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Discovery of Bisamide-heterocycles as Inhibitors of Scavenger Receptor BI (SR-BI)-mediated Lipid Uptake

Chris Dockendorff
Marquette University, christopher.dockendorff@marquette.edu

Patrick W. Faloon
Broad Institute

Andrew Germain
Broad Institute

Miao Yu
Massachusetts Institute of Technology

Willmen Youngsaye
Broad Institute

See next page for additional authors

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A new series of potent inhibitors of cellular lipid uptake from HDL particles mediated by scavenger receptor class B, type I (SR-BI) was identified. The series was identified via a high-throughput screen of the National Institutes of Health Molecular Libraries Small Molecule Repository (NIH MLSMR) that measured the transfer of the fluorescent lipid Dil from HDL particles to CHO cells overexpressing SR-BI. The series is characterized by a linear peptidomimetic scaffold with two adjacent amide groups, as well as an aryl-substituted heterocycle. Analogs of the initial hit were rapidly prepared via Ugi 4-component reaction, and select enantiopure compounds were prepared via a stepwise sequence. Structure–activity relationship (SAR) studies suggest an oxygenated arene is preferred at the western end of the molecule, as well as highly lipophilic substituents on the central and eastern nitrogens. Compound 5e, with (R)-stereocchemistry at the central carbon, was designated as probe ML279. Mechanistic studies indicate that ML279 stabilizes the interaction of HDL particles with SR-BI, and its effect is reversible. It shows good potency (IC50 = 17 nM), is non-toxic, plasma stable, and has improved solubility over our alternative probe ML278.

Keywords: ML279, SR-BI inhibitor, HDL receptor, Cholesterol transport, HCV

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The trafficking of lipids (e.g., cholesterol and its esters) between tissues is critical for lipid homeostasis as well as steriodogenesis. The key players in this transport system include lipoprotein particles (e.g., LDL and HDL) that carry the water-insoluble lipids through the bloodstream, the enzymes that modify the lipid cargos and/or help to transfer them from one particle to another (including CETP and LCAT), and the receptors for the lipoprotein particles that serve to transfer the lipids into and out of cells (i.e., uptake and efflux). The cellular receptor for high-density lipoprotein (HDL) particles, scavenger receptor class B, type I (SR-BI), has been studied in detail in recent years. It serves to transfer the lipids into and out of cells (i.e., uptake and efflux). The cellular receptor for high-density lipoprotein (HDL) particles, scavenger receptor class B, type I (SR-BI), has been studied in detail in recent years.

We herein report our discovery of a second class of SR-BI inhibitors that shows distinct advantages over those previously discovered in our labs (e.g., BLT-1 and BLT-3). Concurrent with this work was our discovery of the indole-thiazole ML278, followed soon after by the discovery of the benzo-fused lactams represented by ML312. Other inhibitors of SR-BI have been reported, including HDL376, ITX-5061, R-138329, and R-154716. Several other inhibitors of SR-BI have been reported, including HDL376, ITX-5061, R-138329, and R-154716. Recently, researchers at iTHERX reported additional HDL entry inhibitors, including ITX-4520, which is postulated to be an inhibitor of SR-BI. Our discovery of the bisamide inhibitors described herein was undertaken as part of the NIH Molecular Libraries Program (MLP).

To elucidate more details about the mechanism of lipid uptake and efflux via SR-BI, and to potentially identify less toxic and more potent small molecule probes, we undertook a high-throughput screening (HTS) campaign measuring the uptake of the fluorescent lipid surrogate 1,1-dioctadecyl-3,3,3-tetramethylindocarbocyanine perchlorate (DiI) from HDL particles into CHO cells overexpressing mouse SR-BI (mSR-BI). 3046 compounds (0.96%)
were classified as inhibitors of DiI-HDL uptake out of 319,533 compounds tested, with inhibition at 12.5 μM of ≥70% relative to 1 μM BLT-1 as positive control. Hit compounds were omitted that were on plates with Z’ <0.3 or that were active in 10% or more of the HTS assays listed in PubChem. A counterscreen was also performed that rejected hit compounds that quenched the fluorescence of Dil-HDL in a dose-dependent manner.

Of the numerous scaffolds verified to inhibit Dil uptake with IC50 of <1 μM, we focused our efforts in part on a bisamide–tetrazole series characterized by the commercially-available compound 1a,29 with IC50 of 0.055 μM in the primary assay (Table 1). We deduced that such compounds could be made rapidly via Ugi 4-component coupling reactions (Eq. 1),30–32 and thus would be amenable to rapid structure–activity relationship (SAR) studies.

1a also showed no measurable cytotoxicity in the cell line used for our assays (ldlA[mSR-BI]) after incubation for 24 h. Additionally, 1a lacked non-selective behavior according to data published in PubChem.33 Finally, in contrast to our hit compounds in the indoline–thiazole series,16,17 members of the bisamide–tetrazole series showed improved aqueous solubility (114 μM for 1a in PBS with 1% DMSO).

We explored the SAR of the dipeptide scaffold by varying each of the components of the Ugi reaction. Reactions were typically performed using a 1:1:1:1 ratio of aldehyde, amine, carboxylic acid, and isocyanide in the Ugi 4-CR (Eq. 1).

### Table 1

<table>
<thead>
<tr>
<th>Compd</th>
<th>Ar1</th>
<th>IC50’ (μM)</th>
<th>Compd</th>
<th>Ar1</th>
<th>IC50’ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td></td>
<td>0.055 ± 0.014</td>
<td>1b</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td>1c</td>
<td></td>
<td>0.81</td>
<td>1d</td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>1e</td>
<td></td>
<td>17</td>
<td>1f</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>1g</td>
<td></td>
<td>7.1</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* Average of at least two measurements in Dil uptake assay, ±standard error of mean when n >2.

### Table 2

<table>
<thead>
<tr>
<th>Compd</th>
<th>Ar1</th>
<th>IC50’ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>MeO</td>
<td>0.055 ± 0.014</td>
</tr>
<tr>
<td>2a</td>
<td></td>
<td>37</td>
</tr>
<tr>
<td>2b</td>
<td>MeO</td>
<td>0.13</td>
</tr>
<tr>
<td>2c</td>
<td>MeO</td>
<td>6.3</td>
</tr>
<tr>
<td>2d</td>
<td>MeO</td>
<td>0.023</td>
</tr>
</tbody>
</table>

* Average of at least two measurements in Dil uptake assay, ±standard error of mean when n >2.
acid, and isonitrile in methanol, and stirring for 18 h. Variation of the isonitrile component allowed us to explore the eastern amide of the scaffold (Table 1). The SAR in this area was very sensitive to modifications, as slight changes in structure from N-cyclopentyl diminished the activity substantially, such as with N-cyclohexyl 1b (IC50 = 0.20 \mu M) and N-isopropyl 1c (0.81 \mu M). The amide N–H is required for high activity, as the tertiary amide 1f had low activity.

Compound 1f was prepared via a sequential peptide synthesis, as selective methylation of 1a proved to be problematic.

Our results exploring tetrazole replacements are summarized in Table 2. Replacement of the aryl tetrazole with a simple phenyl group (2a) abrogated activity. Several heterocyclic replacements for the tetrazole were prepared from commercially available ary lacetic acids: oxazole 2b showed approximately two-fold decreased activity (IC50 = 0.13 \mu M), thiazole 2c possessed poor activity (IC50 = 6.3 \mu M), and isoxazole 2d demonstrated excellent potency (IC50 = 0.023 \mu M). One possible explanation for these results is that a hydrogen-bond acceptor at the 4-position (numbering of tetrazole ring system) is required for optimal activity. Despite the excellent potency with isoxazole 2d, we elected to continue our studies with the tetrazole analogs, for which we had a larger collection of building blocks.

Next, the western end of the scaffold was examined in detail by varying the aromatic substituent (Table 3). Removal of the 4-methoxy group of 1a led to a significant drop in potency (3b, IC50 = 0.18 \mu M). Several 2- and 3-substituted arenes were examined with both electron-withdrawing and donating groups; these were uniformly inferior to the 3,4-dimethoxyarene of 1a. Several 4-substituents were tolerated to some extent, including chlorine (3h, IC50 = 0.17 \mu M) and fluorine (3i, IC50 = 0.13 \mu M). The best results were obtained with the 3,4-methylenedioxy group (3k, IC50 = 0.023 \mu M), which is more potent than 1a. We also examined the difluoromethylene analog of 3k (3l, IC50 = 0.049 \mu M), which showed decreased activity relative to 1a, along with the decreased activity of the 4-methyl analog (3g, IC50 = 0.49 \mu M), suggests that an appropriate 4-aryl substituent may act as a hydrogen bond acceptor with the target.

We examined the effects of varying internal positions of the structure while maintaining the tetrazole along with either a terminal 3,4-dimethoxyphenyl or 3,4-methylenedioxyphenyl group at the western end of the scaffold. Several amines were screened in the Ugi reaction, leading to different substituents at R4 (Table 4). The cyclohexyl substituent of 1a again proved...
optimal. Conservative changes were tolerated to some degree (such as 4c, R4 = cyclopentyl, IC50 = 0.11 μM), but clearly a bulky, lipophilic group is optimal. Inserting a carbon atom between the cyclohexyl group and the backbone nitrogen decreased activity by greater than 20-fold (4d, IC50 = 1.6 μM). Introducing a nitrogen atom expected to be protonated at physiological pH abrogated all activity (4e).

Next, substitution at the alpha carbon atom of the eastern amide was investigated (Table 5). Introducing a second methyl group gave a drastic drop in activity (5a), as did the replacement of the methyl group of 1a with a bulkier isopropyl group (5b). Compound 5c, lacking the methyl group of the parent compound 3k, showed slightly improved activity (IC50 = 0.027 μM).

Finally, each enantiomer of 3k was prepared in a stepwise fashion from (R)- or (S)-alanine (Scheme 1). The required tetrazole acetic acid building block was made by cycloaddition between benzonitrile 6 and sodium azide, then alkylation with methyl bro-moacetate and hydrolysis of the resulting ester to give 8. (R)-N-Boc alanine (9) was then coupled with aminocyclopentane using standard peptide coupling conditions (EDC, HOBt, (i-Pr)2NEt). The resulting amide 10 was treated with TFA to liberate the amine, then subjected to reductive alkylation with cyclohexanone and sodium cyanoborohydride to generate the N-cyclohexylamine 12. Peptide coupling reactions with this relatively hindered secondary amine were sluggish, so 12 was reacted with the acid chloride of tetrazole acetic acid 8 to generate the desired bisamide 5e. The enantiomer 5d was prepared analogously from (S)-N-Boc-alanine. The (R)-isomer 5e (IC50 = 0.017 μM) was 100-fold more potent than its enantiomer 5d.

Additional studies with 5e were performed to study its mode of action. Its inhibitory action is reversible. In experiments where cells were pre-treated with ML279 for 2 h, washed extensively with PBS and then incubated with Dil-HDL, no inhibition was observed. In addition to measuring the uptake of Dil from HDL in the presence of 5e, the uptake of 3H-labeled cholesteryl oleate ester ([3H]CE) from [3H]CE–HDL was measured with an IC50 of 0.005 μM (Supporting information Fig. 2), and is significantly more potent than the clinical compound ITX-5061 in a head-to-head study. The binding of Alexa-488-labeled HDL particles to ldlA[mSR-BI] cells via SR-BI was also measured. As with BLT-1 and other inhibitors, including our recently disclosed probe ML278, 5e enhanced the level of HDL binding to SR-BI, with a measured EC50 of 0.27 μM (Supporting information Fig. 3). One possibility is that such compounds act to inhibit lipid transport by slowing the turnover (release) of the bound HDL particles. Finally, we tested to see if 5e was a general inhibitor of receptor-mediated endocytosis by examining its effects on the endocytosis of Alexa-594-labeled transferrin by [lAI[mSR-BI] cells. 5e showed

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Table 5
SAR analysis of central carbon substituents

<table>
<thead>
<tr>
<th>Compd</th>
<th>Structure</th>
<th>IC50a (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td><img src="image" alt="Structure 1a" /></td>
<td>0.055 ± 0.014</td>
</tr>
<tr>
<td>5a</td>
<td><img src="image" alt="Structure 5a" /></td>
<td>19</td>
</tr>
<tr>
<td>5b</td>
<td><img src="image" alt="Structure 5b" /></td>
<td>&gt;25</td>
</tr>
<tr>
<td>3k</td>
<td><img src="image" alt="Structure 3k" /></td>
<td>0.027 ± 0.008</td>
</tr>
<tr>
<td>5c</td>
<td><img src="image" alt="Structure 5c" /></td>
<td>0.026 ± 0.012</td>
</tr>
<tr>
<td>5d</td>
<td><img src="image" alt="Structure 5d" /></td>
<td>1.6</td>
</tr>
<tr>
<td>5e</td>
<td><img src="image" alt="Structure 5e" /></td>
<td>0.017 ± 0.004</td>
</tr>
</tbody>
</table>

a Average of at least two measurements in Dil uptake assay, ±standard error of mean when n >2.

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Scheme 1. Synthesis of ML279.
no inhibition of this process, at concentrations up to 35 μM. This result is consistent with the previous studies that indicated that SR-BI does not mediate lipid uptake via receptor-mediated endocytosis.  

In summary, potent inhibitors of SR-BI-mediated lipid uptake were discovered as part of the NIH Molecular Libraries Probe Production Centers Network (MLPCN) initiative. Profiling of several top compounds led to the nomination of the bisamide tetrazole 5e (ML279) as a probe compound. ML279 has superior solubility to ML278 (IC50 = 17 vs 6 nM in the diI-uptake assay). It is also not associated with this article can be found, in the online version, at MOL files and InChIKeys of the most important compounds. 

References

29. Purchased from Asinex Ltd.
33. It was listed as a hit in 4 assays not involving SR-BI out of 614 biosays listed on December 10, 2011, and a lack of dose–response data on any of these hits means that none had apparently been confirmed as actives.