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Graphical Abstract

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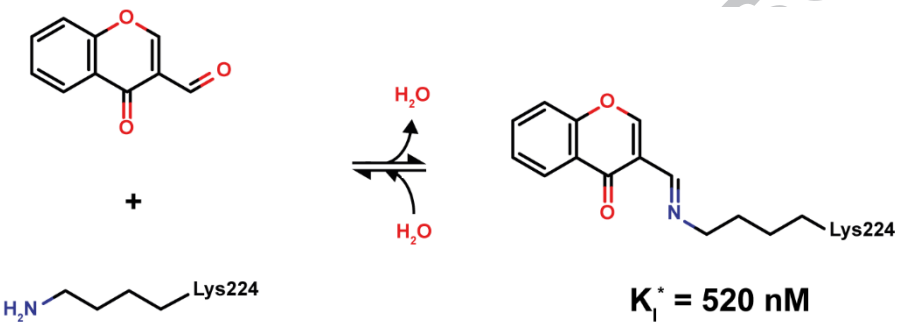

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Discovery of a novel covalent non-β-lactam inhibitor of the metallo-β-lactamase NDM-1

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ABSTRACT

The inhibition of metallo-β-lactamases (MBL) can prevent the hydrolysis of β-lactam antibiotics and hence is a promising strategy for the treatment of antibiotic resistant infections. In this study, we present a novel reversible covalent inhibitor of the clinically relevant MBL New Delhi metallo-β-lactamase 1 (NDM-1). Electrospray ionization-mass spectrometry (ESI-MS) and single site directed mutagenesis were used to show that the inhibitor forms a covalent bond with Lys224 in the active site of NDM-1. The inhibitor was further characterized using an enzyme inhibition assay, a surface plasmon resonance (SPR) based biosensor assay and covalent docking. The determined inhibition constant (K_I^*) was 520 nM and the inhibition constant for the initial complex (K_I) was 76 μM. To our knowledge, this inhibitor is the first example for a reversible covalent non-β-lactam inhibitor targeting NDM-1 and a promising starting point for the design of potent covalent inhibitors.

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1. Introduction

β-Lactam antibiotics are an important class of antibacterial agents and widely used in the treatment of bacterial infections. Therefore, the rapidly increasing number of reported β-lactamases, enzymes able to hydrolyze β-lactam antibiotics and render them ineffective, is a major threat for global public health.[1] β-Lactamases can be divided into two groups, metallo-β-lactamases (MBLs) and serine-β-lactamases (SBLs). The inhibition of β-lactamases is a promising strategy to prevent the hydrolysis of β-lactam antibiotics. For some SBLs, this strategy has been proven successful and SBL inhibitors are already in clinical use. Although several MBL inhibitors have been identified, none of them has reached clinical use so far and hence there is an urgent need for the development of novel inhibitors for this class of enzyme. [2-5]

The New Delhi metallo-β-lactamase (NDM-1) is one of the most abundant MBLs.[6] The enzyme has broad hydrolytic activity, providing bacteria with resistance to almost all β-lactam antibiotics.[7] As most MBLs, NDM-1 is folded in a $\alpha\beta/\beta\alpha$ sandwich with two zinc ions located in the active site.[8] The development of novel inhibitors targeting NDM-1 is of high clinical interest and can help to treat antibiotic resistant infections.

Affinity labels or covalent inhibitors, compounds forming a covalent complex with a drug target, are normally disfavored in drug discovery programs due to concerns about their toxicity and potential side effects.[9] In contrast, 30% of the drugs on the market are covalent inhibitors.[10] Covalent inhibitors have distinct advantages compared to noncovalent inhibitors, e.g. increased ligand efficiencies (LE) and decreased sensitivity to drug resistant mutations, making them interesting as potential MBL inhibitors.[9, 11] The binding of a covalent inhibitor to its target can be described by a two-step binding model as shown in Eq 1.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EI^* \qquad (1)$$

In the first step, the inhibitor rapidly binds to the enzyme forming a non-covalent complex and placing the reactive group in the position to form, in a second step, the covalent bond with the target. The reverse reaction, breaking of the covalent bond, is often much slower than the formation, leading to a slow establishment of the binding equilibrium. Therefore, covalent inhibitors often show a time-dependent inhibition, where the inhibition increases over time, also the inhibitor concentration is constant.[9, 10, 12] Avibactam, a covalent β-lactamase inhibitor, has been recently approved by the U.S. Food and Drug Administration (FDA) for the treatment of antibiotic resistant infections, illustrating the high potential of covalent inhibitors.[13] However, avibactam only inhibits SBLs, but not MBLs. Although several covalent inhibitors for MBLs have been reported, none of them has reached clinical trial. [14-17]

In this study, a novel covalent inhibitor for the clinically relevant MBL NDM-1 was identified. The inhibitor was characterized by elucidating the targeted amino acid in the active site of the enzyme and determining the inhibition constant as well as the rate constant for the bond formation. Furthermore, covalent docking was used to explore the interactions involved in the binding of the inhibitor.

2. Material and Methods

2.1. General procedures

Chemicals and solvents were purchased from standard suppliers and used without further purification. 3-Cyanochromone (**1**) was purchased from Maybridge (Cambridge, UK) and 3-formylchromone (**2**) from Sigma-Aldrich. The class B β-lactamase numbering system is used throughout this paper for the numbering of all residues.[18]

2.2. Protein expression, purification and site directed mutagenesis

Wild type NDM-1 was expressed and purified as previously described.[19] In brief, the gene sequence coding for the residues G29 to R294 was amplified from *Klebsiella pneumonia* and the gene sequences for a 6xHis-tag and a tobacco etch virus (TEV) protease cleaving site were added. The gene construct was transferred into a pDEST14 vector used to transform chemically competent *Escherichia coli* BL21 (DE3)pLysS cells (Invitrogen). The protein was purified by immobilized metal ion affinity chromatography (IMAC) and the His-tag was removed using TEV-protease.

The NDM-1 K224A mutant was generated by using the QuikChange site-directed mutagenesis kit (Agilent Bioscience), the NDM-1 pDEST14 plasmid as a template and the following primers: NDM-1 K224A fwd 5'- TTT GGT GGC TGC CTG ATC GCG GAC AGC -3' and NDM-1 K224A rev 5'- GCT GTC CGC GAT CAG GCA GCC ACC AAA -3' (the mutated nucleotides are underlined). DpnI was used to remove the parental DNA and XL-1 Blue cells transformed with the plasmid. The construct was isolated using the Wizard plus SV minipreps DNA purification system (Promega) and the mutation was confirmed by sequencing using the BigDye Terminator cycle sequencing kit (Applied Biosystems). For protein expression, *Escherichia coli* BL21 (DE3)pLysS cells (Invitrogen) were transformed with the construct and cultured in 500 mL Terrific broth (TB) medium supplied with 100 mg/L ampicillin and 34 mg/L chloramphenicol. Protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cells were harvested by centrifugation after 7 h at 20°C. Purification of the NDM-1 K224A mutant was carried out as described for the NDM-1 wild type.[19] The removing of the 6xHis-tag from wild type NDM-1 and NDM-1 K224A using the TEV-protease leaves an additional glycine residue at the N-terminus prior to G29.

2.3. Mass spectrometric analysis

Mass spectrometric (MS) analyses were performed on a Xevo G2 Q-TOF (Waters) equipped with an electrospray ion source and coupled to a Acquity UPLC I-Class system (Waters). MassLynx V4.1, mMass Version 5.5.0 [20] and ESIprot 1.0 were used for data evaluation.

NDM-1 wild-type and NDM-1 K224A were incubated for 3 h at room temperature with 1.5 mM of compound **2**. The buffer during incubation consisted of 50 mM HEPES, pH 7.2, 150 mM NaCl, 100 µM ZnCl and 1% DMSO. After incubation, the excess of compound **2** was removed and the buffer changed to water using a Amicon ultra centrifugal filter (Merck Killipore Ltd.) with a molecular cut-off of 3 kDa. Samples were further diluted with water to a final concentration of 100 μg/mL protein prior to the mass spectrometric analysis. Mass spectra were recorded by direct injection of a 10 μL sample using a binary solvent system (water/acetonitrile with 0.1% formic acid, 70:30, v/v) at a flow rate of 0.5 mL/min. Soft ionization conditions (Supporting

material, Table S1) yielded mass spectra of the whole protein. Hard ionization conditions (Supporting material, Table S1) were used to fragment the protein in the ion source and selected fragments (NDM-1: *m/z* 774.4 (9+), 871.1 (8+), 995.352 (7+) and NDM-1 compound **2** adduct: *m/z* 791.8 (9+), 890.7 (8+), 1017.7 (7+)) were further analyzed by tandem mass spectrometry (MS/MS). Parameters used for MS/MS experiments are included in supporting material (Table S1).

Tryptic digestion of NDM-1 and the NDM-1 compound **2** adduct were carried out as following: Protein samples were diluted to the concentration 1 mg/mL and urea was added to reach a final concentration of 8 M. Sample aliquots of 1 mL were desalted on Pierce™ polyacrylamide desalting columns (1.8kDa MWCO, Thermo Fisher Scientific) and the buffer exchanged to 50 mM ammonium bicarbonate. Trypsin was added to the protein sample in a 1:30 (trypsin/protein) ratio and the sample was incubated at 37 ºC for 90 min. The digestion process was stopped by adding 10% formic acid to a final concentration of 0.15%.

2.4. Surface plasmon resonance (SPR) based competition studies with meropenem and captopril

Surface plasmon resonance (SPR) experiments were conducted as described by Christopeit *et al.*[19] The experiments were performed using a Biacore T200 (GE Healthcare) and the data analyzed using the Biacore T200 evaluation software (GE Healthcare). NDM-1 was immobilized using streptavidin-biotin capturing. The buffer used in all competition experiments consisted of 100 mM HEPES, pH 7.2, 100 μM $ZnCl_2$, 0.005% surfactant P-20 and 2.5% DMSO.

For competition experiments, captopril (N-[(S)-3-Mercapto-2-methylpropionyl]-L-proline, Sigma-Aldrich), meropenem (Sigma-Aldrich) and compound **2** were each dissolved in buffer at a concentration of 250 μM. At first, meropenem and captopril were successively injected for 1 min, followed by a 5 min injection of compound **2**, and again a successive injection of meropenem and captopril. The sensor surface was flushed for 12 h with buffer and binding of meropenem and captopril was tested again.

2.5. Molecular modelling

The Schrödinger Small-Molecule Drug Discovery Suite 2015-1 (Schrödinger, LLC) was used for the molecular modelling. The NDM-1 structure (PDB: 4HL2, Chain: A) was downloaded from the Protein Data Bank (PDB) and prepared using the protein preparation wizard implemented in Maestro (v. 10.1, Schrödinger, LLC) applying standard defaults settings. The water molecule bridging the two zinc ions was replaced by a hydroxide ion. All other water molecules and the ligand were removed. CovDok [21], part of the Schrödinger Small-Molecule Drug Discovery Suite 2015-1 (Schrödinger, LLC), was used for covalent docking of compound **2** into the active site of NDM-1. Standard default settings were applied and the docking grid centered between the two zinc ions. Imine condensation was used as reaction type and Lys224 was defined as the reactive residue.

2.6. Enzyme inhibition

Enzyme activity measurements were carried out as described before using a Spectra MAX M2^e (Molecular Devices).[19] The SoftMax Pro 5.2 (Molecular Devices) software and GraphPad Prism 5 (GraphPad Software) were used for data analysis. All measurements were performed at 25 °C in buffer consisting of 100 mM HEPES, pH 7.2, 150 mM NaCl, 100 μM $ZnCl_2$, 0.005 % surfactant P-20, 20 μg/ml bovine serum albumin (BSA) and 2.5 % DMSO.

NDM-1 was incubated with compound **2** for 1 min, 5 min, 11 min, 21 min or 31 min. After the incubation, imipenem (MSD) was added to a final concentration of 100 μM and hydrolysis was followed by measuring the absorption at 300 nm every 17 s. The enzyme activity in % was calculated from the initial velocity compared to a control without compound **2**. The concentrations of compound **2** ranged from 0.5 to 250 μM and the final NDM-1 concentration was 1 nM. The observed rate constant (k_{obs}) for every inhibitor concentration was calculated from the slope of a semi-logarithmic plot of the enzyme activity against the time. The data were analyzed using a two-step binding model (Eq. 1). The determined k_{obs} were plotted against the imipenem concentration and Eq. 2 was used to derive the inhibition constant for the initial non-covalent complex (K_I; Eq. 3), the rate constant for the bond formation (k_2), and the rate constant for the bond breaking (k_{-2}).[12, 22] The K_m for imipenem was previously determined by us to be 100 μM.[19] The inhibition constant (K_I^*) was calculated using Eq. 4.

$$k_{obs} = k_{-2} + k_2 \frac{[I]}{K_I\left(1 + \frac{[S]}{K_m}\right) + [I]} \quad (2)$$

$$K_I = \frac{k_{-1}}{k_1} \quad (3)$$

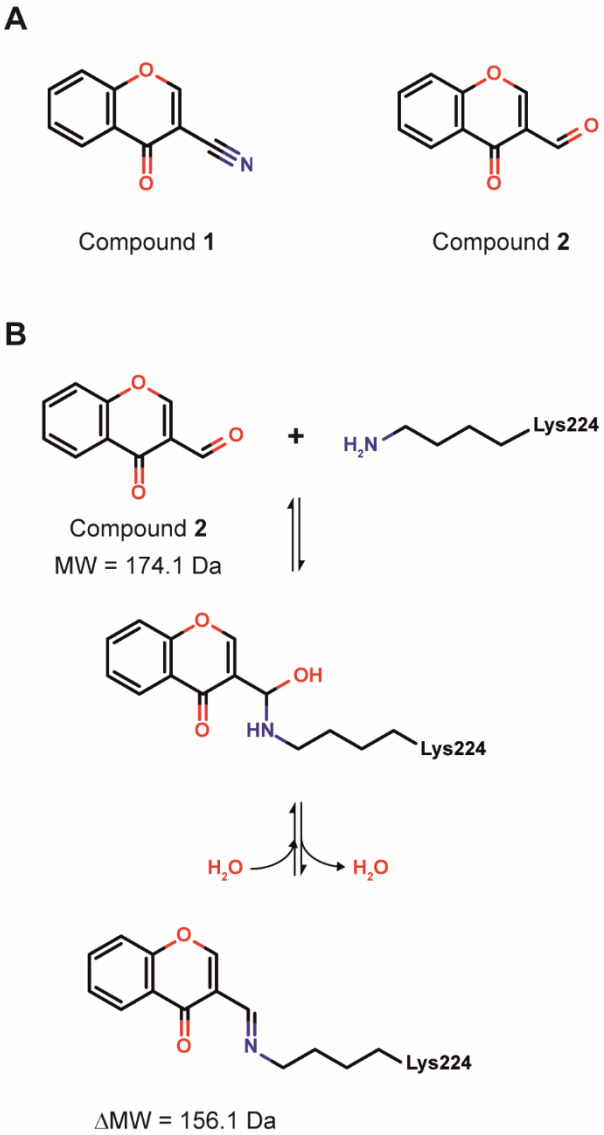

Figure 1: (A) Structure for compound **1** and compound **2**. (B) Proposed mechanism for the covalent inhibition of NDM-1 by compound **2**.

$$K_I^* = \frac{K_I\, k_{-2}}{k_2 + k_{-2}} \qquad (4)$$

Steady-state kinetics were used to calculate k_{cat}/K_m for hydrolysis of imipenem by the NDM-1 K224A mutant. Imipenem was dissolved in buffer in a 2-fold concentration series resulting in a final concentration ranging from 7.8 to 1000 μM. The enzyme was added to a final concentration of 10 nM and hydrolysis was followed by measuring the absorption at 300 nm every 17 s. The initial velocity was determined and plotted against the concentration. From the slope of a linear fit k_{cat}/K_m was calculated. The activity assay was used to determine the inhibition of NDM-1 K224A by compound **2**. The enzyme was incubated for 20 min with concentrations of up to 750 μM compound **2**. The reaction was started by adding imipenem to a final concentration of 100 μM. The final enzyme concentration was 10 nM.

3. Results and Discussion

3.1. Identification of 3-formylchromone as NDM-1 inhibitor

In an previous study, we used a fragment based screening strategy to identify 3-cyanochromone (**1**; Figure 1) as a competitive inhibitor of NDM-1 and promising starting point for the design of potent inhibitors.[19] To further optimize the inhibitor, several analogs were tested leading to the identification of 3-formylchromone (**2**; Figure 1**)** as an inhibitor with an improved affinity. The IC_{50} value for compound **2** showed a strong time-dependency changing from around 200 μM with no incubation time to around 2 μM after 10 min incubation. A time-dependent inhibition is characteristic for Pan Assay Interference Compounds (PAINS) and typically indicates an unspecific inhibition mode like slow protein denaturation, oxidation of amino acid side chains or chelation of important metal cofactors.[23, 24] However, a time-dependent inhibition is also a characteristic of covalent inhibitors or affinity labels. Previously, we have used molecular docking to investigate the interactions involved in the binding of compound **1** to NDM-1.[19] The results show that the nitrile group of compound **1** interacts with the amine group in the side chain of Lys224 in the active site of the enzyme. In compound **2**, the nitrile group is replaced by an aldehyde group. Due to the high similarity between compound **1** and **2**, it can be assumed that the aldehyde group also interacts with the amine group of Lys224. It is well known, that an aldehyde can form a Schiff base with the amine group of a lysine side chain.[25] Hence, we proposed that compound **2** inhibited NDM-1 by forming a covalent bond with the side chain of Lys224 as shown in Figure 1.

3.2. Confirmation of the covalent inhibition mode by ESI-MS

MS is a frequently used method to study the interaction of covalent inhibitors with enzymes. In this study, we implemented electrospray ionization mass spectrometry (ESI-MS) to obtain detailed information about the inhibition mode of compound **2** and confirm the suggested covalent inhibition mechanism. At first, the purified native NDM-1 was analyzed by ESI-MS. The deconvoluted mass spectrum showed a single signal with a mass of 25733.1 ± 0.7 Da (Figure 2A). The theoretical mass of native NDM-1 is 25663 Da (GG29 – R294) and thereby smaller than the experimentally determined mass. NDM-1 has two high affinity Zn^{2+} binding sites and binding of both Zn^{2+} is important for the activity of the enzyme.[8, 26] The increased experimental mass indicates that only one Zn^{2+} (65.4 Da) was bound to the enzyme. However, the purified NDM-1 was active and able to hydrolyze imipenem.[19] Hence it can be assumed that two zinc ions were present in the active site of the enzyme and that the observed monozinc NDM-1 was an artefact from changing the zinc supplemented buffer to water prior to the MS analysis.

In a second experiment, purified NDM-1 was incubated with an excess of compound **2**. After 3 h, compound **2** was removed by centrifugal ultrafiltration, the buffer changed to water and the enzyme analyzed by ESI-MS. Two distinct signals were observed in the deconvoluted mass spectrum (Figure 2B). The first signal had a mass of 25733.8 ± 0.8, corresponding to native NDM-1, whereas the second signal had a mass of 25890.3 ± 0.8 Da. The mass difference between both signals was 156.5 ± 1.1 Da, corresponding to the mass added to NDM-1 by the reaction of compound **2** with a primary amine in the protein (Figure 1). Hence, it can be concluded that the second signal corresponds to NDM-1 modified by the reaction with compound **2**. The fact, that only one additional signal was observed, makes it likely that compound **2** reacts with a specific amine group in the protein. However, it cannot be completely excluded that compound **2** forms covalent bonds with further amine groups, not stable enough to be detected. To obtain more information about which amine group reacts with compound **2**, the modified enzyme was digested with Trypsin and the resulting tryptic peptides analyzed by ESI-MS. The data evaluation revealed that the NDM-1 compound **2** adduct is not stable under the conditions used for digestion (data not shown). The formation of Schiff bases is a reversible reaction.[25] Therefore, it is likely that the digestion of the protein and thereby the removing of stabilizing non-covalent interactions between compound **2** and NDM-1 lead to a rapid release of the inhibitor. The high reversibility of the covalent binding of compound **2** is also reflected by the fact that modified and unmodified NDM-1 existed in equilibrium.

A different approach using tandem mass spectrometry (MS/MS) was chosen to characterize the binding site of compound **2**. Native NDM-1 and the NDM-1 compound **2** adduct

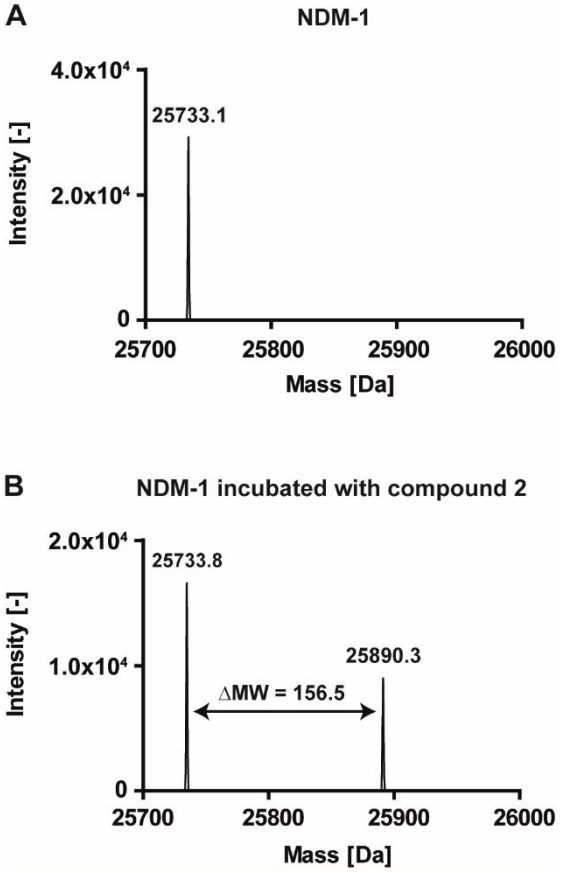

Figure 2: Deconvoluted mass spectrum for (A) native NDM-1 and (B) NDM-1 after incubation with compound **2**. Raw data is shown in the supplementary material (Figure S1).

were fragmented under hard ionization conditions producing fragment 1 with 6960.5 Da and fragment 2 with 7116.9 Da, respectively (ΔMW = 156.4 Da; Supplementary material, Figure S3). The fragments originate from the latter end of the enzyme. Three different charge states of each fragment were selected and further fragmented with MS/MS to characterize their amino acid sequence and the binding site of compound **2**. Data evaluation of characteristic immonium ions originating from amino acids showed that compound **2** binds to a lysine in the enzyme (Supplementary material, Table S2 and Table S4) with possible binding sites at Lys224, Lys227, Lys229, Lys255, and Lys292. Additional MS/MS spectra indicate that the binding site is located at Lys224 (Supplementary material, Tables S3 and Table S5).

3.3. Investigation of the influence of Lys224 on the covalent inhibition

Site-directed mutagenesis was used to replace the Lys224 by an alanine in the active site of NDM-1. The NDM-1 K224A mutant was enzymatic active and hydrolyzed imipenem (Figure 3A). Due to the increased K_m compared to the wild type and the background signal at high imipenem concentration, saturation was not obtained and only k_{cat}/K_m was calculated with $5.7*10^4$ $s^{-1}M^{-1}$. However, the results indicate that NDM-1 K224A had a reduced imipenem affinity and an increased k_{cat}. Similar changes in the kinetics for the hydrolysis of nitrocefin have been observed for the NDM-1 K224A mutant by Thomas *et al*.[14] Lys224 has been shown to participate in the substrate binding of NDM-1 and hence, it is not surprising that K_m for imipenem was increased in the K224A mutant.[8] However, the increase in k_{cat} might be explained by a faster product release after the hydrolysis of imipenem.

To further confirm the mass spectrometric results indicating that compound **2** reacted with Lys224, the NDM-1 K224A mutant was incubated with an excess of compound **2** and analyzed by ESI-MS (Figure 3B). The deconvoluted mass spectrum showed only a single signal with a mass of 25676.2 ± 0.8 Da corresponding to the theoretical mass of the NDM-1 K224A mutant (25671 Da; GG29 - R294 and one Zn^{2+}). No additional signals corresponding to NDM-1 K224A modified by compound **2** were observed. Furthermore, compound **2** had no influence on the hydrolysis of imipenem up to a concentration of 750 μM (data not shown). The fact that compound **2** neither covalently bound to NDM-1 K224A nor has an influence on the enzyme activity shows that the inhibition is specific to the

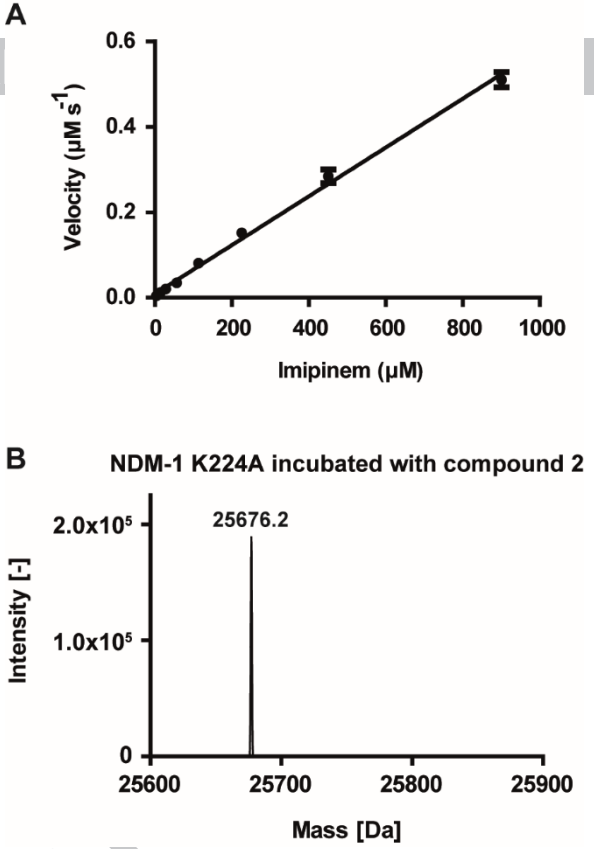

Figure 3: (A) Steady-state kinetics for the NDM-1 K224A mutant hydrolyzing imipenem. (B) Deconvoluted mass spectrum for NDM-1 K224A after incubation with compound **2**. Raw data is shown in the supplementary material (Figure S2).

reaction with Lys224 and not due to the reaction with other amine groups available in the protein.

3.4. Molecular modelling

Molecular modelling was used to explore the interactions involved in the binding of compound **2**. The CovDock algorithm (Schrödinger, LLC) has been shown to successfully predict the binding mode of covalent inhibitors[21] and was applied to dock compound **2** in to the structure of NDM-1 (PDB ID: 4HL2, Chain A). Only a single docking pose was obtained from the algorithm (Figure 4), showing that the carbonyl group of the fragment formed an interaction with Zn2 (D120, C221, H263). Furthermore, compound **2** showed hydrophobic interactions with Trp87, Ile35 and Val67.

3.5. Competition studies with meropenem and captopril using an SPR based biosensor assay

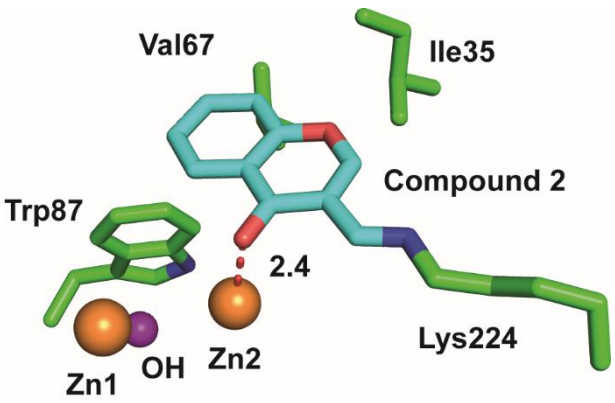

Figure 4: Compound **2** docked to the structure of NDM-1 (PDB ID: 4HL2). NDM-1 carbon atoms are shown in green and carbon atoms of compound **2** in blue. Zn2+ are shown in orange and the hydroxide ion in purple. Interatomic distances are given in Å.

An SPR based biosensor assay for NDM-1 was previously established and used to characterize the interactions with meropenem and captopril.[19] The low molecular weight of compound **2** and the covalent inhibition mode made it difficult to use the assay to directly investigate the interaction of compound **2** with NDM-1. However, to obtain more information about the binding place of compound **2**, the assay was used to carry out competition experiments with meropenem, a substrate of NDM-1, and captopril, a well-characterized MBL inhibitor. NDM-1 was immobilized to the sensor surface and enzyme activity was confirmed by the binding of captopril or meropenem. After compound **2** was injected over the surface, the binding of meropenem was strongly reduced clearly showing that both compounds compete for the same binding site (Figure 5A). It is known that the two zinc ions in the active site as well as Lys224 are important for the meropenem binding.[27] Hence, it is not surprising that the covalent binding of compound **2** prevents meropenem from binding to the active site of NDM-1. Washing the SPR sensor surface for 12 h with buffer lead to an almost complete recovery of the meropenem binding showing that the covalent binding of compound **2** was reversible. In contrast, the binding of captopril was only slightly influenced by the binding of compound **2** (Figure 5B). Captopril binds to the active site of NDM-1 whereby the sulfhydryl group of the inhibitor is located between the two Zn ions and the carboxyl group interacts with Asn233.[27] As indicated by the molecular modeling for compound **2**, both positions are still accessible after the covalent binding of compound **2**. Hence, it can be assumed that captopril and compound **2** can bind at the same time to the active site of NDM-1.

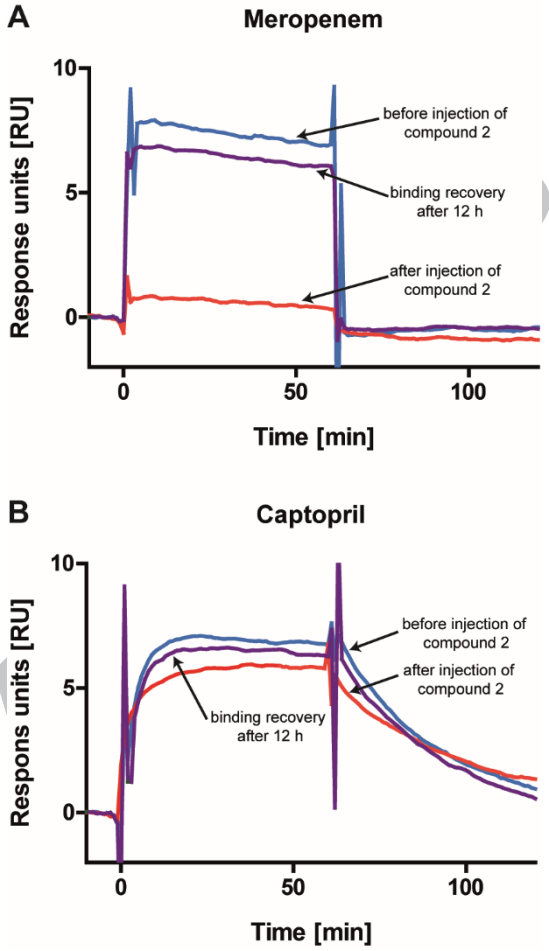

Figure 5: Sensorgrams for the interaction of (A) meropenem and (B) captopril with native NDM-1 (blue), after the injection of compound **2** (red) and after washing the surface with buffer for 12 h (purple).

3.6. Kinetic characterization of the covalent inhibition

Due to the time-dependent inhibition and the slow association of covalent inhibitors, determination of IC_{50} values are of low value to characterize covalent inhibitors.[9] Therefore, the covalent inhibitor was characterized by determining the rate constants for the different reaction steps. A previously established enzyme activity assay[19] using imipenem as a reporter substrate was used to determine the inhibition of NDM-1 at different concentrations of compound **2** and after various incubation times. The observed rate constants (k_{obs}) were calculated and plotted against the inhibitor concentration (Figure 6). The plot showed a saturation at higher concentrations and the data were well fitted by a two-step binding model. The inhibition constant for the initial non-covalent complex K_I was calculated to be 76 ± 9 μM, showing that the binding affinity increased compared to compound **1** (K_D = 181 μM).[19] The rate constant for the bond formation k_2 was determined to be 6.5 ± 0.4*10^{-3} s^{-1} and the rate constant for the bond breaking k_{-2} to be 5 ± 3*10^{-5} s^{-1}. The inhibition constant K_I^* was calculated from the determined values to be 520 nM.

3.7. Evaluation of the compound 2

The ligand efficiency (LE) is a widely used metric in drug discovery, normalizing the affinity of an inhibitor to the molecular size. Normally, inhibitors with a LE below 0.3 kcal/mol per heavy atom are rejected and not considered for a further optimization.[28] The LE of compound **2** was calculated to be 0.66 kcal/mol per heavy atom and clearly exceeds this cut-off.

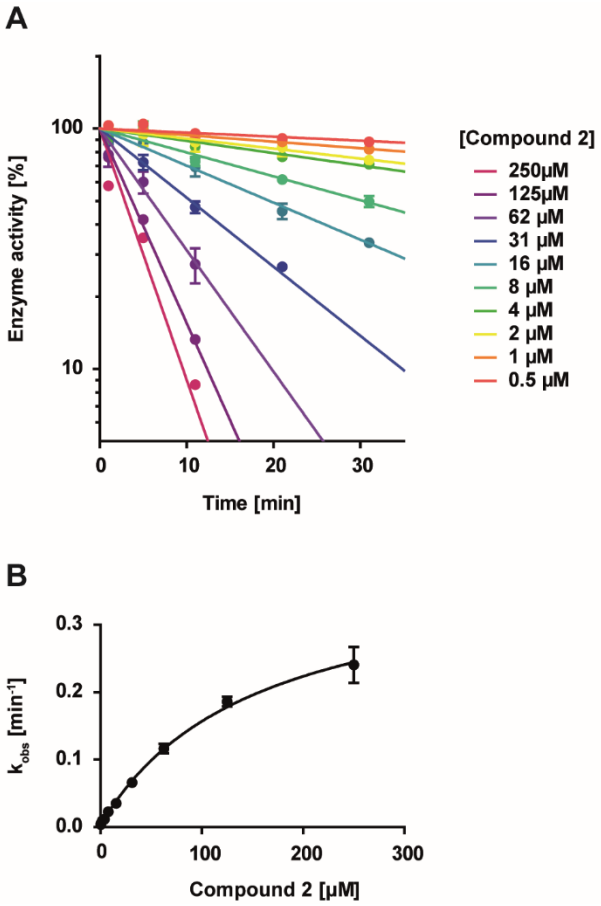

Figure 6: (A) Time-dependent inhibition of NDM-1 by compound **2**. The observed rate constant (k_{obs}) for every concentration was determined from the slope of a linear fit (solid lines). (B) The calculated k_{obs} plotted against the concentration of compound **2**. The data were fitted to Eq. 2 (black line)

Several covalent inhibitors targeting NDM-1 have been recently reported.[14-17] However, compound **2** is the first example of a reversible covalent MBL inhibitor. Such inhibitors are thought to have less toxic side effects compared to irreversible covalent inhibitors.[29] Therefore, compound **2** is a promising starting point for the design of clinical relevant MBL inhibitors. Additionally, compound **2** has a potential as tool compound to characterize MBLs.

For a potential clinical use, compound **2** has to be further optimized. Aldehydes are normally avoided in drug discovery due to their reactivity and susceptible metabolic oxidation. Hence, suitable reactive groups have to be explored, which can replace the aldehyde group in compound **2**. Furthermore, comparing the determined K_I^* and the determined rate constants with similar values reported for avibactam,[30, 31] a recently clinically approved covalent inhibitor for SBLs, shows that these values have to be further optimized. The binding of captopril in close contact to compound **2** might offer an interesting strategy for a further optimization since merging both molecules could lead to a significant increase in the binding affinity.

A clinically relevant inhibitor should inhibit a broad spectrum of MBLs. So far, it has been difficult to design such inhibitors, mainly due to differences in the active sites of MBLs. The influence of compound **2** on the activity of the Verona integron-encoded metallo-β-lactamases 2 (VIM-2) was investigated, but no enzyme inhibition was observed (data not shown). In VIM-2, the Lys224 in the active site of NDM-1 is replaced by a tyrosine. Additionally, VIM-2 has an arginine in positions 228, forming similar substrate interactions as the Lys224. Hence, it was not surprising that VIM-2 was not inhibited by compound **2.** However, other MBLs with the relevant lysine in the active site (e.g., IMP-1) might be inhibited by compound **2**. Therefore, compound **2** can be interesting for the design of broad-spectrum inhibitors targeting MBLs with an active site lysine.

4. Conclusion

To our knowledge, the study presents for the first time a reversible, covalent, non-β-lactam inhibitor targeting the clinically relevant MBL NDM-1. Although the inhibitor has to be further optimized for a therapeutic use, the reversible covalent inhibition mechanism makes the inhibitor interesting for the design of potent inhibitors which can help to treat antibiotic resistant infections. The high potential of reversible, covalent inhibitors for the treatment of antibiotic resistant infection is also reflected in the recent approval of avibactam, a reversible covalent inhibitor of SBLs.

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Supplementary Material

Supplementary data can be found at the publications website.

Author Contributions

Conceived and designed the experiment: HKSL TC AA. Performed the experiments: TC AA. Analyzed the data: TC AA HKSL. Wrote the paper: TC HKSL AA. All authors have given approval to the final version of the manuscript.

Abbreviations

MBL, metallo-β-lactamases; SBL, serine-β-lactamses; NDM-1, New Delhi metallo-β-lactamase; SPR, Surface plasmon resonance; MS, mass spectrometry; TEV, tobacco etch virus; IMAC, immobilized metal ion affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; MS, Mass spectrometric; ESI, electrospray ionization; LE, ligand efficiency; tandem mass spectrometry, MS/MS;

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