Metallo-β-lactamases and a tug-of-war for the available zinc at the host-pathogen interface

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Abstract

Metallo-β-lactamases (MBLs) are zinc-dependent hydrolases that inactivate virtually all β-lactam antibiotics. The expression of MBLs by Gram-negative bacteria severely limits the therapeutic options to treat infections. MBLs bind the essential metal ions in the bacterial periplasm, and their activity is challenged upon the zinc starvation conditions elicited by the native immune response. Metal depletion compromises both the enzyme activity and stability in the periplasm, impacting on the resistance profile *in vivo*. Thus, novel inhibitory approaches involve the use of chelating agents or metal-based drugs that displace the native metal ion. However, newer MBL variants incorporate mutations that improve their metal binding abilities or stabilize the metal-depleted form, revealing that metal starvation is a driving force acting on MBL evolution. Future challenges require addressing the gap between *in cell* and *in vitro* studies, dissecting the mechanism for MBL metalation and determining the metal content *in situ*.

Keywords

Zinc; Metallo-β-lactamases; Antibiotic Resistance; Protein Evolution; Periplasmic Zinc Homeostasis

Introduction

Metallo-β-lactamases (MBLs) are zinc-dependent hydrolases expressed mostly by Gram negative bacteria, able to inactivate almost all β-lactam antibiotics [1]. In contrast to serine-

β-lactamases (SBLs), there are no approved inhibitors for MBLs yet [2]. MBLs present a common protein fold but differ in their metal ligand set and metal stoichiometry. Thus, MBLs are divided into three subclasses (B1, B2, and B3). MBLs have two metal binding sites (Zn1 and Zn2) capable of accommodating exogenous ligands such as water molecules and substrates [1,3] (Figure 1). Either both sites (B1 and B3 subclasses) or only Zn2 (B2 subclass) are occupied in the active form of these enzymes. In B2 enzymes, occupancy of the Zn1 site inhibits the enzyme activity.

The role and essentiality of each metal binding site in the catalytic mechanism of MBLs have been matter of debate along three decades [1]. Despite it is accepted that Zn(II) ions are essential to substrate binding and hydrolysis, MBLs have been mostly studied *in vitro*, without considering their physiological environment. In 2012, the Palzkill [4] and Vila [5] labs showed that active site mutations giving rise to variants with an activity *in vitro* equal or higher than the native enzyme in Zn(II)-excess conditions, were unable to confer resistance in a bacterial cell. This discrepancy was accounted for by a weakened Zn(II) binding affinity leading to an impaired metalation in the periplasm [4,5]. Here we discuss the emerging picture and the new challenges in the field regarding the gap between *in cell* and *in vitro* studies, focusing on the clinically relevant, acquired MBLs.

MBLs bind zinc in the bacterial periplasm

In Gram-negative bacteria, MBLs are translocated to the periplasm. All reported MBLs contain signal peptides targeting them to the Sec system, as demonstrated for the B3 enzyme GOB-18 [6]. Therefore, MBLs traverse the membrane in an unfolded state, and the final folding and Zn(II) acquisition events occur in the periplasm (Figure 2). Zn(II) binding in the periplasm is essential for β-lactamase activity and for enzyme stability *in vivo*. Thus, MBL-mediated antibiotic resistance depends on the Zn(II) availability in the periplasm. This is the case for soluble MBLs and for enzymes from the NDM family, which are membrane-anchored lipoproteins [7,8]. NDMs are lipidated after secretion by the Sec system and then are transported to the inner face of the outer membrane.

Zn(II) is essential for bacteria by serving catalytic and structural roles in many proteins [9]. Accordingly, various systems of metal homeostasis maintain the cytoplasmic Zn(II) levels within a physiologically adequate range (Figure 3) [10,11]. During an infection, the host displays an inflammatory response known as nutritional immunity, in which neutrophils directed to the infection sites secrete large amounts of calprotectin [12]. This metal-chelating protein scavenges Zn(II) and other divalent cations at the host-pathogen interface. Bacteria respond by expressing the Zur regulon (Figure 3) [10]. While these systems maintain the appropriate levels of cytoplasmic Zn(II), the periplasm of most Gram-negative bacteria lacks mechanisms for accumulating and regulating Zn(II) levels. Thus, the available periplasmic Zn(II) and the ability of MBLs to acquire metal ions depend on the extracellular levels of this cation [1]. Therefore, the nutritional immunity response represents a formidable challenge to MBL-mediated antibiotic resistance.

Thermodynamics and kinetics of zinc binding to MBLs

B1 MBLs are active in the periplasm when the binuclear site is fully loaded, a fact that depends on the zinc binding affinities [5]. The metal ligands are highly conserved within each subclass, but the metal binding affinities are tuned by the second coordination sphere [13,14]. Zn(II) binding in B1 enzymes can be described by two macroscopic dissociation constants (Kd1 and Kd2). There are discrepancies in the reported Kd values that can be tracked to the different methods employed, as discussed elsewhere [1]. Kd values determined by competition with chelators range from moderate to moderately high (pM to nM for Kd1 in B1-B3 enzymes; nM for Kd2 in B1 and B3 enzymes; μM for Kd2 in B2 enzymes), implying that these metal sites are thermodynamically stable [15,16]. Instead, Kd2 values in the μM range reported for some B1 MBLs were calculated from activity measurements [17,18]. These low affinities are not consistent with a periplasmic total Zn(II) concentration < 0.1 μM in *E. coli* in rich medium [13]. Under these conditions, binuclear MBLs with μM affinities would not be fully metalated. On the other hand, Zn(II) levels > μM would interfere with the resistance against carbapenems provided by B2 enzymes, that are inhibited at these Zn(II) concentrations [16].

Zn(II) sites in MBLs are kinetically labile compared to other zinc enzymes [14]. Incubation of Zn(II)-loaded IMP-1 either with Cd(II) or with ^{68}Zn(II) results in 60% of metal replacement in 5 minutes [19], suggesting a high k_{off} value. This observation is supported by inhibition experiments of NDM-1 with the chelator AMA [20] and the report that Zn(II) dissociates from BcII during catalytic turnover in the absence of extra Zn(II) in the buffer [5]. This lability makes MBL-mediated antibiotic resistance highly sensitive to variations in the levels of extracellular and periplasmic Zn(II). The k_{on} value for Zn(II) binding to BcII $(1.4 \ \mu M^{-1}s^{-1})$ is not diffusion-controlled, suggesting that metal uptake could be a two-step process or require the involvement of additional molecules in the periplasm to achieve metalation in time scales relevant to *in vivo* processes [14,21]. A better understanding of the kinetics of metal binding to MBLs is required to describe the metalation process within the cell.

The impact of Zn(II) binding on MBL folding after translocation has been scarcely explored. Periplasmic expression of the B3 enzyme L1 in the absence of Zn(II) in *E. coli* rendered an enzyme with an altered quaternary structure [22], disclosing an important role of the metal ion in folding. Metalation of MBLs in the periplasm is a dynamic process modulated by the cellular context, and a trait amenable to protein evolution that requires studies addressing the features of MBLs in their native hosts.

Metal starvation challenges MBLs in the periplasm

MBLs are inhibited in the presence of strong metal chelators such as EDTA and dipicolinic acid (DPA), that also impair MBL-mediated bacterial resistance [1]. This inhibitory effect can be elicited in bacteria either by adding small molecules that diffuse into the periplasm, or by limiting the extracellular Zn(II) by calprotectin or with Chelex [8,23]. This confirms that the levels of periplasmic Zn(II) are minimally regulated and depend on the extracellular

availability of this metal ion. Based on this, metal chelators have been used to mimic the conditions elicited by the nutritional immunity response.

Addition of DPA to cells expressing a set of clinically relevant MBLs showed inhibitory profiles depending on the enzyme [8]. Cells producing SPM-1 or IMP-1 were much less affected than those expressing NDM-1 or VIM-2, both in *E. coli* and in *P. aeruginosa* [24,25]. Zn(II) dissociation in the periplasm inactivated MBLs and led to a time-dependent decrease of the protein levels in this compartment, revealing that metal binding is essential for *in vivo* stability of these enzymes, *i.e.*, apo-MBLs (devoid of metal ions) are unstable in the periplasm [8]. This finding contrasts with the stability of apo-MBLs *in vitro* [26,27]. Upon metal depletion, some apo-MBLs such as NDM-1 are degraded by periplasmic proteases, and it is also likely that other apo-MBLs are prone to aggregation [1].

Metal starvation impacts antibiotic resistance in bacterial infections

The classification of MBL producers as carbapenem-resistant is based on susceptibility tests on cation-adjusted Mueller-Hinton broth (caMHB) [1]. However, Zn(II) levels in these media (*ca.* 15 μM) largely exceed the physiological concentration of free Zn(II) in body fluids, and it varies significantly among different vendors and among batches from the same vendor [28], resulting in variable levels of resistance detected in the lab. This questions the reliability of current phenotypic tests to assess the clinical impact of MBL producers. Nicolau and co-workers reported a significant reduction in the bacterial load upon treatment with meropenem or cefepime in murine infection models with MBL-producing Enterobacterales [23,29]. This finding was accounted for by the almost undetectable levels of Zn(II) in the bronchoalveolar lavage fluid from lung-infected mice, that correlate with the reduction in MICs of meropenem in zinc-depleted caMHB of the same bacterial strains. The authors concluded that MBLs are not a relevant mechanism of resistance against carbapenems or cefepime under physiological Zn(II) concentrations [23,29]. These results conflict with other experiments in Enterobacterales expressing NDM-1 in mice and larvae [30,31]. Furthermore, these experiments do not disprove the efficacy of MBLs in conferring resistance against penicillins or most cephalosporins to Enterobacterales, based on the higher values of MICs compared to carbapenems or cefepime.

The alarming dissemination of MBL-coding genes among clinical isolates suggests that these findings cannot be generalized. Skaar and co-workers have reported that the response to Zn(II) limitation is highly variable depending on the tissue [32]. On the other hand, non-fermenters exhibit markedly higher values of carbapenem MICs compared to Enterobacterales expressing similar levels of MBLs [33]. Thus, it is likely that Zn(II) limitation may not inactivate MBLs in non-fermenters. Indeed, imipenem administration to mice infected by *P. aeruginosa* producing IMP-1 resulted in only 30% survival, but co-administration of Ca-EDTA boosted this rate to 100% [34]. Also, Zn(II) limitation may be relevant only at early stages of infection, and Zn(II) levels in infected tissues may be restored after some time [35]. These controversial issues evidence the need of improving susceptibility tests to achieve clinically meaningful MIC values in MBL producers and pinpoint the nutritional immunity response as one of the major forces shaping the evolution of these enzymes.

Metal starvation is a driving force for the evolution of MBLs

The study of clinical variants from different MBL families provides information of evolution in real time, enabling the identification of the selected biochemical traits. In the case of NDM-1, membrane-anchoring stabilizes the enzyme towards periplasmic degradation during metal restriction (Figure 2) [8]. All known NDM alleles are membrane-bound, highlighting the advantage of this cellular localization [36]. Clinical NDM variants show similar resistance profiles in Zn(II)-replete media, suggesting that the limited number of mutations present in NDM variants are neutral. Upon metal starvation, however, most of these alleles granted higher resistance levels than NDM-1. Remarkably, the most common substitution among NDM variants, M154L (M150aL according to consensus BBL numbering) is crucial in this adaptation by increasing the Zn(II)-binding affinity [17,36,37]. Many NDM variants presented an enhanced stability towards degradation upon metal depletion, with mutations A233V (A248V BBL) and E152K (E149K BBL) conferring the largest improvements (Figure 3).

A similar scenario was reported for a group of closely related VIM variants by Crowder, Bonomo and Fast [38]. Variants containing the H229R (H254R BBL) mutation presented increased resistance under Zn(II) depletion, attributed to an increased stability in the cell due to the formation of a salt bridge by Arg229. These enzymes also presented differences in resistance in Zn(II)-replete conditions towards ceftazidime, considered as a driver for diversification within the VIM family [39].

In contrast, Crowder has shown that IMP allelic variants are being selected based on their improved catalytic performance towards newer carbapenems [40]. Instead, the observed mutations did not elicit better resistance in a Zn(II)-depleted environment. These results reveal that each family evolves driven by different evolutionary pressures, with Zn(II) starvation being a key driving force.

Metal chelation or metal-replacement strategies to inhibit MBLs

Different chelators have been explored as MBL inhibitors, with Ca(II)-EDTA showing efficacy in animal models [34]. However, most chelating agents are not selective towards Zn(II) and may also interfere with essential zinc enzymes from the host. The metal chelator Aspergillomarasmine A (AMA) [30], identified by Wright and co-workers (Figure 4) as a potent MBL inhibitor, displayed a high affinity toward Zn(II) (Kd ~ 0.2 nM), but with a restricted metal selectivity [20]. AMA restored meropenem sensitivity to a panel of clinical isolates producing NDM and VIM, but it was less effective against strains expressing IMP, AIM, SPM and CphA [41]. This profile reproduces the trend of different MBLs in lab strains towards Zn(II) scarcity [8]. The rapid adaptation of MBL alleles to metal starvation challenges this strategy.

AMA acts by sequestering the Zn(II) ions in solution in a process limited by the kinetics of Zn(II) dissociation [20,30,42]. This inhibitory effect has been attributed to depletion of the Zn2 site [20], supporting the proposal that B1 MBLs are active as binuclear enzymes in the periplasm [5]. The potency of AMA to restore β-lactam efficacy depends both on the

antibiotic partner and the MBL [41]. Pairing AMA with carbapenems is the best option since it requires lower concentrations of AMA [41], and was effective in mice infected with a clinical isolate of NDM-1-producing *K. pneumoniae* [30].

The Franz group has explored the use of chelators generated *in situ* upon substrate hydrolysis, such as the modified cephalosporin PcephPT (Figure 4), that liberates a pyrithione group upon hydrolysis and binds the metal site without removing the Zn(II) ions [43]. PcephPT is able to the restore efficacy of meropenem towards NDM-1 producers [43].

The use of metal-based drugs as MBL inhibitors exploits the kinetic lability of the zinc sites in MBLs, leading to replacement of the Zn(II) ions by non-native metal ions that render the enzyme inactive. The Sun group has identified colloidal bismuth subcitrate (CBS) [44], used to treat *Helicobacter pylori* infections, and the anti-rheumatic Au(I) compound auranofin (AUR) [45] as MBL inhibitors. Both drugs were effective inhibitors of various B1 MBLs *in vitro*, significantly reducing β-lactam MICs of MBL producers [44,45]. While treatment with AUR led to binding of Au(I) ions at the Zn1 and Zn2 positions, only one Bi(III) ion was present in NDM-1 treated with CBS, bound to ligands from both metal binding sites (Figure 4). The combination of CBS with meropenem was effective to treat mice infected with an NDM-1 producer [44]. Various Cu(II), Ru(II), Pd(II) and Pt(II) compounds are MBL inhibitors acting by the same mechanism [46,47,48], mostly targeting the Cys221 ligand at the Zn2 site [44–47]. B3 MBLs, lacking a Cys ligand, are not inhibited by these Pd(II) and Pt(II) compounds [47]. The success of these strategies supports the relevance of understanding the kinetics of Zn(II) binding to develop inhibitors.

Perspectives and Future Challenges

Robinson and coworkers provided a thermodynamic description of protein metalation in the bacterial cytosol [49,50]. Knowledge of the thermodynamics and kinetics of Zn(II) binding to MBLs in the periplasm is essential to understand their physiology and their role in resistance. No periplasmic metallochaperones have been identified yet. These studies require tools to quantify the metalation level of MBLs in the periplasm. Different fluorogenic substrates [51,52] and probes binding covalently MBLs [53,54] have been developed (Figure 5). A relevant step forward in this direction is the recent report of a fluorescent thiol-based compound developed by Emily Qués lab (Figure 5) that can be interrogated by confocal microscopy to report on the metal content of NDM-1 in the periplasm of *E. coli* [55]. The reversible binding mode of this compound allows monitoring changes in the metalation state of MBLs. The development of novel compounds is crucial to pursue an integral description of the complex tug-of-war for the available Zn(II) at the host-pathogen interface.

Acknowledgements

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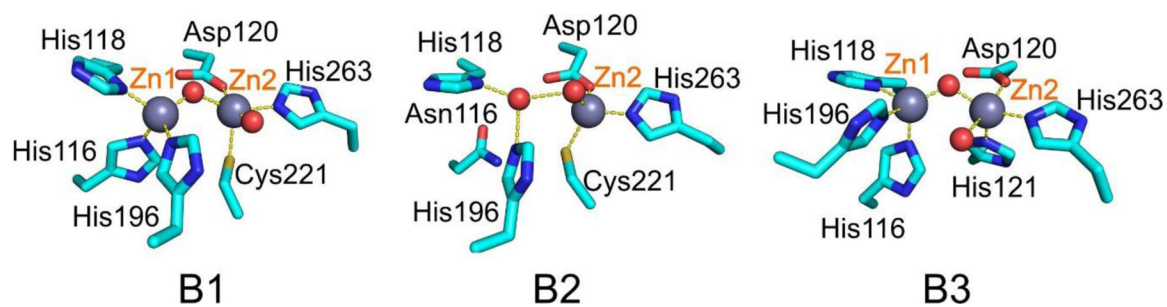
Figure 1.
Active site Zn(II) coordination spheres for B1, B2 and B3 MBLs. Zn(II) ions and water/hydroxide ions are represented as grey and red spheres, respectively. PDB codes for the structures used are: 5N5G (B1, VIM-1), 3SD9 (B2, Sfh-I) and 3LVZ (B3, BJP-1).
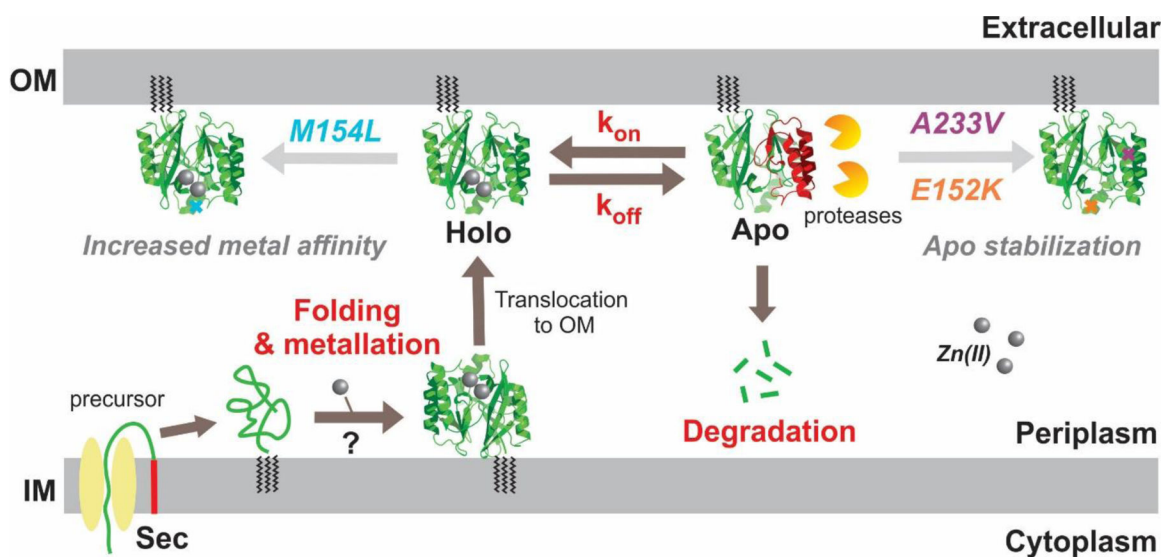
Figure 2.
Metabolism of NDMs in the periplasmic space of Gram-negative bacteria. Once exported, processed and lipidated, NDM enzymes are folded and metalated, and then translocated to the inner-face of the outer membrane. Metal loss from the active site of NDM-1 under extracellular Zn(II) restriction generates a degradation-prone apo-enzyme [8] due to an increased flexibility in the C-terminal region (colored in red), which is targeted by periplasmic proteases [1]. Clinical variants of NDM-1 circumvent periplasmic degradation by accumulating mutations that either increase the metal binding affinities (M154L) or stabilize the apo-enzyme structure (A223V or E152K) [17,36].
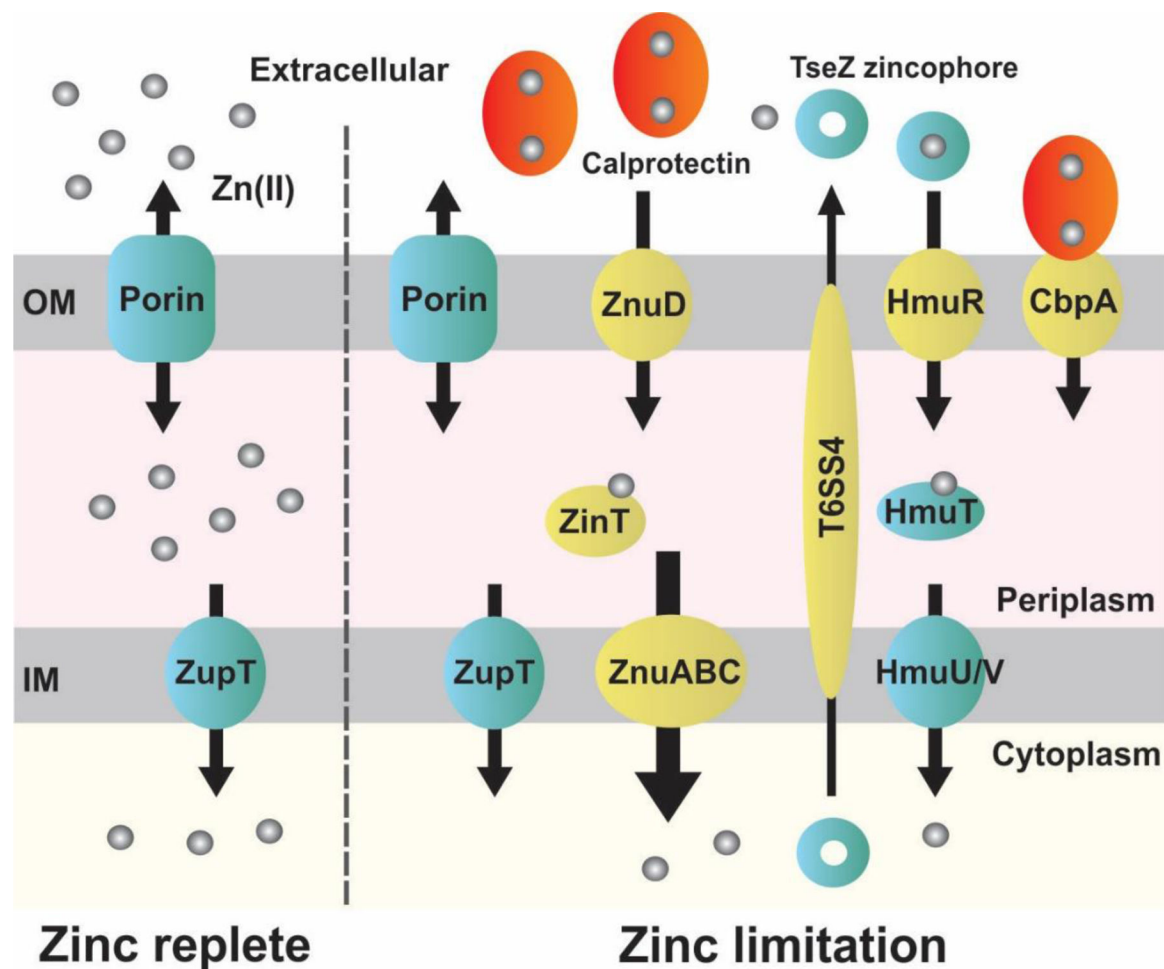
Figure 3.
Mechanisms of Zn(II) internalization in Gram-negative bacteria. In Zn(II)-replete conditions (left), Zn(II) ions enter the periplasmic space through non-specific porins and are transported to the cytoplasm by constitutively expressed antiporters, such as ZupT (in *E. coli*) [56]. Upon Zn(II) limitation (right), the Zur regulon is de-repressed and additional importers and accessory proteins are produced (highlighted in yellow). These include the high-affinity importer ZnuABC [10], widely-conserved in bacteria, the periplasmic zinc-binding protein ZinT from Enterobacterales [57], which delivers Zn(II) ions to ZnuABC, the TonB-dependent ZnuD importers from *Neisseria meningitidis* and non-fermenters [10], the capture of extracellular Zn(II) through secretion and incorporation of zincophores, such as TseZ from *Burkholderia thailandensis* [58], and receptors specific for Zn(II)-bound calprotectin, such as CbpA from *N. meningitidis* [59] or TdfH from *Neisseria gonorrhoeae* [60], that strip Zn(II) from it. IM: inner membrane; OM: outer membrane.
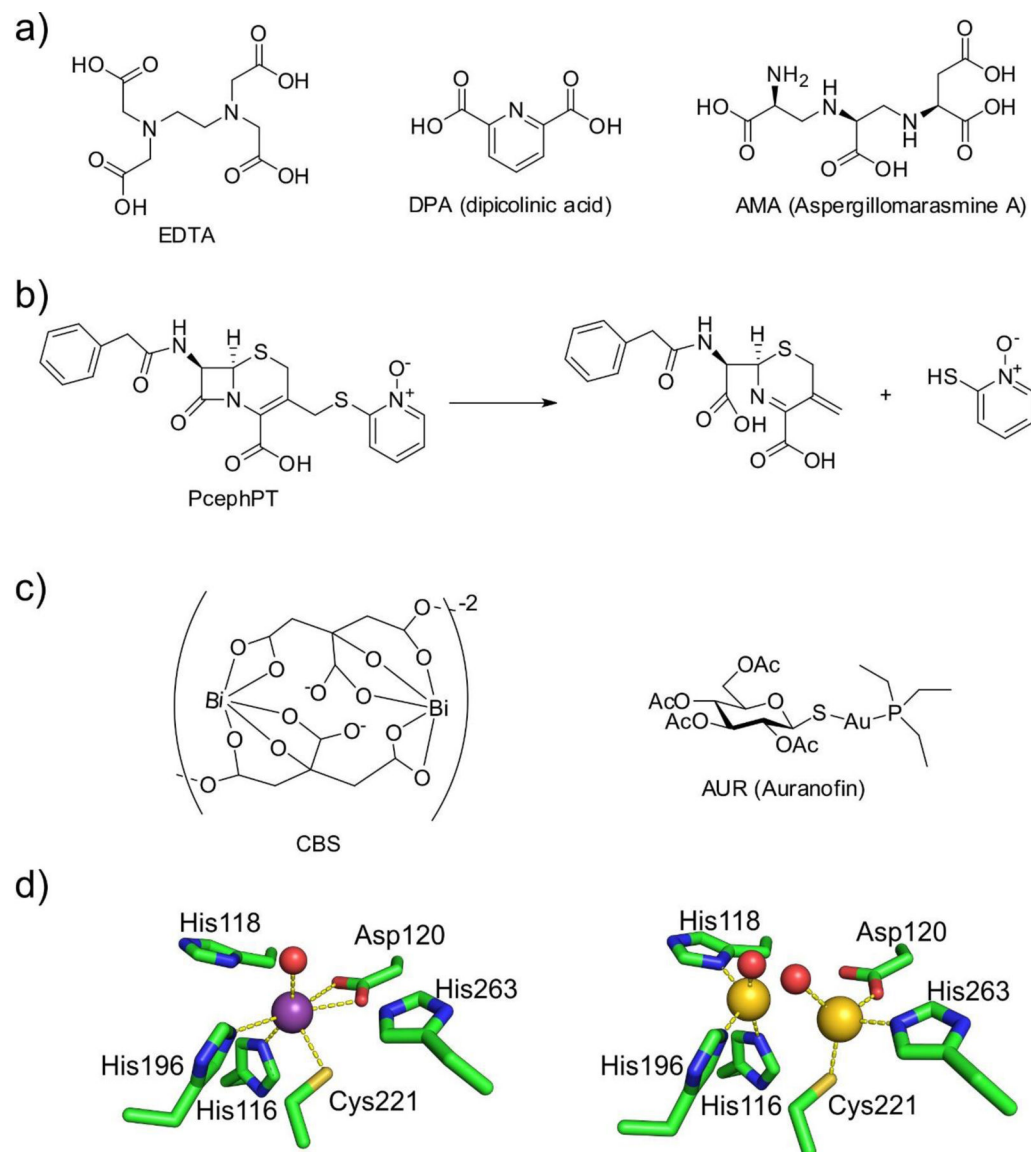
Figure 4.
a) Structures of MBL inhibitors that act by metal chelation. **b)** Scheme showing the structure of the prochelator cephalosporin PcephPT, and the release of a pyrithione group from the drug upon hydrolysis of its β-lactam ring [43] **c)** Structures of metal-based MBL inhibitors. **d)** Active sites in NDM-1 bound to Bi(III) (left) and Au(I) (right), resulting from treatment with CBS [44] and AUR [45], respectively.
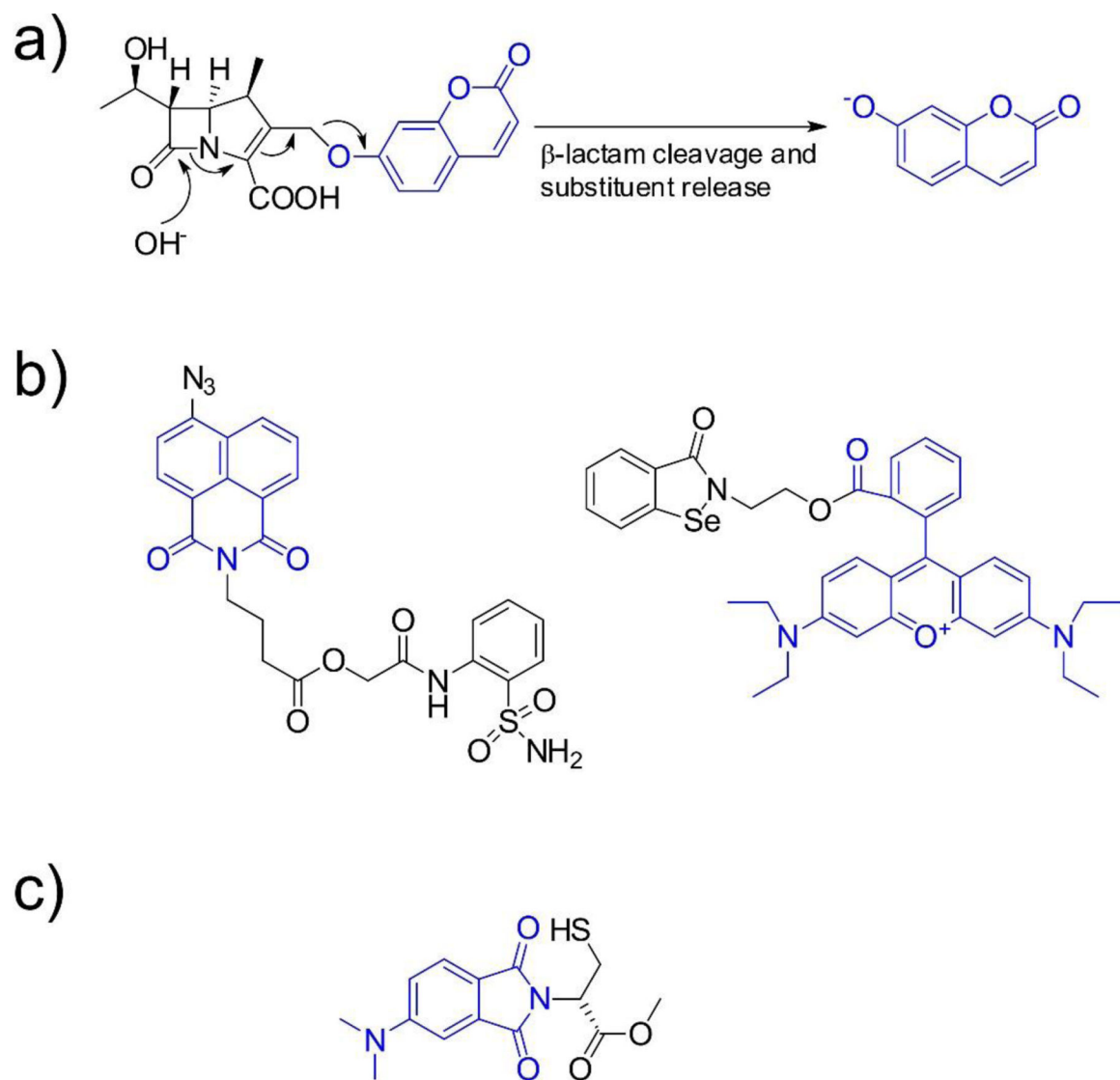
Figure 5.
a) Structure of fluorogenic carbapenem developed by Mao *et al.* [51] **b)** Structures of covalent-binding fluorescent probes, developed by Chen *et al.* [53] (right) and Singha *et al.* [54] (left). **c)** Structure of the reversible fluorescent probe developed by Que *et al.* [55]. In all cases, the fluorescent moiety is shown in blue.