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10.1021/acs.jmedchem.7b01728

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**In silico** fragment based design identifies subfamily B1 metallo-β-lactamase inhibitors


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**ABSTRACT**

Zinc ion dependent β-lactamases (MBLs) catalyze the hydrolysis of almost all β-lactam antibiotics and resist the action of clinically available β-lactamase inhibitors. We report how application of in silico fragment-based molecular design employing thiol-mediated metal
anchorage leads to potent MBL inhibitors. The new inhibitors manifest potent inhibition of clinically important B1 subfamily MBLs, including the widespread NDM-1, IMP-1 and VIM-2 enzymes; with lower potency, some of them also inhibit clinically relevant Class A and D serine-β-lactamases. The inhibitors show selectivity for bacterial MBL enzymes compared to human MBL fold nuclease. Co-crystallization of one inhibitor, which shows potentiation of meropenem activity against MBL-expressing Enterobacteriaceae, with VIM-2 reveals an unexpected binding mode, involving interactions with residues from conserved active site bordering loops.

INTRODUCTION

β-Lactam containing inhibitors remain the single most important antibacterials.1,2 β-Lactams inhibit transpeptidases (or penicillin-binding proteins, PBPs) involved in cell wall biosynthesis by reacting with a catalytically crucial nucleophilic serine residue. Bacteria have developed multiple resistance mechanisms to β-lactams, most importantly by β-lactamase catalysed hydrolysis. There are four classes of β-lactamases:3 classes A, C, and D are serine-β-lactamases (SBLs), which are mechanistically related to the PBPs; class B are metallo-β-lactamases (MBLs), employing (a) zinc ion(s) in catalysis.

Co-administration of a β-lactam antibacterial with a class A β-lactamase inhibitor (clavulanic acid,4 tazobactam,5 sulbactam) has successfully overcome some SBL-mediated resistance. Avibactam,6 a non β-lactam broader spectrum inhibitor of class A, C, and some class D SBLs, has recently been introduced into the clinic.7 As yet there are, however, no reports of clinically useful class B MBL inhibitors (Figure 1a).8-11 MBLs are a concern because they catalyze hydrolysis of almost all β-lactam antibacterials, including the carbapenems, which are commonly used for treatment of severe and highly resistant infections. MBLs are divided into the B1, B2,
and B3 subclasses based on metal site occupancy and sequence similarity.\textsuperscript{12-14} Subclass B1 MBLs, which employ two zinc ions (Zn-1/Zn-2) are the most clinically relevant and include the New Delhi metallo-β-lactamase (NDM-1), Verona integron-encoded MBL (VIM), and Imipenemase (IMP) types of B1 MBLs.

Combating increasing resistance mediated by MBLs requires the identification of either novel antibacterials or MBL resistant β-lactams (both likely substantial undertakings), or developing an MBL inhibitor for co-administration with an existing β-lactam. Here we report how the application of \textit{in silico} fragment-based\textsuperscript{15, 16} molecular design led to identification of potent inhibitors of clinically relevant MBLs, some of which have (lower) activity against certain SBLs.

The \textit{in silico} studies employed a structure-guided approach in which fragments were anchored to the di zinc ion centre \textit{via} a thiol and then optimised for binding \textit{via} hydrophilic and electrostatic interactions. Whilst such metal anchorage (or 'support ligand') based approach has been applied in dynamic combinatorial based identification\textsuperscript{17} of ligands to MBLs, to our knowledge this is the first application of an \textit{in silico} fragment-based approach for production of specific MBL inhibitors.

**RESULTS AND DISCUSSION**

\textit{De Novo Ligand Design}

The \textit{de novo} molecular design program SPROUT\textsuperscript{15, 16} generates potential ligands \textit{via} docking of fragments into targeted regions; subsequent user-directed assembly using template spacers to link these fragments together yields synthetically tractable scaffolds (Figure 1b). Analysis of an crystal structure of NDM-1 structure complexed with hydrolysed ampicillin (PDB code 3Q6X)\textsuperscript{14} using SPROUT\textsuperscript{16} identified active site regions for targeting with \textit{in silico}-generated fragments,
i.e. the regions proximal to: the Lys224 side chain, the Zn-2 metal ion, the nucleophilic hydroxide / water that “bridges” the two zinc ions, and a conserved tryptophan (Trp87) that makes hydrophobic interactions with the aromatic ampicillin C6 side chain\(^{18}\) (Figure 1c). Importantly, while details differ between individual MBLs, these features are predicted to be crucial for β-lactam binding by most, if not all, B1 MBLs.\(^{10}\) This design approach led to the identification of a simple benzyl thiol 1 (Figure 1) as a potential NDM-1 substrate-competitive inhibitor. A detailed, step-by step description of the \textit{in silico} fragment based design novo design is given in the SPROUT utilizing design sections (Supporting Information).

The \textit{in silico} predicted interactions of 1 with NDM-1 include interaction between its carboxylate and the Lys224 side chain and the Zn-2 ion; anchoring of its thiol with both Zn-1 and Zn-2; and between the aryl ring of 1 with Trp87, via π-stacking interactions (Figure 2a). Compound 1 was synthesized using Suzuki coupling (67%)\(^{19}\) of 5-bromophthalide and phenylboronic acid to give a lactone which was ring-opened using Me\(_3\)SiI to give the corresponding iodide (62%). Iodide displacement using thiourea, followed by hydrolysis gave 1 (81%; see Methods and Supporting Information for details).

Using a fluorogenic MBL assay\(^{20}\) 1 was then evaluated for inhibition of recombinant forms of NDM-1, IMP-1, and VIM-2. Inhibition studies with 1 and subsequent compounds were conducted in the presence of 0.01% Triton X-100 to reduce the potential of identifying protein aggregating compounds\(^{21-23}\). The results (Table 1) reveal 1 to be an inhibitor with sub-micromolar IC\(_{50}\) values for all three of the tested B1 MBLs, with IC\(_{50}\) values 232 – 234 nM; these values compare favourably with the thiol-MBL inhibitors such as L-captopril for which IC\(_{50}\) values against B1 MBLs are in the micromolar range.\(^{10}\)
Crystallographic studies

To investigate the binding of 1 with B1 MBLs, a structure of it complexed with VIM-2 was obtained by co-crystallization (1.74 Å resolution; space group C21; see Supporting Information, Figure S1 and Table S1). As predicted by the design studies, analysis of the crystal structure confirms that the thiol of 1 acts as a metal binding ligand that displaces the proposed ‘hydrolytic’ hydroxide ion bridging the two metal ions (Figure 2b). The crystallographically observed conformation of the biphenyl unit in VIM-2 was not, however, as predicted for NDM-1 (Figure 1 and Figure 2a); unexpectedly, the aryl carboxylate of 1 is positioned to interact with Trp87 of VIM-2 and, via a water molecule, with Asp119, rather than with Arg228 (analogous to Lys 224 in NDM-1) (Figure 2b). In the VIM-2 structure, 1 presents two binding faces, as also present in the predicted β-lactam substrate, and observed hydrolysed β-lactam, binding modes. One face is hydrophobic and interacts with the substantially hydrophobic L3 loop, via apparent π-stacking of the aryl ring of 1 with Tyr67 and interactions with Trp87 (Figure 2b). The other binding face of 1 involves electrostatic and hydrogen bonding interactions with residues from L10 loop, apparent interaction between the Arg228 guanidino group and the phenyl ring of 1 (3.8 Å), and potential interaction between one of the carboxylate oxygen’s of 1 with the side chain amide of Asn233 (3.6 Å). The difference in the predicted NDM-1 binding mode for 1 versus the crystallographically observed binding mode of 1 with VIM-2 may, in part, reflect the removal of water molecules from the NDM-1 structure prior to application of SPROUT to simplify the calculations, as well as active site differences between the wo enzymes (see Supporting Information).
Structure Activity Relationship Studies

Following the encouraging levels of inhibition observed with 1, we conducted structure activity relationship (SAR) studies. Modification of the terminal phenyl ring of 1 (Table 1) revealed no clear trend in potency when changing the electron density or steric bulk, via incorporation of bromine (17) or five-membered ring substituents (15 and 16), implying that the π-stacking interaction with Tyr67 is not crucial for affinity. Similarly, positioning substituents in the ortho-position of the phenyl ring (4, 9 and 10), to investigate whether changes to the dihedral angle between the aryl rings might affect affinity, did not yield substantial changes in potency. The importance of the spatial arrangement of functional groups was investigated via 19 (Supporting Information, Figure S5) in which the methylene linking the thiol to the phenyl ring is switched to link the carboxylic acid. The thiolactone 18, (Supporting Information, Figure S4) was synthesised to test whether it undergoes hydrolysis to 1 (Supporting Information, Table S2). Neither 18 nor 19 manifested significant activity against VIM-2 (IC₅₀ > 100 μM) showing that the arrangement of the functional groups is important and indicating that the thiolactone 18 does not appear to be an efficient substrate for VIM-2.

2-17 were evaluated for inhibition against NDM-1 and IMP-1 (Table 1 and Supporting Information, Table S2); selected compounds (4-8) were screened against the model MBL BcII and the ‘hybrid’ B1/B2 like MBL SPM-1.²⁴ The results reveal 4-8 to be active against BcII and SPM-1, with IC₅₀ values 0.78 – 7.8 μM (Supporting Information, Table S3). Moreover, except for 11, all compounds manifested sub-micromolar IC₅₀ values against IMP-1 with several compounds showing sub-micromolar activity against NDM-1 (5, 6, 7, 8 and 13). Binding of selected inhibitors to VIM-2 and NDM-1 was investigated by NMR.²⁵, ²⁶ The ¹H CPMG NMR results validated²⁵ binding of 1 and 9 to VIM-2 and ¹⁹F NMR²⁶ was used to validate binding to
NDM-1 (Supporting Information, Figure S7). Consistent with the inhibition data, the NMR analyses show that 1 and 9 bind with $K_D$ values < 1 µM to VIM-2 and 9 out competes 11 for binding to NDM-1 (Supporting Information, Figure S8).

In the initial assessment of our inhibitors as hits we screened 4 and 9 against the SBL AmpC$^{21}$; 4 and 9 showed activity against AmpC both in the presence and absence of Triton X-100 (Supplementary Information, Table S4). We then screened 1, 7, 8 and 9 against TEM-1, AmpC, and OXA-10, which are representatives of Class A, C, and D SBLs, respectively (Supporting Information, Table S4). Interestingly, 8 and 9 inhibited TEM-1 and 9 inhibited OXA-10 (IC$_{50}$s 32.8, 5.5 and 37.3 µM, respectively, Supporting Information, Table S4). Although we have been unable to obtain structures of these compounds complexed with SBLs, these results show some of the inhibitors could interact with both SBLs and MBLs via related binding modes as shown in Figure 2); indeed, our predicted binding mode of 1 to TEM-1 and OXA-10 is similar to that observed crystallographically for a recently identified biphenyl carboxylic acid obtained through a surface plasmon resonance (SPR) based screen for inhibitors of the OXA-48 SBL$^{27}$. However, the unexpected crystallographically observed binding mode of 1 to VIM-2 (Figure 2) means caution should be exercised in predicting the SBL binding modes of our compounds.

**Selectivity screening**

Clinically relevant MBL inhibitors should show selectivity towards the bacterial MBLs over human metallo-enzymes, including human MBL-fold enzymes.$^{28}$ We tested 1, 5, 13 and 17 against two human MBL fold enzymes involved in DNA repair, DNA cross-link repair enzymes 1A and B (DCLRE1A and DCLRE1B). DCLRE1A was only weakly inhibited by 1 (IC$_{50}$ 247 ± 154 µM) and DCLRE1B was inhibited by 1, 5 and 17 (IC$_{50}$s 149 ± 27, 268 ± 112 and 185 ± 50
μM, respectively), implying, at least partial, selectivity of the compounds towards the bacterial MBLs. Compound 1 showed good aqueous solubility and underwent moderate metabolism in human liver microsomes (see Supporting Information).

**Antibacterial Activity**

Compounds 1 and 11-17 were screened in minimal inhibitory concentration (MIC) antimicrobial assays, in co-administration studies with the clinically important carbapenem meropenem, using MBL producing strains of *E. coli* and *K. pneumoniae* (species where NDM-1 production is a growing clinical problem).\(^2^9\) Tests in the absence of inhibitors manifested meropenem MICs against MBL-producing bacteria of >128 μg/ml, and that when tested alone 1 has no antibacterial activity within limits of detection (Table 2). Encouragingly, at 100 μg/ml all tested compounds, apart from 11, reduced the MIC of meropenem against NDM-1-producing strains. Compound 16 showed the strongest activity, reducing the meropenem MIC to 8 μg/ml against *K. pneumoniae*.

**CONCLUSIONS**

Overall, the results reveal how computer aided design can help generate broad spectrum MBL inhibitors active against three types of clinically important B1 MBLs, as well as some SBLs. The inhibitors can reduce MICs of meropenem against MBL-producing bacteria, indicating they are suitable for further development. Unlike some reported MBL inhibitors,\(^3^0\) our compounds bind directly to the MBL active sites, i.e. do not function by removing the zinc ions, as shown by NMR and crystallographic analyses.\(^3^1\) While utilizing its thiol to bind the MBL active site by intercalating between the two B1 MBL subclass zinc ions has precedent,\(^8\) the
crystallographically observed mode of binding 1 with VIM-2 (Figure 1), has not been previously observed. The difference between the crystallographically observed binding mode for 1 with VIM-2 and that predicted for NDM-1 (Figure 2) indicates how differences in closely related metallo-enzymes can apparently enable different binding modes of the same inhibitors, even when they are anchored in the same manner, i.e. to the two active site zinc ions of the B1 MBLs. It may also reflect of limitations in our current computational methodology, including with an inability to deal with solvation.

The observation of activity for the designed compounds against several different types of clinically relevant B1 MBLs is notable (Table 1) because inhibition of a spectrum of MBLs is a likely prerequisite for clinical application of an MBL inhibitor. Although the compounds described here are unlikely to be of direct clinical utility, the work suggests that computational chemistry can have an important role in designing inhibitors that are active for use against both current and future predicted antibacterial resistance mediated by β-lactamases. The need for an ability to rapidly optimise against emerging resistance is highlighted by the identifications of the multiple class B1 MBL variants already identified. Our approach may have utility in efficiently identifying other inhibitor chemotypes involving alternative metal-coordinating moieties, as well as inhibitors not relying on metal coordination.

EXPERIMENTAL SECTION

The Supporting Information contains a complete general experimental section, including all procedures and equipment used. Chemicals were from commonly used suppliers (Aldrich, Acros, and Alfa Aesar) and used without purification. The purity of compounds submitted for screening
was ≥95% as determined by UV analysis of liquid chromatography (HPLC) chromatograms at 254 nM.

**General procedure for the Suzuki cross coupling reaction**

The boronic acid (1.1 equiv.) was added to a stirred solution of 5-bromophthalide (1.0 equiv.), potassium carbonate (1.0 equiv.) and tetrakis(triphenylphosphine) palladium (0) (0.05 equiv.) in THF (4 mL) and water (2 mL). The reaction mixture was heated at reflux for 12 h, then filtered through Celite®, and concentrated in vacuo. The residue was diluted with water (20 mL), then extracted using dichloromethane (3 × 20 mL). The combined organic extracts were dried (MgSO₄) and concentrated in vacuo to give a pale-yellow solid, which was purified using flash column chromatography to afford the coupled product.

**General procedure for lactone opening with iodontrimethylsilane forming the iodides**

The benzoﬂuranone (1.0 equiv.) was dissolved in dichloromethane (4.5 mL); TMSI (1.5 equiv.) was then added and the reaction was refluxed under nitrogen for 3 h. After this time, the mixture was cooled to room temperature and quenched with water (3 mL). The precipitate was isolated by filtration washing with water (2 × 10 mL) to give the desired iodide product as an off-white solid, which was used without further purification.

**General procedure for introduction of the thiol”**

A mixture of thiourea (1.1 equiv.) in THF (3 mL) was heated to reflux. To this mixture was added the iodide product of the previous procedure (1.0 equiv.). The reaction mixture was heated at reflux for 16 h under nitrogen. The reaction mixture was then cooled to room temperature and concentrated in vacuo. The residue was re-suspended in 2M aqueous NaOH (5 mL) and the solution reﬂuxed for 3 h. The reaction mixture then acidified with 2M aqueous HCl to ~pH2. The colorless precipitate was isolated by filtration.
Representative synthesis: for compound 1

Synthesis of 5-phenyl-1,3-dihydro-2-benzofuranone

Phenyl boronic acid (0.32 g, 2.58 mmol, 1.1 equiv.) was added to a stirred solution of 5-bromophthalide (0.50 g, 2.35 mmol, 1.0 equiv.), potassium carbonate (0.33 g, 2.35 mmol, 1.0 equiv.) and tetrakis(triphenylphosphine) palladium (0) (0.14 g, 0.12 mmol, 0.05 equiv.) in THF (4 mL) and water (2 mL). The reaction mixture was heated to reflux for 12 h, then filtered through Celite®, and concentrated in vacuo. The residue was diluted with water (20 mL) and extracted using dichloromethane (3 × 20 mL). The combined organic extracts were dried (MgSO₄), then concentrated in vacuo to give a pale-yellow solid, which was purified by flash column chromatography to afford the desired product as a cream colored solid, which was used without further purification. (0.33 g, 1.58 mmol, 67%) m.p.: 124.4-125.8 °C; Rf: 0.5 (2:1 petrol-EtOAc); δH (300 MHz, CDCl3): 7.98 (1H, d, J 7.6, 3-H), 7.75 (1H, d, J 7.6, 4-H), 7.68 (1H, s, 6-H), 7.62 (2H, d, J 7.1, 2’-H and 6’-H), 7.47 (2H, app. t, J 7.1, 3’-H and 5’-H) 7.45 (1H, t, J 7.1, 4’-H), 5.38 (2H, s, CH2O); δC (75 MHz, CDCl3): 147.5 (C=O), 147.3 (C1), 139.6 (C3), 129.1 (C3’’ and C5’’), 128.6 (C2), 128.5 (C5), 127.5 (C2’ and C6’) 126.1 (C4’), 124.5 (C1’), 120.6 (C6), 69.9 (CH2O); vmax/ cm⁻¹ (solid): 3473, 3070, 2924, 1747, 1615, 1452; HPLC: Tr= 3.00 (100% rel. area); m/z (ES): (Found: MNa+, 233.0573. C14H10O2 requires MNa, 223.0570).

Synthesis of 2-iodomethyl-4-phenyl-benzoic acid

5-Phenyl-1,3-dihydro-2-benzofuranone (0.20 g, 0.95 mmol, 1.0 equiv.) was dissolved in dichloromethane (4.5 mL). TMSI (0.20 mL, 1.43 mmol, 1.5 equiv.) was added and the reaction was refluxed under nitrogen for 3 h. The mixture was then cooled to room temperature and quenched with water (3 mL). The precipitate was isolated by filtration, washing with water (2 ×
10 mL) to give the desired product as a pale brown solid, which was used without further purification. (0.20 g, 0.59 mmol, 62%) m.p.: 81.3-82.4 °C; Rf: 0.75 (CHCl3); δH (300MHz, DMSO-d6): 7.94 (1H, d, J 8.3, 6-H), 7.88 (1h, d, J 1.8, 3-H), 7.73 (2H, d, J 7.3, 6’-H and 2’-H), 7.68 (1H, dd, J 8.3 and 1.8, 5-H), 7.51 (2H, app. t, J 7.3, 5’-H and 3’-H), 7.44 (1H, t, J 7.3, 4’-H), 5.09 (2H, s, CH2); vmax/ cm-1 (solid): 2806, 2630, 1750, 1603, 1257, 909; m/z (ES): (Found: [M+2Na-H]+, 382.9516. C14H11IO2 requires [M+2Na-H], 382.9515).

**Synthesis of 4-phenyl-2-sulfonylmethylbenzoic acid (I)**

A mixture of thiourea (0.16 g, 2.11 mmol, 1.1 equiv.) and 2-Iodomethyl-4-phenyl-benzoic acid (0.65 g, 1.92 mmol, 1.0 equiv.) in THF was heated at reflux for 16 h under an atmosphere of nitrogen, then cooled to room temperature. The mixture was then concentrated in vacuo, then re-suspended in 2M aqueous NaOH (5 mL) and refluxed for 3 h. The resultant mixture then acidified with 2M aqueous HCl to ~pH2. The pale yellow microcrystals were isolated by filtration. C14H10Cl2O2S requires [M-H], 310.9706). (0.25 g, 1.01 mmol, 53%). m.p.: 168.2-169.9 °C; Rf: 0.20 (19:1 DCM–MeOH); δH (300 MHz, DMSO-d6): 7.95 (1H, d, J 8.2, 6-H), 7.77 (1H, d, J 2.0, 3-H), 7.73 (2H, d, J 7.4, 6’-H and 2’-H), 7.65 (1H, dd, J 8.2 and 2.0, 5-H), 7.52 (2H, app. t, J 7.4, 5’-H and 3’-H), 7.44 (1H, t, J 7.4, 4’-H), 4.13 (2H, d, J 8.5, CH2), 2.87 (1H, t, J 8.5, SH); δC (75 MHz, DMSO-d6): 168.0 (C=O), 144.3 (C4), 143.6 (C1’), 138.7 (C2), 131.5 (C6), 129.0 (C5’ and C3’), 128.9 (C4’), 128.2 (C5), 127.8 (C1), 126.9 (C6’ and C2’), 124.9 (C3), 26.4 (CH2); vmax/ cm-1 (solid): 2976, 1681, 1350, 1278, 756; HPLC: Tr= 2.88 (95% rel. area); m/z (ES): (Found: [M-H]–, 243.0477 C14H12O2S requires [M-H], 243.0485).
ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Experimental procedures, characterization of intermediates and target compounds, description of protein production and purification, biological assays and determination of IC$_{50}$ values. (PDF)

Accession Codes

PDB ID code: VIM-2 with bound 1 is 5K48. Authors will release the atomic coordinates and experimental data upon article publication.

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Author Contributions

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Notes

The authors declare no competing financial interests.

Acknowledgements
We thank the Medical Research Council (MRC)/Canadian Grant G1100135 (RC, JS, CWGF, JB and CJS), the Wellcome Trust, and the Biotechnology and Biological Research Council for funding (BBSRC). This work was supported by Cancer Research UK (CR-UK) grant number C5255/A18085, through the Cancer Research UK Oxford Centre (JB).

ABBREVIATIONS USED
MBL, metallo-β-lactamase; SBL, serine-β-lactamase; TMSI, iodotrimethylsilane.
References


Figure 1. Design of broad spectrum MBL inhibitors. A) Known MBL binders (inhibitors) and compound 1.\textsuperscript{8-11} B) Key elements used in the SPROUT computational design process leading to the identification of putative broad spectrum MBL inhibitors. C) Outline mechanism for SBLs and MBLs as shown for a penicillin substrate.
**Figure 2.** Predicted potential binding modes for the designed NDM-1 inhibitors and the crystallographically observed binding mode for VIM-2. (A), (C), and (D) shows views from modelling of 1 into the active sites of NDM-1, TEM-1, and OXA-10, respectively (see Supporting Information for details); (B) shows view from a crystal structure of 2 co-crystallized with VIM-2 (PDB ID: 5K48).
Table 1. IC50 values of *de novo* designed thiols against clinically relevant B1 MBLs. Assays were conducted as reported20.

![Chemical structure](image)

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a cLogP values were calculated using MarvinSketch 17.28 (ChemAxon).

b The IC50 values are shown as the mean ± SD from minimum three separate experiments.
Table 2. Determination of Meropenem MICs against bacterial strains when co-administered with the designed β-lactamase inhibitors. Assays were conducted as reported\textsuperscript{11}.

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>Compound</th>
<th>Meropenem MIC (μg/mL)</th>
<th>Meropenem MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NDM-1 producing <em>K. pneumoniae</em> (ATCC 5055)</td>
<td>NDM-1 producing <em>E. coli</em> (MG1655)</td>
</tr>
<tr>
<td>Meropenem</td>
<td></td>
<td>&gt; 128</td>
<td>&gt; 128</td>
</tr>
<tr>
<td>1</td>
<td>C\textsubscript{6}H\textsubscript{5}</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>17</td>
<td>Br</td>
<td>64</td>
<td>32</td>
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<tr>
<td>11</td>
<td>3,5-CF\textsubscript{3}C\textsubscript{6}H\textsubscript{3}</td>
<td>&gt;128</td>
<td>&gt;128</td>
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<tr>
<td>12</td>
<td>2-Naphthyl</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>13</td>
<td>4-Et-C\textsubscript{6}H\textsubscript{4}</td>
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<tr>
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<td>3-EtO-tC\textsubscript{6}H\textsubscript{4}</td>
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<tr>
<td>15</td>
<td>2-Furyl</td>
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<tr>
<td>16</td>
<td>3-Thienyl</td>
<td>8</td>
<td>16</td>
</tr>
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</table>
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