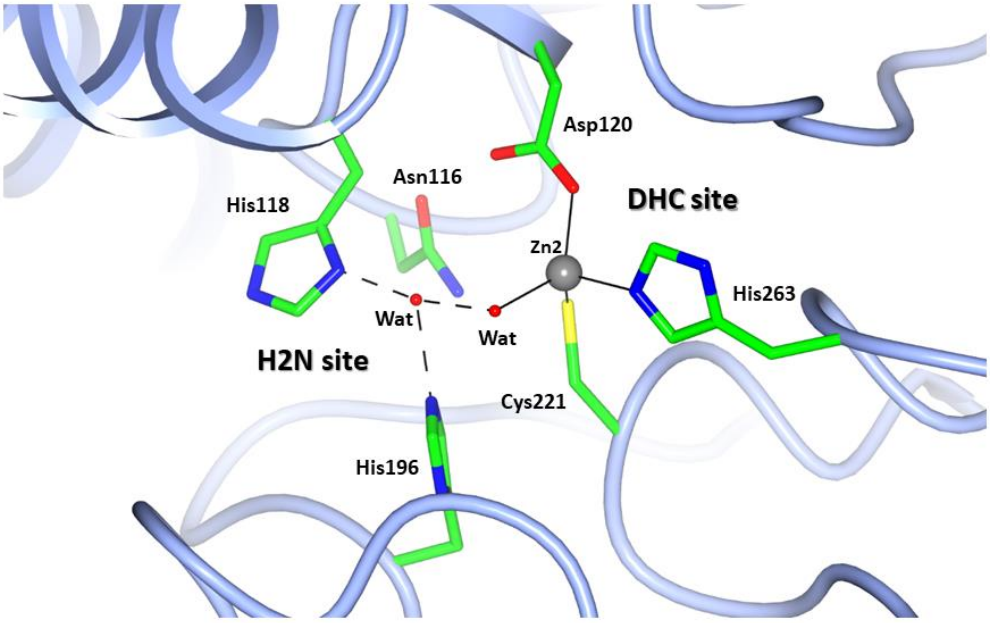


Figure 4C.

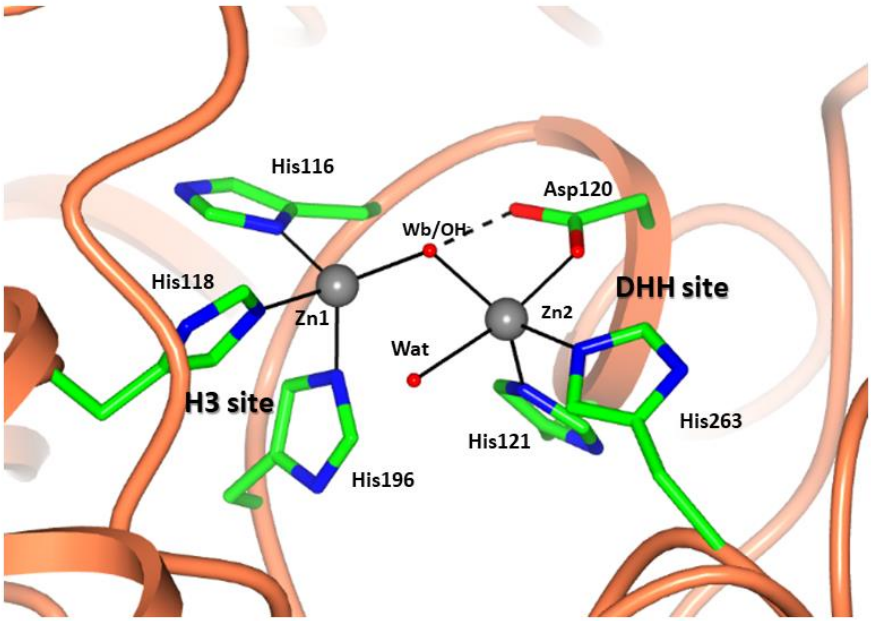


Figure 4D.

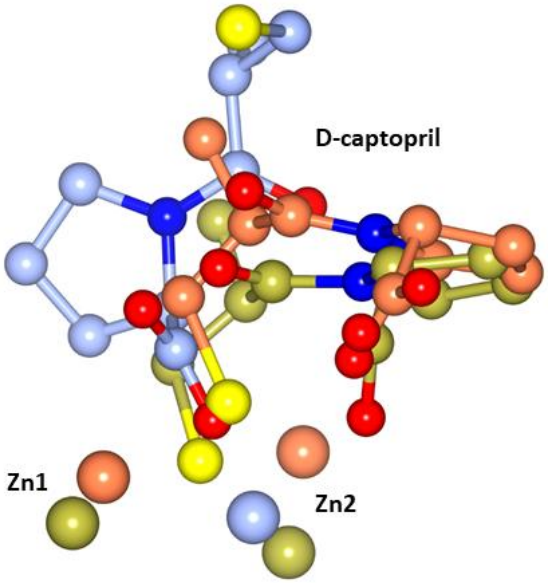


Figure 5.

1270

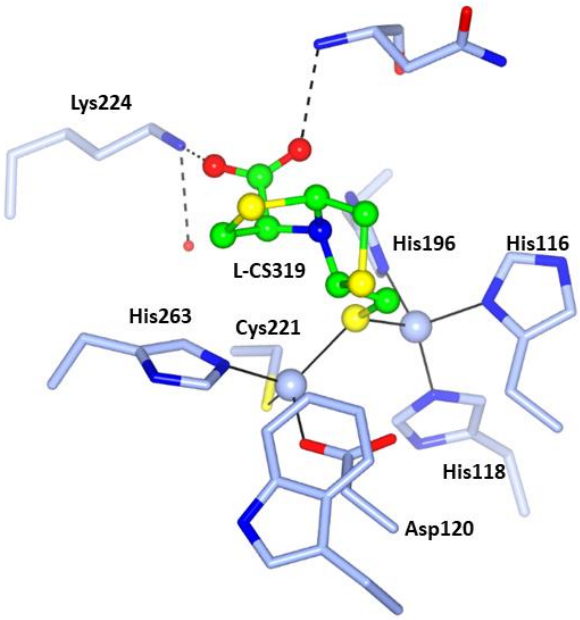


Figure 6A.

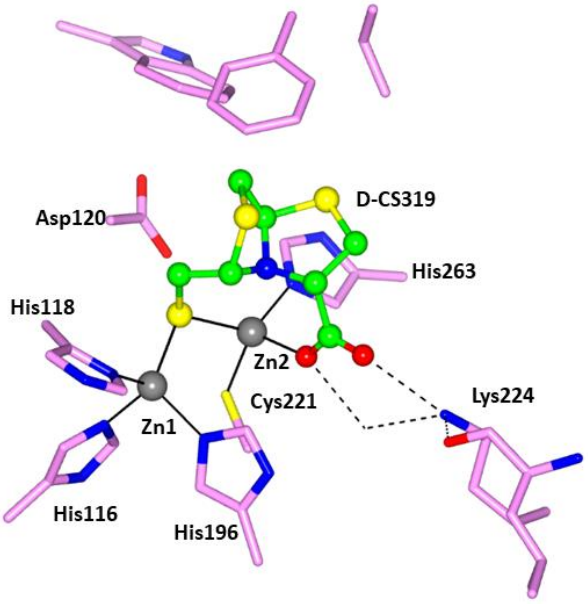
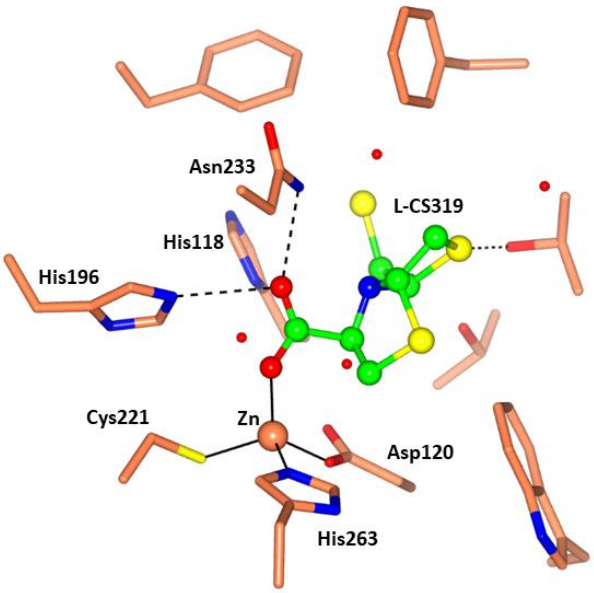


Figure 6B.



1275

Figure 6C.

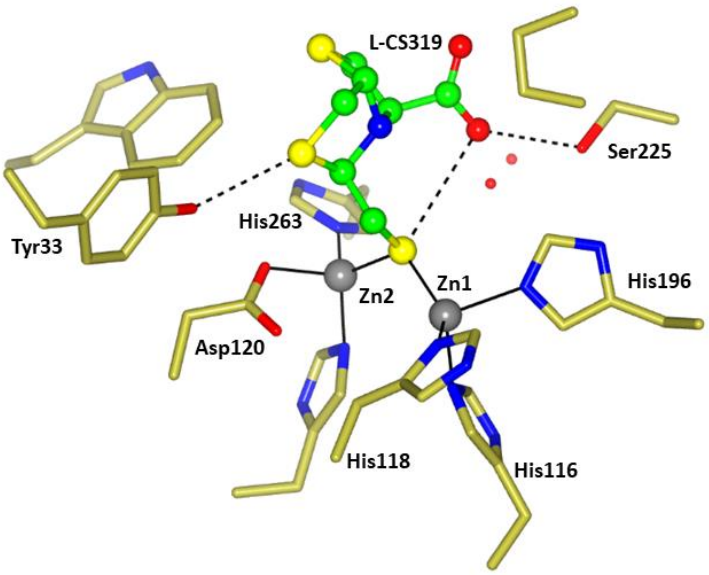
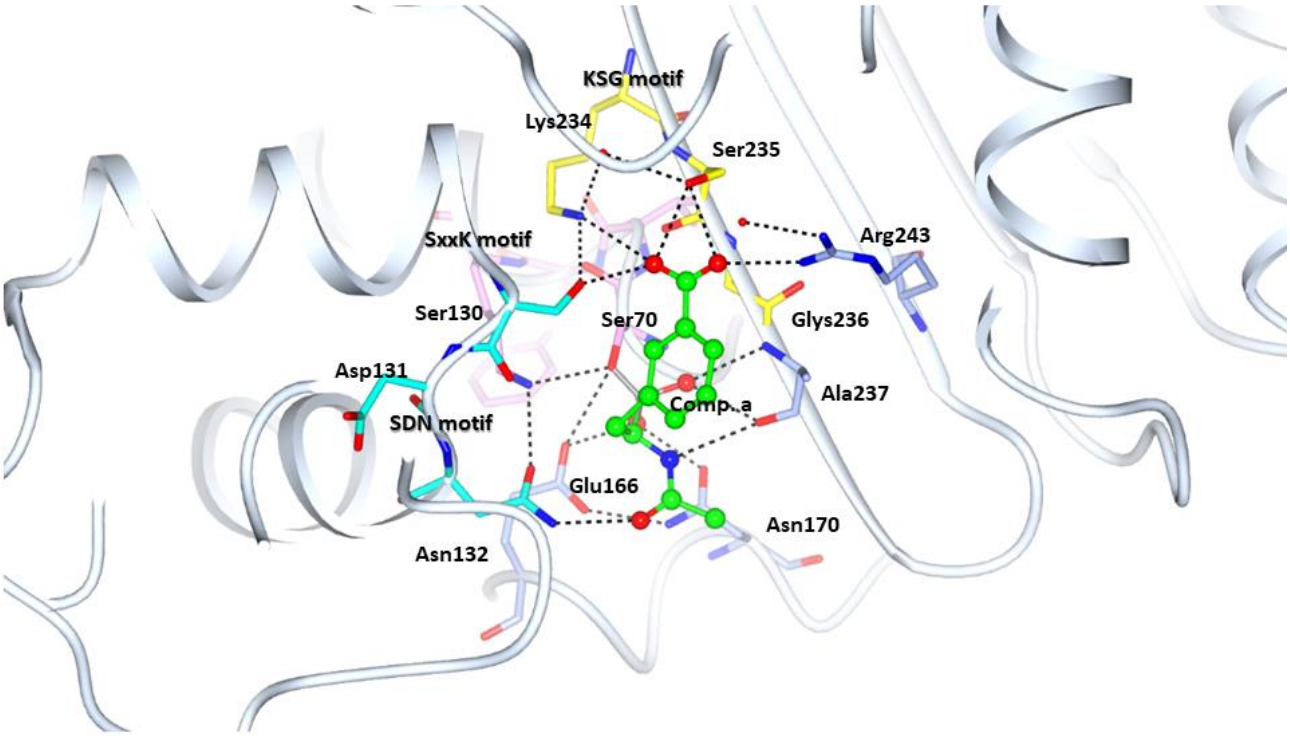


Figure 6D.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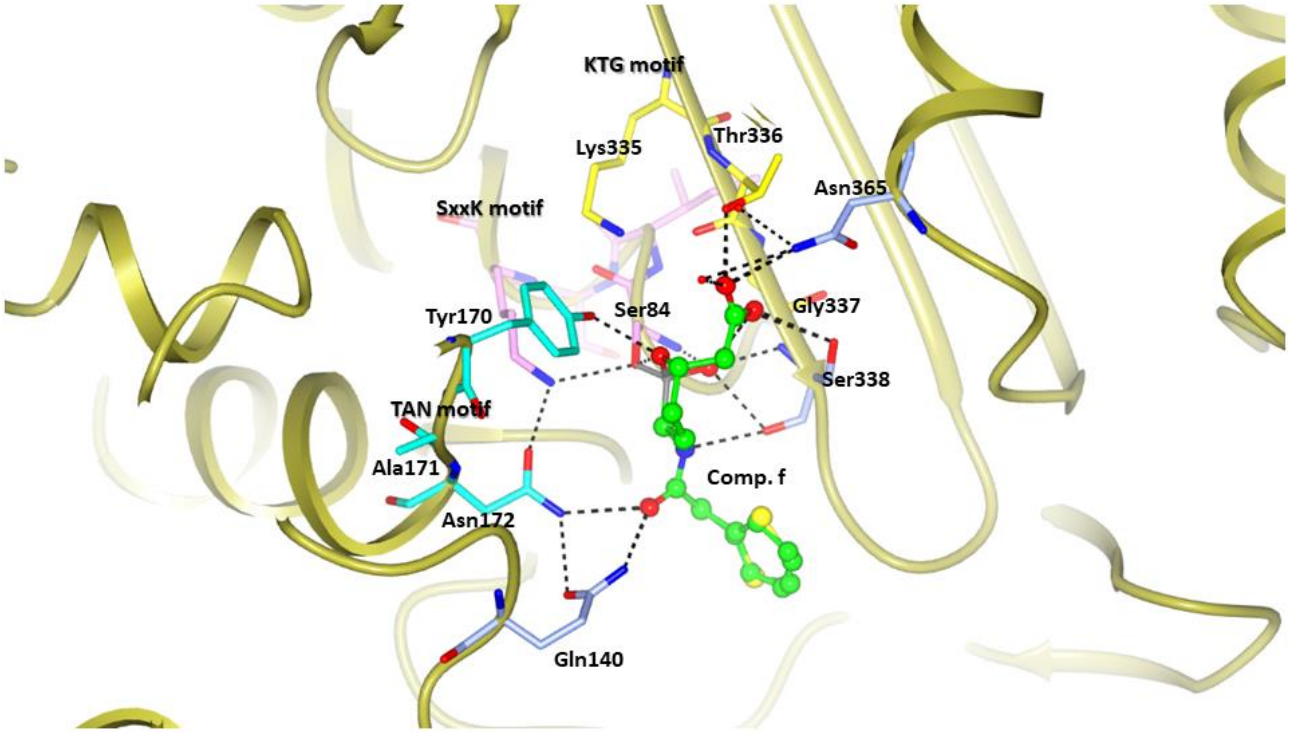
Ser235
Arg243
SxxK motif
Ser130
Ser70
Glys236
Asp131
Comp. a
Ala237
SDN motif
Glu166
Asn170
Asn132

Figure 7A.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Thr336
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SxxK motif
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Gly337
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TAN motif
Ala171
Comp. f
Asn172
Gln140

Figure 7B.

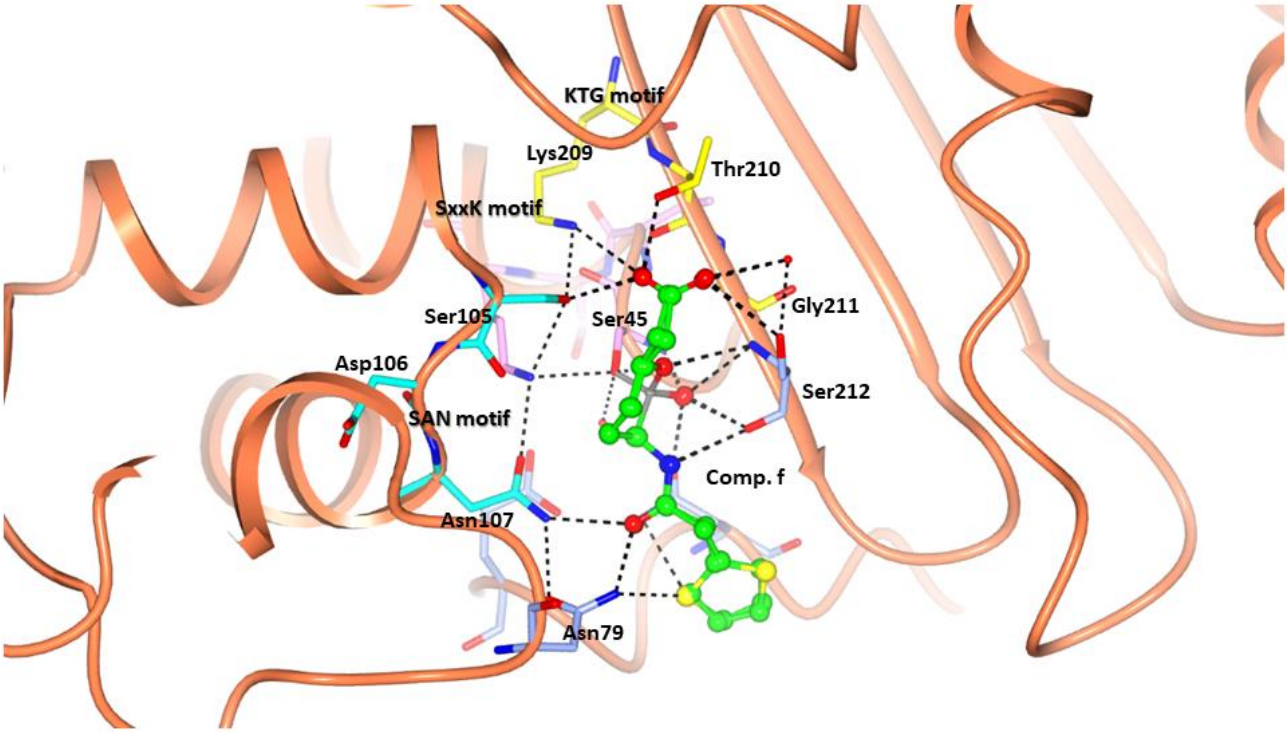
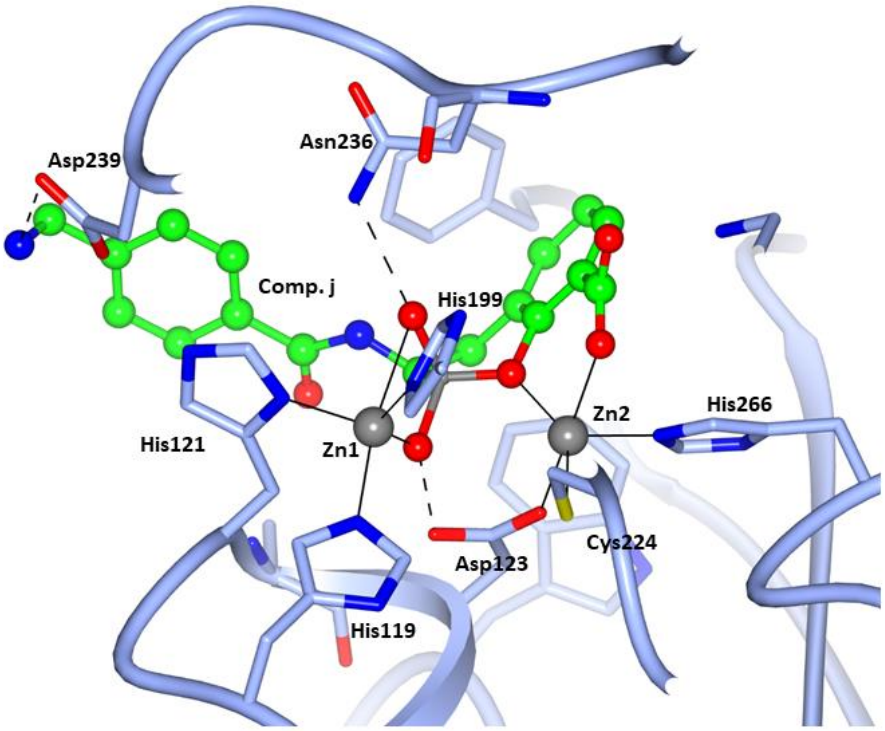


Figure 7C.

1285


Asp239
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His121
Zn1
Zn2
His266
Asp123
Cys224
His119

Figure 8.