Published in final edited form as:

Bioorg Med Chem. 2013 June 1; 21(11): 3138–3146. doi:10.1016/j.bmc.2013.03.031.

An altered zinc-binding site confers resistance to a covalent inactivator of New Delhi metallo-beta-lactamase-1 (NDM-1) discovered by high-throughput screening

Pei W. Thomas^a, Timothy Spicer^b, Michael Cammarata^c, Jennifer S. Brodbelt^c, Peter Hodder^{b,*}, and Walter Fast^{a,*}

^aDivision of Medicinal Chemistry, College of Pharmacy, University of Texas, Austin, TX 78712, USA

^bLead Identification, Translational Research Institute, The Scripps Research Institute, Scripps Florida, 130 Scripps Way #1A1, Jupiter, 33458 FL, USA

^cDepartment of Chemistry and Biochemistry, University of Texas, Austin, TX 78712

Abstract

Due to the global threat of antibiotic resistance mediated by New Delhi metallo-beta-lactamase-1 (NDM-1) and the lack of structurally diverse inhibitors reported for this enzyme, we developed screening and counter-screening assays for manual and automated formats. The manual assay is a trans-well absorbance-based endpoint assay in 96-well plates and has a Z' factor of 0.8. The automated assay is an epi-absorbance endpoint assay in 384-well plates, has a Z' factor of 0.8, good signal / baseline ratios (> 3.8), and is likely scalable for high-throughput screening (HTS). A TEM-1-based counter-screen is also presented to eliminate false positives due to assay interference or off-target activities. A pilot screen of a pharmacologically characterized compound library identified two thiol-modifying compounds as authentic NDM-1 inhibitors: p-hloromecuribenzoate (p-CMB) and nitroprusside. Recombinant NDM-1 has one Cys residue that serves as a conserved active-site primary zinc ligand and is selectively modified by p-CMB as confirmed by LC-MS/MS. However a C208D mutation results in an enzyme that maintains almost full lactamase activity, yet is completely resistant to the inhibitor. These results predict that covalent targeting of the conserved active-site Cys residue may have drawbacks as a drug design strategy.

1. Introduction

Beta-lactams are a mainstay for clinical treatment of bacterial infections.¹ In particular, carbapenems are among the most broad-spectrum beta-lactams known and are often reserved for treatment of hospital-acquired infections in patients that have already been exposed to a number of different classes of antibiotics.² Therefore, the emergence of

© 2013 Elsevier Ltd. All rights reserved.

*Corresponding authors. Tel: +1 512 232 4000; fax: +1 512 232 2606. WaltFast@mail.utexas.edu (W.F.); Tel: +1 561 228 2100; fax: +1 561 3054. hodderp@scripps.edu (P.H.).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Supplementary data

Supplementary data associated with this article can be found in the online version, at

carbapenem-resistant organisms is a major health concern.³ The carbapenemases of greatest clinical threat are bacterial metallo-beta-lactamase enzymes, which can hydrolyze all beta-lactam drugs except the monobactams.⁴ Of special concern is the recent (2008) emergence of community-acquired *Escherichia coli* and *Klebsiella pneumoniae* expressing plasmid-encoded New Delhi metallo-beta-lactamase-1 (NDM-1).^{5, 6} Some plasmids encoding NDM-1 also carry genes for resistance to macrolides, aminoglycosides, rifampicin, sulfamethoxazole and monobactams.⁵ Other isolates show additional resistance to colistin and tigecycline, making them pan-resistant bacteria.⁷ To put this in perspective, the emergence of NDM-1 has been called “possibly the most worrying development since Fleming gave us penicillin in 1929.”⁷ Since their recent discovery, NDM-1 bearing “superbugs” have already spread worldwide.⁷ Because there are no clinically-approved drugs that target NDM-1, or that target any other metallo-beta-lactamase, NDM-1 remains an alarming health concern that threatens to render obsolete a major class of therapeutics.

NDM-1 belongs to the B1 family of metallo-beta-lactamases, which also contains the most prevalent metallo-beta-lactamases worldwide: IMP-1 (Japan), IMP-4 (China), and VIM-2 (Turkey, Greece, India).⁷ These enzymes share a similar fold, but are quite diverse in primary sequence, in active-site loop conformations, and in metal stoichiometry, with mono- or dinuclear zinc sites leading to large differences in mechanism and inhibitor potency.⁸ At the time of writing, only two inhibitors have been reported for NDM-1, and these have only moderate potencies ($> 8 \mu\text{M}$).^{9, 10} More broadly, a number of inhibitors have been developed for other metallo-beta-lactamase isoforms, but the inhibitors that are effective *in vitro* with most clinically-relevant enzymes are limited to a narrow range of chemical space. Even the design of inhibitors for a single metallo-beta-lactamase isoform is difficult because the flexibility of the active-site zinc cluster and adjacent loop structures complicates structure-based design. The development of novel and useful NDM-1 inhibitors is clearly needed to stimulate the development of targeted therapeutics and to understand how the flexibility of this enzyme contributes to ligand recognition and the evolution of antibiotic resistance.

In response to the need for structurally-diverse NDM-1 inhibitors, we report here the development of sensitive, robust and scalable high-throughput screening (HTS) and counter-screening methodologies applicable to both manual and automated systems. A pilot screen is used to discover a covalent inactivator of NDM-1 that targets a conserved active-site Cys that has been previously suggested as a target for drug design.¹¹ However, we show that a single amino acid replacement results in a variant of NDM-1 that is almost fully active, yet is completely resistant to this covalent inactivator. The implications for NDM-1-targeted drug design are discussed.

2. Results

2.1 Trans-absorbance endpoint assay in 96-well format

The enzymatic activity of NDM-1 is measured using the colorimetric substrate nitrocefin in a cuvette-based continuous assay and has steady-state rate constants of $k_{\text{cat}} = 15.4 \pm 0.4 \text{ s}^{-1}$; $K_{\text{M}} = 1.3 \pm 0.2 \mu\text{M}$; $k_{\text{cat}}/K_{\text{M}} = 1.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$.¹² The continuous assay shows acceptable co-solvent tolerance: NDM-1 maintains $> 90 \%$ activity at DMSO concentrations up to 2% (v/v) (Supplementary Data Figure S1), and the purified enzyme is quite stable and can be stored at 4°C for more than several months with no significant loss in activity.

This continuous assay was converted into a plate-based endpoint assay conducted by manual pipetting. The reaction was initiated by mixing two equal volumes of reagents, one containing enzyme and inhibitor, the other containing substrate. After a set incubation time, the reaction was quenched upon addition of EDTA, which chelates the zinc ions in NDM-1

and rapidly stops all enzyme activity.¹³ Product formation is monitored by trans-well absorbance of the hydrolyzed nitrocefin product at 490 nm. The concentration of NDM-1 was adjusted such that linear product production was observed and < 10 % of substrate was consumed when using nitrocefin at concentrations of approximately $2 \times K_M$ during 20 min reactions, insuring initial rate conditions throughout the assay. In the 96-well format, this assay gives excellent Z' factors¹⁴ of 0.8 (Figure 1A). Plate-based IC_{50} determinations for the known inhibitor L-captopril¹⁰ showed acceptable day-to-day reproducibility (Figure 1B). Therefore, the assay was deemed suitable for manual screening and also amenable for further miniaturization and automation.

2.2 Epi-absorbance endpoint assay in 384-well format

To increase the throughput of this assay, it was converted into an epi-absorbance endpoint assay using 384-well plates and optimized using automated liquid handling instruments. Briefly, the epi-absorbance assay is set up similar to a fluorescence assay, using the apparent “excitation” of each well at 485 nm, and detecting an apparent “emission” at 535 nm that stems from the inherent fluorescent properties of white plastic plates. Hydrolysis of nitrocefin within each well produces a product that absorbs at 485 nm and results in a subsequent decrease of the apparent “emission” signal monitored at 535 nm. This assay is a very sensitive, non-radioactive, homogenous, “mix-and-read” format that is compatible with low volume 384- and 1,536-well plates, and has a number of advantages described elsewhere.¹⁵ This assay format has been successfully used to screen for inhibitors of other beta-lactamase enzymes.¹³ Using a reduced reaction volume (10 μ L), we demonstrated that the assay response remains linear up to 20 min. To gauge reproducibility, the IC_{50} value of L-captopril for NDM-1 was measured in separate experiments (see below). IC_{50} values determined using different assay times also indicated that a 20 min window was optimal, with minor decreases in sensitivity occurring at later times (data not shown).

Using a DMSO concentration of 1.2 % (v/v) allows screening of library compounds (stock concentration 2.5 mM in DMSO) at a final assay concentration of 30 μ M. Results from representative 384-well plates show clear separation between positive and negative controls (Figure 2A), with some evidence of minor plate-to-plate variation. No significant differences in the performance of the assay is observed when run on three different days, demonstrating excellent batch-to-batch reproducibility. The average S/B ratio for days 1, 2 and 3 were 3.8 ± 0.02 , 3.9 ± 0.04 , and 4.3 ± 0.2 , respectively. The average Z' values for days 1, 2 and 3 were 0.91 ± 0.01 , 0.88 ± 0.02 , and 0.83 ± 0.05 , respectively. All reagents exhibited excellent stability during the time necessary for all screening-related activities (> 3 h). Separate experiments using L-captopril as an inhibitor gave acceptable reproducibility, with the aggregate values being $IC_{50} = 6.1 \pm 0.7 \mu$ M, and Hill coefficient = 0.58 ± 0.03 (Figure 2B).

2.3 Screen of 1,280-compound LOPAC library

The 384-well format epi-absorbance endpoint assay was then used to screen the 1,280-compound LOPAC library (Sigma-Aldrich Library of Pharmacologically Active Compounds) with the final concentration of test compounds at 30 μ M and nitrocefin at 3 μ M (approximately $2 \times K_M$). Each plate contained controls for inhibited (substrate with no enzyme) and uninhibited (no inhibitor) reactions, which were used in Z' factor calculations. An activity scatter-plot of all compounds tested, as well as inhibited and uninhibited controls, is shown (Figure 3A). The assay has a high Z' factor (0.87 ± 0.03) for the entire pilot screen, indicative of an excellent assay window. Further, the complete absence of hits from the wells containing the DMSO control indicates that the assay is not very susceptible to this type of false positive. Comparison of two replicate plates demonstrates high reproducibility in hit identification, with an $r^2 = 0.984$ (Figure 3B). Using a typical cutoff¹⁶

(assay average plus three times the standard deviation: 42 % inhibition) we calculated an approximate primary hit rate of 1 % (14 hits / 1280 total compounds).

2.4 Counter-screen

A counter-screen was developed to identify and eliminate false positives. This counter-screen uses the exact same nitrocefin substrate and assay format, but NDM-1 is substituted by the serine-beta-lactamase TEM-1. TEM-1 and NDM-1 are not homologous enzymes. They do not share any significant sequence homology.¹⁷ They do not work by the same enzymatic mechanisms; TEM-1 proceeds through a covalent intermediate with an active-site Ser,¹⁸ but NDM-1 does not.¹⁹ Therefore, NDM-1 inhibitors that achieve potency through specific molecular interactions with the enzyme would not be expected to also show potent inhibition of TEM-1. Based on this design, compounds shown to inhibit NDM-1 that also inhibit TEM-1 are flagged as compounds that are likely to be false positives. This counter-screen has proven to be successful in screens of other metallo-beta-lactamases (PubChem AID: 1866).

Here, we flagged the top 13 hits from the primary screen for further validation. An initial LC-MS characterization of 10 / 13 hit compounds (compounds **1** – **10**) obtained from secondary stock solutions were deemed to be > 94 % homogeneous and displayed their expected masses (Table 1). The other 3 hits did not show the expected masses or purity and so were discarded from further consideration.

One hit is a maleate salt (compounds **6**). Since dicarboxylates are known inhibitors of metallo-beta-lactamases,⁹ we tested sodium maleate as an inhibitor. However, no inhibition of NDM-1 was observed up to 1 mM maleate (using 20 μ M nitrocefin), and no time-dependent inhibition was observed.

We applied the counter-screen by re-testing 10 hits from the primary screen in 10-point concentration-response plots for inhibition of either NDM-1 or TEM-1 (Table 1, Figure 4). Of the hits, five compounds (**4**, **5**, **6**, **7**, **8**) showed NDM-1 IC₅₀ values $\geq$ 30 μ M and were discarded from further consideration. Three compounds (**3**, **9**, **10**) showed apparent inhibition of NDM-1, but also showed a maximum percent inhibition that was much greater than 100%. This result is typically indicative of assay interference, often due to compound fluorescence. Two of these compounds (**9**, **10**) also showed apparent inhibition of TEM-1. Therefore these compounds were flagged as likely false positives and were also discarded.

The two remaining compounds, **1** (p-chloromercuribenzoic acid, pCMB) and **2** (sodium nitroprusside) show inhibition of NDM-1, but not TEM-1, and their maximum percent inhibition is < 100 %, results consistent with those expected for authentic NDM-1 inhibitors. See Table 1 and Figure 4 for a summary of the validation results.

2.5 Variants of Cys208 in NDM-1

Since two of the validated hits (**1**, **2**) are known to modify thiols, we sought to mutate the sole Cys residue of purified recombinant NDM-1, Cys208.¹² This residue is a conserved primary zinc ligand in the metallo-beta-lactamase B1 family (Figure 5),²⁰ so we chose variants that might also serve as metal ion ligands. Substitution of Cys with Ser or His was deleterious both to activity and to zinc content (Table 2). However the C208D variant maintains approximately 2 equivalents of zinc ions and maintains approximately 75 % of the activity of tagged wild-type NDM-1 ($k_{\text{cat}} / K_{\text{M}}$ values), stemming mostly from a decrease in k_{cat} that is somewhat offset by a smaller decrease in K_{M} .

2.6 Time-dependent Inactivation Assay

To test a proposed covalent inactivation mechanism, a preincubation / dilution assay was used to test for a progressive loss of activity that is not reversible upon dilution into excess substrate. Incubation of the p-CMB (**1**) inhibitor with wild-type NDM-1 leads to time-dependent loss of activity relative to a control incubation mixture lacking the inhibitor (Figure 6). In contrast, the C208D variant does not show a time dependent loss of activity after incubation with the same inhibitor (Figure 6).

2.7 Mass Spectrometry of NDM-1

High mass accuracy electrospray ionization mass spectrometry (ESI-MS) was used to determine the molecular weights of wild-type NDM-1 before and after treatment with the p-CMB (**1**) inhibitor. The ESI mass spectra of the intact proteins are shown in Supplemental Figure 2. In each case only one major molecular ion was found (in multiple charge states). The unmodified NDM-1 shows a mono-isotopic mass of $24,823.3 \pm 0.2$ Da, which matches the theoretical monoisotopic mass calculated for the protein (24,823.3 Da). Treatment of NDM-1 with the inhibitor results in an intact mass of $25,143.3 \pm 0.2$ Da. This ~320 Da mass shift is consistent with the addition of one equivalent of the inhibitor p-CMB (**1**) (monoisotopic mass 357.9684 Da) in conjunction with the loss of a chloride atom (monoisotopic mass 34.9688 Da) and two additional hydrogen losses (1.0078 Da per hydrogen atom, specific location unknown). The low abundance of the unmodified protein in the ESI mass spectrum acquired for the p-CMB-modified NDM-1 protein indicates that the reaction went to near completion.

Through the use of a bottom-up LC-MS/MS strategy, the unambiguous site of covalent adduction of p-CMB (**1**) to the NDM-1 protein was elucidated at residue Cys208. After trypsin proteolysis of the p-CMB-modified protein, LC-MS/MS characterization of the resulting digest yielded the identification of a single modified peptide: $^{182}\text{VFYPGPGHTSDNITVGIDGTDIAFGGcLIK}^{211}$ for which the lower case c indicates the location of the modified residue. The fragmentation pattern of this key peptide is shown in Supplemental Figure 3A, and the corresponding MS/MS spectrum of the unmodified peptide is shown in Supplemental Figure 3B for comparison. The broad series of *b* and *y* fragment ions confirms the sequence of this NDM-specific peptide as well as the modification of the Cys208 residue by p-CMB. In particular, the N-terminal *b*₂₈ and *b*₂₉ ions retain the modification and thus are shifted by ~321 Da, whereas the smaller fragment ions (*b*₆ through *b*₂₇, ones which do not contain the cysteine residue) are not mass shifted. Likewise, the *y*³ ion is not shifted for the p-CMB-modified peptide, but the next *y* ion (*y*₄ which contains the key cysteine residue) includes the mass shift corresponding to incorporation of the inhibitor.

3. Discussion

In response to the need to discover structurally diverse inhibitors of NDM-1, we report two screening assays suitable for either manual or automated use. Both methods are based on known metallo-beta-lactamase assays that use a colorimetric substrate and a metal ion chelator to stop the reaction due to chelation of the active-site zinc ions.²¹ A particular issue with NDM-1 is its low *K*_M value for the nitrocefin substrate,¹² but the use of decreased enzyme concentrations and slightly elevated substrate concentrations (*[S]* ≈ 2 *K*_M value) allows for linear initial reaction rates to be determined over the entire assay time course. Because substrate concentrations are not fully saturating, and because a pre-equilibration step is used, these conditions theoretically allow for the balanced detection of inhibitors that are rapidly reversible, time-dependent, competitive, or not competitive.²²

Here, the benchtop endpoint assay uses manual pipetting in a 96-well plate format and follows product formation by an increase in trans-well absorbance values. This assay gives excellent separation between the uninhibited reaction (enzyme + substrate) and the fully inhibited reaction (enzyme omitted) (Figure 1A), as evidenced by a Z' factor of 0.8. This assay also has good day-to-day reproducibility and is suitable for determining IC_{50} values as shown in the concentration-response plots for NDM-1 inhibition by L-captopril measured on three separate days (Figure 1B). The aggregate IC_{50} value ($72 \pm 3 \mu M$) and Hill coefficient (0.92 ± 0.03) determined using this manual method matches reasonably well with the reported IC_{50} value ($202 \mu M$)¹⁰ and the known stoichiometry of inhibition,²³ although the previously published value¹⁰ does not give an associated substrate concentration to allow a direct comparison. Therefore, this assay is sensitive, reproducible, and suitable for NDM-1 inhibitor screening and characterization using manual methodology.

To increase throughput, we modified the endpoint assay format for use with automated equipment in a 384-well plate format that follows product formation by epi-absorbance. Briefly, each well is “excited” at a wavelength that corresponds to the product’s absorbance, and we monitor the apparent “emission” due to the inherent fluorescent properties of the white plastic plate. Product formation results in a decrease in the monitored “emission.” This assay gives excellent separation between the uninhibited reaction (enzyme + substrate) and the fully inhibited reaction (enzyme omitted) (Figure 2A), as evidenced by a Z' factor of 0.8 or greater. This assay has good reproducibility and is suitable for determining IC_{50} values as shown in the concentration-response plots for NDM-1 inhibition by L-captopril measured using four separate experiments (Figure 2B). The aggregate IC_{50} value ($6.1 \pm 0.7 \mu M$) and Hill coefficient (0.58 ± 0.03) determined using this automated method are somewhat lower than that determined using the manual method (above), possibly due to the direct dilution from DMSO stocks, or due to some oxidation of the inhibitor upon storage. However, the values are highly reproducible and suitable for sensitive detection of inhibitors during a screen. In this case, an increase in assay sensitivity is not a drawback since false positives can be identified using an effective counter screen (below). In sum, this automated HTS assay format is shown to be sensitive, reproducible, and suitable for NDM-1 inhibitor screening. Based on previous work (PubChem AID: 1866) and the excellent assay statistics, we anticipate this format will be readily scalable to a 1536-well format to further increase throughput.

The automated epi-absorbance assay was used to screen triplicates of a 1,280-member commercial library (LOPAC¹²⁸⁰, Sigma-Aldrich) to gauge assay performance in a pilot screen and to identify any NDM-1 inhibitors. The assay maintained excellent discrimination between positive and negative controls (Z' factor = 0.8), and resulted in a reasonable distribution of responses for the library compounds (Figure 3A). Assay reproducibility was also excellent, with a linear correlation ($r^2 = 0.984$) observed between responses for corresponding samples in duplicate wells (Figure 3B). The top 13 primary hits were selected for validation as authentic NDM-1 inhibitors (Table 1).

The validation procedure consisted of confirming each compound’s identity and purity by LC-MS, and then collecting a concentration-response plot for inhibition of NDM-1 and also for the unrelated serine-beta-lactamase TEM-1. Compounds with IC_{50} values $> 30 \mu M$ (the largest concentration tested) for inhibition of NDM-1 (**4 – 8**) were discarded from consideration due to their lack of potency (Table 1). Compounds that were apparent inhibitors of both NDM-1 and TEM-1 were also discarded (**9, 10**). Due to the divergent structures and mechanisms for these two enzymes, we reasoned that apparent inhibition of both enzymes is more likely due to an artifact in their shared detection method rather than overlapping pharmacophores. Admittedly, since both enzymes do process beta-lactams, this counter screen could possibly eliminate a small subset of hits that are dual inhibitors.

Finally, compounds that show a maximum inhibition > 100 % are discarded (**3**, **9**, **10**) from consideration (Table 1). This typically arises from fluorescent library compounds that increase the apparent “emission.”

To further illustrate the counter screen, the concentration-response plots for two compounds (**1**, **10**) are shown (Figure 4). Compound **1** shows inhibition of NDM-1 with a reasonably potent apparent IC₅₀ value of 2 μM and a Hill coefficient of 1.7. A large Hill coefficient, which is not observed with compound **1**, would be a possible indication for a promiscuous aggregating inhibitor.²⁴ No inhibition of TEM-1 by **1** is observed and the maximum percent NDM-1 inhibition is 84 %; both results are consistent with a lack of assay interference. Therefore compound **1** is deemed to be an authentic NDM-1 inhibitor. In contrast, compound **10** shows potent inhibition of NDM-1, but also inhibits TEM-1 and has a maximum NDM-1 inhibition that greatly exceeds 100 %; the latter two results are consistent with assay interference. Therefore, compound **10** is deemed to be a false positive. Structurally-related naphthalimides have known fluorescent properties,²⁵ which also reinforce this interpretation.

After counter screening the top primary hits, we assigned two compounds (**1**, **2**) as authentic NDM-1 inhibitors (Table 1). Both of these compounds, *p*-chloromecuribenzoic acid (*p*-CMB, **1**) and nitroprusside (**2**) are well-known thiol-modifying compounds.^{26, 27} Recombinant NDM-1 has only one Cys residue (Cys208), which is located at the active site and serves as a primary zinc ligand (Figure 5). Therefore, we propose that NDM-1 inactivation through active-site Cys modification is a reasonable mechanism of inhibition for each of these compounds. Please note that the apparent IC₅₀ values for time-dependent inhibitors are strongly dependent on incubation time, and are not, by themselves, an accurate measure of affinity.²² Additionally, the non-selective thiol-reactivity of these compounds rules them out as lead compounds for future drug development. However, they do clearly demonstrate the ability of our HTS to identify authentic NDM-1 inhibitors. The discovery that the nitric oxide donor nitroprusside (**2**) can inhibit NDM-1 also suggests a possible role for macrophage-derived reactive nitrogen species in fighting NDM-1 bearing infections.

Like compounds **1** and **2**, other published inhibitors of metallo-beta-lactamases have been shown to target a Cys residue for covalent modification. For example: the metallo-beta-lactamases from *Aeromonas sobria* AER 14M and from *Pseudomonas aeruginosa* are susceptible to inactivation by *p*-CMB (**1**)^{21, 28}; the metallo-beta-lactamase from *Aeromonas hydrophila* is inactivated by moxalactam through formation of a mixed disulfide²⁹; a variant of the metallo-beta-lactamase from *Fluoribacter (Legionella) gormanii* is inactivated by cefuroxime by formation of a mixed disulfide³⁰; and the metallo-beta-lactamase from *Bacillus cereus* is inactivated by mercaptoacetic acid thiol ester derivatives through formation of a mixed disulfide.³¹ Formation of the mixed disulfide does not readily occur with thiol inhibitors, and seems to require an undefined “mechanism-based” delivery.³¹ The inactivation mechanism might use an active-site zinc to facilitate disulfide formation through disulfide exchange, as has been demonstrated with small molecule mimics.¹¹ Since the primary zinc ligand Cys208 (Figure 5) is conserved throughout the B1 family of metallo-beta-lactamases, it would therefore seem to be an attractive site to target with covalent therapeutics - this strategy has been previously proposed.¹¹ However, a recent study with another B1 family member (IMP-1) indicates that, despite its sequence conservation, the Cys residue at this site can be substituted by Asp or Gly and still confer resistance to β-lactams.²⁰ If variation of a single residue can also confer resistance against Cys-directed inhibitors, it may suggest that this is not a suitable strategy for therapeutic development. Therefore, we built on our HTS results to test this possibility with NDM-1 by using *p*-CMB (**1**) as a model inhibitor that covalently targets Cys residues.

In order to find a functional variant of NDM-1 in which the sole Cys residue (Cys208) was replaced by a less reactive side chain, we prepared three site-directed mutants: C208D, C208S and C208H (Table 2). The His substitution displayed the worst catalytic efficiency ($k_{\text{cat}} / K_{\text{M}}$) and only bound 1 equivalent of zinc ion, possibly due to the increased steric bulk of this substitution. The Ser substitution maintained a moderate amount of activity (≈ 44 -fold lower $k_{\text{cat}} / K_{\text{M}}$), which is an activity greater than has been observed with an analogous mutations in a different B1 superfamily member,³² possibly reflecting an enhanced plasticity of the zinc site in NDM-1. In contrast, the Asp substitution maintained binding of both zinc ions and displayed a catalytic efficiency that was only slightly lower ($\approx 75\%$) than the similarly tagged wild type enzyme. Therefore, variation of a single residue in NDM-1 (C208D) can create a nearly fully functional enzyme that does not contain any Cys residues.

Co-incubation of the p-CMB (**1**) inhibitor with wild-type NDM-1 leads to a progressive, time-dependent loss of activity that is not reversible upon dilution into excess substrate (Figure 6). ESI-MS of NDM-1 before and after incubation with p-CMB (**1**) reveals a mass increase that reflects covalent addition of one equivalent of the inhibitor to the protein and this addition is mapped specifically to Cys208, consistent with the proposed mechanism (Supplementary Figures S2, S3). However, co-incubation of p-CMB (**1**) with the C208D NDM-1 variant does not show any time-dependent inhibition (Figure 6). These findings are consistent with the proposal that p-CMB (**1**) targets Cys208. More importantly, these results demonstrate that a single amino acid change can result in an enzyme that maintains almost complete activity, yet becomes resistant to a Cys-directed covalent inhibitor. These results indicate that inhibitor design strategies that target the active-site Cys may be inherently flawed due to an acquirable resistance mutation and should be avoided. Although the C208D variant requires accumulation of two point mutations, other variants (e.g. C208G) are more easily acquired and might also confer inhibitor resistance without compromising activity. Compounds discovered through HTS that target Cys208 could be triaged by incorporating the C208D NDM-1 variant into the counter-screen.

4. Conclusion

In response to the global threat of NDM-1-mediated antibiotic resistant bacteria and the lack of structurally-diverse NDM-1 inhibitors, we report here a robust, sensitive, reproducible and scalable HTS assay with an associated counter-screen to facilitate inhibitor discovery using either manual or automated formats. From a pilot screen, two Cys-modifying compounds were authenticated as NDM-1 inhibitors. Although the sole Cys residue in recombinant NDM-1 is a conserved primary zinc ligand, we found that substitution by an Asp residue results in a variant that maintains almost full enzymatic activity, yet is no longer susceptible to the inhibitor. Due to this acquirable resistance mutation, targeting the active-site Cys zinc ligand in NDM-1 may have drawbacks as a drug design strategy.

5. Experimental

5.1 Trans-absorbance endpoint assay in 96-well format

For NDM-1 studies, we use the substrate nitrocefim, a cephem with a highly conjugated dinitrostyrene group that changes absorbance upon hydrolysis of the lactam C-N bond to shift colors from “yellow” ($\epsilon_{390\text{ nm}} = 14,920\text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{490\text{ nm}} = 1,520\text{ M}^{-1}\text{cm}^{-1}$) to “red” ($\epsilon_{390\text{ nm}} = 5,840\text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{490\text{ nm}} = 17,200\text{ M}^{-1}\text{cm}^{-1}$).³³ This continuous assay was converted into a 96-well plate-based endpoint assay as described below.

Immediately before assays, several stock solutions are freshly prepared. An NDM-1 stock solution (0.4 nM) is prepared in HEPES (0.1 M), ZnSO_4 (20 μM), Tween-20 (0.02 % v/v) at pH 7.0. For L-captopril studies, a stock solution (0.1 M) is prepared in water. A nitrocefim

stock solution (6 μM) is prepared in water containing Tween-20 (0.02 % v/v). To screen inhibitors, 2.5 μL of DMSO alone (negative control) or the same volume containing inhibitor is added to each well of a 96-well plate (Nunc flat bottomed untreated 96 well microtiter plate, Fisher Scientific part number 260895). A positive control can also be prepared using a final concentration of 1 mM L-captopril as a known inhibitor of NDM-1. After aliquoting library compounds, 125 μL of the NDM-1 stock is added to each well and incubated at 25 $^{\circ}\text{C}$ for 10 min to allow equilibration with the inhibitor. To initiate the reaction, 125 μL of the nitrocefin stock solution is added to each well (the final substrate concentration is approximately $2 \times K_M$) and the reaction allowed to proceed for 20 min. Final assay conditions are nitrocefin (3 μM), NDM-1 (0.2 nM), HEPES (50 mM), ZnSO_4 (10 μM), Tween-20 (0.02 % v/v) at pH 7.0.

After completion of the 20 min incubation, 50 μL is added from a quench solution containing EDTA (0.5 M) in water. Wells are mixed and, after a short 3 min incubation, the absorbance for each well is measured at 490 nm to determine the extent of product formation.

5.2 Epi-absorbance endpoint assay in 384-well format

The NDM-1 nitrocefin assay began by dispensing 5 μL of 0.4 nM NDM-1 in assay buffer (0.1 M HEPES, 20 μM ZnSO_4 , 0.02% (v/v) Tween-20 at pH 7.0) to the appropriate wells of a 384-well microtiter plate (part 784075 Greiner, USA). Following enzyme addition, 120 nL of controls or test compounds were added to the appropriate wells. The plates were then incubated at room temperature for 10 min. Nitrocefin was prepared from powder at 50 mM in neat DMSO and then diluted to 6 μM in 0.02% (v/v) Tween-20. To start the reaction, 5 μL of 6 μM nitrocefin was dispensed into all wells. Final concentrations of substrate and enzyme were 3 μM nitrocefin and 0.2 nM NDM-1, respectively. The plate was then incubated for 20 min at room temperature and the reaction stopped by the addition of 2 μL of 0.5 M EDTA. Epi-Absorbance readings at 485 nm excitation and 535 nm emission were performed using a Viewlux plate reader (Perkin-Elmer, Finland).

5.3 Compound validation

Compounds were assayed for mass confirmation and purity using an Agilent 1200 series LC-MS with multimode mass spectrometry (ES, APCI, ELSD).

5.4 Counter screen

The TEM-1 nitrocefin assay was run in an identical fashion to the NDM-1 384-well assay, with the following exceptions: After addition of substrate and enzyme (at final concentrations of 100 μM nitrocefin and 0.15 nM TEM-1), the plate was incubated for 25 min at room temperature and the reaction stopped by the addition of 2 μL of 20 μM clavulanate.

5.5 Variants of Cys208 in NDM-1

Overlap-extension PCR was used to generate three site-directed mutations. As described elsewhere,¹² the pET27b-Strep-NDM1 plasmid template encodes an N-terminal pelB leader sequence to direct export of NDM-1 to the periplasm, followed by a sequence encoding an eight-amino acid *StrepII* affinity tag, a tobacco etch virus (TEV) protease cleavage site, and, finally, the NDM-1 sequence starting from residue Gly36. The first step of the PCR generated a 5' DNA fragment using the outside forward primer NDMP3¹² and a reverse primer specific for each mutant, and 3' DNA fragment using the outside reverse primer NDMP4¹² and a forward primer specific for each mutant (Supplementary Data, Table S1). The PCR temperature sequence was 94 $^{\circ}\text{C}$ for 2 min followed by 30 cycles of 30 s at 94 $^{\circ}\text{C}$,

30 s at 62 °C, and 60 s at 72 °C in 25 μ L reactions. 1 μ L of each 5'- and 3'-PCR reaction mixture were then combined to serve as a template for a second PCR using NDMP3 and NDMP4 as outside primers to generate the full length DNA product encoding the mutated NDM-1 sequences. After QIAquick purification, the PCR products were digested with *Nco*I and *Bam*HI and ligated into the corresponding sites of an identical digested pET27b(+) plasmid. The ligated mixtures were transformed into *E. coli* BL21(DE3) by electroporation and selected for resistant transformants using LB agar supplemented with kanamycin (30 μ g / mL) antibiotic. Final plasmid DNA derived from a single colony in each set of transformants was purified and the DNA sequence was verified as described previously to result in the plasmids pET27b-Strep-NDM1-C208D, pET27b-Strep-NDM1-C208S, and pET27b-Strep-NDM1-C208H.

The NDM-1 variant proteins were expressed and purified from growth media, as described earlier for wild-type protein¹² with the following minor changes. For each of the variants, 20 mL of the LB growth media from expression cultures was diluted with an equal volume of 1 $\times$ PBS buffer, and gravity loaded onto a spin column containing 1.5 mL of Strep-Tactin resin. Lactamase activity in fractions of the flow-through were monitored using nitrocefin as substrate, and loading was stopped when this activity exceeded that of background. Each column was washed with 6 mL of 1 $\times$ PBS, and eluted with 1 $\times$ PBS + 2.5 mM desthiobiotin. The eluate was concentrated using Ultra-10K centrifugal filter concentrators and buffer exchanged into 50 mM Tris-HCl and 150 mM NaCl. The resulting proteins were not cleaved by TEV protease, but instead were purified as intact fusion proteins as previously described.¹² The total protein and zinc concentration of each preparation was determined as previously described.¹² Using this method, each protein variant was purified to approximately 95 % homogeneity as gauged by Coomassie-stained SDS-PAGE.

5.6 Steady-State and Time-dependent Inactivation Assay

Steady-state kinetic assays of NDM-1 were performed as previously described.¹² Time-dependent inactivation assays were performed using a preincubation / dilution assay. Briefly, wild-type WT or C208D NDM-1 (4 μ M) was incubated with or without 0.1 mM p-CMB (**1**) at room temperature in HEPES (50 mM) at pH 7.0. At various timepoints, 10 μ L of this preincubation mixture was removed and diluted into a final assay mixture (1 mL) containing a saturating concentration of nitrocefin (20 μ M) and the reaction rate was monitored as describe above. The rates at time 0 (immediately after making the preincubation mixture) was used as 100 % activity reference for each experiment.

5.7 Mass Spectrometry

Wild type NDM-1 (16 μ M) was prepared with or without p-CMB (**1**) (0.4 mM) in HEPES (50 mM) at pH 7. After 10 min of incubation, the enzymatic activity of NDM-1 was almost completely eliminated. These samples were subsequently used for mass spectrometry.

Sample Preparation for MS analysis—40 μ g of the NDM-1 protein before and after inhibition was prepared for digestion by performing a buffer exchange using a 10 KDa molecular weight cutoff filter. The filtered protein was diluted in 150 mM ammonium bicarbonate at a pH of 7.8. For trypsin digestion, 1 μ L of trypsin (1 μ g/ μ L) was added to 20 μ g of buffer-exchanged protein and incubated overnight at 37 °C. Following digestion, the solutions was desalted using C18 spin columns (Thermo/Pierce) and reconstituted in 98% water, 2% acetonitrile for LC-MS/MS analysis. For analysis of the intact proteins, each filtered protein was diluted to 15 μ M in a solvent mixture of 49.75:49.75:0.5 by volume with respect to water:acetonitrile:formic acid.

LC-MS/MS Analysis—LC-MS/MS analysis was undertaken using a Dionex Ultimate 3000 NSLC nano liquid chromatograph interfaced to a Thermo LTQ mass spectrometer. 1 pmol of the digest was injected onto a New Objective IntegraFrit 100 μ m inner diameter trap column (Woburn, MA) packed with 3 μ m Michrom Magic C18 AQ (Auburn, CA) to 2 cm. Samples were preconcentrated for 3 min using 2% acetonitrile / 0.1% formic acid at a flow rate of 5 μ L/min. The preconcentration column was then switched in-line with a New Objective PicoFrit analytical column (Woburn, MA) (75 μ m $\times$ 15 cm) packed with 3 μ m Michrom Magic C18 AQ resin. Separation was performed with mobile phase A consisting of 0.1% formic acid in water and mobile phase B consisting of 0.1% formic acid in acetonitrile applied as a 60 min linear gradient from 2% to 35% eluent B at a flow rate of 300 nL/min. MS/MS analysis was performed on a Thermo Scientific LTQ mass spectrometer using a normalized collision energy of 35%. For the LCMS/MS data acquired for the tryptic digests, the raw LCMS files were converted to mzXML files and then processed using the MassMatrix database search engine (ver. 2.4.0). All MS/MS spectra were searched against the NDM-1 protein sequence. Oxidation of methionine and pCMB (1) were set as variable modifications. The peptide mass tolerance was set to ± 1.25 Da. The peptide length was constrained by a minimum length of 6 amino acids and a maximum length of 40 amino acids. Two missed cleavages were allowed for trypsin. For CID mass spectra, the fragment mass tolerances were set to ± 0.80 Da. The minimum score, pp value, pp2 value, and pptag value were 10, 5.0, 5.0, and 3.0, respectively, for the MassMatrix database search of the CID data. Mass measurement of the intact proteins was undertaken on a Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Each protein was infused and ionized by electrospray ionization in the positive mode.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by grants from the Robert A Welch Foundation (F1572 to W.F.; F1155 to J.S.B.) and the National Institutes of Health (R21 GM099028 to J.S.B.; MH084512 to T.P.S. and P.H.). We thank Peter Chase and Louis Scampavia (Lead Identification, Scripps Florida) for management of compounds screened in the 384 well assays.

Reference and notes

1. Goodman, LS.; Gilman, A.; Brunton, LL. Goodman & Gilman's manual of pharmacology and therapeutics. New York: McGraw-Hill Medical; 2008.
2. Gallagher, JC.; MacDougall, C. Antibiotics, simplified. Sudbury, Mass: Jones and Bartlett Publishers; 2009.
3. Shahid M, Sobia F, Singh A, Malik A, Khan HM, Jonas D, Hawkey PM. Crit Rev Microbiol. 2009; 35:81. [PubMed: 19514910]
4. Oelschlaeger P, Ai N, Duprez KT, Welsh WJ, Toney JH. J Med Chem. 2010; 53:3013. [PubMed: 20121112]
5. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR. Antimicrob Agents Chemother. 2009; 53:5046. [PubMed: 19770275]
6. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N. Lancet Infect Dis. 2010; 10:597. [PubMed: 20705517]
7. Walsh TR. Int J Antimicrob Agents. 2010; 36(Suppl 3):S8. [PubMed: 21129630]
8. Crowder MW, Spencer J, Vila AJ. Acc Chem Res. 2006; 39:721. [PubMed: 17042472]

9. Livermore DM, Mushtaq S, Morinaka A, Ida T, Maebashi K, Hope R. *J Antimicrob Chemother.* 2013; 68:153. [PubMed: 22945917]
10. Guo Y, Wang J, Niu G, Shui W, Sun Y, Zhou H, Zhang Y, Yang C, Lou Z, Rao Z. *Protein Cell.* 2011; 2:384. [PubMed: 21637961]
11. Boerzel H, Koeckert M, Bu W, Spingler B, Lippard SJ. *Inorg Chem.* 2003; 42:1604. [PubMed: 12611529]
12. Thomas PW, Zheng M, Wu S, Guo H, Liu D, Xu D, Fast W. *Biochemistry.* 2011; 50:10102. [PubMed: 22029287]
13. Minond D, Saldanha SA, Spicer T, Qin L, Mercer BA, Roush WR, Hodder P. Probe Reports from the NIH Molecular Libraries Program. 2010 PMID: 21961119.
14. Zhang JH, Chung TD, Oldenburg KR. *J Biomol Screen.* 1999; 4:67. [PubMed: 10838414]
15. Zuck P, O'Donnell GT, Cassaday J, Chase P, Hodder P, Strulovici B, Ferrer M. *Anal Biochem.* 2005; 342:254. [PubMed: 15949786]
16. Hodder P, Cassaday J, Peltier R, Berry K, Inglese J, Feuston B, Culbertson C, Bleicher L, Cosford ND, Bayly C, Suto C, Varney M, Strulovici B. *Anal Biochem.* 2003; 313:246. [PubMed: 12605861]
17. Massova I, Mobashery S. *Antimicrob Agents Chemother.* 1998; 42:1. [PubMed: 9449253]
18. Adachi H, Ohta T, Matsuzawa H. *J Biol Chem.* 1991; 266:3186. [PubMed: 1993691]
19. Yang H, Aitha M, Hetrick AM, Richmond TK, Tierney DL, Crowder MW. *Biochemistry.* 2012; 51:3839. [PubMed: 22482529]
20. Horton LB, Shanker S, Mikulski R, Brown NG, Phillips KJ, Lykissa E, Venkataram Prasad BV, Palzkill T. *Antimicrob Agents Chemother.* 2012; 56:5667. [PubMed: 22908171]
21. Minond D, Saldanha SA, Subramaniam P, Spaargaren M, Spicer T, Fotsing JR, Weide T, Fokin VV, Sharpless KB, Galleni M, Bebrone C, Lassaux P, Hodder P. *Bioorg Med Chem.* 2009; 17:5027. [PubMed: 19553129]
22. Copeland, RA. *Evaluation of enzyme inhibitors in drug discovery : a guide for medicinal chemists and pharmacologists.* Hoboken, N.J: Wiley-Interscience; 2005.
23. King DT, Worrall LJ, Gruninger R, Strynadka NC. *J Am Chem Soc.* 2012; 134:11362. [PubMed: 22713171]
24. Shoichet BK. *J Med Chem.* 2006; 49:7274. [PubMed: 17149857]
25. Parkesh R, Clive Lee T, Gunnlaugsson T. *Org Biomol Chem.* 2007; 5:310. [PubMed: 17205175]
26. Lundblad, RL. *Chemical reagents for protein modification.* Boca Raton: CRC Press; 2005.
27. Wang, PG.; Cai, TB.; Taniguchi, N., editors. *Nitric Oxide Donors: for Pharmaceutical and Biological Applications.* Weinheim: Wiley-VCH; 2005.
28. Yang Y, Bush K. *FEMS Microbiol Lett.* 1996; 137:193. [PubMed: 8998985]
29. Zervosen A, Valladares MH, Devreese B, Prosperi-Meys C, Adolph HW, Mercuri PS, Vanhove M, Amicosante G, van Beeumen J, Frere JM, Galleni M. *Eur J Biochem.* 2001; 268:3840. [PubMed: 11432752]
30. Mercuri PS, Garcia-Saez I, De Vriendt K, Thamm I, Devreese B, Van Beeumen J, Dideberg O, Rossolini GM, Frere JM, Galleni M. *J Biol Chem.* 2004; 279:33630. [PubMed: 15159411]
31. Payne DJ, Bateson JH, Gasson BC, Proctor D, Khushi T, Farmer TH, Tolson DA, Bell D, Skett PW, Marshall AC, Reid R, Ghosez L, Combret Y, Marchand-Brynaert J. *Antimicrob Agents Chemother.* 1997; 41:135. [PubMed: 8980769]
32. Yang Y, Keeney D, Tang X, Canfield N, Rasmussen BA. *J Biol Chem.* 1999; 274:15706. [PubMed: 10336469]
33. Wang Z, Fast W, Benkovic SJ. *Biochemistry.* 1999; 38:10013. [PubMed: 10433708]
34. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. *J Comput Chem.* 2004; 25:1605. [PubMed: 15264254]

Figure 1.

Trans-absorbance endpoint assay for NDM-1 in manual 96-well format. A. Z' factor determination: Assays of controls for uninhibited (substrate + enzyme, ○) and inhibited (substrate and no enzyme, ●) reactions show good separation of their means (0.056 ± 0.01 and 0.022 ± 0.001 Abs units, respectively). Solid lines are the mean values for each set, dashed lines are at $\pm 3 \sigma$. The calculated Z' factor = 0.8. Two points were discarded before analysis due to pipetting errors. Sample numbers were scrambled, so no row or column effects can be obtained from this plot. B. Day-to-Day replication: Using triplicate experiments, IC_{50} and Hill Coefficient values were determined for L-captopril on three separate days (Day 1: $65 \pm 2 \mu M$, 0.88 ± 0.03 ; Day 2: $58 \pm 2 \mu M$, 0.94 ± 0.03 ; Day 3: 83 ± 3

μM , 0.97 ± 0.04), and combined here ($n = 9$) to give an aggregate $\text{IC}_{50} = 72 \pm 3 \mu\text{M}$; Hill = 0.92 ± 0.03 .

Figure 2.

Epi-absorbance endpoint assay for NDM-1 in an automated 384-well format. A. Z' factor determination: To present results in parallel with Figure 1, the fluorescence Em_{535} intensity is converted into absorbance units. Assays of controls for inhibited (substrate and no enzyme, ●) and uninhibited (substrate + enzyme, ○) reactions show good separations of their means (0.038 and 0.15 absorbance units, respectively). Solid lines are the mean values for each set, dashed lines are at $\pm 3\sigma$. The calculated Z' factor = 0.8. B. Reproducibility: IC_{50} and Hill Coefficient values were determined for L-captopril for four separate experiments and combined here to give an aggregate $IC_{50} = 6.1 \pm 0.7 \mu M$; Hill = 0.58 ± 0.03 .

Figure 3.

Screen of 1,280-compound LOPAC library. A. Scatter plot of HTS of the LOPAC library in triplicate for inhibition of NDM-1 activity. The means of the controls for inhibited (□, substrate, no enzyme, no inhibitor) and uninhibited (○, substrate + enzyme, no inhibitor) reactions are well separated and give a Z' factor of 0.87 ± 0.03 , and a broad range of apparent inhibition is seen in the library compounds (●). A primary hit cutoff value of 42 % inhibition (assay average + 3σ) is shown as a dashed line. B. Replicates: The % inhibition measured for each of the duplicate library wells is plotted against its paired sample and shows a linear response ($r^2 = 0.984$), indicating good reproducibility.

Figure 4. Counter-screen of Primary Hits. Concentration-response plots are shown for two representative primary hits, **1** (A) and **10** (B). Assay results for NDM-1 (●) or TEM-1 (○) are fit to determine IC_{50} values, Hill coefficients, and maximum percent inhibition (see Table 1).

Figure 5.

Primary Zinc Ligands of NDM-1. A structure of NDM-1 (grey) bound to hydrolyzed ampicillin (light blue) is shown. The primary zinc ligands conserved throughout the B1 family of metallo-beta-lactamases. Notably, Zn2 bridges between Cys208 and the product carboxylate. The figure was prepared using coordinates from protein databank accession code 3Q6X²³ and the molecular visualization program UCSF Chimera³⁴.

Figure 6. Susceptibility of NDM-1 Cys108 Variants to Inactivation by p-CMB (**1**). Treatment of wild-type NDM-1 by 0 (●, dashed line) or 100 μ M p-CMB (**1**) (○, solid line), indicates time-dependent inactivation of NDM-1 activity. In contrast, treatment of C108D by 0 (□, dashed line) or 100 μ M p-CMB (**1**) (■, solid line), does not show time-dependent inactivation. Each is fit to an exponential decay.

Table 1

HTS and Counter-Screen for Top Hits in the Primary NDM-1 Inhibition Assay

#	Structure	Name	NDM-1			TEM-1			LC-MS % Purity
			IC ₅₀ (μ M)	Max % Inhibit	Hill	IC ₅₀ (μ M)	Max % Inhibit	Hill	
1		p-CMB	2.3	84 $\pm$ 6	1.7	> 30	8 $\pm$ 5	ND	95
2		Sodium nitroprusside dihydrate	9.0	70 $\pm$ 1	2.6	> 30	3 $\pm$ 1	ND	98
3		Dipyridamole	> 10	261 $\pm$ 7	ND	> 30	26 $\pm$ 1	ND	94
4		SB 216763	28	57 $\pm$ 1	1.7	> 30	2 $\pm$ 1	ND	95
5		Ellipticine	> 30	ND	ND	> 30	ND	ND	99
6		R(+)-Lisuride hydrogen maleate	> 30	28 $\pm$ 2	ND	> 30	6 $\pm$ 1	ND	96

#	Structure	Name	NDM-1			TEM-1			LC-MS % Purity
			IC ₅₀ (μ M)	Max % Inhibit	Hill	IC ₅₀ (μ M)	Max % Inhibit	Hill	
7		S-(-)-Lisuride L-122	> 30	7 $\pm$ 2	ND	> 30	4 $\pm$ 1	ND	97
8		R(+)- Terguride T-165	> 30	ND	ND	> 30	ND	ND	99
9		Idarubicin I 1656	0.7	590 $\pm$ 10	1.1	7.3	100 $\pm$ 20	1.4	99
10		4-Amino-1,8- naphthalimide A 0966	0.7	409 $\pm$ 8	0.9	5.2	131 $\pm$ 4	0.9	95

Table 2

Kinetic Constants for Nitrocefin Hydrolysis and Zinc Content for C108X Variants of NDM-1

Variant	[Zinc] / [Protein]	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1} s^{-1}$)
WT ^a	1.8	1.3 ± 0.2	15.4 ± 0.4	$1.2 \cdot 10^7$
Strep-WT ^b	1.8	1.1 ± 0.3	12.6 ± 0.7	$1.1 \cdot 10^7$
C208D	1.9	0.9 ± 0.1	7.0 ± 0.2	$8.2 \cdot 10^6$
C208S	1.5	16 ± 1	4.1 ± 0.2	$2.5 \cdot 10^5$
C208H	1.0	> 20	0.1	$6 \cdot 10^3$

^aData for wild-type (WT) NDM-1 is taken from reference (12).^bStrep stands for an *N*-terminal eight-amino acid *StrepII* affinity tag fusion