Letter

Indolinyl-thiazole based inhibitors of Scavenger Receptor-BI (SR-BI)-mediated lipid transport

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Indolinyl-thiazole based inhibitors of Scavenger Receptor-BI (SR-BI)-mediated lipid transport

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Supporting Information Placeholder

ABSTRACT: A potent class of indolinyl-thiazole based inhibitors of cellular lipid uptake mediated by Scavenger Receptor, class B, type I (SR-BI) was identified via a high-throughput screen of the National Institutes of Health Molecular Libraries Small Molecule Repository (NIH MLSMR) in an assay measuring the uptake of the fluorescent lipid DiI from HDL particles. This class of compounds is represented by ML278 (**17-11**), a potent (average IC_{50} = 6 nM) and reversible inhibitor of lipid uptake via SR-BI. ML278 is a plasma-stable, non-cytotoxic probe that exhibits moderate metabolic stability, thus displaying improved properties for in vitro and in vivo studies. Strikingly, ML278 and previously described inhibitors of lipid transport share the property of increasing the binding of HDL to SR-BI, rather than blocking it, suggesting there may be similarities in their mechanisms of action.

The inverse correlation between human plasma HDL cholesterol (HDL-C) levels and risk for adverse events from atherosclerotic coronary artery disease (CAD)[1,2] has generated considerable interest in developing novel therapies that increase HDL-C levels by several different mechanisms,[3] most prominently by CETP (cholesteryl ester transfer protein) inhibition.[4] Despite massive investments in the clinical study of these inhibitors, the strategy of decreasing CAD risk by artificially boosting HDL-C levels has yet to be validated, and our understanding of lipid trafficking and regulation remains incomplete. New pharmacologic tools that selectively modulate HDL-C via different targets would be valuable for in vitro mechanistic studies and in vivo functional analyses. Here we attempted to identify compounds that can modulate the actions of Scavenger Receptor, class B type I (SR-BI),[5] a mammalian high density lipoprotein (HDL) receptor that binds HDL particles on the cell surface and mediates transport of unesterified cholesterol (UC) or cholesteryl esters (CE). Unlike LDL-receptor mediated uptake of lipids, this process is independent of endocytosis.[5,6] In vivo, the structure and composition of plasma HDL, and the metabolic fates of its cholesterol, are controlled by SR-BI.[7] In fact, SR-BI has important influences on the gastrointestinal, endocrine and reproductive systems, as well as development, inflammation/host defense, Hepatitis C Virus (HCV) infection, and cardiovascular physiology.[7–13]

Thus, SR-BI is an interesting drug target, particularly for compounds that may modulate cholesterol levels or inhibit HCV infection. However, despite several informative, mechanistic studies[14–16] the precise details of HDL recognition by SR-BI and consequent lipid uptake and efflux remain unclear; new chemical probes may help further our understanding of these processes.[17]

We and others have previously discovered small molecule inhibitors of SR-BI (some examples in Figure 1), which have subsequently proven to be valuable tools in the study of the activities of SR-BI in vitro and in vivo, including lipid transport and lipoprotein metabolism (BLTs,[18] HDL376,[19,20] R-138329[21]), and HCV infection (ITX-5061[22,23]). Unfortunately, the reported disadvantages of these tool compounds limit their utility, including toxicity (BLT-1), weak potency (BLT-3, BLT-4), specificity (mitogen-activated protein kinase activity; ITX-5061), and potential safety issues for humans (HDL376). ITX-5061 also recently showed disappointing results in a Phase 1b clinical trial.[24] It would be valuable to identify additional potent SR-BI chemical modulators that lack detrimental side effects[25] and that might selectively modulate lipid uptake or efflux.

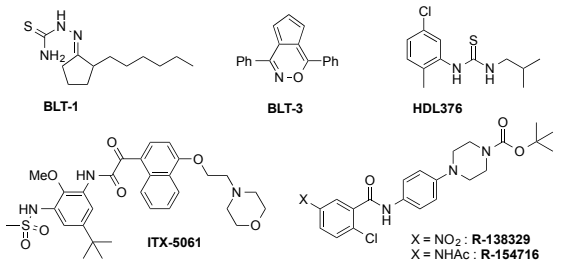
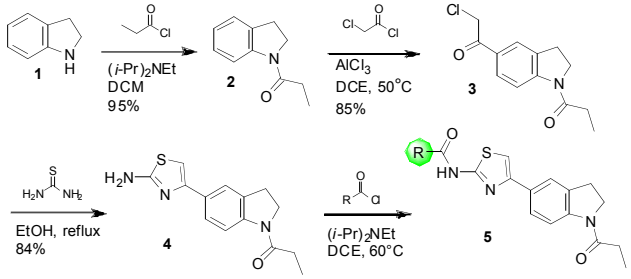


FIGURE 1. Some reported inhibitors of SR-BI

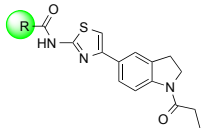
Scheme 1. Synthesis of amide analogs

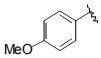
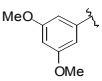


Under the auspices of the NIH's Molecular Libraries Probe Production Centers Network (MLPCN), we used a high-throughput screening approach to identify potent SR-BI modulators with low toxicities and favorable physicochemical properties that may be useful as in vitro and in vivo probes of SR-BI mechanism and function, and that might also be promising lead compounds for drug discovery. The screen involved measuring the effects of cellular uptake of the fluorescent lipid 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) from HDL particles into CHO cells overexpressing mouse SR-BI (ldlA[mSR-BI]).[26] An initial probe report is available,[27] and assay data may be found in the PubChem database (http://pubchem.ncbi.nlm.nih.gov, AID 488952).

Of the 319,533 compounds tested in duplicate at 12.5 μM, 3,046 compounds (0.96%) reduced cellular fluorescence with inhibition of ≥ 70% of that of 1 μM BLT-1, the positive control and one of the most potent inhibitors readily available. Hit compounds that were on plates with Z' < 0.3 or that were active in 10% or more of the HTS assays listed in PubChem were eliminated as either unreliable or too nonspecific. From the primary screen, a variety of scaffolds was verified to inhibit DiI uptake with IC_{50}s of < 1 μM. 127 out of 186 of the selected hits that showed dose-dependent inhibition of DiI uptake were rejected for further study because secondary screening established that they quench the intrinsic fluorescence of DiI in DiI-HDL. We describe here our structure–activity analysis of one of the most potent scaffolds, represented by the commercially available indolinyl-thiazole **5-1**, with an IC_{50} of 0.047 μM (Table 1). This scaffold was chosen for further analysis in part for its modularity and synthetic tractability, and for its lack of measurable toxicity after incubation with CHO cells for 24 h. Furthermore, **5-1** appeared to lack non-selective behavior based on data in PubChem.[28]

Table 1. Amide analogs

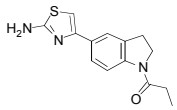
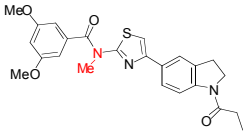
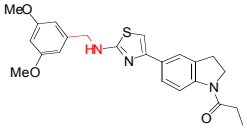
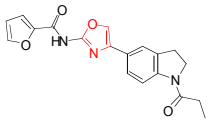
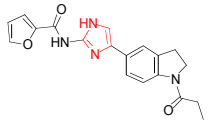
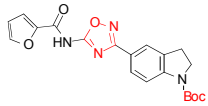
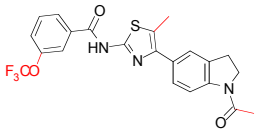


Cmp	R	IC_{50} (μM)[a]	Cmp	R	IC_{50} (μM)[a]
5-1		0.047 ±0.009	**5-14**		0.14
5-2		9.8	**5-15**		0.97
5-3		5.4	**5-16**		2.2
5-4		2.1	**5-17**		1.4
5-5		0.90	**5-18**		0.77
5-6		14.1	**5-19**		1.5
5-7		1.6	**5-20**		0.17
5-8		0.58	**5-21**		0.06 ±0.02
5-9		0.26	**5-22**		NT[b]
5-10		0.29	**5-23**		0.09 ±0.06
5-11		NT[b]	**5-24**		0.03 ±0.01
5-12		0.53	**5-25**		0.22
5-13		0.26	**5-26**		0.12 ±0.09

[a] Average of at least two measurements in DiI uptake assay, ± standard error of mean when n > 2. [b] Insoluble in DMSO. NT = not tested.

We explored structure–activity relationships (SAR) of the scaffold by first varying the *N*-substituent of the aminothiazole. The indolinyl-aminothiazole core of **5-1** was prepared via a simple 3-step sequence (Scheme 1). Indoline **1** was acylated with propionyl chloride, and the resulting amide **2** was subjected to Friedel-Crafts acylation with chloroacetyl chloride. The chloroketone **3** was condensed with thiourea to provide the desired aminothiazole **4**. The poor solubility and nucleophilicity of **4** required heating with the acid chloride coupling partners for optimal preparation of a series of amide derivatives of **5** (Table 1). Caution should be taken with the interpretation of the SAR analysis in this series, as the compounds nearly all have measured solubility in PBS of <1 μM. Despite these issues, the top compounds in this report showed reproducible inhibition and were very potent, providing low nanomolar IC_{50} values.

Table 2. Functional group modifications and central ring SAR

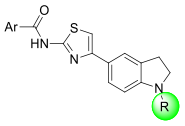
Cmp	Structure	IC_{50} (μM)[a]
4		16.5
6		0.12
7		0.37
8		18.9
9		10.1
10		14.3
11		0.3 ±0.3

[a] Average of at least two measurements in DiI uptake assay, ± standard error of mean when n > 2. [b] Insoluble in DMSO. NT = not tested.

A number of heterocyclic analogs (Table 1, **5-2**–**5-14**) were examined to find a replacement for the furan of **5-1**, which is a potential toxicophore.[29] None of these compounds provided a level of inhibition comparable to **5-1**. A number of aliphatic (**5-15**) and aromatic (**5-16**–**5-26**) analogs were prepared, and analogs with a 3-alkoxybenzene substituent (**5-21**, **5-23**, **5-24**, **5-26**) provided high levels of inhibition with IC_{50}s in the range of 30 to 120 nM.

We next modified the central heterocyclic ring, as well as the adjacent amide functionality (Table 2). The parent amino-thiazole **4** showed poor activity. *N*-methylation of **5-24** (**6**) or reduction of the amide (**7**) gave compounds with attenuated activity. A number of heterocyclic replacements for the central thiazole were prepared, including oxazole **8**, imidazole **9**, and 1,2,4-oxadiazole **10**. The activities sharply decreased in all cases. A 5-methyl group was tolerated on the thiazole (**11**), which may decrease the potential of thiazole ring oxidation by CYP enzymes to give toxic metabolites.[30] Compound **11** also demonstrates that a trifluoromethoxy group could be a potential replacement of the 3-methoxy substituent.

Table 3. Modifications to indolinyl amide



Cmp	Ar	R	IC_{50} (μM)[a]
5-1	2-furyl		0.047 ±0.009
5-24	3,5-(MeO)Ph		0.03 ±0.01
17-1	3,5-(MeO)Ph		0.11
17-2	3,5-(MeO)Ph		0.08 ±0.06
17-3	2-furyl		0.24
17-4	2-furyl		0.39
17-5	2-furyl		0.44
17-6	2-furyl		4.1
17-7	3,5-(MeO)Ph		0.046 ±0.13
17-8	3,5-(MeO)Ph		0.004 ±0.003
17-9	3,5-(MeO)Ph		0.002
17-10	3,5-(MeO)Ph		0.013 ±0.007
17-11	3,5-(MeO)Ph		0.006 ±0.0003

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

SAR studies were continued by modifying the indoline *N*-substituent; a representative synthesis is provided in Scheme 2. Protection of the indoline nitrogen with a phenylsulfonyl group provided an intermediate (**12**) that underwent Friedel-Crafts acylation with chloroacetyl chloride to yield ketone **13** in high yield. The sulfonamide could be hydrolyzed in the presence of the chloroketone by heating in sulfuric acid. The resulting indoline **14** was subsequently condensed with thiourea to generate a 2-aminothiazole, which reacted with Boc_2O exclusively at the indoline nitrogen to generate carbamate **15**. The free amine of **15** was acylated with the desired acid chlorides, the Boc group was removed with TFA, and the indoline nitrogen was acylated to provide compounds **17** (Table 3).

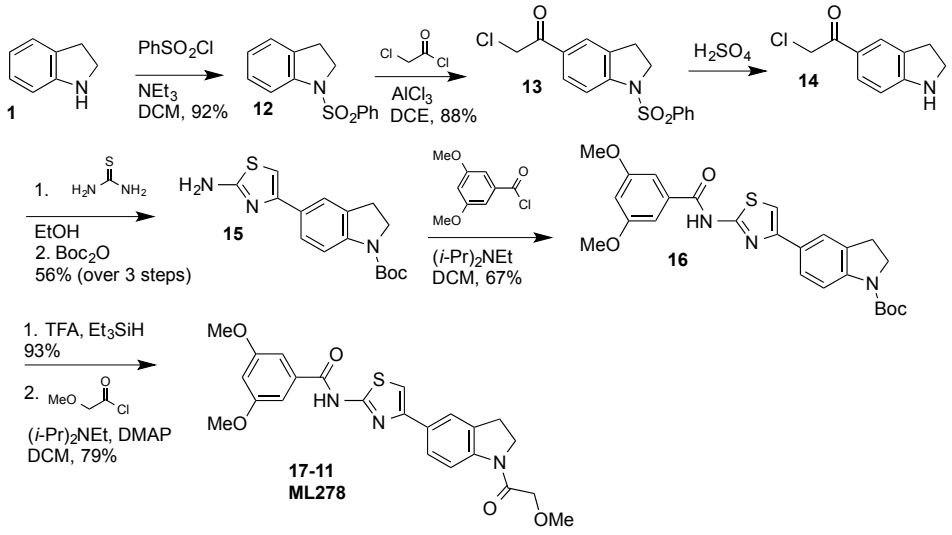
Removal of (**17-1**) or shortening (**17-2**) the indolinyl acyl group of **5-24** did not improve activity; whereas, the addition of a methyl group to the ethyl chain of compound **5-1** (**17-3**) decreased potency. The sulfonamides **17-4** and **17-5** showed an approximately 10-fold drop in potency relative to **5-24**, and the *N*-allyl indoline **17-6** showed only weak inhibition. More

positive results were obtained with compounds **17-7** to **17-11**. Both smaller and bulkier substituents were well tolerated with compounds possessing the western 3,5-dimethoxybenzene moiety. The *N*-Boc compound **17-8** showed excellent potency in the DiI-uptake assay (4 nM), as did the urea **17-9** (2 nM) and the methoxyacetamide **17-11** (6 nM).

Modifications to the indoline ring itself were also examined. A selection of our results is provided in the Supporting Information (Table S1). A range of anilines and oxindoles showed good to excellent potencies, though none were superior to the top indoline compounds, and they also suffered from very low solubilities (<1 μM).

Several of our more promising compounds were profiled in secondary assays to gain insights into the mode of action and potential for further development of the indolinyl-thiazole compound class. None of the compounds showed any significant cytotoxicity after incubation with the ldlA[mSR-BI] cells for 24 h, and in fact compounds **6** (CC_{50} = 15 μM) and **17-10** (CC_{50} = 20 μM) were the only ones with measurable cytotoxicities.[31] Solubility is an issue with this series of compounds, as all of the compounds tested with low nanomolar IC_{50}s have solubilities of < 1 μM. The methoxyacetamide **17-11** showed excellent potency (IC_{50} = 6 nM), measurable solubility (0.57 μM), and excellent stability in human plasma (>99% remaining after 5 h, with 94% plasma protein-bound). **17-11** was nominated as a probe (ML278) as part of the NIH Molecular Libraries Probe Production Centers Network (MLPCN) initiative.

Scheme 2. Representative synthesis of analogs with alternative indoline *N*-substituents



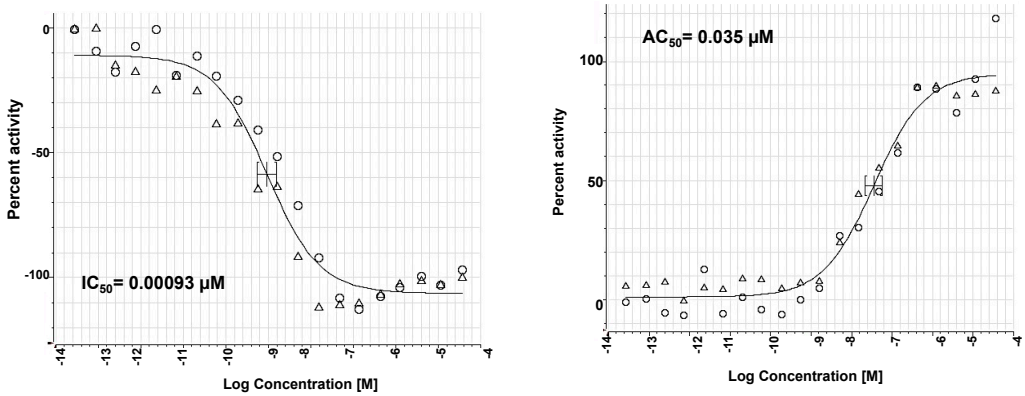


FIGURE 2. DiI-HDL uptake assay with ML278 (left); Alexa488-HDL binding assay with ML278 (right).

Additional mechanistic studies with ML278 were performed to obtain details on its mode of action. First, in experiments where cells were pre-treated with ML278 for 2 hours, washed extensively with PBS and then incubated with DiI-HDL, sharply reduced levels of inhibition were observed. This demonstrates that the inhibitory action of ML278 is reversible. In addition to inhibiting the selective uptake of the synthetic lipid tracer DiI from HDL into ldlA[m-SR-BI] cells (Table 3 and Figure 2, left frame), ML278 inhibited uptake of the physiological relevant [^{3}H]labeled cholesteryl oleate ester ([^{3}H]CE) from [^{3}H]CE-HDL (calculated IC_{50} = 7 nM, Supporting Information Figure S1). Its potency in these assays is far greater than the clinical compound ITX-5061 (IC_{50} = 0.94 μM , see comparison in Table S2). We also showed that, as was the case for BLT-1 and other SR-BI inhibitors, ML278 enhanced the binding of fluorescent Alexa448-HDL to SR-BI (EC_{50} = 0.035 μM) (Figure 2, right frame). Thus, ML278 joins a growing list of small molecules that inhibit lipid transport mediated by SR-BI yet increase the binding of HDL to SR-BI.[18]

The potential utility of ML278 as a probe for in vivo studies was investigated by measuring its metabolic stability in the presence of liver microsomes (Table S2). The compound shows moderate stability, with 75% remaining after 1 h incubation with CD-1 mouse liver microsomes, and 48% remaining with human liver microsomes. Additionally, competitive binding studies were performed with a panel of 67 different receptors and secondary targets (Eurofins Panlabs). At a concentration of 10 μM, 11 targets were inhibited by 20% or more, with the highest level of inhibition observed with the Adenosine A3 receptor (43% inhibition).

In summary, potent inhibitors of SR-BI-mediated lipid (DiI) uptake were discovered as part of the NIH Molecular Libraries Program. Profiling of several top compounds led to the nomination of the indolinyl-thiazole **17-11** (ML278) as a probe compound. SAR studies demonstrated that the thiazole of ML278 was required for activity, flanked by a benzamide, optimally with a 3-methoxy substituent. ML278 shows superior potency in the uptake of the synthetic lipid tracer DiI , as well as [^{3}H]CE, compared to the prior art compounds BLT-1 and ITX-5061. ML278 also shows no cytotoxicity, has no significant chemical liabilities, shows reversible inhibition, and appears to act selectively at SR-BI. Additionally, it has excellent plasma stability and moderate metabolic stability. ML278 is expected be a valuable tool compound for further in vitro and in vivo studies involving SR-BI.

ASSOCIATED CONTENT

Supporting Information. Additional SAR data; preparation and characterization of **17-11** (ML278); probe comparison table; representative dose-response curves of ML278 and ITX-5061 in [^{3}H]CE uptake assay; compound profiling and assay protocols. This material is available free of charge via the internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

Boc, *tert*-butoxycarbonyl; CC_{50}, half-maximal cytotoxic concentration; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CHO, Chinese Hamster Ovary; CYP, cytochrome P450; DCM, dichloromethane; DCE, 1,2-dichloroethane; DMAP, 4-(*N*,*N*-dimethylamino)-pyridine; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; EC_{50}, half-maximal effective concentration; EtOH, ethanol; HCV, Hepatitis C virus; HDL, High Density Lipoprotein; HDL-C; High Density Lipoprotein Cholesterol; HTS, high-throughput screen; IC_{50}, half-maximal inhibitory concentration; MAPK, mitogen-activated protein kinase; MLP, Molecular Libraries Program; MLPCN, Molecular Libraries Probe Production Centers Network; MLSMR, Molecular Libraries Small Molecule Repository; NIH, National Institutes of Health; NO, nitric oxide; NT, not tested; PPR, Pattern Recognition Receptor; SAR, structure-activity relationship; SR-BI, Scavenger Receptor Class B, Type I; TFA, 2,2,2-trifluoroacetic acid; Z', Z'-factor, a measure of assay quality calculated from the variability of positive and negative controls.[32]

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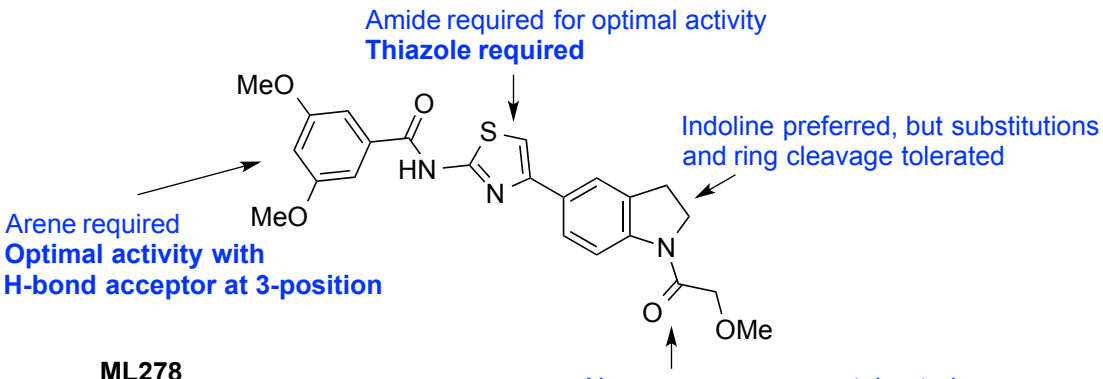

ML278

dil-HDL uptake assay: IC_{50} = 6 nM