Synthesis and Preclinical Evaluation of TPA-Based Zinc Chelators as Metallo-\(\beta\)-lactamase Inhibitors

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Abstract: The rise of antimicrobial resistance (AMR) worldwide and the increasing spread of multi-drug-resistant organisms expressing metallo-\(\beta\)-lactamases (MBL) require the development of efficient and clinically available MBL inhibitors. At present, no such inhibitor is available, and research is urgently needed to advance this field. We report herein the development, synthesis, and biological evaluation of chemical compounds based on the selective zinc chelator tris-picolylamine (TPA) that can restore the bactericidal activity of Meropenem (MEM) against Pseudomonas aeruginosa and Klebsiella pneumoniae expressing carbapenemases Verona integron-encoded metallo-\(\beta\)-lactamase (VIM-2) and New Delhi metallo-\(\beta\)-lactamase 1 (NDM-1), respectively. These adjuvants were prepared via standard chemical methods and evaluated in biological assays for potentiation of MEM against bacteria and toxicity (IC\(\text{50}\)) against HepG2 human liver carcinoma cells. One of the best compounds, 15, lowered the minimum inhibitory concentration (MIC) of MEM by a factor of 32–256 at 50 \(\mu\)M within all tested MBL-expressing clinical isolates and showed no activity toward serine carbapenemase expressing isolates. Biochemical assays with purified VIM-2 and NDM-1 and 15 resulted in inhibition kinetics with \(k_{\text{act}}/K_i\) of 12.5 \(\text{min}^{-1} \text{mM}^{-1}\) and 0.500 \(\text{min}^{-1} \text{mM}^{-1}\), respectively. The resistance frequency of 15 at 50 \(\mu\)M was in the range of 10\(^{-7}\) to 10\(^{-9}\). 15 showed good tolerance in HepG2 cells with an IC\(\text{50}\) well above 100 \(\mu\)M, and an in vivo study in mice showed no acute toxic effects even at a dose of 128 mg/kg.

Keywords: antimicrobial resistance, zinc chelator, metallo-\(\beta\)-lactamase inhibitor, enzyme inhibition kinetics, resistance frequency, toxicity

Antibiotics are considered to be one of the cornerstones of modern medicine.\(^1\) The rising threat of antimicrobial resistance\(^2,3\) (AMR), however, has become a global public health challenge that has been accelerated by the overuse of antibiotics worldwide\(^4,5\) as well as other factors.\(^6,7\) AMR results in serious and more complicated infections, which leads to longer hospital stays and increased mortality.\(^8\) Few genuinely new antibiotics have been introduced during the past few decades,\(^9\) demanding...
research and development as well as international initiatives to raise awareness and fight the threat of AMR.20

The β-lactamases are one of the most important bacterial defense systems against β-lactam antibiotics.11,12 These enzymes are classified according to sequence criteria (Ambler classes A–D) and can be structurally grouped into two superfamilies; the serine β-lactamases (classes A, C, and D) and metallo-β-lactamases (MBLs, class B).13–15 MBLs require divalent zinc ions as a metal cofactor for enzyme activity16 and are emerging as one of the most clinically important families of β-lactamases.17 The IMP- (Imipenemase), VIM- (Verona integron-mediated metallo-β-lactamase), and NDM- (New Delhi metallo-β-lactamase) groups are now widespread in a variety of gram-negative species,18,19 and since first being reported in 2008, NDM has spread globally.20,21 Furthermore, MBLs are able to inactivate almost all β-lactam antibiotics, including the carbapenems, which are among the “last resort antibiotics” used in clinics.22 Recent research afforded inhibitors of serine β-lactamases;23 however, no clinically available inhibitor against MBLs currently exists, despite ongoing research.24,25

The mechanism by which β-lactam antibiotics are inactivated by MBLs is based on the hydrolysis of the β-lactam ring catalyzed by a Zn2+-coordinated water molecule in the active site of the enzymes.19 Zinc chelators could potentially be used as adjuvants26 to inhibit MBLs by the removal of zinc27 and thus restore the antibacterial activity of β-lactam antibiotics. A high degree of selectivity for zinc chelation would ensure the inhibition of MBLs without affecting host metalloenzymes.28

The aim of this study is to further increase the scope and chemical diversity of chelators with potential application as MBLs inhibitors. We have developed and studied compounds based on the tris-picolylamine (TPA) scaffold31 (the chelator), something that modulates the physicochemical properties of the combination (modulator), and a linker that connects the two (Figure 1). We evaluated the biological activity in combination

However, we also explored other small peptides as modulators in order to change the solubility of the constructs. The straightforward chemical synthesis of our putative inhibitors facilitates varying each of the three main components (Figure 1).

### RESULTS AND DISCUSSION

**Chemical Synthesis.** To synthesize a variety of TPA analogs, we started with the preparation of building blocks based on the TPA scaffold, which were then further functionalized using standard chemical transformations. The synthesis of the key fragment, TPA methyl ester (3), is shown in Scheme 1 and was previously reported by starting from the methyl nicotinate 1.36 Methyl(6-bromomethyl)nicotinate 2 was commercially acquired from BocSci (Shirley, USA) and used as received for amination with dipicolylamine in the presence of DIPEA in THF to afford the TPA methyl ester (3) after precipitation from cold Et2O as a pale-yellow solid. This reaction was convenient and scalable to afford multigram quantities of 3 as our main building block.

The TPA methyl ester (3) could then be further converted, giving access to the TPA acid36 (4), TPA alcohol37 (5), TPA amine (6), and TPA amide (7) (Scheme 2), with the first two being described in the literature. These TPA analogs were tested in biological assays to obtain the initial results (see the discussion and Table 1) and subsequently served as starting materials for the synthesis of the compounds discussed below. The TPA acid (4) was isolated once for characterization but otherwise used in subsequent reactions without further purification after the saponification of 3 with LiOH in H2O/THF and neutralization with 2 M HCl. The TPA alcohol was prepared via the reduction of 3 with NaBH4 in EtOH and was further reacted in a sequence of mesylation, nuclophilic substitution with NaN3, and Staudinger reaction to TPA amine hydrochloride 6. This sequence required no chromatography and afforded TPA amine 6 as the hydrochloride salt from the water phase cleanly in high yield after acidic extraction.

The first series of compounds containing modulators directly attached to the chelator was then prepared from TPA acid 4 or amine 6 via standard peptide coupling conditions with dipptides or other amines (Scheme 3). The low solubility of TPA acid 4 in nonpolar and aprotic solvents limited the applicable reaction conditions. HATU and N-methylmorpholine (NMM) or EDCl, HOAt, and NMM in DMF were thus used, and the isolation of the coupling products was performed via C18 solid-phase extraction (SPE) or column chromatography on neutral alumina, affording mediocre to good product yields. The C18 SPE was in most cases the method of choice for the efficient chromatographic removal of byproducts from the peptide couplings, namely, tetramethylurea, DMF, and HOAt. Alternative methods would suggest the preparation of an activated ester of 4 as succinimidyl ester, acid chloride, or acid fluoride.38,39 Neither of those variations improved the method above.

Compounds 14 and 16 were prepared via couplings facilitated by EDCl with the corresponding protected anilines, followed by deprotection of TFA or LiOH, respectively. The obtained N- and C-terminal TPA-linker fragments were then coupled via HATU with Boc-d-aladala-OH or H-d-aladala-OMe, affording after another deprotection corresponding compounds 15 and 17 (Scheme 4).

By analogy to 15 and 17, two aliphatic linkers were implemented using similar peptide chemistry and deprotection methods (Scheme 5). Compounds 19 and 21, derived from the

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**Figure 1.** Design principle of the putative MBL inhibitors presented in this publication as exemplified by compound 15.
couplings with the diamino linkers, deprotection and coupling with Boc-D-ala-D-ala-OH were obtained in 79 and 62% yields, respectively.

Compound 12, previously prepared in Scheme 3 containing the piperazine moiety as a linker, was furthermore used and coupled to Boc-D-ala-D-ala-OH by analogy, affording 22 (Scheme 6).

**In Vitro and in Vivo Biological Evaluation of the Synthesized Zinc Chelators as MBL Inhibitors.** Antimicrobial resistance can be targeted in different ways depending on the mechanism of action of the active compounds. Parameters such as selectivity, potency, membrane permeability, resistance development, pharmacokinetics, and toxicity are critical and need to be taken into account for the development of any drug or pharmaceutically active compound. The MBL inhibitors could be a prodrug, have additional bactericidal or bacteriostatic activity or function solely as an adjuvant being coadministered to an already existing antibiotic, rendering it active again. We considered the removal of zinc from the zinc-dependent MBLs as an efficient way to inhibit these enzymes and aimed to develop a selective and strong zinc chelator. These selective zinc chelators should additionally provide the possibility of chemical modification to limit toxicity, vary lipophilicity (clogP), and find SAR trends leading to the development of efficient candidates with a wide therapeutic window. Synthesis simplicity, employing standard and scalable methods, was considered to be an advantage compared to the often stepwise and demanding synthesis of other putative MBL inhibitors.

To be effective, compounds should have a specific zinc binding strength (K_d) of between 10^{-9} and 10^{-12} M. Earlier studies in our laboratories resulted in the initial observations that a K_d below 10^{-12} M results in toxicity, and values higher than 10^{-9} M are not effective. Two potentially active and reported Zn^{2+} chelators are N,N,N',N'-tetrais(2-pyridylmethyl) ethylenediamine (TPEN) and tris(2-pyridylmethyl)amine (TPA). TPEN has a K_d of about 10^{-16} M while TPA has a K_d of about 10^{-11} M and this seemed to be the ideal starting point for our biological testing against the MBL harboring strains. Further advantages of TPA are that it coordinates Zn^{2+}...
with a higher kinetic rate and that it is less toxic than TPEN.\(^{48}\)

TPEN was included in our biological studies for comparison. The easily available derivatives of TPA, namely, the TPA methyl ester (3), TPA acid (4), TPA alcohol (5), and TPA amine (6) were first tested in a biological assay to obtain the initial results for activity (Table 1). \(\text{clogP}\) values for the compounds as a parameter for trends and further ongoing SAR studies and possible relations to toxicity are displayed and included in the tables.

First, the putative inhibitors were tested alone against the MBL-producing strains at concentrations of up to 1000 \(\mu\)M and showed no intrinsic antibacterial activity. They were then tested in vitro at 50 \(\mu\)M (except 13, 19, and 21 which were tested at 125 \(\mu\)M) in combination with MEM in a standard MIC assay on clinical isolates of \(P.\) \textit{aeruginosa} and \(K.\) \textit{pneumoniae} expressing either the VIM-2 or NDM-1 MBLs, which hydrolyze MEM.\(^{49}\) The initial MIC value of MEM alone was between 32 and 64 mg/L for both strains. The addition of the zinc chelators reduced the MIC values markedly, which were in all cases equal to or less than 2 mg/L (except for 7, which was insoluble in the medium, and 9, Table 2), showing that the compounds behave as adjuvants.\(^{50}\)

The TPA derivatives were additionally tested against HepG2 cells to determine their IC\(_{50}\) values, which corresponded to the relative toxicity toward this cell line, a critical factor in determining a therapeutic window. As expected from the
literature, the IC_{50} of TPEN (7 μM) was half that of 3 (16 μM). The low IC_{50} values of the compounds in Table 1 required chemical modification to lower the toxicity.

Peptide- or non-peptide-based side chains (modulators) were then attached to the chelators, spanning a range of clogP values (0.22–2.54), to obtain insight into the parameters that influenced toxicity. We started with the direct attachment of peptide side chains to the TPA moiety resulting in compounds 8, 9, 10, and 13, which contain D-ala-D-ala-OMe, L-ala-L-ala-OMe, gly-gly-OMe, and D-ala-D-ala-NH₂ as modulators (Table 2). Additionally, allylamine and piperazine were introduced to give compounds 11 and 12. Piperazine was also used as a linker in the compounds discussed below.

All of the compounds tested in Table 2 held potential for the activity of MEM, with compounds 8–10 (Table 2), TPA-D-ala-D-ala-OMe, TPA-L-ala-L-ala-OMe, and TPA-gly-gly-OMe being slightly less effective at reducing the MIC for MEM compared to that for 11–13 (Table 2). The compounds in Table 2 were markedly less toxic than the TPA derivatives tested in Table 1 (IC_{50} > 50 μM) with the exception of compound 11 (IC_{50} = 25 μM). However, no direct correlation between the clogP values and toxicity could be obtained for this series of compounds.

In parallel to the synthesis of the compounds shown in Table 2, linkers of different physical properties and molecular flexibility were introduced between the chelator and modulator D-ala-D-ala to examine the dependency of activity and toxicity on the molecular structure. Four different diamine linkers were used, giving access to the N-terminal D-ala-D-ala products as well as one aminocarboxylate linker for the corresponding C-terminal derivative. Lipophilic hexyldiamine and piperidin-4-yl ethylamine as well as aromatic 4-aminophenethylamine and 2-(4-
Table 2. In Vitro Testing of 8–13 as Putative MBL Inhibitors in Clinical Isolates Together with MEM and Toxicity towards HepG2 Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \log P )</th>
<th>( \text{MIC MEM (mg/L)} )</th>
<th>( \text{IC}_{50} ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aeruginosa</td>
<td>K. pneumoniae</td>
<td></td>
</tr>
<tr>
<td>MEM</td>
<td>–1.79</td>
<td>32–64</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>1.21</td>
<td>2</td>
<td>55.5</td>
</tr>
<tr>
<td>9</td>
<td>1.21</td>
<td>0.25</td>
<td>87.2 †</td>
</tr>
<tr>
<td>10</td>
<td>0.22</td>
<td>0.5</td>
<td>127.7</td>
</tr>
<tr>
<td>11</td>
<td>2.54</td>
<td>0.125</td>
<td>24.7</td>
</tr>
<tr>
<td>12</td>
<td>1.46</td>
<td>0.125</td>
<td>89.2</td>
</tr>
<tr>
<td>13</td>
<td>0.43</td>
<td>0.125</td>
<td>–</td>
</tr>
</tbody>
</table>

“MIC assay performed as one biological replicate and two technical replicates. †Estimated using ChemDraw Ultra 15.1 from Cambridgesoft. For the MIC determination, all compounds were tested at 50 µM except 13, which was tested at 125 µM in coadministration with MEM. ‡MIC values of P. aeruginosa harboring MBLs VIM-2. ‡MIC values of K. pneumoniae harboring MBLs NDM-1. §IC50 could not be accurately determined from the data.

aminophenyl) carbamate were chosen along with the piperazine from 12 (Table 3).

Table 3. In Vitro Testing of 15, 17, 19, 21, and 22 as Putative MBL Inhibitors in Clinical Isolates Together with MEM and Toxicity towards HepG2 Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \log P )</th>
<th>( \text{MIC MEM (mg/L)} )</th>
<th>( \text{IC}_{50} ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aeruginosa</td>
<td>K. pneumoniae</td>
<td></td>
</tr>
<tr>
<td>MEM</td>
<td>–1.79</td>
<td>32–64</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>2.48</td>
<td>0.125</td>
<td>&gt;100</td>
</tr>
<tr>
<td>17</td>
<td>1.96</td>
<td>0.125</td>
<td>&gt;100</td>
</tr>
<tr>
<td>19</td>
<td>1.03</td>
<td>0.125</td>
<td>&gt;100</td>
</tr>
<tr>
<td>21</td>
<td>0.67</td>
<td>0.125</td>
<td>&gt;100</td>
</tr>
<tr>
<td>22</td>
<td>0.82</td>
<td>0.25</td>
<td>162.6</td>
</tr>
</tbody>
</table>

“The MIC assay was performed as one biological replicate and two technical replicates. †Estimated using ChemDraw Ultra 15.1 from Cambridgesoft. For the MIC determination, all compounds were tested at 50 µM except 19 and 21, which were tested at 125 µM in coadministration with MEM. ‡MIC values of P. aeruginosa harboring MBLs VIM-2. §MIC values of K. pneumoniae harboring MBLs NDM-1. ‡IC50 could not be accurately determined from the data because cell viability did not decline to 50%. Poor solubility might have affected the results.

The compounds from Table 3 were among the most potent inhibitors with MIC values for 1 mg/L against the VIM-2-producing P. aeruginosa strain and 0.125–0.25 mg/L for the NDM-1-producing K. pneumoniae strain. It was not possible to fit data obtained for the HepG2 toxicity measurements of the compounds in Table 3 to a sigmoidal pattern, and thus IC50s could not be accurately determined, only estimated. A relative comparison of the IC50 values obtained for the compounds in Tables 1–3 allows the observation that the inhibitors in Table 3 showed lower toxicity (IC50 > 100 µM) as compared to the compounds in Tables 1 and 2. The estimated IC50 values in Table 3 corresponded to a concentration much higher than needed for activity in the MIC assay (max. 50 µM). The compounds reported herein did not show a correlation between the \( \log P \) values and toxicity in the HepG2 assays. More importantly, the activity increased, and the toxicity decreased with increasing molecule size, as compounds including linkers were the least toxic and most active. Compound 15 was further tested at 50 µM against six clinical isolates harboring carbapenemases (Table 4). Three strains expressed MBLs (VIM-1, NDM-1, and VIM-29), while three strains carried other serine carbapenemases (GES-5, KPC-3, and OXA-48). Resensitization of all MBL-carrying strains could be achieved, and no activity against serine-carbapenemases-expressing strains could be demonstrated.

We performed enzyme kinetics based on purified protein to confirm that MBLs are the main target of our designed inhibitors. Both NDM-1 (\( K_{\text{cat}}/K_{\text{M}} = 0.83 \times 10^{-6} \text{ s}^{-1} \text{ M}^{-1} \)) and VIM-2 (\( K_{\text{cat}}/K_{\text{M}} = 20.8 \times 10^{-6} \text{ s}^{-1} \text{ M}^{-1} \)) were inhibited by 15 (Table 5). The corresponding graphs for the enzyme inhibition kinetics can be found in the Supporting Information.

Compound 15 was chosen for in vivo toxicity studies in mice as well as the frequency of resistance development and zinc binding ability in solution by NMR titration.

\( \text{ZnCl}_2 \) NMR Titration of 15. The addition of increasing amounts of \( \text{ZnCl}_2 \) to the test vials with dissolved 15 in DMSO-\( d_6 \) showed a linear increase in complex formation. The peaks at 10.32 ppm (15) and 10.67 ppm (complex) were used to visualize the ratios between complexed and uncomplexed 15, revealing the quantitative and equimolar binding of Zn\(^{2+} \) by the TPA chelator. The amount of complex formation corresponded to the amount of \( \text{ZnCl}_2 \) added for each sample within experimental tolerances. No further changes in the signals were detected by the addition of 1.5 equiv of \( \text{ZnCl}_2 \) compared to 1.0 equiv. The detailed experimental data (NMR spectra and tables) can be found in the Supporting Information.

In Vivo Toxicity Testing of 15. Compound 15 was administered to female mice at doses doubling each week, ranging from 4 to 128 mg/kg. No acute toxicity was observed as shown by the lack of relative body mass loss, dead mice, or any other obvious health concerns (Supporting Information). In particular, the weight gain of the treated mice did not drop below 90% of the control group. This initial in vivo evaluation indicates that 15 is well tolerated in mice.

Table 4. In Vitro Testing of 15 against an Extended Panel of Clinical Isolates

<table>
<thead>
<tr>
<th>( \text{MIC MEM (mg/L)} )</th>
<th>( \text{K. pneumoniae VIM-1} )</th>
<th>( \text{E. coli NDM-1} )</th>
<th>( \text{E. coli VIM-29} )</th>
<th>( \text{P. aeruginosa GES-5} )</th>
<th>( \text{K. pneumoniae KPC-3} )</th>
<th>( \text{E. coli OXA-48} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>64–256</td>
<td>1–8</td>
<td>8–32</td>
<td>128–256</td>
<td>32–64</td>
<td>0.5–2</td>
</tr>
<tr>
<td>MEM + 15</td>
<td>0.5</td>
<td>0.06</td>
<td>0.125</td>
<td>&gt;64</td>
<td>32</td>
<td>2</td>
</tr>
</tbody>
</table>

“The MIC was assay performed as one biological replicate and two technical replicates. †For the MIC determination, compound 15 was tested at 50 µM in coadministration with MEM. ‡MIC values of K. pneumoniae harboring MBLs VIM-1. ‡MIC values of E. coli harboring MBLs NDM-1. ‡MIC values of P. aeruginosa harboring MBLs VIM-29. ‡MIC values of P. aeruginosa harboring MBLs GES-5 (Guiana extended spectrum of metalloβ-lactamase 5). ‡MIC values of K. pneumoniae harboring carbapenemases KPC-3 (Klebsiella pneumoniae carbapenemase 3). ‡MIC values of E. coli harboring carbapenemases OXA-48 (oxacillinase 48).
Table 5. Enzyme Inhibition Kinetics for 15 against VIM-2 and NDM-1

<table>
<thead>
<tr>
<th>compound</th>
<th>VIM-2</th>
<th>NDM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{inact}}$ (s$^{-1}$)</td>
<td>$K_i$ (μM)</td>
</tr>
<tr>
<td>15</td>
<td>41.62</td>
<td>0.56</td>
</tr>
<tr>
<td>CI95</td>
<td>1.99</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Abbreviations: $K_i$, inhibitor concentration that produces the half-maximal rate of inactivation; $k_{\text{inact}}$, maximum inactivation rate; CI95, confidence interval 95%.

Frequency of Resistance Development. During the development of new antimicrobials, it is common to assess the frequency of spontaneous resistant mutants within a bacterial population. For NDM-1-expressing strain K. pneumoniae K66-45, compound 15 reduced the MIC of MEM to 0.125 mg/L (Table 3). We therefore decided to determine the frequency of resistance of K. pneumoniae K66-45 grown from a single colony to approximately 10$^9$ CFU/mL in at least 50 µM compound 15 in a single-step selection experiment in combination with several concentrations of MEM (1, 2, 4, and 8 mg/L). The frequency of resistance against 50 µM compound 15 and 1 mg/L MEM was $10^{-7}$. Importantly, colonies were only present at the lowest concentration of MEM tested (1 mg/L), indicating that at concentrations of 2 mg/L and above the frequency of resistant mutants was less than $10^{-6}$. The very low rates of resistant mutants observed at these concentrations of MEM suggest that the mechanism of MBL-inhibition by compound 15 is not easily overcome by spontaneous mutation, an important consideration for the future clinical development of any putative MBL inhibitors. Even more encouraging, from the perspective of developing a clinically useful MBL inhibitor, is that EUCAST clinical breakpoints for MEM define ≤2 mg/L as susceptible. While the colonies growing on the 50 µM compound 15 and 1 mg/L combination plate were resistant when compared to the microbroth dilution MIC value of 0.125 mg/L MEM (Table 3), these mutants were still classified as susceptible on the basis of the clinical breakpoints.

**CONCLUSIONS**

This work presents the development, synthesis and biological evaluation of new selective zinc chelators as putative MBL inhibitors. The key structural elements include the TPA chelator modified with a linker and a modulator. The chemical synthesis of these compounds involves standard methods and allows flexible functionalization. All new compounds lowered the MIC of MEM against two gram-negative bacterial strains harboring carbapenemases VIM-2 and NDM-1. The toxicity of the compounds was evaluated as IC$_{50}$ values on human liver cells (HepG2). The most promising compounds, those displaying the highest potentiation of MEM and lowest HepG2 toxicity, were 10, 15, 17, 19, 21 and 22, with IC$_{50}$ values above or equal to 100 µM. 15 was able to inhibit NDM-1 and VIM-2 and showed activity against an extended panel of gram-negative clinical isolates with MBLs, while no reduction in MIC for MEM was seen for serine-$ß$-lactamase harboring bacteria. Initial in vivo toxicity testing (up to 128 mg/kg) of compound 15 in mice showed no acute toxic effects on the animals except a slight reduction in weight gain. The frequency of the spontaneous resistance of the NDM-1 expressing strain K. pneumoniae K66-45 against 15 was low ($10^{-7}$) for concentrations of MEM > 2 mg/L. On the basis of the high potency, low initial toxicity, and low frequency of resistance of these novel zinc chelators, we are currently undertaking further SAR studies to identify and advance compounds with suitable therapeutic windows and the potential for clinical development.

**METHODS**

**Chemistry.** Experimental procedures and spectral characterizations are described below and in the Supporting Information.

**Evaluation of the Potentiation of Meropenem.** The MICs of MEM alone or in combination with the synthesized compounds were determined using premade broth microdilution plates containing MEM in 2-fold dilution steps ranging from 0.03 to 64 mg/L (TREK Diagnostic Systems/Thermo Fisher Scientific, East Grinstead, U.K.). The compounds were tested at a final concentration of 50 or 125 µM. The MIC assays were performed using two MBL-producing MEM-resistant clinical strains: one NDM-1-producing K. pneumoniae and one VIM-2-producing P. aeruginosa. Compound 15 was also evaluated against a VIM-1-producing K. pneumoniae, an NDM-1-producing E. coli, a VIM-29-producing E. coli, a GES-5-producing P. aeruginosa, a KPC-3-producing K. pneumoniae, and an OXA-48-producing E. coli from the collection of carbapenemase-producing strains at the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance. In brief, the compounds were diluted in MH broth (Becton Dickinson, Franklin Lakes, NJ, USA), and 50 µL of the suspension was added to each well of the MEM plate. Bacterial colonies were suspended in 0.9% saline buffer to 0.5 McFarland and further diluted 1:100 in MH broth (Thermo Fisher Scientific, East Grinstead, U.K.). The diluted bacterial suspension (50 µL) was added to the MEM plates to a final volume of 100 µL. The plates were incubated for 20 h at 37 °C. Each MIC was determined in duplicate. To investigate any intrinsic antibacterial activity of the compounds, the MIC of each compound was determined in a 2-fold serial dilution series in MH broth (Becton Dickinson, Franklin Lakes, NJ, USA) ranging from 2 to 1000 µM. Each dilution step (50 µL) was then mixed with 50 µL of a bacterial inoculum, prepared as described above, in 96-well microtiter plates (Thermo Fisher Scientific, Roskilde, Denmark). The plates were incubated, and the MIC was determined as described above.

**In Vitro Toxicity in HepG2 Liver Cells.** Human hepatocarcinoma cell line HepG2 (HB-8065, ATCC, Manassas, VA, USA) was cultivated in DMEM-Glutamax (5.5 mM glucose) supplemented with 10% fetal bovine serum (Gibco, Life Technologies AG, Basle, Switzerland), 100 µg/mL streptomycin, and 100 units/mL penicillin (both from Gibco, Life Technologies AG, Basle, Switzerland). Cells were incubated at 37 °C under a 5% CO$_2$ atmosphere. For viability assays, cells were seeded in white 96-well Nunc plates at a density of 20 000 cells/well and left overnight to adhere before experiments were conducted.

**Cell Viability Assay.** The Zn chelators were dissolved in DMSO at concentrations ranging from 1 to $10^{-6}$ mM and were added to white 96-well plates (maximum DMSO concentration in wells lower than 1%) containing 20.000 HepG2 cells/well.
Plates were incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. After 24 h, AlamarBlue cell viability reagent (Thermo Fisher, Carlsbad, CA, USA) was added as a 10% solution, and plates were placed back in the incubator for 4 h. AlamarBlue is a red-ox indicator yielding a fluorescence signal proportional to the number of viable cells in each well. The fluorescence signal was measured in a microplate reader (Clariostar, BMG Labtech, Ortenberg, Germany) at 550 nm/603 nm (excitation/emission). Data from eight replicates were used to calculate the half-maximal inhibitory concentration (IC₅₀) using Sigmoidal, 4PL, where X is log(concentration) analysis, and a four-parameter logistic regression from GraphPad Prism 6 (GraphPad Software Inc., USA).

ClogP Calculations. The partition coefficient for all compounds was calculated using PerkinElmer Informatics ChemDraw Professional, version 15.1.0.144.

Time-Dependent Inactivation Kinetics. Stock solutions of NDM-1 and VIM-2 were prepared in 50 mM HEPS buffer at pH 7.5. The inhibition of MBLs by zinc chelators has been shown to be time-dependent. Therefore, the enzyme inhibition for compound 15 was measured at different concentrations of inhibitor after preincubation times of 2, 8, 15, 25, and 32 min in 50 mM HEPS buffer at pH 7.5 supplemented with 1 μM ZnSO₄ and BSA (final concentration 2 μg/mL) at 25 °C. Concentrations of 1 nM VIM-2 and 30 nM NDM-1 were used, and the reaction was initiated by the addition of 30 μM nitrocenin (VIM-2) or 100 μM imipenem (NDM-1). The reaction was measured at 482 nm (VIM-2) or 300 nm (NDM-1) in either standard (VIM-2) or UV-transparent (NDM-1) 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark and Corning, Kennebunk, ME, USA) at 25 °C in a SpectraMax Plus plate reader (Molecular Devices). All enzyme and substrate concentrations indicated are final concentrations in the assay. The % enzyme activity was calculated on the basis of the initial velocity and compared to the control without inhibitor. All tests were performed at least in duplicate.

The observed rate constant (kₗ) per inhibitor concentration was calculated from the slope of the semilog plot of % enzyme activity versus preincubation time. The individual values of kₗ were plotted against the inhibitor concentration, and saturation kinetics were fitted into eq 1 by using Graph Pad Prism 4 based on the following model (eq 2)

\[ k_{\text{obs}} = \frac{k_{\text{max}}[I]}{K_i + [I]} \]  

(1)

where E:Zn + I \rightleftharpoons E:Zn:I \rightarrow E + Zn:I or E*:Zn:I

(2)

where Kᵢ represents the inhibitor concentration that leads to a half-maximum inactivation of the enzyme, kₗ is the first-order rate constant, E:Zn is the holoenzyme, I is the inhibitor, E:Zn:I is the enzyme:Zn:inhibitor ternary complex, E is the inactive Zn-depleted enzyme, Zn:I is the zinc-inhibitor complex, and E*:Zn:I is the inactive enzyme:Zn:inhibitor ternary complex.

By fitting these values (eq 1), the irreversible kinetic parameters’ maximum inactivation rate (kₗ) and the inhibitor concentration that produces the half-maximal rate of inactivation (Kᵢ) were obtained. Finally, the inhibitors were characterized by calculating kₗmax/Kᵢ. Where no saturation curve could be observed, Kᵢ and kₗmax were determined from the linear part of the plot of 1/kₗobs versus 1/[I].

In Vivo Toxicity of Compound 15 in Mice. In vivo toxicity experiments were performed by Antineo (www.antineo.fr).

Female Balb/c mice (4 weeks old, approximately 20 g, Charles River, L’Arbresle, France) were acclimatized for 4 days in the animal facility before the initiation of experiments. A group of six mice were treated with 200 μL of 15 intraperitoneally once a week, with increasing doses each time from 4–128 mg/kg. Another group, also containing six mice, was left untreated and used as control group. Individual weights were followed 4 days a week. Relative weight was calculated as the ratio between the weight of the day and the weight of the first day. The protocol for experiments in mice was approved by the University of Lyon Animal Ethics Committee (Comité d’Ethique en Expérimentation Animale de l’Université Claude Bernard Lyon 1, authorization number DR2015-09).

Frequency of Resistance Determination for Compound 15. To determine the frequency of resistant mutants, a modified single-step selection experiment was carried out as previously described. Briefly, K. pneumoniae K66-45 was grown from a single colony to approximately 10⁶ CFU/mL and plated on Mueller-Hinton (Becton Dickinson, Franklin Lakes, NJ, USA) agar containing 50 μM compound 15 and 1, 2, 4, or 8 mg/L MEM. Colonies were counted after overnight incubation at 37 °C. The concentrations of MEM were chosen on the basis of the EUCAST clinical breakpoints for Enterobacteriaceae that define ≤2 mg/L as susceptible and >8 mg/L as resistant to MEM.

Synthesis Procedures and Characterization. All reagents and solvents were of analytical grade and were used as received, without further purification. 1H spectra were recorded with a Bruker DRX400, DRX300, or AVI 600 Fourier transform spectrometer using an internal deuterium lock, operating at 400, 300, or 600 MHz. 13C NMR spectra were recorded with a Bruker DRX400, DRX300, or AVI 600 Fourier transform spectrometer using an internal deuterium lock, operating at 100, 75, or 150 MHz. All spectra were recorded at 25 °C. Chemical shifts are reported in parts per million (ppm) relative to residual protons or carbons of deuterated solvents (δ = 2.50 ppm for 1H NMR and δ = 39.52 ppm for 13C NMR for DMSO-δ₆, δ = 7.26 ppm for 1H NMR and δ = 77.16 ppm for 13C NMR for CDCl₃, δ = 3.31 ppm for 1H NMR and δ = 49.00 ppm for 13C NMR for CD₂OD). The carbon multiplicity was determined by DEPT experiments. Mass spectra were recorded at 70 eV on a Waters Prospec Q or Micromass QTOF 2W spectrometer using ESI or APCl as the method of ionization. High-resolution mass spectra were recorded at 70 eV on a Waters Prospec Q or a Micromass QTOF 2W spectrometer using ESI or APCl as the method of ionization. TLC analyses were carried out using Merck Aluminum Oxide 60 F₂₅₆ or Merck Silica gel 60 RP-18 plates visualized by UV light. Agilent Bondesil C18-OH or Versaflash C18 column material supplied by Sigma-Aldrich was used as the stationary phase for reverse-phase dry vacuum chromatography. The yields reported are of isolated material and are uncorrected for purity.

Methyl 6-(((Bis(pyridin-2-ylmethyl)amino)methyl)nicotinate (3)). Methyl 5-((bromomethyl)picolinate (9.52 mg, 64 mmol, 1.0 equiv) was suspended in 400 mL of THF at room temperature. Dipicolylamine (13.49 g, 12.15 mL, 67.7 mmol, 1.2 equiv) and DIPEA (16.7 mL, 95.89 mmol, 1.7 equiv) were added, and the reaction mixture was stirred at room temperature for 16 h and then concentrated under reduced pressure to approximately 200 mL. The suspension was filtered through a paper filter, the solid was washed with THF (2 × 50 mL), and the obtained solution was concentrated under reduced pressure. The residual dark-brown oil was dissolved in 100 mL of diethyl...
H2O was added, and the solution was stirred at 0 °C. An aqueous solution was adjusted to pH 6 using 4 N HCl. The mixture was heated to 50 °C and stirred for 3 h until TLC (alumina, 3% MeOH in CH2Cl2) indicated full conversion. The mixture was concentrated under reduced pressure to give 221 mg (69%) of 6-((Bis(pyridin-2-ylmethyl)amino)methyl)nicotinic acid (5-Mes) in DMF was used in the next reaction without further purification. NMR data were in agreement with the reported data.

6-((Bis(pyridin-2-ylmethyl)amino)methyl)nicotinic Acid (4). Methyl 6-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinate (3, 330 mg, 0.95 mmol, 1.0 equiv) was dissolved in absolute ethanol (10 mL) and placed under argon. NaBH4 pellets (250 mg, 6.95 mmol, 7.0 equiv) were added to the stirring mixture, and the slurry was held at 50 °C for 48 h. The mixture was then quenched by the addition of NH4Cl solution and concentrated under reduced pressure to give a sticky white solid. The crude material was suspended in 1 M H2SO4 (25 mL) and extracted with DCM (3 × 25 mL). The combined organic phases were dried over K2CO3, filtered, and concentrated under reduced pressure to give 221 mg (69%) of the title product as a pale-yellow oil. NMR data were in agreement with the published data.

6-((Bis(pyridin-2-ylmethyl)amino)methyl)pyridin-3-yl)methanamine Hydrochloride (7). NH3 (7 M) in MeOH (10.29 mL, 72 mmol, 50 equiv) was slowly added to solid methyl 6-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinate (3, 502 mg, 1.44 mmol, 1 equiv) under rapid stirring. The reaction mixture stirred for 24 h at room temperature before another 50 equiv of NH3 was added, and the reaction mixture was stirred for another 24 h at room temperature. The mixture was concentrated under reduced pressure to give 475 mg (1.43 mmol, >99%) of the title compound as a clear yellow liquid/oil. 1H NMR (300 MHz, DMSO-d6) δ 8.94 (d, J = 2.0 Hz, 1H), 8.53–8.46 (m, 2H), 8.18 (dd, J = 8.2, 2.2 Hz, 1H), 8.09 (s (b), 1H), 7.77 (td, J = 7.7, 1.8 Hz, 2H), 7.68 (d, J = 8.1 Hz, 1H), 7.58 (d, J = 7.8 Hz, 2H), 7.53 (s (b), 1H), 7.29–7.20 (m, 2H), 3.84 (s (b), 2H), 3.79 (s (b), 3H). 13C NMR (101 MHz, DMSO-d6) δ 166.4, 161.9, 158.8, 148.9, 148.1, 136.6, 135.7, 128.1, 122.6, 122.2, 121.9, 59.4, 59.2, 48.6. MS: m/z calcd for C19H14N4O·HCl, 320.1785; found, 320.1869.

6-((Pyridin-2-ylmethyl)amino) methyl)nicotinic acid (8). To the solution of 6-((bis(pyridin-2-ylmethyl)amino)methyl)pyridin-3-yl)methanesulfonate prepared in the previous reaction (5-Mes, 3.51 g, 8.81 mmol, 1.0 equiv) in 80 mL of DMF was added NaN3 (2.864 g, 44.04 mmol, 5.0 equiv) at room temperature. The mixture was stirred at room temperature for 20 h and then filtered into a new flask and concentrated under reduced pressure to a volume of approximately 30 mL. The mixture was diluted with 100 mL of H2O, transferred to a separation funnel, and extracted with EtOAc (2 × 100 mL). The combined organics were washed with saturated aqueous K2CO3 solution (50 mL) and brine (50 mL), dried over K2CO3, filtered, and concentrated under reduced pressure. The compound was used in the next reaction without further treatment. 1H NMR (300 MHz, methanol-d4) δ 8.45–8.40 (m, 3H), 7.82–7.74 (m, 3H), 7.66 (dd, J = 8.0, 2.4 Hz, 3H), 7.26 (ddd, J = 7.0, 5.0, 1.2 Hz, 2H), 4.42 (s, 2H), 3.86 (s, 6H).

1-(5-(Aminomethyl)pyridin-2-yl)-N,N-bis(pyridin-2- ylmethyl)methanamine Hydrochloride (6). The 1-(5- (Azipydino)methyl)pyridin-2-yl)-N,N-bis(pyridin-2-yl)methanamine prepared in the previous reaction (TPA-N3, 2.783 g, 8.06 mmol, 1.0 equiv) was dissolved in 50 mL of THF. To this solution was added 5 mL of distilled H2O followed by PPh3 (4.228 g, 16.12 mmol, 2.0 equiv) in one portion. The mixture was heated to 50 °C and stirred for 3 h until TLC (alumina, 3% MeOH in CH2Cl2) indicated full conversion. The mixture was concentrated under reduced pressure, the residue was treated with CH2Cl2 and H2O (100 mL each) and transferred to a separation funnel, and the phases were separated. The pH of the aqueous phase was adjusted to 1 with conc HCl under rapid stirring and was transferred to a separation funnel. The aqueous phase was washed with CH2Cl2 (50 mL) and concentrated under reduced pressure to afford 2.825 g (7.93 mmol, 98%) of 1-(5-(Aminomethyl)pyridin-2-yl)-N,N-bis(pyridin-2-yl)methanamine 6 as the hydrochloride salt. 1H NMR (400 MHz, DMSO-d6) δ 9.00 (s (b), 3H), 8.91 (d, J = 1.6 Hz, 1H), 8.81 (dd, J = 5.7, 0.9 Hz, 2H), 8.47 (dt, J = 9.3, 8.1, 1.4 Hz, 3H), 8.16 (d, J = 7.9 Hz, 2H), 8.09 (d, J = 8.2 Hz, 1H), 7.93–7.84 (m, 2H), 4.39 (s (b), 4H), 4.30 (s (b), 2H), 4.17 (g, J = 5.3 Hz, 2H). 13C NMR (101 MHz, DMSO-d6) δ 153.2, 152.5, 145.4, 142.6, 131.7, 127.0, 125.9, 125.8, 56.2, 55.6, 38.7. APCI-HRMS m/z calcd for C25H20N4O·HCl, 320.1875; found, 320.1869.

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mixture was stirred at 0 °C for 1 h and then at room temperature for 16 h. The reaction mixture was concentrated under reduced pressure, and product purification was achieved by way of dry column vacuum chromatography on C18 material using a stepwise elution from 10 to 90% methanol in water affording 195 mg (0.398 mmol, 75%) of product 8. 1H NMR (400 MHz, methanol-d4) δ 8.91 (d, J = 2.3 Hz, 1H), 8.84-8.38 (m, 2H), 8.20 (dd, J = 18.2, 2.3 Hz, 2H), 7.78 (dd, J = 11.9, 8.5 Hz, 2H), 7.66 (d, J = 7.8 Hz, 2H), 7.27 (dd, J = 7.4, 5.0 Hz, 1H), 4.58 (q, J = 7.2 Hz, 1H), 4.44 (q, J = 7.3 Hz, 1H), 3.92 (s, 2H), 3.88 (s, 4H), 3.71 (s, 3H), 1.48 (d, J = 7.2 Hz, 3H). 13C NMR (101 MHz, methanol-d4) δ 174.9, 174.6, 167.7, 163.5, 159.9, 149.6, 148.9, 138.7, 137.4, 124.9, 124.3, 123.9, 61.2, 60.9, 52.8, 43.8, 41.8. APCLI-HRMS m/z calcd for C26H24N6O5 [M + H]+, 491.2401; found, 491.2399.

(S)-Methyl 2-[(S)-2-(6-(bis(pyridin-2-ylmethyl)amino)nicotinamido)propanamido]propanoate (9). 6-[(Bis(pyridin-2-ylmethyl)amino)nicotinoyl]piperazine-1-carboxylate (10). 1H NMR (400 MHz, methanol-d4) δ 7.98 (s, 1H), 8.44-8.38 (m, 2H), 8.21 (dd, J = 6.0, 2.7 Hz, 2H), 7.77 (dd, J = 18.2, 2.0 Hz, 2H), 7.65 (d, J = 7.8 Hz, 2H), 7.27 (t, J = 5.7 Hz, 2H), 4.11 (s, 2H), 3.98 (s, 2H), 3.91 (d, J = 7.4 Hz, 2H), 3.88 (s, 4H), 3.71 (s, 3H), 1.48 (d, J = 7.3 Hz, 3H). 13C NMR (101 MHz, methanol-d4) δ 172.1, 171.7, 166.1, 163.6, 159.9, 149.6, 148.9, 138.7, 137.4, 129.8, 124.9, 124.3, 123.9, 61.2, 60.9, 52.8, 43.8, 41.8. APCLI-HRMS m/z calcd for C26H24N6O5 [M + H]+, 466.2088; found, 463.2087.

N- Allyl-6-[(bis(pyridin-2-ylmethyl)amino)methyl]nicotinamide (11). 6-[(Bis(pyridin-2-ylmethyl)amino)methyl]nicotinic acid (4, 180 mg, 0.537 mmol, 1.0 equiv) was dissolved in 3 mL of dry DMF at room temperature. tert-Butyl piperazine-1-carboxylate (12-Boc). 6-[(Bis(pyridin-2-ylmethyl)amino)methyl]nicotinic acid (4, 180 mg, 0.537 mmol, 1.0 equiv) was dissolved in 3 mL of dry DMF at room temperature. tert-Butyl piperazine-1-carboxylate (150 mg, 0.806 mmol, 1.5 equiv), EDCl (134 mg, 0.806 mmol, 1.5 equiv), HOAt (109.6 mg, 0.806 mmol, 1.5 equiv), and NMM (89 μL, 0.806 mmol, 1.5 equiv) were added, and the mixture was stirred at room temperature for 16 h. The reaction mixture was concentrated under reduced pressure, and the purification of the product was achieved by way of dry column vacuum chromatography on C18 material using stepwise elution from 10 to 90% methanol in water affording 93 mg (0.189 mmol, 54%) of product 9. 1H NMR (300 MHz, MeOH-d4) δ 8.91 (d, J = 1.6 Hz, 1H), 8.48-8.38 (m, 2H), 8.20 (dd, J = 18.2, 2.3 Hz, 2H), 7.78 (dd, J = 11.9, 8.5 Hz, 2H), 7.66 (d, J = 7.8 Hz, 2H), 7.27 (dd, J = 7.4, 5.0 Hz, 1H), 4.58 (q, J = 7.2 Hz, 1H), 4.44 (q, J = 7.3 Hz, 1H), 3.90 (d, J = 5.4 Hz, 2H), 3.87 (s, 4H), 3.71 (s, 3H), 1.48 (d, J = 7.2 Hz, 3H), 1.41 (d, J = 7.3 Hz, 3H).

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The N-Boc-protected amine (13-Boc, 84 mg, 0.015 mmol, 1.0 equiv) was dissolved in 1 mL of CH₂Cl₂ and cooled to 0 °C in an ice-water bath. Trifluoroacetic acid (0.09 mL, 9.0 mmol, 60 equiv) in 1 mL of CH₂Cl₂ was then slowly added to the stirring mixture. The reaction was left at 0 °C for 20 min before warming to room temperature. The mixture was stirred for an additional 3 h at room temperature until TLC (2% MeOH in CH₂Cl₂, alumina plates) indicated the consumption of the carbamate. After solvent removal under reduced pressure, excess 1 M aqueous K₂CO₃ (50 mL) was added to the mixture, and the compound was extracted with CH₂Cl₂ (3 × 20 mL). The combined organic layers were washed with fresh 0.5 M K₂CO₃ and, after centrifugation, and evaporated to a small volume under reduced pressure. The orange oily residue was dissolved in CH₂Cl₂ and purified by column chromatography on neutral alumina (1–2% MeOH in CH₂Cl₂) to afford the title compound. Yield (89 mg, 69%). ¹H NMR (400 MHz, CDCl₃) δ 8.50 (dd, J = 5.0, 1.8 Hz, 2H), 8.40 (dd, J = 2.3 Hz, 1H), 7.67–7.49 (m, 7H), 7.12 (dd, J = 4.9, 1.3 Hz, 2H), 6.76 (d, J = 7.6 Hz, 1H), 5.12 (broad s, 1H). 4.48 (pentet, J = 7.5 Hz, 1H), 1.43 (d, J = 5.9 Hz, 2H), 4.05 (m, 1H), 3.84 (s, 4H). 13C NMR (100 MHz, CDCl₃) δ 172.8, 172.3, 162.6, 159.3, 158.5, 149.2, 148.4, 136.6, 136.1, 132.4, 123.1, 122.9, 122.2, 80.7, 60.2, 59.9, 51.0, 49.1, 40.8, 28.3, 19.01, 18.0. HRMS (ESI) calcd for Cₗ₃H₉₅N₇O₄ [M + H]+, 462.2612; found, 462.316.13; 1.21 (m, 15H). 13C NMR (100 MHz, CDCl₃) δ 172.8, 172.3, 162.6, 159.3, 158.5, 149.2, 148.4, 136.6, 136.1, 132.4, 123.1, 122.9, 122.2, 80.7, 60.2, 59.9, 51.0, 49.1, 40.8, 28.3, 19.01, 18.0. HRMS (ESI) calcd for Cₗ₃H₉₅N₇O₄ [M + H]+, 462.2612; found, 462.316. (R)-2-Amino-N-((R)-(R)-4-((4-((6-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamido)phenethylcarbamate (14-Boc, 4.69 g, 3.07 mmol, 1 equiv) prepared in the previous reaction was dissolved in 10 mL of DCM at room temperature. To this solution was added TFA (5 mL), and the mixture was stirred at room temperature until TLC (Al₂O₃, 5% MeOH in DCM) or NMR indicated full conversion. The mixture was then concentrated under reduced pressure, and the residue was dissolved in a mixture of CHCl₃/dest. H₂O/sat. aq. K₂CO₃ (100 mL/10 mL/100 mL) and transferred to a separation funnel. The organic phase was separated, the aqueous phase was extracted twice with 50 mL of CHCl₃, and the combined organics were washed with 100 mL of brine, dried over K₂CO₃/Na₂SO₄, filtered, and concentrated under reduced pressure to afford N-(4-((2-aminoethyl)phenyl)-6-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamide (14) in quantitative yield. ¹H NMR (300 MHz, MeOH-d₄) δ 8.97 (d, J = 1.7 Hz, 1H), 8.45 (dd, J = 5.0, 1.7, 0.9 Hz, 2H), 8.26 (dd, J = 8.2, 3.2 Hz, 1H), 7.80 (td, J = 7.6, 1.6 Hz, 3H), 7.68 (d, J = 7.8 Hz, 2H), 7.62 (d, J = 8.5 Hz, 2H), 7.28 (dd, J = 7.4, 5.0, 1.2 Hz, 2H), 7.23 (d, J = 8.5 Hz, 2H), 3.94 (s, 2H), 3.90 (s, 4H), 2.89 (dd, J = 10.6, 4.1 Hz, 2H), 2.76 (t, J = 6.9 Hz, 2H). ¹C NMR (100 MHz, methanol-d₄) δ 163.6, 160.0, 149.6, 148.9, 138.7, 137.5, 131.0, 130.2, 124.5, 123.9, 122.5, 61.2, 61.0, 44.0, 39.2. APCId HRS m/z calcd for C₂₉H₂₉N₁₀O [M + H]+, 543.2927; found, 543.2936.

tert-Butyl (R)-1-((R)-4-((6-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamido)phenethylcarbamate (15-Boc). N-(4-((2-aminophenyl)phenyl)-6-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamide prepared in the previous reaction (14, 218 mg, 0.48 mmol, 1.0 equiv) was dissolved in 3 mL of dry DCM and cooled to 0 °C in an ice bath. To this solution was added Boc-N-Ala-N-Ala-OBzl (132 mg, 0.506 mmol, 1.05 equiv), HATU (192 mg, 0.506 mmol, 1.05 equiv), and NMM (116 µL, 1.056 mmol, 2.2 equiv), and the solution was stirred at 0 °C for 2 h.
30 min and then at room temperature for 16 h. The mixture was concentrated under reduced pressure, and the product was purified by dry column vacuum chromatography on C18 material using stepwise elution from 10 to 90% methanol in water, affording 217.7 mg (0.313 mmol, 65%) of the product as a pale-yellow oil. 1H NMR (600 MHz, methanol-d4) δ 8.96 (d, J = 1.7 Hz, 1H), 8.44 (d, J = 4.3 Hz, 2H), 8.25 (dd, J = 8.2, 2.2 Hz, 1H), 7.78 (ddd, J = 10.3, 6.1, 2.2 Hz, 3H), 7.67 (d, J = 7.9 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 7.27 (ddd, J = 7.3, 5.1, 0.8 Hz, 2H), 7.20 (d, J = 8.5 Hz, 2H), 4.30 (q, J = 7.0 Hz, 1H), 4.03 (q, J = 7.0 Hz, 1H), 3.92 (s, 2H), 3.88 (s, 4H), 3.84–3.41 (m, 3H), 3.35 (dd, J = 20.2, 7.1 Hz, 1H), 2.78 (t, J = 7.0 Hz, 2H), 1.44 (s, 9H), 1.30 (t, J = 6.6 Hz, 6H). 13C NMR (151 MHz, methanol-d4) δ 175.6, 174.9, 166.3, 163.5, 159.9, 149.6, 149.2, 138.4, 137.5, 136.9, 131.0, 130.6, 124.2, 123.9, 122.3, 80.7, 61.2, 60.9, 52.0, 50.4, 42.0, 35.9, 28.7, 18.2, 18.0. APCLI-HRMS m/z calcd for C34H38N7O5 [M + H]+, 624.2929; found, 624.2927.

**N-(4-[(2-((R)-2-Aminopropanamido)propanamido)ethyl]phenyl)-6-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamide Hydrochloride (15).** (R)-1-((R)-1-((R)-2-(4-(6-((Bis(pyridin-2-ylmethyl)amino)methyl)nicotinamido)-1-oxopropan-2-ylamino)-1-oxopropan-2-ylcarbamate prepared in the previous reaction (15-Boc, 176 mg, 0.253 mmol, 1.0 equiv) was dissolved in 5 mL of CH2Cl2 and cooled to 0 °C in an ice bath. To this solution was added TFA (0.97 mL, 12.6 mmol, 50 equiv), and the mixture was stirred at room temperature until full conversion as monitored by 1H NMR. This oil was dissolved in warm H2O, collected in a funnel, and concentrated under reduced pressure to a residue. The residue was dissolved in a mixture of EtOAc/distilled H2O/saturated aqueous K2CO3 (30 mL/10 mL/30 mL) and transferred to a separation funnel. The organic phase was separated and the aq. phase was filtered, and concentrated under reduced pressure. The THF was removed under reduced pressure, and the residual aqueous solution was adjusted to pH 6 using 4 N HCl. The solvent was removed under reduced pressure, affording the product in quantitative yield, which was used in the next step without further purification.

(R)-Methyl-2-((R)-2-((R)-2-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamido)phenyl)acetamido)propanamido)propanoate (17). 2-((R)-2-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamido)phenyl)acetamido acid prepared in the previous reaction (16, 94 mg, 0.202 mmol, 1.0 equiv) was dissolved in 3 mL of dry DMF at room temperature. d-Ala-d-Ala-OMe hydrochloride (44.6 mg, 0.212 mmol, 1 equiv) was then added, and the mixture was cooled to 0 °C in an ice bath. HATU (80.6 mg, 0.212 mmol, 1.05 equiv) and NMM (91 μL, 0.828 mmol, 4.2 equiv) were added, and the mixture was stirred at 0 °C for 30 min and then at room temperature for 16 h. The mixture was concentrated under reduced pressure, and the product was purified by dry column vacuum chromatography on C18 material using stepwise elution from 10 to 90% methanol in water, affording 97 mg (0.155 mmol, 77%) of product. 1H NMR (400 MHz, methanol-d4) δ 8.44 (d, J = 4.8 Hz, 2H), 8.25 (dd, J = 8.2, 2.2 Hz, 1H), 7.79 (dd, J = 8.4, 5.9, 2.0 Hz, 3H), 7.67 (d, J = 7.9 Hz, 2H), 7.64 (d, J = 8.5 Hz, 2H), 7.33–7.24 (m, 4H), 4.38 (qd, J = 7.2, 2.7 Hz, 2H), 3.94 (s, 2H), 3.89 (s, 4H), 3.70 (s, 3H), 3.54 (s, 2H), 1.36 (s, 3H), 1.35 (d, J = 0.8 Hz, 3H). 13C NMR (101 MHz, methanol-d4) δ 174.8, 174.5, 173.7, 166.3, 163.5, 159.9, 149.6, 149.0, 148.9, 138.7, 137.9, 137.5, 137.0, 130.1, 130.3, 124.9, 124.3, 123.9, 122.3, 61.2, 60.9, 51.4, 50.3, 41.9, 35.9, 21.2, 18.5. APCLI-HRMS m/z calcd for C33H34N8O4 442.2187; found, 442.2184.

**3-(4-((2-((R)-2-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamido)phenyl)acetamido)propanamido)phenyl)acetate (16).** Methyl 2-((R)-2-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamido)phenylacetate (16-OMe). 6-((Bis(pyridin-2-ylmethyl)amino)methyl)nicotinic acid (4, 976 mg, 2.92 mmol, 1.0 equiv) was dissolved in 20 mL of dry DMF and cooled to 0 °C in an ice bath. Methyl 2-((4-aminophenyl)acetate hydrochloride (883 mg, 4.38 mmol, 1.5 equiv), EDCI (839 mg, 4.38 mmol, 1.5 equiv), and HOAt (596 mg, 4.38 mmol, 1.5 equiv) were added, followed by NMM (740 μL, 6.71 mmol, 2.3 equiv) dropwise over a period of 30 min at 0 °C. The mixture was allowed to warm to room temperature, stirred for 16 h, and concentrated under reduced pressure. The residue was dissolved in 100 mL of CHCl3 and transferred to a separation funnel. The organic phase was washed with a mixture of saturated aqueous K2C2O4 solution and H2O (50 mL each) followed by brine (50 mL). The organic phase was separated, dried over Na2SO4, and concentrated under reduced pressure, and the product was purified by dry column vacuum chromatography on C18 material using stepwise elution from 10 to 90% methanol in water, affording 724 mg (1.5 mmol, 51%) of product as a yellow oil. 1H NMR (400 MHz, methanol-d4) δ 9.01 (s, 1H), 8.56 (d, J = 4.6 Hz, 2H), 8.24 (d, J = 8.1 Hz, 1H), 7.89 (t, J = 7.7 Hz, 2H), 7.62 (t, J = 8.6 Hz, 2H), 7.42–7.35 (m, 2H), 7.26 (d, J = 8.0 Hz, 2H), 4.14 (s, 6H), 3.67 (s, 3H), 3.63 (s, 2H). 13C NMR (101 MHz, methanol-d4) δ 173.9, 166.1, 161.5, 157.7, 157.7, 149.2, 148.6, 140.2, 138.5, 137.7, 132.1, 131.1, 130.8, 125.5, 124.7, 124.5, 122.2, 60.5, 60.2, 60.1, 52.5, 41.1. APCLI-HRMS m/z calcd for C39H35N10O4 [M + H]+, 595.3138; found, 595.3135.

**Methyl 2-((Bis(pyridin-2-ylmethyl)amino)methyl)nicotinamido)phenylacetate (16-OMe).** 6-((Bis(pyridin-2-ylmethyl)amino)methyl)nicotinic acid (4, 772 mg, 1.45 mmol, 1.0 equiv) was dissolved in 60 mL of dry DMF and cooled to 0 °C in an ice-water bath. tert-Butyl-6-aminoheptylcarbamate (313.7 mg, 1.45 mmol, 1.0 equiv) and HATU (551.5 mg, 1.45 mmol, 1.0 equiv) were added before NMM (318 μL, 2.9 mmol, 2 equiv) was added to the stirring mixture. The mixture was...
stirred in an ice-water bath for 15 min before slowly warming to room temperature and was left to stir overnight. The mixture was diluted with 0.5 M K₂CO₃ (30 mL) and extracted with EtOAc (3 × 20 mL). The combined extracts were washed with 0.5 M K₂CO₃ (3 × 20 mL), dried over K₂CO₃, and concentrated under reduced pressure. The orange oily residue was dissolved in CH₂Cl₂ and purified by column chromatography on reverse-phase (1–2% MeOH in CH₂Cl₂) or C₁₈ reverse-phase chromatography (20–75% MeOH in water). Yield (436 mg, 72%). ¹H NMR (400 MHz, CDCl₃) δ 8.95 (d, J = 2.3 Hz, 1H), 8.51 (dd, J = 4.9, 1.8 Hz, 2H), 8.14 (dd, J = 8.2, 2.3 Hz, 1H), 7.68–7.61 (m, 3H), 7.53 (dt, J = 7.8, 1.1 Hz, 2H), 7.14 (dd, J = 7.5, 4.9, 1.2 Hz, 2H), 7.02 (broad s, 1H), 6.82–6.67 (m, 2H), 5.23 (broad s, 1H), 4.41 (pentet, J = 7.2 Hz, 1H), 4.08 (m, 1H), 3.91 (s, 2H), 3.86 (s, 4H), 3.51–3.36 (m, 2H), 3.30 (m, 1H), 3.15 (m, 1H), 2.13 (broad s, 2H), 1.66–1.55 (m, 2H), 1.55–1.46 (m, 2H), 1.41 (s, 9H), 1.38–1.29 (m, 8H). ¹³C NMR (100 MHz, CDCl₃) δ 172.8, 172.2, 165.9, 162.4, 159.2, 156.1, 149.3, 147.6, 136.6, 135.9, 129.1, 123.1, 122.8, 122.3, 80.8, 60.3, 60.0, 51.1, 39.4, 39.0, 29.2, 28.4, 25.8, 25.6, 18.1. HRMS (ESI) calcd for C₃₉H₅₅N₁₀O₅ [M + H⁺], 675.3977; found, 675.3977.

N-(6-((R)-2-((R)-2-Aminopropanamido)propyl)amino)-6-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinamide (18). N-Boc-protected amine 19-Boc (412 mg, 0.61 mmol, 1.0 equiv) was dissolved in 3.0 mL of CH₂Cl₂ and cooled to 0 °C in an ice-water bath. Trifluoroacetic acid (2.8 mL, 36.6 mmol, 60.0 equiv) in 3.0 mL of CH₂Cl₂ was then slowly added to the stirring mixture. The reaction was left at 0 °C for 20 min before warming to room temperature. The mixture was stirred for an additional 3 h at room temperature until TLC (2% MeOH in CH₂Cl₂, alumina plates) indicated the consumption of the carbamate. After solvent removal under reduced pressure, excess 1 M aqueous K₂CO₃ (50 mL) was added to the mixture, and the compound was extracted with CH₂Cl₂ (3 × 20 mL). The combined organic layers were washed with fresh 0.5 M K₂CO₃ (3 × 50 mL), dried on K₂CO₃, and filtered, and the solvent was removed under reduced pressure to give 280 mg (79%) of the title compound as a brown oil. ¹H NMR (400 MHz, CDCl₃) δ 8.95 (d, J = 2.3 Hz, 1H), 8.51 (dd, J = 4.9, 1.8 Hz, 2H), 8.14 (dd, J = 8.2, 2.3 Hz, 1H), 7.78 (d, J = 7.7 Hz, 1H), 7.68–7.59 (m, 3H), 7.52 (dt, J = 7.9, 1.1 Hz, 2H), 7.13 (dd, J = 7.5, 4.9, 1.2 Hz, 2H), 7.05 (t, J = 5.9 Hz, 1H), 6.72 (t, J = 6.0 Hz, 1H), 4.38 (pentet, J = 7.1 Hz, 1H), 3.90 (s, 2H), 3.85 (s, 4H), 3.50–3.32 (m, 3H), 3.22 (m, 2H), 2.27 (broad s, 2H), 1.64–1.42 (m, 4H), 1.42–1.22 (m, 4H), 1.35 (d, J = 7.0 Hz, 3H), 1.29 (d, J = 7.0 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 176.2, 172.5, 165.8, 162.5, 159.1, 149.3, 147.6, 136.6, 135.9, 129.0, 123.1, 122.7, 122.2, 60.3, 60.0, 50.7, 48.7, 39.4, 38.8, 29.4, 29.7, 25.8, 25.6, 21.7, 17.9. HRMS (ESI) calcd for C₃₉H₅₅N₁₀O₅ [M + H⁺], 575.4353; found, 575.4353.

tert-Butyl ((R)-1-((R)-1-((6-((Bis(pyridin-2-ylmethyl)amino)methyl)nicotinyl)amido)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamate (19-Boc). Free amine 18 (389 mg, 0.9 mmol, 1.0 equiv) was dissolved in 4 mL of dry DMF and cooled to 0 °C in an ice-water bath. tert-Butyl-4-(pipеридин-4-ил)этилкарбамат (313.1 mg, 1.45 mmol, 1.0 equiv) and HATU (551.5 mg, 1.45 mmol, 1.0 equiv) were added before NMM (318 μL, 2.9 mmol, 2.0 equiv) was added to the stirring mixture. The mixture was stirred in the ice-water bath for 15 min before slowly warming to room temperature and was left to stir overnight. The mixture was diluted with 0.5 M K₂CO₃ (30 mL) and extracted with EtOAc (3 × 20 mL). The combined extracts were washed with 0.5 M K₂CO₃ (3 × 20 mL), dried over K₂CO₃, and concentrated under reduced pressure. The orange oily residue was dissolved in CH₂Cl₂ and purified by column chromatography on neutral alumina (1–2% MeOH in CH₂Cl₂) to afford 505 mg (64% of the title compound as an orange oil. The title compound was isolated as a mixture of rotamers (conformational isomers). ¹H NMR (400 MHz, CDCl₃) δ 8.59–8.55 (m, 3H), 7.73–7.66 (m, 3H), 7.59 (d, J = 8.0 Hz, 1H), 7.51 (d, J = 8.0 Hz, 2H), 7.20 (dd, J = 7.7, 5.1, 1.3 Hz, 2H), 4.78–4.44 (m, 2H), 4.07 (m, 6H), 3.77 (m,
1H), 3.15 (q, J = 6.8 Hz, 2H), 3.00 (dd, J = 13.4, 12.4, 2.8 Hz, 1H), 2.51 (td, J = 12.9, 2.9 Hz, 1H), 1.82–1.64 (m, 2H), 1.64–1.38 (m, 12H), 1.19–1.00 (m, 2H). 13C NMR (100 MHz, CDCl3) δ 171.3, 168.9, 167.6, 162.6, 156.1, 149.2, 147.4, 137.2, 135.8, 123.3, 122.9, 122.8, 79.4, 60.5, 59.6, 46.7, 41.8, 38.1, 36.8, 33.7, 32.7, 31.9, 28.5. HRMS (ESI) calcd for C31H41N6O3 [M + H]+, 545.3283; found, 545.3285.

(R)-(2-Amino-N-((R)-1-((2-(1-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinoyl)piperazin-1-yl)-1-oxopropan-2-yl)carbamate (21)). The tert-butylic ((R)-1-((2-(1-((bis(pyridin-2-ylmethyl)amino)methyl)nicotinoyl)piperazin-1-yl)-1-oxopropan-2-yl)carbamate prepared in the previous reaction (22-Boc, 78 mg, 0.12 mmol, 1.0 equiv) was dissolved in CH2Cl2 and cooled to 0 °C in an ice-water bath. Trifluoroacetic acid (1.24 mL, 16.2 mmol, 60.0 equiv) in 1 mL of CH2Cl2 was then slowly added to the stirring mixture. The reaction was left at 0 °C for 20 min before warming to room temperature. The mixture was stirred for an additional 3 h at room temperature until TLC (2% MeOH in CH2Cl2, aluminia plates) indicated the consumption of the carbamate. After solvent removal under reduced pressure, excess 1 M aqueous K2CO3 (50 mL) was added to the compound, and the compound was extracted with CH2Cl2 (3 × 20 mL). The combined organic layers were washed with fresh 0.5 M K2CO3 (3 × 50 mL), dried on K2CO3, and filtered, and the solvent was removed under reduced pressure to give 97 mg (62%) of the title compound as a brown oil. The title compound was isolated as a mixture of rotamers (conformational isomers). 1H NMR (400 MHz, CDCl3) δ 8.57–8.47 (m, 3H), 7.73 (d, J = 7.8 Hz, 1H), 7.71–7.60 (m, 4H), 7.53 (d, J = 7.9 Hz, 2H), 7.16–7.09 (m, 2H), 6.62 (m, 1H), 4.65 (m, 1H), 4.36 (pentet, J = 7.1 Hz, 1H), 3.89 (s, 2H), 3.87 (s, 4H), 3.69 (m, 1H), 3.46 (q, J = 7.0 Hz, 1H), 3.33–3.16 (m, 2H), 2.99 (m, 1H), 2.74 (m, 1H), 2.27–2.06 (m, 2H), 1.89–1.38 (m, 5H), 1.35 (d, J = 7.0, 9.0 Hz, 3H), 1.32 (d, J = 7.0, 1.0 Hz, 3H), 1.25–1.06 (m, 2H). 13C NMR (100 MHz, CDCl3) δ 176.2, 172.2, 167.9, 161.0, 159.2, 149.3, 147.2, 136.6, 135.6, 130.5, 123.1, 122.7, 122.2, 60.4, 60.0, 50.6, 48.6, 48.2, 42.6, 37.0, 36.0, 33.9, 32.9, 31.8, 21.7, 17.6. HRMS (ESI) calcd for C31H41N6O3 [M + H]+, 587.3453; found, 587.3453.

tert-Butyl ((R)-1-((R)-1-(((2-(1-((6-(((bis(pyridin-2-ylmethyl)amino)methyl)nicotinoyl)piperazin-1-yl)-1-oxopropan-2-yl)carbamate (21). 5

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temperature for 16 h. The mixture was then concentrated under reduced pressure, the residue was dissolved in distilled H2O, the pH was adjusted to 8 with saturated aqueous K2CO3 solution, and the mixture was concentrated under reduced pressure. Purification of the product was achieved by way of dry column vacuum chromatography on C18 material using stepwise elution from 10 to 90% methanol in water, affording 48 mg (0.088 mmol, 74%) of product.1H NMR (400 MHz, methanol-d4) δ 8.54 (s, 1H), 8.44 (d, J = 4.7 Hz, 2H), 7.85 (d, J = 8.0 Hz, 1H), 7.79 (t, J = 7.7 Hz, 2H), 7.74 (d, J = 8.0 Hz, 1H), 7.67 (d, J = 7.8 Hz, 2H), 7.28 (t, J = 6.0 Hz, 2H), 3.91 (s, 2H), 3.90 (s, 4H), 3.86−3.39 (m, 9H), 1.33 (d, J = 6.3 Hz, 3H), 1.28 (d, J = 6.7 Hz, 3H).13C NMR (101 MHz, methanol-d4) δ 177.6, 173.2, 169.8, 162.3, 160.0, 149.6, 148.2, 138.7, 137.2, 131.3, 123.0, 124.4, 123.9, 61.4, 61.1, 51.3, 46.4, 21.3, 17.9. APCI-HRMS m/z calcd for C29H37N8O3 [M + H]+, 545.2983; found, 545.2981.

■ ASSOCIATED CONTENT

2 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.8b00137.

1H and 13C spectra, NMR titration experimental details for compound 15, HepG2 toxicity curves, and in vivo toxicity data (PDF)

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Notes

The authors declare the following competing financial interest(s): OAHA, ØS, PR, AB, HKSL, GKA and CS have filed a patent on these results.

■ REFERENCES


