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Virtual Screening and Experimental Testing of B1 Metallo- β -lactamase Inhibitors

Joon S. Kang^{a,b,#}, Antonia L. Zhang^{a,#}, Mohammad Faheem^a, Charles J. Zhang^a, Ni Ai^c, John D. Buynak^d, William J. Welsh^e, and Peter Oelschlaeger^{a,*}

^aDepartment of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, Pomona, California, 91766-1854

^bDepartment of Biological Sciences, California State Polytechnic University, Pomona, California, 91768-2557

^cPharmaceutical Informatics Institute, School of Pharmaceutical Sciences, Zhejiang University, Zhejiang, People's Republic of China, 31005

^dDepartment of Chemistry, Southern Methodist University, Dallas, Texas, 75275-0314

^eDepartment of Pharmacology, Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey, and Division of Chem Informatics, Biomedical Informatics Shared Resource, Rutgers-Cancer Institute of New Jersey, Piscataway, New Jersey 08854-8021

Abstract

The global rise of metallo- β -lactamases (MBLs) is problematic due to their ability to inactivate most β -lactam antibiotics. MBL inhibitors that could be co-administered with and restore the efficacy of β -lactams are highly sought after. In this study, we employ virtual screening of candidate MBL inhibitors without thiols or carboxylates to avoid off-target effects using the *Avalanche* software package, followed by experimental validation of the selected compounds. As target enzymes we chose the clinically relevant B1 MBLs NDM-1, IMP-1, and VIM-2. Among 32 compounds selected from a ~1.5 million compound library, 6 exhibited IC₅₀ values < 40 μ M against NDM-1 and/or IMP-1. The most potent inhibitors of NDM-1, IMP-1, and VIM-2 had IC₅₀ values of 19 ± 2 μ M, 14 ± 1 μ M, and 50 ± 20 μ M, respectively. While chemically diverse, the most potent inhibitors all contain combinations of hydroxyl, ketone, ester, amide, or sulfonyl groups. Docking studies suggest that these electron-dense moieties are involved in Zn(II) coordination and interaction with protein residues. These novel scaffolds could serve as the basis for further development of MBL inhibitors. A procedure for renaming NDM-1 residues to conform to the class B β -lactamase (BBL) numbering scheme is also included.

Graphical Abstract

*Address correspondence to Peter Oelschlaeger, poelschlaeger@westernu.edu, Phone: 909-469-8232, Fax: 909-469-5600.

#These authors contributed equally to this study.

SUPPORTING INFORMATION

Supporting Information Available: Details on virtual screen, chemical structures, SMILES format, and PAINS assessment of tested compounds; BBL numbering of NDM-1; kinetic constants of NDM-1 and VIM-2; coordinates and images of docking poses.

INTRODUCTION

β -Lactam antibiotics have been effective for decades in targeting transpeptidases involved in the last step of bacterial peptidoglycan synthesis,¹ but we have now arrived at the long predicted future of antibiotic resistance,² when bacterial infections that were treatable pose a threat to humankind again.³ While antibiotic resistance can occur through various evolutionary processes, the main contributing factor for the increase in antibiotic resistance is bacterial production of β -lactamases.⁴ β -Lactamases have been grouped into classes based on homology:⁵ enzymes of classes A, C, and D, also known as serine β -lactamases (SBLs) utilize a serine-dependent mechanism, while class B enzymes, also known as metallo- β -lactamases (MBLs), employ Zn(II) ions to facilitate binding of antibiotics and activation of the Zn(II)-bound water/hydroxide for nucleophilic attack on and eventually cleavage of the β -lactam amide bond.^{6–7} It was not until the 1990's, when plasmid-mediated imipenemase (IMP)- and Verona Integron-borne MBL (VIM)-type MBLs were discovered in Gram-negative pathogens, that MBLs were recognized as a rising global threat in antibiotic resistance.⁸ New Delhi MBL (NDM)-type enzymes have been known for less than a decade⁹ and have since spread globally.¹⁰ The encoding MBL genes are often located on mobile genetic elements that allow for the rapid horizontal transmission of antibiotic resistance genes.^{4, 11} The encoded enzymes confer resistance to Gram-negative bacteria like *Serratia marcescens*, *Pseudomonas aeruginosa*, members of the Enterobacteriaceae family, and *Acinetobacter* spp..^{12–13}

IMP-, VIM-, and NDM-type enzymes belong to the B1 subclass,¹⁴ enzymes of which have two Zn(II) sites in common, one with Zn(II) ligands H116, H118, and H196 (3H or Zn1 site) and one with Zn(II) ligands D120, C221, and H263 (DCH or Zn2 site). B1 MBLs have a broad substrate spectrum, including penams, cepheids, and carbapenems. They are the most clinically significant, wide-spread, and diverse MBLs^{12–13} with 54 IMP¹⁵ (IMP-8 and IMP-47 are identical¹⁶), 48 VIM,¹⁷ and 17 NDM¹⁸ variants reported to date. There are also clinically important MBLs in the other subclasses, such as CphA¹⁹ and Sfh-1²⁰ (B2) and L1²¹ and AIM-1²² (B3). B2 enzymes feature a Zn1 site made up of N116, H118, and H196 that is unoccupied and a Zn2 site identical to that of B1 enzymes. They prefer carbapenems as substrates.⁶ B3 enzymes have an occupied 3H Zn1 site like B1 enzymes, but their Zn2 site deviates from that of B1 and B2 enzymes in that the C221 Zn2 ligand is replaced by His121.⁶ Their substrate preference is similar to that of B1 enzymes.^{23–24} There is some

variation in Zn(II) affinity and also catalytic mechanism between different MBLs.^{6–7, 25–26} In this contribution, we will focus on the clinically most important B1 enzymes.

MBLs are insensitive to all clinically available inhibitors, clavulanic acid, sulbactam, tazobactam,¹¹ and avibactam,²⁷ which inhibit serine β -lactamases exclusively. MBLs cannot inactivate aztreonam, but are often co-produced with extended-spectrum serine β -lactamases that have this ability.²⁸ Currently, no inhibitors of MBLs are available in the clinic, although several good candidates have been reported, many of which coordinate the Zn(II) ions through thiolates,^{29–31} carboxylates,^{29, 32–33} boronates,³⁴ azoles,^{35–37} or are Zn(II) chelators.^{38–39} Other scaffolds that have been shown to inhibit MBLs are pyrroles,^{40–42} thiosemicarbazides,³⁵ tetrahydropyrimidine-2-thiones,⁴¹ and rhodanines.^{43–44} What makes finding a clinically viable MBL inhibitor challenging is the non-covalent nature of substrate hydrolysis, making the design of mechanism-based covalent inhibitors difficult, as well as the fact that inhibitor binding is typically based on coordination to the Zn(II) ions. Compounds that are good Zn(II) ligands due to containing thiols or carboxylates are prone to also inhibit other essential metalloenzymes, potentially causing toxicities. In this study, we report the virtual screening of compounds as potential inhibitors of NDM-1, IMP-1 and VIM-2 using the virtual screening program *Avalanche*.⁴⁵ This program selects compounds based on shape complementarity and surface features, and compounds that would bind primarily due to the Zn(II)-ligating properties, such as thiols and carboxylates, can be filtered out during the screening process. Inhibitory activity of selected compounds was validated by *in vitro* experiments. This approach provides an avenue to identify novel scaffolds as MBL inhibitors.

MATERIALS & METHODS

Virtual Screening.

Avalanche (Snowdon Inc., Princeton, NJ)⁴⁵ is a virtual screening program that represents the shape and pharmacophoric character of molecular conformations as histograms which can be rapidly compared. First, *Avalanche* creates a histogram based not only on rays emanating between points on the Connolly surface of the query or hit molecule but also based on rays emanating between points on the Connolly surface and points on exterior surfaces representing interaction surfaces of potential receptors. The second major difference to other virtual screening programs is that the top hits, typically <1 %, from the histogram matching stage undergo a full three-dimensional (3D) alignment to the query conformation. The histogram comparison is used only as a first pass filter to eliminate the vast majority of molecular conformations that have little chance of demonstrating a high degree of 3D similarity. The 3D alignment enables the user to visualize the 3D similarity and to select and rank those that offer the greatest potential to address issues beyond primary receptor activity such as novelty, solubility, etc. For this study, inhibitor compounds were identified from a filtered eMolecules database consisting of ~1.5 million molecules using the inhibitor conformations found in crystal structures as queries. The filtered library was prepared as described and excluded molecules with unfavorable traits, such as carboxylate and thiol groups.⁴⁵

Antibiotics.

Antibiotics were purchased from the suppliers mentioned previously.⁴⁶ Cefepime was purchased from USP (Rockville, MD).

Plasmids and Subcloning.

pET26b-*bla*_{IMP-1} coding for IMP-1 including its native leader sequence was a gift from Dr. James Spencer (University of Bristol, UK). pET24a-*bla*_{VIM-2} coding for VIM-2 excluding its native leader sequence and pET26b-*bla*_{NDM-1} coding for NDM-1 including its native leader sequence were gifts from Dr. Michael Crowder (Miami University, Oxford, OH). pBC SK(-)-*bla*_{VIM-2} coding for VIM-2 with its native leader sequence was a gift from Dr. Robert Bonomo (Veterans Affairs Medical Center, Cleveland, OH). PCR-based site-directed mutagenesis was used to create pET26b-*bla*_{NDM-1}-C26A. pBC SK(+)-*bla*_{IMP-1} was reported previously.⁴⁷ pBC SK(+)-*bla*_{NDM-1} was generated by subcloning *bla*_{NDM-1} from pET26b-*bla*_{NDM-1} and pBC SK(+)-*bla*_{VIM-2} by subcloning *bla*_{VIM-2} from pBC SK(-)-*bla*_{VIM-2}⁴⁸ into pBC SK(+) (Agilent, La Jolla, CA).

Expression and Purification.

E. coli OverExpress C43 (DE3) cells (Lucigen, Middleton, WI) were used to overexpress NDM-1, IMP-1, and VIM-2 under control of the T7 promoter. IMP-1 was purified as previously reported using cell lysis by sonication, cation exchange chromatography, and gel filtration, resulting in an enzyme preparation in 50 mM MOPS, pH 7.0, 100 mM NaCl, 100 μ M Zn₂SO₄. The purification of NDM-1 and VIM-2 followed a similar procedure, except that the first purification step was anion exchange chromatography using three connected 5 ml HiTrapTM DEAE FF columns (GE Healthcare, Piscataway, NJ) and the buffer was 30 mM Tris, pH 7.6, 100 mM NaCl, containing 10 μ M Zn₂SO₄ for NDM-1⁴⁹ and 100 μ M Zn₂SO₄ for VIM-2. Purity of the final preparations was assessed by SDS-PAGE and protein concentration was determined using UV-Vis absorption measurement at 280 nm and published molar extinction coefficients (28,500 M⁻¹ cm⁻¹ for NDM-1,⁵⁰ 49,000 M⁻¹ cm⁻¹ for IMP-1,⁵¹ and 28,500 M⁻¹ cm⁻¹ for VIM-2.⁵²

Biophysical Characterization.

The molecular masses of purified NDM-1 and VIM-2 were determined by electrospray ionization mass spectrometry (ESI-MS) as described previously.⁴⁶ Purified NDM-1 and VIM-2 enzymes were dialyzed against zinc-free 30 mM Tris, pH 7.6, and the zinc content was determined with the 4-(2-pyridylazo) resorcinol (PAR) assay,⁵³ as described previously.^{54–55} All three enzymes were dialyzed against 50 mM phosphate buffer pH 7.0 and circular dichroism (CD) scans and thermal denaturation curves were recorded as described previously⁴⁶ except that the melting curves were recorded at 222 nm. Following dialysis in preparation of the PAR assay and CD experiments, protein was quantified as described above.

Steady-state Kinetic Assay.

Steady-state kinetic experiments were conducted as described previously,⁴⁶ except that the purified NDM-1 and VIM-2 enzymes were prepared in 30 mM Tris, pH 7.6, 10 μ M ZnSO₄

for NDM-1 and 100 μ M ZnSO₄ for VIM-2 (the buffers in which each enzyme was purified), plus 10 μ g/ml BSA. Previously described^{56–58} wavelengths and extinction coefficients were used. The enzyme concentration was adjusted to obtain less than 10% substrate hydrolysis within the first minute at the highest substrate concentration. The absorbance at specific wavelengths was measured at eight different substrate concentrations and three times per each substrate concentration. The kinetic constants were obtained for each of the three data sets using the Michaelis-Menten equation in Prism 7 (GraphPad Software, Inc., La Jolla, CA) and the data are presented as means $\pm$ standard deviations.

Inhibition Assay.

The eight top-ranked compounds obtained from the virtual screens for each query that were identified to be commercially available were purchased through RyanScientific, Inc. (Mount Pleasant, SC) for experimental testing. Compounds 1497396, 2006888, and 2003020 were supplied by ChemBridge (San Diego, CA), 2994990, 3331076, 11789171, 11990632, 11993658, 30009082, 11800316, 13548110, 30050820, 11802828, 15270308, 23978304, and 13527883 by Enamine (Monmouth, NJ), 4857944 by Key Organics (Camelford, United Kingdom), 1073834 by TimTec (Newark, DE), 17049647, 1098072, 8606972, 6821770, and 10101108 by Vitas-M Laboratory (Champaign, IL), 13601557 and 24897966 by Life Chemicals (Kyiv, Ukraine), 625527 and 4805083 by Princeton BioMolecular Research (Monmouth, NJ), and 16213040, 16213170, 16345258, and 16358780 from ChemDiv (San Diego, CA). RyanScientific stated that compound purity was guaranteed to be 90% and likely 95%. Additional compounds were purchased directly from suppliers: 1361183 (90% purity) from Vitas-M Laboratory (Champaign, IL), 179441547 and 179441565 (95% purity) from Otava, LTD (Toronto, Canada). The compounds were prepared by initially dissolving them in a small volume of DMSO and then diluting them into the assay buffer that was used for steady-state kinetic assays for each enzyme, resulting in a final percentage of DMSO of 0.1 % (vol/vol). No inhibition by DMSO was detectable under these conditions. For percent inhibition assays carried out at 30°C, the final compound concentration was 40 μ M, nitrocefin was 10 μ M, and the different MBLs were 320 pM. Percent inhibition was determined as full activity (initial velocity in the absence of any inhibitor) – residual activity (initial velocity in the presence of 40 μ M of compound). To determine IC₅₀ values, the compound concentration was tested at serial dilutions from 80 μ M to 0.625 μ M. All experiments were carried out in triplicate and are presented as means $\pm$ standard deviations.

Molecular Docking.

The three-dimensional structures of the 7 most potent compounds (see below) were built using ArgusLab (www.arguslab.com). The docking simulations were conducted using the AutoGrid 4.0 and AutoDock 4.2 programs,⁵⁹ as described previously,⁴⁶ but generating 100 conformations per complex. Adjustments were made to the charges of the Zn(II) ions and the Zn(II)-coordinating residues⁶⁰ and the Zn(II) van der Waals parameters.⁶¹ Carboxylic acids and thiols were treated as deprotonated and negatively charged. The highest-ranked clusters (and second- and third-highest, if those contained more conformations than the highest-ranked cluster) were analyzed based on binding energies and visually using Swiss PDB Viewer.⁶² Images of molecular models were created with VMD.⁶³

RESULTS & DISCUSSION

Virtual Screening.

Avalanche (Snowdon Inc., Princeton, NJ)⁴⁵ is a virtual screening program that identifies compounds from libraries that have shape complementarity with a query compound.⁶⁴ The program automatically incorporates both shape- and pharmacophore-based features comparison with three-dimensional (3D) alignment between the query molecule and test compounds residing in chemical databases. The following inhibitors obtained from crystal structures of MBL/inhibitor complexes were used as queries: L-captopril co-crystallized with NDM-1 (PDB code 4EXS⁶⁵), a mercaptocarboxylate co-crystallized with IMP-1 (1DD6⁶⁶), a biaryl succinate co-crystallized with IMP-1 (1JJT⁶⁷), and a mercaptocarboxylate co-crystallized with VIM-2 (2YZ3⁵¹). The eMolecules (www.emolecules.com) screening library, consisting of >7 million compounds was pre-filtered as previously described⁴⁵ to a library of about 1.5 million compounds, which was used for the virtual screen. This library excluded pharmacologically unfavorable traits and compounds with certain functional groups like thiols and carboxylates, as these have the potential to inhibit off-target metalloenzymes. The eight highest-ranked and commercially available compounds for each query (32 compounds total) were purchased from various suppliers (see Table S1 and Figs. S1–S4 in the Supporting Information for details). The most potent MBL inhibitors identified in this way and confirmed by experiment (below) are shown in Table 1.

As expected based on the screening library, none of the compounds contained thiols or carboxylates, but a few displayed other interesting functional groups: sulfonamides (11993658, 24897966, 6821770, 23978304), esters in β position to an amide (2994990) or a ketone (3484004, 3331076), phthalimides (11993658, 3484004, 24897966), N-substituted tetrazoles (6821770, 23978304), and amides (24897966, 23978304). Functional groups involved in Zn(II) coordination and/or characteristic interaction with enzyme residues as observed by molecular docking (see below) are colored red in Table 1. No pan-assay interference compounds (PAINS)^{68–69} were identified (see Supporting Information for details).

Expression and Purification of MBLs.

A pET26b-*bla*_{NDM-1} expression vector was subjected to PCR-based site-directed mutagenesis to obtain pET26b-*bla*_{NDM-1-C26A}, with the goal of removing a lipidation signal^{70–71} and obtaining soluble NDM-1 in the periplasm. pET26b-*bla*_{NDM-1-C26A}, pET26b-*bla*_{IMP-1}, and pET24a-*bla*_{VIM-2(-)} were used to overexpress NDM-1 and IMP-1 in the periplasm and VIM-2 in the cytoplasm of *Escherichia coli* C43 cells (see Experimental Section for details). Cells were harvested and lysed by sonication and the MBLs purified from the soluble fractions by anion (NDM-1 and VIM-2) or cation (IMP-1) exchange chromatography followed by gel filtration and finally concentrated before use or storage at –20°C. These preparations contained the enzymes of >95% purity, as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the yields were between 2 and 15 mg of purified protein per liter of culture (Table 2).

Biophysical Characterization.

Mass spectrometry.—The purified proteins were subjected to electrospray ionization mass spectrometry (ESI-MS). The molecular mass of NDM-1 was 25,607 (Table 2), consistent with the calculated mass of 25,606 of the enzyme missing the first 28 residues (G₂₉EIRP...), indicating that the C26A mutation altered the signal peptide cleavage site from between residues 25 and 26 (25|26)⁷⁰ to 28|29, which is in agreement with the prediction by the LipoP 1.0 server.⁷³ The molecular mass of IMP-1 expressed from pET26b-*bla*_{IMP-1} was previously reported as 25,112,⁷² in agreement with the calculated mass of 25,113 of the mature protein missing the first 18 residues (A₁₉ESLP...). The LipoP 1.0 server also predicts 18|19 as the predominant cleavage site in the preprotein. pET24a-*bla*_{VIM-2(-)} codes for VIM-2 missing the signal peptide and with A₂₀ replaced by M₂₀. ESI-MS yielded molecular masses of 26,034 (~95%) and 26,253 (<5%), indicating that the majority of the protein is missing 2 residues of the truncated construct (P₂₂LAFS... ; calculated mass of 26,031), and a small portion consists of the full-length truncated construct (M₂₀SPLA... ; calculated mass of 26,249). The LipoP 1.0 server predicts several cleavage sites for the natural VIM-2 preprotein, including 26|27, 20|21, 18|19, and 24|25. Thus, all our proteins as isolated are very good representatives of the naturally occurring enzymes with deviations of no more than 3 residues on the N-termini.

Circular dichroism experiments.—Circular dichroism scans of the purified proteins revealed that they all contain the mixture of α helices and β sheets typical for proteins of the $\alpha\beta/\beta\alpha$ metallo- β -lactamase fold⁷⁴ (data not shown). The melting temperatures of NDM-1, IMP-1, and VIM-2 were determined to be 61°C, 70°C, and 63°C (Table 2), in good agreement with the previously reported values of 59.5°C,⁷⁵ 72°C,⁷⁶ and 62°C,⁴⁸ respectively.

Zinc content.—The number of Zn(II) ions bound per molecule MBL was determined using the 4-(2-pyridylazo) resorcinol (PAR) assay.^{53–54} NDM-1 bound 2.45 ± 0.04 Zn(II) ions per molecule (Table 2), half an equivalent higher than expected. While most crystal structures of NDM-1 containing Zn(II) have two Zn(II) ions bound at the two Zn(II) binding sites, one high resolution structure (PDB code 4HL2, 1.05 Å resolution) with two molecules in the asymmetric unit contains 5 Zn(II) ions, 4 at the expected Zn(II) binding sites plus one at the interface between the two enzyme molecules coordinated by E242 of molecule A and E149 and D236 of molecule B (residue numbering according to class B β -lactamase (BBL) standard numbering scheme;^{14, 77} see Supporting Information for details). It is possible that under our experimental conditions (1–2 μ M enzyme) NDM-1 formed similar dimers with an additional Zn(II) ion. As expected and as reported previously, IMP-1 bound approximately 2 (1.8 ± 0.2) Zn(II) ions.⁷² VIM-2 bound 0.9 ± 0.1 Zn(II) ions, significantly less than the expected 2. VIM-2 was reported previously to display a uniquely high tendency to be oxidized at the Zn²⁺-coordinating C221 under crystallization conditions, which may contribute to a lower zinc affinity at the Zn²⁺ site.⁷⁸ Other studies have reported between 0.4 and 2 equivalents of Zn(II) for VIM-2,^{48–49, 79} indicating that its Zn(II) content may vary depending on the experimental conditions. The subsequent experiments were either supplemented with micromolar Zn(II) in the buffer and carried out at picomolar to nanomolar enzyme concentrations (kinetic and inhibition assays) or complex medium was

used (minimum inhibitory concentration (MIC) assays), so that all enzymes were expected to bind the full complement of 2 Zn(II) equivalents.

Biochemical Characterization.

Kinetic constants of IMP-1 were determined previously and found to be comparable to those of other groups.⁵⁵ Kinetic constants of NDM-1 and VIM-2 were determined by the same method except for enzyme-specific buffers at 30°C with a panel of β -lactam antibiotics representing penicillins, cephalosporins, a cephamycin, carbapenems, and the monobactam aztreonam. Some representative catalytic efficiencies, $k_{\text{cat}}/K_{\text{M}}$, are presented and compared to previously published values in Figure 1, while detailed kinetic constants of all β -lactams tested are listed in Tables S2 and S3 in the Supporting Information. Although the activities are higher, the activity profiles of our enzymes are comparable to previously published data.^{49, 52, 75} All three enzymes have very broad substrate spectra with relatively low catalytic efficiencies against the cephamycin cefoxitin (FOX) and the third- and fourth-generation cephalosporins, ceftazidime (CAZ) and cefepime (FEP). In addition, the current results as well as the previously published reports mentioned demonstrate that nitrocefin is the substrate converted most efficiently by the three enzymes, making it a suitable substrate for the subsequent inhibition studies.

Experimental Inhibition Study.

The 32 candidate compounds identified by the virtual screening described above were tested initially by determining percent inhibition at 40 μM . This was done not only against their specific MBL (the enzyme that their query molecule was bound to in the crystal structure), but also against the other two MBLs, resulting in 96 data points (Figure 2).

The data provide several insights: 1) Six compounds inhibited at least one enzyme more than 50% and are in the following referred to as inhibitors (Table 1). 2) Some inhibitors identified were relatively specific to their original target from the crystal structure (2994990 to NDM-1, 3484004 and 3331076 to IMP-1), while others inhibited other enzymes as well, in some cases more than their original target from the crystal structure (11993658 IMP-1 more than NDM-1, 6821770 and 23978304 NDM-1 and IMP-1 more than VIM-2). 3) A potent inhibitor with >50% inhibition of VIM-2 could not be found, although some came close with 24897966 being the closest. 4) Most inhibitors of NDM-1 are also inhibitors of IMP-1 and *vice versa* (11993658, 3484004, 6821770, and 23978304).

Insight 1 indicates that the general approach of using conformations from crystal structure complexes as queries works with a success rate of 19% when defining >50% inhibition at 40 μM as our threshold for success. The inclusion of thiols and carboxylates would have likely resulted in a higher success rate. Insight 2 suggests that the specificity of the query compound correlates with the specificity of the virtual screening hits. While we are not aware of experimental studies of the biaryl succinate inhibitor with other MBLs than IMP-1, it seems that this query is the most specific, as it only resulted in the identification of an IMP-1 inhibitor, but not NDM-1 or VIM-2 inhibitors (Fig. 2C). The elaborate mercaptocarboxylate bound to IMP-1 also seems to be quite specific to IMP-1 for the same reason, although the identified inhibitor 3484004 also inhibited NDM-1 (Fig. 2B). Captopril,

reported to be a broad-spectrum, typically micromolar, MBL inhibitor^{65, 80–81} (IC_{50} values for the D and L isomers, respectively, are of 20.1 μ M and 157.4 μ M against NDM-1, 7.2 μ M and 23.3 μ M against IMP-1, and 0.072 μ M and 4.4 μ M against VIM-2⁸²), unsurprisingly resulted in an IMP-1 inhibitor in addition to NDM-1 inhibitors when used as a query (Fig. 2A). The fact that the mercaptocarboxylate inhibitor bound to VIM-2 is much smaller than the one bound to IMP-1 may explain why this query resulted in the selection of inhibitors not specific to VIM-2. In fact, 6821770 and 23978304 are better inhibitors of NDM-1 and IMP-1 than of VIM-2 (Fig. 2D). Insights 3 and 4 combined suggest that VIM-2 deviates more from NDM-1 and IMP-1 than those enzymes from each other in their ability to be inhibited. This is somewhat unexpected, because at the amino acid sequence level VIM-2 is the closest relative to NDM-1 with 32.4% sequence identity,⁹ while IMP-1 and NDM-1 only share 28.7% identity. In terms of overall structure (PDB codes 4EXS, 1DD6, 3YZ3), VIM-2 is more similar to IMP-1 (2.54 Å Ca root mean square deviation, RMSD) than IMP-1 to NDM-1 (2.66 Å RMSD). However, a closer look at the active site reveals a significant difference between VIM-2 and IMP-1, namely the insertion of W87, which is mostly important for the enzyme's proper structure and folding,⁸³ between the active-site mobile loop and the active site, which may make the active site narrower. NDM-1 contains the identical residue at this position, but it does not constrict the active site as much as in VIM-2.

In order to further establish the identified compounds as MBL inhibitors, the concentrations resulting in 50% inhibition (IC_{50}) were determined for the six compounds reaching at least 50% inhibition at 40 μ M plus the most potent inhibitor of VIM-2, 24897966. The results are summarized in Table 3 and IC_{50} curves for the most potent inhibitors of each enzyme are provided in the Supplemental Information Figure S6. The results corroborate the percent inhibition data from Figure 2 with IC_{50} values of compounds identified as inhibitors below 40 μ M. As expected, compound 24897966 exhibited an IC_{50} value slightly above 40 μ M against VIM-2.

The IC_{50} values obtained in this study are more favorable than or comparable to those found in previous fragment-based screening studies. The most potent fragment identified as an IMP-1 inhibitor from a 500 molecule library had a K_i value of 410 μ M,⁸⁴ about 30 times higher than the IC_{50} of 6821770. In another fragment-based screening study using surface plasmon resonance, the most potent VIM-2 inhibitor exhibited an IC_{50} value of 14 μ M,⁸⁵ three times more potent than our most potent VIM-2 inhibitor 24897966 and identical to our most potent IMP-1 inhibitor 6821770.

Molecular Docking Study.

Curious about possible binding modes of these novel MBL inhibitors, molecular docking simulations using AutoDock 4.2⁵⁹ were carried out following unsuccessful attempts to obtain crystal structures of MBL/inhibitor complexes. The docking protocol followed previous reports.^{36, 46, 86} One hundred conformations were generated for 28 enzyme/inhibitor complexes (the four MBL crystal structures used for the initial screening and the seven compounds listed in Table 3), positioning the docking grid around the substrate-binding site, as the inhibitors were designed to be orthosteric. For each complex, the highest

ranked cluster was analyzed; in cases where the second- or third-ranked clusters were more populated than the first, those were evaluated as well. Plotting the IC₅₀ data for inhibitor binding to NDM-1 and IMP-1 *versus* the energies of the lowest-energy conformations (ranging from -9.9 to -7.7 kcal/mol) as well as the average energies of all conformations in those clusters (ranging from -9.3 to -6.8 kcal/mol) resulted in acceptable correlations (Figure 3).

Interestingly, the binding energies of the VIM-2 complexes were generally overestimated, ranging from -10.3 to -7.8 kcal/mol for the lowest-energy conformations and from -9.7 to -7.2 kcal/mol for the average energies. Thus, they were often more negative than those of the NDM-1 and IMP-1 complexes, even though none of the compounds were VIM-2 inhibitors but the majority of them NDM-1 and IMP-1 inhibitors according to our definition. One possible explanation is that when placed at the right location in the docking procedure, the compounds bind relatively tightly to the VIM-2 active site, but in reality, they might not be able to reach the active site efficiently, possibly due to the W87 side chain making the active site narrower. Interestingly, the more potent VIM-2 inhibitor identified by Christopheit *et al.*⁸⁵ is smaller and more planar than our compounds (in addition to having a carboxylate), which would be conducive for easier entry into a narrow binding site.

As an additional quality control, the co-crystallized inhibitors were re-docked into their respective enzymes. The conformations of the mercaptocarboxylate inhibitor and the biaryl succinate inhibitor docked into IMP-1 were superimposable with those observed in the crystal structures 1DD6 and 1JJT, respectively. Their binding energies were much more negative than those of the presently investigated inhibitors with -18.6 (-15.4) kcal/mol [lowest-energy conformation (average energy of top rank)] for the mercaptocarboxylate inhibitor and -16.1 (-14.3) kcal/mol for the biaryl succinate inhibitor, consistent with their very low experimentally observed IC₅₀ values of <90 nM⁶⁶ and 9 nM,⁶⁷ respectively, and the presence of thiolate and/or carboxylate Zn(II) ligands. L-captopril and the mercaptocarboxylate docked into NDM-1 and VIM-2 both had the thiolate at the expected position, bridging both Zn(II) ions, while the carboxylate interacted with K224/R228 instead of facing toward solvent as in the crystal structures. This may be attributed to the particular crystallization conditions or the treatment of solvent and particular charges used in the docking procedure. In summary, the binding energies observed, and the very realistic binding conformations of the query compounds provide some confidence in the validity of the docking procedure and results described in the following.

As the compounds are chemically diverse, so were the binding conformations. The coordinates and images of the 28 docking conformations that were selected for further analysis are provided in the Supporting Information. However, one general theme that was observed in potent inhibitors is a pair of electron-dense moieties 4–6 Å apart, one coordinating one or both Zn(II) ions and the other interacting with K224 in NDM-1 and IMP-1 and R228 or N233 in VIM-2. All groups that were found to interact with the Zn(II) ions and/or these residues are colored red in Table 1. We will discuss in more detail compound 3331076 as an example of an IMP-1-specific inhibitor, 6821770 as an example of an NDM-1- and IMP-1-specific inhibitor, and 24897966 as an example of a broad-spectrum inhibitor with moderate but comparable potency against all three enzymes.

Compound 3331076, an IMP-1-specific inhibitor.—The docked conformations to both IMP-1 crystal structures (1DD6 and 1JJT) showed the aromatic hydroxyl group acting as a bridging ligand between the Zn(II) ions, while the keto group interacted with K224 (Figure 4B and D). Thus, the hydroxyl mimicked the function of the thiolate in the mercaptocarboxylate inhibitor in 1DD6 and one of the carboxylates of the biaryl succinate inhibitor in 1JJT, while the keto group mimicked the (other) carboxylate (Figure 4A and C). It is also conceivable that the aromatic hydroxyl ($pK_a \sim 10$) would be deprotonated in the vicinity of two Zn(II) ions. The binding mode of this compound to NDM-1 was quite different with the ring ether oxygen bridging the two Zn(II) ions and the ester carbonyl oxygen interacting with K224 (Figure S20). In VIM-2, the compound interacted with Zn1 via its ketone carbonyl and with R228 via the ring amide carbonyl (Figure S23). These data suggest that interaction of the aromatic hydroxyl group with the Zn(II) ions and the keto group with K224 could result in efficient binding in IMP-1, while the other binding modes to NDM-1 and VIM-2 are less efficient.

Compound 6821770, an NDM-1- and IMP-1-specific inhibitor.—This compound is the most potent inhibitor identified in this study of both NDM-1 and IMP-1 with IC_{50} values of 19 ± 2 and 14 ± 1 μ M, respectively. When docked into NDM-1, the two sulfonamide oxygens of this compound coordinated the two Zn(II) ions, while the sulfonamide nitrogen hydrogen-bonded with D120 (Figure 5A). When docked into IMP-1 (1DD6), the interactions were almost identical, except that the sulfonamide oxygen was more distant from Zn1 (Figure 5B). Docked into the other IMP-1 structure (1JJT), the dibenzofuran oxygen bridged the Zn(II) ions (Figure 5C), indicating that there may be different binding modes in IMP-1. When docked into VIM-2, the sulfonamide of 6821770 interacted with R228, but there was no specific interaction with the Zn(II) ions (Figure S27), consistent with the fact that it is not a VIM-2 inhibitor. Interestingly, neither 6821770 nor the other inhibitor identified with the same query (23978304, Figures S28–S31) interacted with the Zn(II) ions *via* their tetrazoles, even though a biphenyl tetrazole has previously been reported as a potent inhibitor of the B1 MBL CcrA.³⁷ The fact that the previously reported compound was C-substituted, while the current compounds are N-substituted, might explain this discrepancy, as the C-substituted tetrazole may be deprotonated ($pK_a \sim 8$), especially in the vicinity of Zn(II) ions, making it an excellent Zn(II) ligand, while the N-substituted tetrazole is unlikely to be deprotonated ($pK_a \sim 27$).

Compound 24897966, a moderate broad-spectrum inhibitor.—Even though this compound was not as potent as some of the others, we selected it, because it shows the same percent inhibition of all three enzymes. Accordingly, we expected that docking poses of this compound to all three enzymes would be similar. In NDM-1, one of the sulfonamide oxygens bridged the two Zn(II) ions and the amide nitrogen interacted with K224 (Figure 6A). The same pose of 24897966 was observed in both crystal structures of IMP-1, 1DD6 (Figure 6B) and 1JJT (Figure 6C) with the small modification that the amide interacted with K224 *via* its carbonyl oxygen rather than the nitrogen. In VIM-2, a sulfonamide oxygen interacted with Zn1, but the amide did not interact with R228, which corresponds to K224 in NDM-1 and IMP-1. Instead, hydrogen bonds were formed with E149 and N150a (Figure 6D). In addition, the phthalimide formed hydrogen bonds with N233, a residue conserved in

most B1 MBLs. These interactions could be responsible for the relatively tight binding of this compound to VIM-2.

In summary, the docking study can help hypothesize on potential binding modes of the virtually screened inhibitors. Sulfonyl groups found in sulfonic acid,⁸⁷ sulfonyl hydrazones,⁸⁸ and sulfonamides^{89–90} seem to be interesting moieties. The sulfonyl oxygens can interact with the Zn(II) ions, while an appropriately spaced second electron-dense group can interact with K224 in NDM-1 and IMP-1 (Figure 5A and B, Figure 6A–C), but also other residues, as seen in VIM-2 (Figure 6D). Replacement of a Zn(II)-bridging thiolate, which is observed in numerous MBL inhibitors, with another bridging moiety (such as the aromatic hydroxyl of 3331076 and the sulfonyl oxygens of 6821770 and 24897966) has been proposed to be a “significant advance in the design of potent MBL inhibitors”.⁹¹ Aromatic groups on the termini of the molecules also seem to be favorable, as they nicely fill the MBLs’ substrate binding cleft formed by the hydrophobic L3 loop.⁶⁷ We cannot exclude the possibility that some of the compounds containing esters or amides were hydrolyzed by the enzymes and the products (e.g., carboxylates) caused inhibition. However, as these groups were not activated by being in a strained ring, like the amide group in a β -lactam ring, we consider this possibility to be rather unlikely.

Structure-Activity-Relationship of 11990632 and 11993658 and Derivatives.

We observed that 11993658, which is identical to 11990632 except that a fluorine atom in *para* position on a phenyl ring is replaced by a chlorine atom (see Figure S1), resulted in significantly higher % inhibition of all three enzymes (about 2-fold for NDM-1 and VIM-2 and 4-fold for IMP-1). Therefore, we rationalized that removing the electron-withdrawing group (chlorine is less electronegative than fluorine) or replacing it with an electron-donating group might improve the potency of the inhibitor. Thus, we also tested the compounds without a substituent (eMolecules ID 1361183), a methyl (179441547), and a methoxy group (179441565) in *para* position of the phenyl ring. In docking calculations, the poses of these molecules were indistinguishable (In NDM-1 and VIM-2, the phthalimide coordinating the Zn(II) ions and the sulfonamide interacting with K224 and R228, respectively; in IMP-1, the sulfonamide coordinating the Zn(II) ions and the phthalimide interacting with K224). In experiments, none of these molecules were inhibitors of the three enzymes (no more than 16% inhibition at 40 μ M). While disappointing, these results suggest that *Avalanche* was effective in selecting the most potent compounds from the available library.

Minimum Inhibitory Concentration (MIC) Assays.

The ability of the identified MBL inhibitors to restore the antibacterial activity of a clinically significant β -lactam antibiotic, ceftazidime, was assessed by performing MIC assays with *Escherichia coli* DH10B cells expressing the three MBLs as well as the appropriate controls not expressing any MBLs. Unfortunately, at inhibitor concentrations of 160 μ M (50–70 μ g/ml) no difference could be detected in ceftazidime MIC *versus* no inhibitor with cells expressing the MBLs. Specifically, the MICs of ceftazidime were 256, 512, and 32 μ g/ml for cells expressing NDM-1, IMP-1, and VIM-2, respectively, with or without the inhibitors present, while MICs with cells not expressing any MBL were 0.5 μ g/ml. The lower than

expected MICs for cells expressing NDM-1 and VIM-2 relative to IMP-1 based on their kinetic constants (Tables S4 and S5,⁵⁵) may be due to relatively low expression levels and thermal stability of NDM-1 and VIM-2 (Table 2) as well as lipidation in the case of NDM-1 and lower Zn(II) occupancy in the case of VIM-2 (Table 2).

The observation that none of the inhibitors could inactivate MBLs inside *E. coli* cells led us to assume that either the compounds could not penetrate into the bacterial periplasm or were efficiently exported, or that their affinity was not high enough, although this concentration is at least four-fold that of the IC₅₀ values of the investigated compounds. Also, at such high inhibitor concentrations some issues with solubility were observed when the inhibitor solutions were mixed with the growth medium. All these issues would have to be examined in more detail in the quest for more potent derivatives of these compounds.

CONCLUSIONS

We have demonstrated here the utility of *Avalanche*, which represents a further development of the *Shape Signatures* algorithm.⁶⁴ The underlying idea is to select compounds based on shape and surface complementarity with known inhibitors rather than building inhibitors from pharmacophores that are known to tightly bind to Zn(II) ions. This approach was chosen to avoid off-target interactions with other metalloenzymes and also opens up the search space to novel scaffolds that fit these criteria. The validation of one to two out of eight tested compounds per query as actual inhibitors presents an acceptable success rate and can significantly narrow down the number of molecules that would typically be tested in an uninformed high throughput screen. While the compounds identified were not as potent as previously described MBL inhibitors and apparently could not penetrate the bacterial periplasm sufficiently, the identification of novel scaffolds can mean an important step in the development of clinically useful inhibitors. We identified both enzyme-specific inhibitors (2994990 and 3331076) and inhibitors with a broad spectrum (24897966, 6821770, and 23978304). Probably more promising for a clinical application, the latter could serve as a starting point for improving potency and penetration into the bacterial periplasm. Specific moieties could be modified rationally to optimize the fit of these molecules into the MBL active sites, while penetration could perhaps be addressed through linkage to a siderophore.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of catalytic efficiencies obtained in the present study to those of previous studies. (A) Data for NDM-1 from the present (white bars) and a previous study (black bars).⁷⁵ (B) Data for VIM-2 from the present (white bars) and a previous study (black bars).⁵²

The data of the current study (white bars) are means $\pm$ standard deviations of triplicate experiments. PEN, benzylpenicillin; AMP, ampicillin; NIT, nitrocefin; CEF, cephalothin; FOX, ceftazidime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; MEM, meropenem; DOR, doripenem.

Figure 2. Percent inhibition data with virtually screened compounds (indicated by their eMolecules IDs on the x axes) at 40 μ M against all three MBLs (320 pM) using nitrocefin (10 μ M) as substrate. The headings in panels A–D indicate how the compounds were obtained, e.g., the compounds in panel A were obtained using L-captopril from an NDM-1/L-captopril complex crystal structure (PDB code 4EXS) as the query. Percent inhibition of the three different MBLs are indicated by the differently colored columns: red, NDM-1; blue, IMP-1; green, VIM-2. The experiments were carried out in triplicate and data indicate means $\pm$ standard deviations.

Figure 3.

Plot of the experimental IC_{50} values obtained with NDM-1 and IMP-1 *versus* the docking energies of the lowest-energy conformations (red) and the average energy of all conformations (black) of a given cluster. R^2 for the logarithmic correlation of the lowest-energy data is 0.61 and that of the average energies is 0.59. The values for the VIM-2 complexes did not correlate with the other data and are not included.

Figure 4. Graphical representation of inhibitors (query compounds) bound to IMP-1 (crystal structures), (A) and (C), and lowest-energy docking conformations of compound 3331076, (B) and (D). The protein backbone (PDB code 1DD6 in (A) and (B) and 1JJT in (C) and (D)) are represented as a cartoon in cyan with the K224 side chain shown as licorice (C, cyan; N, blue). Zn(II) ions are shown as gray spheres. The query and screened compounds are shown as licorice (H, white; C, gray; N, blue; O, red; S, yellow) and distances indicating characteristic interactions between them and the proteins are shown as dashed lines with distances in Å. L3, mobile active-site loop; MCI, mercaptocarboxylate inhibitor; BSI, biaryl succinate inhibitor.

Figure 5. Compound 6821770 docked into NDM-1 (PDB code 4EXS) (**A**) and IMP-1 (1DD6 (**B**) and 1JJT (**C**)). Rendering of protein, zincs, and compound is as described for Figure 3. For clarity, (**A**) shows a view looking into the active site of NDM-1 from above, while (**B**) and (**C**) show the active site of IMP-1 from a side view, because the active site is occluded by the L3 mobile loop.

Figure 6.

Compound 24897966, a moderately potent broad-spectrum inhibitor docked into NDM-1 (PDB code 4EXS, (A)), IMP-1 (1DD6, (B); 1JJT (C)), and VIM-2 (2YZ3 (D)). Rendering of protein, zincs, and compound is as described for Figures 3 and 4. The docking poses in NDM-1 and IMP-1 are comparable, while that in VIM-2 is rotated by $\sim 180^\circ$.

Table 1.

Structures of query compounds and virtually screened MBL inhibitors exhibiting >50% inhibition of MBL activity at 40 μ M.

PDB code, Ref.	MBL	Query inhibitor structure/name	Screened inhibitor structures/eMolecules IDs
4EXS, ⁶⁵	NDM-1	L-Captopril
1DD6, ⁶⁶	IMP-1	Mercaptocarboxylate
1JJT, ⁶⁷	IMP-1	Biaryl succinate
2YZ3, ⁵¹	VIM-2	Mercaptocarboxylate

^aCompound 24897966 was included, even though it did not reach the 50% inhibition threshold, because it was the most potent inhibitor of VIM-2. Atoms/groups in red were involved in Zn(II) coordination and/or interaction with enzyme residues.

Table 2.

Biophysical characterization of the purified enzymes.

Enzyme	Yield (mg/liter)	Molecular mass from ESI-MS (calculated)	N-terminal amino acid sequence ^a	No. of Zn(II) ions/enzyme molecule ^b	T _m (°C)
NDM-1	2.05	25,607 (25,606)	G ₂₉₍₂₃₎ EIRP...	2.45 ± 0.04	61
IMP-1	14.8	25,112 ^c (25,113)	A ₁₉₍₃₇₎ ESLP...	1.8 ± 0.1 ^b	70
VIM-2	4.6	26,034 ^d (26,031)	P ₂₂₍₂₁₎ LAFS...	0.9 ± 0.1	63

^aN-terminal numbering is as in the PDB file and according to standard numbering in brackets (see Ref.¹⁴ and Supporting Information).^bData represents average of three measurements ± standard deviations.^cData for IMP-1 are from reference.⁷²^dOnly major species representing ~95% shown.

Table 3.

IC₅₀ values (in μM) of the most potent inhibitors against their respective MBL targets.

Compound ID (Enzyme) ^a	NDM-1	IMP-1	VIM-2
2994990 (NDM-1) ^a	31 $\pm$ 7	-	-
11993658 (NDM-1) ^a	33 $\pm$ 12	29 $\pm$ 4	-
3484004 (IMP-1) ^a	21 $\pm$ 4	16 $\pm$ 1	-
3331076 (IMP-1) ^a	-	39 $\pm$ 3	-
6821770 (VIM-2) ^a	19 $\pm$ 2	14 $\pm$ 1	-
23978304 (VIM-2) ^a	19 $\pm$ 4	15 $\pm$ 1	-
24897966 (IMP-1) ^a	-	-	50 $\pm$ 20

^a Enzyme that was co-crystallized with its query.

- Not determined (less than 50% inhibition at 40 μM compound).