Comparison of Verona Integron-Borne Metallo-β-Lactamase (VIM) Variants Reveals Differences in Stability and Inhibition Profiles

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Metallo-β-lactamases (MBLs) are of increasing clinical significance; the development of clinically useful MBL inhibitors is challenged by the rapid evolution of variant MBLs. The Verona integron-borne metallo-β-lactamase (VIM) enzymes are among the most widely distributed MBLs, with >40 VIM variants having been reported. We report on the crystallographic analysis of VIM-5 and comparison of biochemical and biophysical properties of VIM-1, VIM-2, VIM-4, VIM-5, and VIM-38. Recombinant VIM variants were produced and purified, and their secondary structure and thermal stabilities were investigated by circular dichroism analyses. Steady-state kinetic analyses with a representative panel of β-lactam substrates were carried out to compare the catalytic efficiencies of the VIM variants. Furthermore, a set of metalloenzyme inhibitors were screened to compare their effects on the different VIM variants. The results reveal only small variations in the kinetic parameters of the VIM variants but substantial differences in their thermal stabilities and inhibition profiles. Overall, these results support the proposal that protein stability may be a factor in MBL evolution and highlight the importance of screening MBL variants during inhibitor development programs.
key (27). This conservative substitution at the C terminus (second-to-last residue) is present in ~50% of VIMs. The greatest variability in amino acid sequence across the five VIM variants is found in the N-terminal leader sequence and, to a lesser extent, in the C-terminal residues (see Fig. S1 in the supplemental material). VIM-2, one of the most widely reported MBLs, shares ~90% amino acid similarity with the VIM-1 cluster (28). VIM-5 and VIM-38 belong to the VIM-1 cluster and differ from VIM-1 by 5 and 6 residues, respectively (Ala130Lys, His224Leu, Glu225Ala, Ser228Arg, and Lys291Thr, with an additional Ala316Val substitution for VIM-38). Both VIM-5 and VIM-38 contain His224Leu and Ser228Arg substitutions relative to VIM-1; these residues are positioned on the L10 loop and are proposed to influence the substrate specificity of VIM variants (13, 29–32).

Unlike the serine-β-lactamases, as yet, there are no clinically useful MBL inhibitors. The development of broad-spectrum MBL inhibitors is challenging, in part because of structural variation across (and even within) subclasses but also because of the requirement for selective inhibition of bacterial MBLs over human MBL-fold enzymes, some of which have important physiological roles. The continued emergence of new MBL variants with altered substrate selectivity presents a further challenge to inhibitor development. Despite the increased number of reports of VIM variants, structural information is available for only five of these variants (VIM-2, VIM-4, VIM-7, VIM-26, and VIM-38), and biochemical characterization has been carried out for <10 VIM variants (15, 19, 30, 32, 33). Biochemical studies have reported that VIM-5 manifests a level of carbapenemase activity similar to those of VIM-1 and VIM-2 but with greater efficiency toward imipenem than meropenem (26). However, as exemplified by studies with NDM variants (34), it is desirable to compare the activities of MBL variants under the same experimental conditions. To date, the important question of whether all the clinically relevant MBL variants are similarly inhibited has not been addressed.

Here, we report studies on the biochemical and biophysical properties of VIM-4, VIM-5, and VIM-38, in comparison with those of the VIM-1 and VIM-2 enzymes, carried out under the same experimental conditions. We observe modest differences in the kinetic parameters for β-lactamase activities against a variety of β-lactam substrates; however, the results reveal clear differences in the thermal stabilities of the VIM variants, as recently reported for NDM variants (34). Interestingly, the tested variants show clear differences in their inhibition profiles, with one isoquinoline derivative selectively inhibiting VIM-5 and VIM-38 more potently than VIM-1, VIM-2, or VIM-4. Crystallographic studies on VIM-5 reveal structural differences that rationalize the observed differences in inhibition potency.

MATERIALS AND METHODS

Cloning and mutagenesis. DNA encoding full-length VIM-38, lacking its N-terminal periplasmic signaling sequence, was cloned into the pET-28a vector (Novagen) for the production of recombinant protein with an N-terminal His6 tag (pET28a- blaVIM-38). Site-directed mutagenesis (Ala316Val) was performed to generate VIM-5 using pET28a- blaVIM-38 as a template. Insertion PCR was carried out to incorporate a cleavage site for human rhinovirus 3C (HRV3C) protease into the N terminus of the VIM-5 and VIM-38 sequences. Primers used for mutagenesis are listed in Table S1 in the supplemental material.

For the production of N-terminally Hisx6-tagged VIM-1, Escherichia coli codon-optimized VIM-1 coding sequences were inserted into pNIC28-Bsa4 by using standard procedures (35).

Protein production and purification. E. coli BL21(DE3) cells were transformed with plasmids encoding VIM-1, VIM-5, and VIM-38 for protein production. The cells were cultured in 2× TY medium supplemented with kanamycin (50 μg/ml), until mid-log phase (optical density at 600 nm [OD600] of ~0.7) was reached. The production of the recombinant proteins was then induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were cultured for a further 16 h at 18°C. Cells were harvested by centrifugation (7,000 × g for 10 min) and lysed by sonication. Three-step protein purification was carried out using nickel ion affinity chromatography followed by size exclusion chromatography, as previously reported (34). The purified VIM-5 and VIM-38 proteins were incubated overnight at 4°C with His-tagged HRV3C protease or with His-tagged tobacco etch virus (TEV) for VIM-1, to remove the N-terminal His6 tag, and further purified by using a second nickel ion affinity column to obtain the untagged enzymes. VIM-2, VIM-4, and C-terminally Hisx6-tagged VIM-1 were produced and purified as previously described (19, 36, 37). The purity of the proteins was ascertained by SDS-PAGE; mass spectrometric analysis under both denaturing and nondenaturing conditions was used to verify the masses and metal contents of the purified VIM-5 and VIM-38 enzymes (see Fig. S2 and S3 in the supplemental material).

Analysis of secondary structure content and melting temperature. Circular dichroism (CD) analyses were carried out using a Chirascan CD spectrophotometer (Applied Photophysics) equipped with a Peltier temperature-controlled cell holder. CD measurements were collected in the range of 185 to 260 nm; spectra were baseline corrected and smoothed using a Savitzky-Golay filter. Data were normalized at 207 nm to account for differences in protein concentrations (38), and the estimation of secondary structure content was performed with DichroWeb (39) using the CONTIN Analysis Programme (reference set 6) (40). Melting temperatures ($T_m$) of the recombinant enzymes were determined by monitoring temperature-induced changes in the CD signal at 222 nm. The temperature was increased by 1°C per min, and the CD signal was recorded at temperatures ranging from 25°C to 92°C. The samples were then cooled.

FIG 1 Outline scheme for MBL-mediated β-lactam hydrolysis.
from 92°C to 25°C at the same rate, and a CD spectrum of the refolded proteins was determined. The activity of the enzymes was recorded before thermal denaturation and after refolding by monitoring nitrocefin hydrolysis. The thermal denaturation data were fitted to a Boltzmann sigmoidal curve by using GraphPad Prism 5.02 software. CD spectra and thermal denaturation curves are shown in Fig. 2 and in Tables S2 and S3 in the supplemental material.

Determination of kinetic parameters. The hydrolysis of a representative panel of β-lactam substrates was monitored at their respective absorbance wavelengths. The wavelengths and extinction coefficients used were previously described (34). The assays were carried out using 50 mM HEPES buffer (pH 7.2) supplemented with 1 μg/ml bovine serum albumin (BSA), 1 μM ZnSO₄, and 0.01% Triton X-100. In the case of VIM-1, C-terminally His₆-tagged VIM-1 was used for kinetic analyses. Note that no differences were observed between the activities of untagged VIM-1 and C-terminally His₆-tagged VIM-1 for the selected antibiotics (data not shown). The initial rates were obtained from the changes in absorbance at various substrate concentrations. Steady-state kinetic parameters (K_m, V_max) were determined by fitting the initial velocity data to the Michaelis-Menten equation using GraphPad Prism 5.01 software (see Table S4 in the supplemental material).

Inhibition assays. Inhibitors were prepared as reported previously (36, 41). Enzyme-mediated hydrolysis of nitrocefin was monitored by determining the changes in absorbance at 495 nm, as previously described (36, 37). Residual activities were first determined at a 100 μM inhibitor concentration. For the determination of 50% inhibitory concentrations ([I]₅₀), the reporter substrate (nitrocefin) was used at near-K_m values, and the enzyme was preincubated with the inhibitors for 10 min at room temperature prior to the addition of the substrate (42). The residual activities were obtained at increasing inhibitor concentrations (0.2 to 2,000 μM); the data were analyzed using GraphPad Prism 5.01 software.

NMR binding assays. Binding assays were carried out using ¹H-edited Carr-Purcell-Meiboom-Gill (CPMG) nuclear magnetic resonance (NMR) analyses. Spectra were recorded by using a Bruker AVIII 600-MHz NMR spectrometer equipped with a BB-F/1H Prodigy N2 cryoprobe using 3-mm Match NMR tubes (Cortextnet). The PROJECT-CPMG sequence (90°-x-[τ-180°-y-τ-90°-τ-180°-y-τ]ₙ-[n-acq]) was applied, and water suppression was achieved by presaturation. Data were collected with a sweep width of 12,019 Hz, an acquisition time of 2.7 s, and a filter width of 125,000 s. Assays were conducted using 50 mM Tris-D11 (pH 7.5) supplemented with 0.02% NaN₃ in 90% H₂O and 10% D₂O. Equilibrium dissociation constant (K_D) measurements for the reporter ligand (23 mg/ml) and the reservoir buffer at a 2:1 ratio (protein/reservoir). Crystals formed in wells containing 0.1 M Tris buffer (pH 8.5), 25% polyethylene glycol 3350 (PEG 3350), and 0.2 M NaCl. A cryoprotectant solution was prepared by diluting the well solution with glycerol to a final concentration of 25% (vol/vol) glycerol. An ~10% drop volume of cryoprotectant was then added to the top of a drop containing crystals, and the crystals were harvested using a nylon loop, followed by plunging into liquid nitrogen. Data were then collected for a single crystal at the Diamond Light Source synchrotron beamline. Data were processed by using XDS and CCP4-SCALA in XIA2 (44–46). Initial phases were obtained by molecular replacement (MR) (47) using the PHASER (48) subroutine within PHENIX (49, 50), with the structure of VIM-4 (Protein Data Bank [PDB] accession number 2WRS) (51) as the search model. Crystallographic structure refinement was carried out by iterative rounds of model building using WinCoot (52) and maximum likelihood restrained refinement using PHENIX. Data collection and refinement statistics are given in Table S6 in the supplemental material.

Protein structure accession number. Coordinates and structure factors for VIM-5 have been deposited in the PDB under accession number 5A87.

RESULTS

Recombinant VIM-1, VIM-2, VIM-4, VIM-5, and VIM-38 were efficiently produced and purified to near homogeneity as determined by SDS-PAGE analysis (see Fig. S2 in the supplemental material). The molecular masses of the recombinant proteins as determined by liquid chromatography-mass spectrometry (LC-MS) analyses were in close agreement with the theoretical values. Data from nonnondenaturing electrospray ionization mass spectrometric analyses validated the identities of the VIM-5 and VIM-38 variants and were consistent with the binding of two metal (zinc) ions for each of the purified proteins (see Fig. S3 in the supplemental material).

Biophysical characterization. To compare the secondary structural content of the VIM variants, far-UV circular dichroism (CD) analyses were carried out on the untagged recombinant proteins. All five VIM variants exhibited very similar CD spectra, with a slight deviation in ellipticity at lower wavelengths, indicating that the amino acid substitutions do not cause major perturbations in secondary structure (Fig. 2a).

We then carried out temperature-dependent CD spectroscopy to investigate the relative thermal stability of the VIM variants, because some substitutions in VIM and NDM MBLs have been associated with variations in protein stability (28, 34). With the exception of VIM-1 and VIM-4, the CD spectra of the cooled samples were nearly identical to those obtained before heating.
For all the tested VIM variants, the refolded samples retained substantial activity (<2-fold differences as judged by \( k_{\text{cat}}/K_m \) values), indicating that proteins regained their native structure upon refolding (see Table S3 in the supplemental material). The observed reversibility allowed further thermodynamic analysis to obtain melting temperature (\( T_m \)) values. Significant differences in the \( T_m \) values of the five VIM variants were observed. VIM-5 and VIM-38 were the most stable variants, with an apparent \( T_m \) of \( >83^\circ \text{C} \), which is \( \sim 5^\circ \text{C} \) higher than that determined for VIM-2 (78°C). VIM-1 and VIM-4 were the least stable of the tested variants, with apparent \( T_m \) values of 60°C and 64°C, respectively, which are \( >20^\circ \text{C} \) lower than those of VIM-5 and VIM-38 and \( >14^\circ \text{C} \) lower than that of VIM-2 (Fig. 2b).

**Functional properties of VIM variants.** The hydrolytic activities of the VIM variants against a representative set of \( \beta \)-lactam substrates were then compared under similar assay conditions. Overall, the tested variants showed relatively small differences in their catalytic efficiencies toward the tested \( \beta \)-lactam substrates, with VIM-5 and VIM-38 showing very similar kinetic parameters (Table 1). In some cases, differences in individual kinetic parameters were observed, sometimes contributing to changes (albeit relatively small ones) in the overall hydrolytic efficiency as defined by \( k_{\text{cat}}/K_m \) values. For instance, for VIM-1, the \( K_m \) value for meropenem was 6-fold higher than that for VIM-2, indicating a lower affinity for this substrate. This difference was, however, counterbalanced by high \( k_{\text{cat}} \) values for VIM-1 (\( >7 \)-fold higher than those for VIM-2), resulting in similar \( k_{\text{cat}}/K_m \) values. Despite VIM-1 and VIM-2 variants having similar \( K_m \) values for imipenem (likely within experimental error), VIM-2 exhibited higher \( k_{\text{cat}} \) values, resulting in a 6-fold increase in \( k_{\text{cat}}/K_m \) values compared to that for VIM-1. Relatively high apparent \( K_m \) values (\( >300 \mu \text{M} \)) were recorded for all VIM variants with ampicillin, indicating a relatively low affinity for this penicillin. Notably, VIM-5 and VIM-38 had \( >10 \)-fold-higher \( k_{\text{cat}} \) values for cefoxitin than did VIM-1, resulting in a \( >7 \)-fold increase in \( k_{\text{cat}}/K_m \) values. Cefazidime and nitrocefin were efficiently hydrolyzed by all the tested VIM variants, recording the highest \( k_{\text{cat}}/K_m \) values compared to those with the other \( \beta \)-lactam substrates. Cefazidime was a poor substrate for the variants, with \( k_{\text{cat}}/K_m \) values being \( <0.1 \text{s}^{-1}/\mu \text{M} \) for the tested VIM variants. Apparent kinetic parameters were reported for cefazidime and ampicillin hydrolysis, since the initial velocity remained proportional to the substrate concentration up to 300 \( \mu \text{M} \) (see Table S4 in the supplemental material).

**Inhibition of VIM variants.** To investigate if the VIM variants might manifest different degrees of inhibition, we screened a set of compounds containing different potential metal-chelating motifs (36, 41), including isoquinolines and pyridine-2-carboxylates, to compare their inhibitory effects on the VIM variants. An interesting result to emerge from this work was that the isoquinoline derivative (compound 1) is a substantially better inhibitor of VIM-5 and VIM-38 (IC\( _{50} \) = 2 μM) by \( >500 \)-fold than VIM-1 (IC\( _{50} \) of \( >1 \text{mM} \)). VIM-2 and VIM-4 also had \( >40 \)-fold-higher IC\( _{50} \) (IC\( _{50} \) of 90 μM and 200 μM, respectively) with inhibitor 1 than did VIM-5 and VIM-38 (Table 2). The configuration of the stereocenter of the inhibitor side chain is important with respect to the degree of inhibition, with the (\( R \))-enantiomer (compound 1) being a more potent inhibitor than the (\( S \))-enantiomer (compound 2) for all the variants, except for VIM-1, for which the (\( S \))-enantiomer (compound 2) was a more potent inhibitor. The isoquinoline ring system is also apparently important, as the pyridine-2-carboxylate derivative (compound 3) with an (\( R \))-tryptophan side chain analogous to that of compound 1 inhibited all VIM variants similarly and with only modest potency (Fig. 3). Interestingly, VIM-4 showed IC\( _{50} \) similar to those of VIM-2 for the tested compounds despite differing from VIM-1 by only one residue (Ser228 in VIM-1 and Arg228 in VIM-4).

To validate the inhibition results, we carried out \(^1\text{H} \) CPMG NMR binding assays (43). The NMR analyses showed a similar trend, with both isoquinoline inhibitors 1 and 2 binding more strongly to VIM-5 than to VIM-2 (see Table S5 in the supplemental material). The (\( R \))-enantiomer (compound 1) was also shown to be a stronger binder to VIM-2 (apparent \( K_D \) \([K_D \text{ app}] = 180 \mu \text{M} \)) than the (\( S \))-enantiomer (compound 2) \([K_D \text{ app}] = 330 \mu \text{M} \)), highlighting the importance of the configuration of the side-chain stereocenter in the potency of inhibition.

**Crystalllographic analysis.** To explore the structural features contributing to the observed differences in inhibition of the VIM variants, a crystal structure of recombinant VIM-5 was determined to a 1.5-Å resolution (P2\(_1\) space group), having 2 molecules per asymmetric unit. The overall fold of VIM-5 was almost identical to that described for the previously reported VIM-2 structure (PDB accession number 4BZ3), with a root mean square distance (RMSD) of 0.206 Å. The overall fold of VIM-5 has the canonical MBL \( \beta \)-sandwich structure, with two zinc(II) ions (3.5 Å apart) being bound in the active site located in a shallow cleft formed by the interface of the two \( \beta \)-sheets (Fig. 4A). The proposed nucleophilic hydroxide/water molecule bridges between the two zinc(II) ions and is positioned 1.9 Å and 2.0 Å from Zn1 and Zn2, respectively. The zinc(II) ion coordination geometry is similar to the coordination observed for VIM-2 (Fig. 4B) (53).

### Table 1: Kinetic parameters for VIM-1, VIM-2, VIM-5, and VIM-38 with a representative panel of \( \beta \)-lactam substrates

| Substrate | Mean \( K_m \) (μM) ± SD \( ^a \) | \( k_{\text{cat}} \) (s\(^{-1} \)) | \( k_{\text{cat}}/K_m \) (s\(^{-1} \)μM) |
|-----------|---------------------------------|-----------------|---------------------------------|
| VIM-1     | 130 ± 20                        | 70 ± 10         | 30 ± 4                          |
| VIM-2     | 60 ± 10                         | 60 ± 6          | 150 ± 20                        |
| VIM-5     | >1,000                          | >1,000          | 380 ± 80                        |
| VIM-38    | 170 ± 50                        | 320 ± 60        | 230 ± 60                        |
| VIM-1     | 180 ± 60                        | 340 ± 80        | 430 ± 140                       |
| VIM-2     | 140 ± 10                        | 100 ± 8         | 60 ± 8                          |
| VIM-5     | 15 ± 1                          | 50 ± 5          | 70 ± 10                         |
| VIM-38    | Meropenem                       | 50              | 7                               |
|           | 20                              | 20              | 10                              |
|           | 40                              | 40              | 40                              |
|           | 60                              | 60              | 60                              |
|           | 80                              | 80              | 80                              |
|           | 100                             | 100             | 100                             |
|           | 120                             | 120             | 120                             |
|           | 180                             | 180             | 180                             |

\( ^a \) \( K_m \) values are reported as the means of data from three independent measurements ± standard deviations. Apparent kinetic parameters are reported for ampicillin and ceftazidime hydrolysis. Standard deviation values for \( k_{\text{cat}} \) did not exceed 10%.

\( ^b \) C-terminally His\(_6\)-tagged VIM-1 was used for kinetic analyses (36).
Despite having identical sequences, the VIM-5 L3 loop was closer to the active site than observed for VIM-2, as was reported previously for VIM-4 and VIM-31 (19, 32). It is also notable that a 4.8-Å shift in the carbonyl oxygen position of Ala231 was observed in comparison to the VIM-2 structure (PDB accession number 4BZ3) (see Fig. S4 in the supplemental material).

DISCUSSION

Overall, the kinetic results reveal relatively small differences in the catalytic efficiencies of the tested VIM variants (Table 1), the biological relevance of which, if any, requires further investigation. VIM-5 was found to have activity toward carbapenems similar to the active site than observed for VIM-2, as was reported previously for VIM-4 and VIM-31 (19, 32). It is also notable that a 4.8-Å shift in the carbonyl oxygen position of Ala231 was observed in comparison to the VIM-2 structure (PDB accession number 4BZ3) (see Fig. S4 in the supplemental material).

The VIM variants tested in this study exhibited similar CD spectra, implying that their secondary structures are not substantially affected by the differences in their sequences (Fig. 2a).

| Inhibitor | IC<sub>50</sub> (μM) (K<sub>D,app</sub> [μM])<sup>a</sup> |
|-----------|-----------------------------|---|---|---|---|
| 1 | >1,000 | 90 (180) | 200 | 2 (140) | 2 |
| 2 | 60 | 300 (330) | 600 | 50 (180) | 80 |
| 3 | 50 | 100 | 125 | 50 | 50 |

<sup>a</sup> IC<sub>50</sub> determinations were performed in triplicate over a range of inhibitor concentrations from 0.2 to 2,000 μM. K<sub>D,app</sub> values were determined by 1H CPMG NMR experiments (see Table S5 in the supplemental material).

The hydrolytic efficiencies presented here are in agreement with those reported previously for some of the substrates (e.g., VIM-2 hydrolyzes imipenem better than meropenem) (21) but differ with regard to other substrates; e.g., in our study, VIM-5 hydrolyzes ceftazidime with a 10-fold-higher efficiency than that previously reported (26). The discrepancies observed may be attributed to differences in enzyme preparation and assay conditions, emphasizing the need to carry out comparative kinetic studies under similar experimental conditions (see Table S7 in the supplemental material for a comparison of our data with data from previous studies).

The VIM variants tested in this study exhibited similar CD spectra, implying that their secondary structures are not substantially affected by the differences in their sequences (Fig. 2a).

**FIG 3** Inhibition of VIM variants by selected inhibitors. RA, residual activity.
ever, marked differences in the thermal stabilities of the variants were observed, with VIM-5 and VIM-38 having the highest melting temperatures (84°C and 83°C, respectively) compared to VIM-2 (78°C). VIM-1 (60°C) and VIM-4 (64°C) showed significantly lower melting temperatures (Fig. 2b). These results are in agreement with previously reported low melting temperatures for VIM-4 (19). The results showing increased thermal stability of at least some VIM variants is interesting, as we have observed similar trends with NDM variants (34). As proposed in the case of the NDM variants, it is possible that the increased thermal stabilities of emergent MBL variants could reflect increased cellular lifetimes, directly due to the improved thermal stability or a reduced propensity for proteolysis or aggregation (34). Thus, although “evolutionary drift” cannot be ruled out and further work is required, the increased thermal stability of some MBL variants presents an interesting line of investigation.

Unlike the relatively small differences in kinetic parameters observed, very clear differences were observed for the inhibition of different VIM variants with some (but not all) types of inhibitors tested (Fig. 3). These differences are most clearly exemplified by the relatively low IC_{50} reported for VIM-5 and VIM-38 with isooquinoline compound 1 compared to those for VIM-1, VIM-2, and VIM-4 (Table 2). A crystal structure of VIM-5 reveals interesting active-site differences compared to VIM-2 and provides some possible insights into the differences in the catalytic and inhibitory profiles of the VIM variants (Fig. 4).

The substitutions at residue 224 (His, Tyr, and Leu in VIM-1/VIM-4, VIM-2, and VIM-5/VIM-38, respectively), which is located on the L10 loop, likely contribute to the differential inhibition profiles observed. Comparison of the crystal structures of the VIM variants reveals that replacement of Tyr224, as in VIM-2, with Leu224, as in VIM-5 and VIM-38, likely provides a more spacious active site that may better accommodate the bulky tryptophan side chains of the tested inhibitors, as reflected by the increased inhibition of VIM-5 and VIM-38 by inhibitors 1 and 2. The smaller side chain of Leu224 in VIM-5 also enables a “flipping” of the main-chain carbonyl of Ala231, altering the entrance to the active site and likely affecting inhibitor binding. Notably, the main-chain flipping of Ala231 is also observed for other enzymes with Leu224, such as VIM-26 (30) (see Fig. S4 in the supplemental material).

The bicyclic isooquinoline ring system of inhibitors 1 and 2 may
interact with the hydrophobic residues on the mobile L3 loop (residues 60 to 66) (15), leading to better binding than with pyridine-2-carboxylate inhibitor 3 for VIM-5 and VIM-38. It is proposed that VIM-2 residue Glu225 forms electrostatic interactions that rigidify and partially neutralize the side chain of Arg228, which interacts with the carboxylate of β-lactam substrates (15, 19). The replacement of Glu225 with Ala225, as in VIM-5 and VIM-38, may enable more flexibility of the Arg228 side chain, contributing to the observed differences in substrate/inhibitor binding. Additionally, the hydrogen bond formed between the Glu225 side chain and the main chain of Leu265 is disrupted in the VIM-5 structure with the replacement of Glu225 with Ala225. This may also have implications for substrate-inhibitor interactions.

The overall results reveal that the tested VIM variants show relatively small differences in their catalytic efficiencies with the tested substrates, suggesting that changes in substrate selectivity are not their sole evolutionary driving force, as was recently proposed for NDM variants (34). We observed a marked difference in thermal stability for the VIM variants, as observed for NDM variants, suggesting that this may reflect a selection pressure (34). Perhaps most importantly, the results reveal that clinically observed MBL variants can manifest different inhibition profiles, a factor that we propose should be taken into account at an early stage in inhibitor development programs.

ACKNOWLEDGMENTS

This work was supported by the Rhodes Trust (United Kingdom), the Scientific and Technological Research Council of Turkey (TUBITAK), Recep Tayyip Erdogan University Research Fund grant BAP-2013.102.03.13, the Biochemical Society Krebs Memorial Award, Medical Research Council (MRC) grant MR/L007665/1, and MRC/Canadian grant G1100135.

FUNDING INFORMATION

The Rhodes Trust provided funding to Anne Makena. Scientific and Technology Council of Turkey provided funding to Cemal Sandalli. Recep Tayyip Erdogan University Research Fund provided funding to Aysegul Saral, Asyguel C. Cicek, and Cemal Sandalli under grant number BAP-2013.102.03.13. Medical Research Council provided funding to Jurgen Brem, Michael A. McDonough, Anna M. Rydzik, and Christopher J. Schofield under grant number MR/L007665/1. Medical Research Council/Canadian Grant provided funding to Jurgen Brem, Michael A. McDonough, Anna M. Rydzik, and Christopher J. Schofield under grant number G1100135. Biochemical Society Krebs Memorial Award provided funding to Martine I. Abboud.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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