Identification of the Molecular Target of Small Molecule Inhibitors of HDL Receptor SR-BI Activity[†,‡,§]

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ABSTRACT: Scavenger receptor, class B, type I (SR-BI), controls high-density lipoprotein (HDL) metabolism by mediating cellular selective uptake of lipids from HDL without the concomitant degradation of the lipoprotein particle. We previously identified in a high-throughput chemical screen of intact cells five compounds (BLT-1−5) that inhibit SR-BI-dependent lipid transport from HDL, but do not block HDL binding to SR-BI on the cell surface. Although these BLTs are widely used to examine the diverse functions of SR-BI, their direct target(s), SR-BI itself or some other component of the SR-BI pathway, has not been identified. Here we show that SR-BI in the context of a membrane lipid environment is the target of BLT-1, -3, -4, and -5. The analysis using intact cells and an in vitro system of purified SR-BI reconstituted into liposomes was aided by information derived from structure−activity relationship (SAR) analysis of the most potent of these BLTs, the thiosemicarbazone BLT-1. We found that the sulfur atom of BLT-1 was crucially important for its inhibitory activity, because changing it to an oxygen atom resulted in the isostructural, but essentially inactive, semicarbazone derivative BLT-1sc. SAR analysis also established the importance of BLT-1's hydrophobic tail. BLTs and their corresponding inactive compounds can be used to explore the mechanism and function of SR-BI-mediated selective lipid uptake in diverse mammalian experimental models. Consequently, BLTs may help determine the therapeutic potential of SR-BI-targeted pharmaceutical drugs.

The HDL[1] receptor SR-BI plays a key role in lipoprotein metabolism (*1*). The physiological functions of SR-BI have been most thoroughly studied in mice, because of the facile genetic manipulation of gene expression in this species. In mice, SR-BI is expressed in hepatocytes, where it controls plasma lipoprotein metabolism and the structure and composition of plasma HDL particles, and in steroidogenic cells, into which it delivers cholesterol for storage and steroidogenesis (*2*−*4*). Hepatic expression of SR-BI protects against atherosclerosis in several murine models (*5*−*11*), and SR-BI-deficiency in homozygous null knockout mice results in female infertility (*12*, *13*) and defects in red blood cell maturation (*14*). Although it seems likely that SR-BI plays similarly important roles in lipoprotein metabolism in other mammals, including humans, direct experimental evidence is very limited.

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[§] T.J.F.N. participated in the conception of the study and the design and interpretation of all experiments and designed, performed, and interpreted all SR-BI assays, as well as cowrote the paper. J.T.S. and F.A.J. were primarily responsible for the design of the SAR approach and were responsible for the synthesis and characterization of novel derivatives of BLT-1. J.L.D. and A.N.K. designed, performed, and interpreted the surface plasmon resonance binding experiments. S.B. and V.I.Z. prepared the tetracycline-inducible SR-BI-t1 vector, and S.B. generated HEK293S GnTI(−) cells that stably express inducible SR-BI-t1 and purified SR-BI-t1 protein. T.K. was involved in the interpretation of liposome experiments in the early phase of this work. M.K. participated in the conception of the study and the design and interpretation of all experiments and cowrote the paper.

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[1] Abbreviations: [^{3}H]CE, [^{3}H]cholesteryl oleyl ether; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; HDL, high-density lipoprotein; SR-BI, scavenger receptor, class B, type I; LDL, low-density lipoprotein; CE, cholesteryl ester or cholesteryl ether; apoA, apolipoprotein A; BLT, blocker of lipid transport; IC_{50}, concentration of the compounds that caused a half-maximal decrease in (selective) uptake.

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SR-BI mediates the binding of HDL to cells and the subsequent efficient transfer into the cells of cholesteryl esters from HDL's core, but not the net transfer of HDL's protein or most of the lipid components of its outer shell. This two-step process, called selective lipid uptake (*1*, *2*, *15*−*17*), occurs on the cell surface and does not require endocytosis (*18*−*25*), and thus markedly differs from the receptor-mediated endocytic delivery of lipoprotein cholesterol to cells via the LDL receptor (*26*). SR-BI also mediates bidirectional movement of unesterified cholesterol between cells and lipoproteins (*27*−*29*). The detailed molecular mechanism underlying SR-BI-mediated lipid transport into and out of cells is not understood.

We previously employed a high-throughput screen in intact cells to identify small-molecule inhibitors of SR-BI-dependent selective lipid uptake (*30*). Five compounds discovered in this screen were named BLT-1 to BLT-5, because they block lipid transport mediated by SR-BI. This includes inhibition of selective uptake from HDL of either cholesteryl ethers (nonhydrolyzable analogues of cholesterol esters) or DiI (a fluorescent lipid), as well as efflux of unesterified cholesterol from cells to HDL (*30*, *31*). The BLTs were the first pharmacologic tools identified that can directly probe the physiologic functions of the SR-BI pathway and can be used to provide additional insight into the molecular mechanisms underlying SR-BI-mediated HDL binding and lipid transport. Indeed, BLTs have been used to study a plethora of SR-BI activities (*24*, *32*−*42*), including viral infection (*43*−*47*), vitamin E transport (*48*), lipoprotein endocytosis (*21*, *23*, *49*), and lipid transport mediated by ABC superfamily proteins (*31*).

Unexpectedly, inhibition of SR-BI-mediated lipid transport by BLTs is accompanied by an increase in SR-BI's binding affinity for HDL (*30*). The novel effects of BLTs on SR-BI's activity suggest that they may help probe the mechanism of SR-BI-mediated lipid transport. However, the potential utility of the BLTs was limited, because their direct molecular target(s) had not been identified. One candidate is SR-BI itself, but because the BLTs were identified in an intact cell-based screen, other cellular components required for normal SR-BI activity were also possible targets. Indeed, a comparison of the effects of the BLTs on cholesterol efflux mediated by SR-BI and the structurally distinct transporter ABCA1 (*50*) raised the possibility that the direct target of BLT-4 might not be SR-BI (*31*).

In the current study we identify the molecular target of BLT-1 by using a two-step approach. BLT-1, a thiosemicarbazone (Figure 1A), was chosen for detailed analysis because it is the most potent of the BLTs. First, we performed structure−activity relationship (SAR) analysis to gain insight into the chemical features underlying the nanomolar potency by which BLT-1 blocks cellular lipid transport. This highlighted a key feature of BLT-1 required for its inhibitory activity—the sulfur of the thiosemicarbazone moiety. Substitution of an oxygen atom for this sulfur resulted in an inactive semicarbazone derivative, BLT-1sc (Figure 2B). Second, we showed that BLT-1, but not BLT-1sc, inhibits selective uptake mediated by virtually homogenously pure, epitope-tagged SR-BI reconstituted into phosphotidylcholine/cholesterol liposomes. BLT-3, -4, and -5 also inhibited SR-BI-mediated selective lipid uptake in the reconstituted liposomes. Thus, SR-BI in the context of a membrane lipid environment is the molecular target of these four chemically distinct BLTs, suggesting that these BLTs can be used to explore further the molecular mechanisms underlying SR-BI function.

EXPERIMENTAL PROCEDURES

Compounds. Compounds tested for their effects on SR-BI activity were obtained either by synthesis (see below) or by purchase from Oakwood Products, Inc. (1-decyl-2-thiourea), Karl Industries, Sapon Laboratories Division (*n*-octylurea), and Sigma/Aldrich (BLT-1nac, catalog number R397857, and 1-decanethiol). BLT-4 was either obtained from Chembridge Corp. (San Diego) or synthesized as previously described (*31*). The remaining compounds, which were purchased from Chembridge Corp., were (Chembridge ID numbers in parentheses) compounds **1**−**9** (5136875, 5136871, 5234224, 5234954, 5554654, 8016115, 8016113, 5153618, 5136957, respectively), BLT-1 (5234221), BLT-2 (5234225), BLT-3 (5106931), BLT-4 (5109586), BLT-5 (5117098), and BLT-1sc (5152562). All compounds synthesized specifically for this study (BLT-1, ref *31* and see below) were prepared as stock solutions in DMSO (25−500 mM), and their identities and purities were verified by LC−MS and NMR analysis. The purities of all compounds purchased from Chembridge Corp. and BLT-1nac were verified by LC−MS. In addition, we selected several of the compounds we deemed most critical for this study (BLT-1, BLT-1ncp7, 1-decyl-2-thiourea, BLT-1sc, compound **5**, compound **9**) and determined whether there were any signs of degradation under conditions of our standard assays: a 3 h incubation in assay medium (Ham's F12/0.5% DMSO/25 mM HEPES, pH 7.4) containing 0.5% (w/v) bovine serum albumin (BSA; Sigma catalog no. A3059; see below). After this treatment the compounds were extracted from the medium with ethyl acetate and characterized by LC−MS. The only masses we could identify in the LC−MS trace were those of the unmodified compounds of interest as well as those of the solvent (DMSO, Ham's F12, albumin-associated molecules). Although it is possible that some degradation of the compounds of interest could have produced products hidden under peaks of the assay medium components, we conclude that the compounds remain stable under the conditions used for the receptor assays in this study.

Synthesis and Chemical Characterization of Chemical Compounds. (A) BLT-1NMe [2-(2-Hexylcyclopentylidene)-N-methylhydrazinecarbothioamide]. To a solution of 2-*n*-hexylcyclopentanone (0.200 g, 1.19 mmol) in ethanol (4 mL) at room temperature were added 4-methylthiosemicarbazide (0.125 g, 1.19 mmol) and 6 M hydrochloric acid (0.22 mL, 1.31 mmol). The reaction mixture was stirred at reflux for 2 h. It was cooled to room temperature and neutralized with aqueous sodium bicarbonate. The aqueous layer was extracted with ethyl acetate (2 × 50 mL). The combined organic layer was washed with water (30 mL) and saturated aqueous (satd aq) NaCl (30 mL) and dried over sodium sulfate. The solvent was evaporated under reduced pressure and the crude product purified by flash column chromatography on silica gel using 5% MeOH/CH_2Cl_2. The desired product was obtained as a light yellow solid (0.155 g, 0.608 mmol, 51%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 (s, 1H), 7.43 (br s, 1H), 3.18 (d, 3H, J = 4.8 Hz), 2.41−2.31 (m, 2H), 2.24−2.12 (m, 1H), 2.07−1.87 (m, 2H), 1.75−1.56 (m,

2H), 1.38−1.26 (m, 10H), 0.862 (t, 3H, J = 6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.7, 163.8, 44.6, 32.1, 31.7, 31.2, 31.0, 29.3, 27.9, 27.5, 22.7, 22.6, 14.0; RP-LC−MS (gradient/column/ionization) m/z for $C_{13}H_{25}N_3S$ $(M + H)^+$ 256.0, t_R = 1.36 min. LC−MS data for all synthesized compounds were collected using a 5:95 to 95:5 acetonitrile/water (0.01% formic acid) solvent gradient on an XTerra MS C18 3.5 μm, 4.6 × 30 mm column using electrospray ionization.

(B) BLT-1SMe [Methyl 2-(2-Hexylcyclopentylidene)hydrazinecarbimidothioate]. To a solution of 2-*n*-hexylcyclopentanone (0.200 g, 1.09 mmol) in ethanol (4 mL) at room temperature were added *S*-methylisothiosemicarbazide hydroiodide (0.277 g, 1.19 mmol) and 6 M hydrochloric acid (0.22 mL, 1.31 mmol). The reaction mixture was stirred at reflux for 4 h. It was cooled to room temperature and neutralized with aqueous sodium bicarbonate. The aqueous layer was extracted with ethyl acetate (2 × 50 mL). The combined organic layer was washed with water (30 mL) and satd aq NaCl (30 mL) and dried over sodium sulfate. The solvent was evaporated under reduced pressure and the crude product purified by flash column chromatography on silica gel using 5% MeOH/CH_2Cl_2. The desired product was obtained as a light yellow solid (0.181 g, 0.709 mmol, 60%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.19 (br s, 2H), 2.75−2.65 (m, 1H), 2.49−2.37 (m, 2H), 2.43 (s, 3H), 2.06−1.96 (m, 1H), 1.88−1.79 (m, 2H), 1.67−1.52 (m, 1H), 1.42−1.29 (m, 10H), 0.887 (t, 3H, J = 6.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.8, 158.0, 44.8, 32.9, 32.1, 31.7, 30.0, 29.7, 28.1, 22.9, 14.3, 12.8; RP-LC−MS (gradient/column/ionization) m/z for $C_{13}H_{25}N_3S$ $(M + H)^+$ 255.9, t_R = 1.40 min.

(C) BLT-1ncp7 (Octanal Thiosemicarbazone). To a solution of octanal (0.200 g, 1.56 mmol) in 95% ethanol (4 mL) at room temperature was added thiosemicarbazide (0.142 g, 1.56 mmol). The reaction mixture was sonicated for 1 h, and the solvent was removed under reduced pressure to yield a white solid (0.298 g, 1.48 mmol, 95%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.53 (s, 1H), 7.28 (t, 1H, J = 6 Hz), 7.09 (br s, 1H), 6.31 (br s, 1H), 2.24−2.31 (m, 2H), 1.48−1.59 (m, 2H), 1.25−1.34 (m, 8H), 0.90 (t, 3H, J = 6.9 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.1, 148.3, 32.1, 31.6, 29.0, 28.9, 26.1, 22.5, 14.0; RP-LC−MS (gradient/column/ionization) m/z for $C_9H_{19}N_3S$ $(M + H)^+$ 202.0, t_R = 1.60 min.

(D) BLT-1ncp9 (Decanal Thiosemicarbazone). To a solution of decanal (0.200 g, 1.28 mmol) in 95% ethanol (4 mL) at room temperature was added thiosemicarbazide (0.117 g, 1.28 mmol). The reaction mixture was sonicated for 1 h, and the solvent was removed under reduced pressure to yield a white solid (0.271 g, 1.18 mmol, 92%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.89 (s, 1H), 7.33 (t, 1H, J = 5.4 Hz), 7.09 (br s, 1H), 6.41 (br s, 1H), 2.22−2.29 (m, 2H), 1.49−1.57 (m, 2H), 1.25−1.32 (m, 12H), 0.89 (t, 3H, J = 6.9 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.2, 148.6, 32.2, 31.8, 29.4, 29.3, 29.2, 29.1, 26.1, 22.6, 14.0; RP-LC−MS (gradient/column/ionization) m/z for $C_{11}H_{23}N_3S$ $(M + H)^+$ 230.0, t_R = 1.81 min.

(E) BLT-1ncp11 (Dodecanal Thiosemicarbazone). To a solution of dodecanal (0.200 g, 1.09 mmol) in 95% ethanol (4 mL) at room temperature was added thiosemicarbazide (0.099 g, 1.09 mmol). The reaction mixture was sonicated for 1 h, and the solvent was removed under reduced pressure to yield a white solid (0.268 g, 1.04 mmol, 96%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.70 (s, 1H), 7.30 (t, 1H, J = 5.4 Hz), 7.09 (br s, 1H), 6.36 (br s, 1H), 2.23−2.30 (m, 2H), 1.48−1.58 (m, 2H), 1.24−1.34 (m, 16H), 0.89 (t, 3H, J = 6.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.3, 148.4, 32.1, 31.9, 29.5, 29.4, 29.3, 29.1, 26.1, 22.6, 14.0; RP-LC−MS (gradient/column/ionization) m/z for $C_{13}H_{27}N_3S$ $(M + H)^+$ 258.1, t_R = 1.98 min.

Lipoproteins and Cells. Human HDL (density of ∼1.09−1.16 g/mL) was isolated and labeled as previously described (*2*, *51*−*54*). Its protein components were labeled with ^{125}I (^{125}I-HDL), and it was labeled with lipids, either [^{3}H]-cholesteryl oleyl ether ([^{3}H]CE; [^{3}H]CE-HDL) or DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (from Molecular Probes); DiI-HDL). Most intact cell assays were performed using control LDL receptor-deficient Chinese hamster ovary (CHO) cells that express low levels of endogenous SR-BI (ldlA-7) (*55*) or ldlA-7 cells stably transfected to express high levels of wild-type murine SR-BI (ldlA[mSR-BI]) (*2*). These cells were maintained in culture medium A (Ham's F12 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 50 units/mL penicillin/50 μg/mL streptomycin, and, for ldlA[mSR-BI] cells only, 0.25 mg/mL G418) (*51*). HEK293 cells were used for experiments involving transient transfection of plasmids encoding wild-type and cysteine mutant forms (see below) of mSR-BI. For these experiments, the HEK293 cells were plated in poly-L-lysine-coated 24-well dishes at a density of 150 000 cells/well in 0.5 mL/well of medium B (DMEM/Ham's F12 (1:1 from Gibco BRL) supplemented with 10% (v/v) fetal bovine serum and 50 units/mL penicillin/50 μg/mL streptomycin). On day 1, the cells were washed once with medium C (medium B containing 5% fetal bovine serum) and then transfected using lipofectamine 2000 (Invitrogen) following the manufacturer's instructions with the following expression vectors: (1) wild-type murine SR-BI (mSR-BI) in pcDNA1 (*2*); (2) a mutant mSR-BI in which both Cys^{462} and Cys^{470} were replaced by serines (mSR-BI (CC → SS); double cysteine mutant) in pcDNA3 (*53*), or (3) a pcDNA3 (Invitrogen) control vector (no cDNA insert). SR-BI activity assays in these transiently transfected cells were performed on day 3 as described below. All experiments with cells were conducted at 37 °C.

Cellular Lipid Transport and ^{125}I-HDL Binding Assays. As previously described, assays of the uptake of [^{3}H]CE from [^{3}H]CE-HDL or uptake of DiI from DiI-HDL, as well as ^{125}I-HDL binding, were performed using ldlA[mSR-BI] cells and control untransfected ldlA-7 cells (*2*, *27*, *30*, *52*) or using transiently transfected HEK293 cells generated as described above. For [^{3}H]CE uptake and ^{125}I-HDL binding assays in ldlA[mSR-BI] and ldlA-7 cells, cells were seeded in 24-well plates on day 0 in medium A at a density of 75 000 cells/well. In the case of HEK293 cells, cells were plated on day 0 at a density of 150 000 cells per well and transfected on day 1 (see above), and [^{3}H]CE-HDL uptake and ^{125}I-HDL binding assays were performed on day 3. For DiI-HDL uptake assays, ldlA[mSR-BI] cells and ldlA-7 cells were seeded on day 0 in medium A in 96-well plates (Costar, black with a clear bottom) at a density of 35 000 cells/well in the same medium. Assays were performed on day 2. On the day of the assay, the cells were washed twice with Ham's F12 without supplements and then preincubated for 1 h at 37 °C with compounds at the indicated concentrations in assay

medium (Ham's F12/0.5% DMSO/25 mM HEPES, pH 7.4) containing 0.5% (w/v) BSA (Sigma catalog no. A3059). The cells were then incubated for 2 h (ldlA[mSR-BI] and ldlA-7 cells) or 4 h (HEK293 cells) with the same concentrations of small molecules together with 10 μg of protein/mL of ^{125}I-HDL (binding), [^{3}H]CE-HDL, or DiI-HDL (uptake). In experiments using radiolabeled lipoproteins, the cells were washed (4 °C) one time with PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS+) and 0.5% (w/v) BSA and two times with PBS+ without BSA. Cellular accumulation of [^{3}H]CE was measured following isopropyl alcohol extraction, and binding of ^{125}I-HDL was determined as previously described (*2*). To determine cellular uptake of DiI after incubation with DiI-HDL, we placed the cells on ice and washed them three times with ice-cold PBS+ containing 0.5% (w/v) BSA and then measured cellular DiI using a Molecular Devices SpectraMax GEMINI XS fluorescence plate reader or an Analyst Plate fluorescence plate reader (Molecular Devices). For all experiments, the values presented were normalized so that 100% of the control value represents receptor-specific activity in ldlA[mSR-BI] or HEK293[SR-BI] cells in the presence of 0.5% DMSO without compounds, and 0% activity is defined as the activity determined in ldlA-7 or control pcDNA3-transfected HEK293 cells. The amounts of cell-associated [^{3}H]CE are expressed as the equivalent amount of [^{3}H]CE-HDL protein (ng) (*56*). For experiments using HEK293 cells transiently transfected with either the wild-type (wt) mSR-BI or the CC → SS double cysteine mutant, values (ng of protein (equivalent)/mg of cell protein) determined in the absence of BLT-1 of [^{3}H]CE-HDL selective uptake were as follows: SR-BI (wt), 2526; SR-BI (CC → SS), 2762; pcDNA3 empty vector control, 448. Values (ng of protein/mg of cell protein) determined in the absence of BLT-1 of ^{125}I-HDL binding were as follows SR-BI (wt) cells, 234; SR-BI (CC → SS) 257; pcDNA3 empty vector control 46. Results averaged from two independent experiments are presented. In the DiI-HDL assay, background nonspecific uptake was determined by inclusion of a 40-fold excess (400 μg of protein/mL) of unlabeled HDL in the media (*2*). Data analysis was performed using Graphpad Prism4 software from GraphPad Software, Inc., San Diego, CA. The IC_{50} values are the concentrations of the compounds that caused a half-maximal inhibition of SR-BI-mediated lipid transport. Selective uptake data presented are representative of results from three or more independent experiments, unless otherwise indicated. DiI-HDL data presented are averaged results from two or three experiments. We observed that the inhibition of cellular SR-BI activity by BLT-1−BLT-5 is fully reversible after a 3−6 h wash out of each of the compounds as measured in the 2 h cellular DiI-HDL uptake assay (data not shown). In addition, the IC_{50} and extent of inhibition by these BLTs are similar for experiments in which the cells are preincubated for 1 h with the compounds prior to addition of the HDL ligand (standard assay in which cells are incubated with DiI-HDL for 2 h) and for those in which the BLTs are only added essentially simultaneously with the HDL ligand (no preincubation) (not shown).

Lipid Transport and ^{125}I-HDL Binding Assays in Liposomes. Purification of mSR-BI-t1 with uniform, truncated N-linked oligosaccharide chains: We have previously described the isolation of HEK293S cells that overexpress a C-terminally epitope-tagged murine SR-BI (mSR-BI-t1) and the single-step immunoaffinity purification of mSR-BI-t1 to virtual homogeneity (*57*). The detergent-solubilized purified protein can be reconstituted into liposomes that exhibit SR-BI-mediated ^{125}I-HDL binding and selective uptake of [^{3}H]-CE from [^{3}H]CE-HDL (*57*). We used a similar approach in this study to examine the potential direct effects of SR-BI lipid transport inhibitors on this receptor in liposomes, but with one key difference (S. Banakos, P. J. Reeves, V. Zannis, and M. Krieger, manuscript in preparation). mSR-BI-t1 was expressed in an *N*-acetylglucosaminyltransferase I (GnTI)-defective HEK293S derivative, HEK293S GnTI(−), that generates a glycoprotein with uniform, truncated N-linked oligosaccharide chains under the control of a tetracycline inducible promoter (*58*, *59*). A clone of HEK293S GnTI(−) cells expressing high levels of mSR-BI-t1 was isolated and grown to mass culture in a Celligen Plus bioreactor (New Brunswick Scientific, Edison, NJ) and the receptor purified to homogeneity as previously described (*57*). mSR-BI-t1 with truncated N-linked chains was reconstituted into liposomes as previously described (*57*, *60*, *61*). Briefly, 20 μg of mSR-BI-t1 (or an equivalent volume of protein-free buffer to generate control liposomes that are devoid of mSR-BI-t1) was reconstituted into liposomes by acetone precipitation. mSR-BI-t1 liposomes were washed once by resuspension of the acetone precipitate in protein-free assay medium followed by centrifugation (48000*g*) for 25 min at 4 °C. The pellet was first reconstituted in assay medium without protein, and then an equal volume of assay medium with 1% fatty acid free (FAF) BSA (Sigma catalog no. A6003) was added to yield liposomes at a nominal final concentration of ∼18 ng of mSR-BI-t1/mL. In each reaction, 30 μL of the liposome preparation was preincubated with 30 μL of assay medium containing 0.5% FAF BSA and DMSO with the indicated compounds (0.5% DMSO final concentration) for 60 min at 37 °C. Subsequently, 20 μL of [^{3}H]CE-HDL (five replicates per sample) or ^{125}I-HDL (four replicates per sample) was added to a final concentration of 10 μg of protein/mL. Incubation was continued for 4 h at 37 °C, and then selective uptake of [^{3}H]CE into liposomes and binding of ^{125}I-HDL to liposomes were determined using the previously described filter binding assays (*57*). SR-BI specific activity was determined by subtracting uptake and binding values in control liposomes from those in mSR-BI-t1-containing liposomes, and selective uptake values were determined as described above for cellular assays. A value 100% of the control value represents receptor-specific activity in mSR-BI-t1 containing liposomes in the presence of 0.5% DMSO without compounds, and 0% of the control value represents selective uptake in control liposomes devoid of mSR-BI-t1. Representative results from two or three independent experiments are shown.

Surface Plasmon Resonance Analysis of Compound Binding to BSA. BSA is included in the assays reported here to reduce nonspecific interactions of lipoproteins with cells or liposomes. Albumin can bind a variety of small molecules (reviewed in ref *62*); thus, binding to BSA might reduce the bioavailability of the compounds tested here, depending on the concentration of a compound and its affinity for binding to BSA. To calculate the value of the amount of free compound required to inhibit SR-BI activity by 50% [IC_{50}-(corrected)] and compare it to the uncorrected value (IC_{50}),

we determined the affinity of binding of compounds to BSA using surface plasmon resonance analysis with a Biacore S51 instrument, as previously described for binding studies using human serum albumin (*63*−*65*). BSA was diluted to 115 μg/mL in 10 mM acetate buffer, pH 5.0, and immobilized on a Biacore CM5 sensor chip using standard 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride/*N*-hydroxysuccinimide coupling chemistry. On each sensor detection spot, 5000−9000 response units (RU; corresponding to ~1 pg of protein/mm^2 sensor area) of BSA was immobilized.

The Biacore binding assay was conducted two or three times, depending on the compound, in Biacore buffer, TBS-P, pH 7.4 (25 mM Tris−HCl, 137 mM NaCl, 3 mM KCl, 0.005% (v/v) P20 surfactant (Biacore AB, catalog number BR-100-54)) containing either 2% (v/v) DMSO or 5% (v/v) DMSO. The compounds were tested using 4-fold serial dilutions, starting at either 50 μM (to cover the lower concentration range, with 781 nM being the lowest dose tested) or 200 μM (to cover the 50−200 μM range). The compounds were then sequentially injected with an association time of 60 s, a dissociation time of 60 s, a flow rate of 30 μL/min, and an assay temperature of 25 °C. Naproxen and warfarin (381 nM to 200 μM, both from Sigma) were used as positive BSA binding controls. Data were analyzed using Scrubber software (University of Utah's Center for Biomolecular Interaction Analysis, http://www.cores.utah.edu/interaction/). Data were double reference subtracted and normalized so that the theoretical maximum response from the sensor equals 100 RU for each compound. Equilibrium binding affinities assuming binding to a single site on BSA were determined using GraphPad Prism 4 with a nonlinear regression, one-site binding model. The K_d values (μM) were as follows: BLT-1, 161 ± 41; BLT-1sc, 80 ± 26; BLT-2, 113 ± 34; compound **3**, 43 ± 2; compound **4**, 33 ± 4; compound **6**, 72 ± 3; compound **7**, 177 ± 8; and compound **8**, 95 ± 4. None of the other compounds were found to bind to BSA up to a concentration of 200 μM, the highest concentration tested in these experiments. The K_d values for compound binding to BSA were used to calculate the concentrations of compound "free" (not bound to BSA) in the incubation media, and these free concentrations were then used to calculate IC_{50}(corrected) values. The free concentrations of compounds, $[A]_{free}$, were calculated using eq 2 from the total concentration ($[A]_0$), the total concentration of BSA ($[B]_0$ = 75.76 μM), the concentration of the 1:1 complex formed between BSA and compound ([AB]; calculated using eq 4, which was derived from eqs 1 and 3)), and the equilibrium dissociation constant for compound/BSA binding as measured by surface plasmon resonance (K_d) assuming a 1:1 binding ratio and no competition for albumin binding with other components of the assay media.

$$[B]_{free} = [B]_0 - [AB] \quad (1)$$

$$[A]_{free} = [A]_0 - [AB] \quad (2)$$

$$K_d = \frac{[A]_{free}[B]_{free}}{[AB]} \quad (3)$$

$$0 = [A]_0[B]_0 - [AB]([A]_0 + [B]_0 + K_d) + [AB]^2 \quad (4)$$

The corrected IC_{50} for inhibition of DiI-HDL uptake was calculated (see above) by replacing the total concentration, $[A]_0$, by the free concentration of the compound, $[A]_{free}$. The corrected (and uncorrected) values for DiI-HDL uptake (nM, with the exception of BLT-1sc) were as follows BLT-1, 15 (21); BLT-1sc, >500 μM (>500 μM); compound **3**, 110 (320); compound **4**, 72 (240); compound **6**, 33 (69); compound **7**, 116 (220); and compound **8**, 400 (720). The corrected value for the IC_{50} for BLT-2 is 220 nM, which is based on the uncorrected value of 350 nM reported by Nieland et al. (*30*). IC_{50} values (nM) for BLT-1 for [^{3}H]CE-HDL selective uptake were 39 (57) in cells and 68 (98) in liposomes. The effects of the correction are relatively small and do not influence the conclusions. Therefore, we have presented the uncorrected values throughout this paper.

Data Analysis. The statistical significance of differences was determined by two-tailed unpaired Student's *t* test using Graphpad Prism 4 software. Average values are presented ± the standard error of the mean. Differences were considered significant for $P < 0.05$. Hill coefficients were calculated using Graphpad Prism 4 software. cLogP values were determined using ChemBioDraw Ultra version 11 from Cambridgesoft.com.

RESULTS

Here we first present the results of SAR analysis of BLT-1 for the inhibition of mSR-BI and then use the results to help identify the molecular target of BLT-1 in cells.

SAR Analysis of BLT-1 in Cultured Cells. Our SAR analysis of BLT-1 has focused on three features of its structure: the cyclopentyl group, the alkyl chain, and the thiosemicarbazone group (Figure 1A). To assess the ability of BLT-1 and its derivatives and analogues to inhibit lipid transport mediated by SR-BI in cultured cells, we pretreated (1 h, 37 °C) stably transfected cells expressing mSR-BI, ldlA-[mSR-BI] (*2*), or their untransfected essentially SR-BI-negative controls, ldlA-7 (*55*), with varying concentrations of the compounds. We then used standard assays (2 h incubations, 37 °C), conducted in the presence of the same concentrations of compounds to measure SR-BI-mediated (i) cellular uptake of the lipophilic dye DiI from DiI-HDL (10 μg of protein/mL) or (ii) selective uptake of [^{3}H]CE from [^{3}H]CE-HDL (10 μg of protein/mL) and (iii) receptor-specific binding of ^{125}I-HDL (10 μg of protein/mL), which was used to calculate selective lipid uptake. Inhibitory activity was characterized by both potency (concentrations of the compounds that caused a half-maximal inhibition of SR-BI-mediated lipid transport, IC_{50}) and the extent of inhibition (absolute reduction in SR-BI-mediated lipid transport). The principle differences in inhibitory activity we observed for different compounds were altered IC_{50} values; unless indicated otherwise, the extent of inhibition for a compound with IC_{50} < 500 μM was comparable to that for the positive control (e.g., BLT-1).

(A) Cyclopentyl Group. We initiated our SAR analysis of BLT-1 by altering its cyclopentane moiety. Figure 1C shows that when the cyclopentane was removed by substitution of three of its methylenes with two protons (no cyclopentane (ncp), compound BLT-1ncp7; Figure 1B) inhibition of SR-

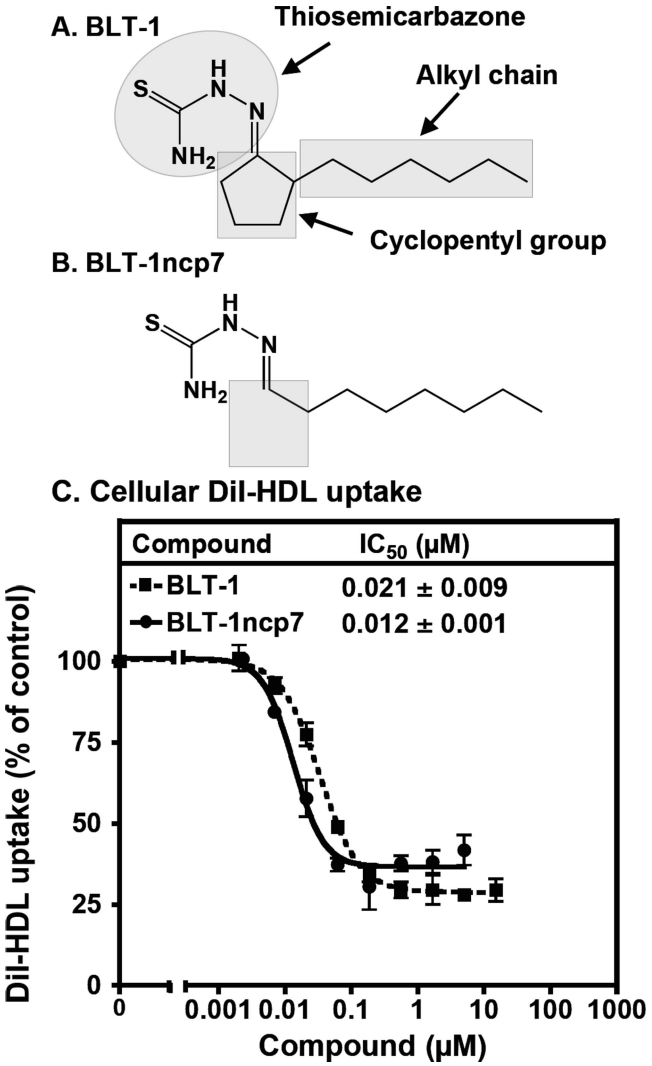


FIGURE 1: Effect of BLT-1's cyclopentane on inhibition of SR-BI-mediated lipid transport. Cells (ldlA[mSR-BI] and ldlA-7) were preincubated with BLT-1 (A) or BLT-1ncp (B) at the indicated concentrations for 1 h at 37 °C in medium containing 0.5% (v/v) DMSO. The cells were then incubated for an additional 2 h with the same medium containing DiI-HDL (10 μg of protein/mL) in the presence of the same concentrations of BLT-1 or BLT-1ncp. The cells were then washed, and the amount of DiI incorporated into the cells was measured using a fluorescence plate reader. The values were normalized so that 100% of the control represents receptor-specific DiI-HDL uptake by ldlA[mSR-BI] cells without compounds and 0% activity is defined as the activity determined in ldlA[mSR-BI] cells in the presence of a 40-fold excess of unlabeled HDL. Panel C shows for BLT-1 (squares) one of four representative experiments in which the values were determined from duplicate incubations and for BLT-1ncp (circles) the average of two independent experiments. IC_{50} values ± the standard error of the mean are derived from an average of four (BLT-1) or two (BLT-1ncp) experiments using nonlinear regression analysis. In ldlA[mSR-BI] cells, DiI-HDL uptake in the presence of excess of unlabeled HDL was 23% of that without the unlabeled HDL.

BI-mediated uptake of DiI-HDL was similar (IC_{50} = 12 ± 1 nM) to that with unmodified BLT-1 (IC_{50} = 21 ± 9 nM). It is noteworthy that BLT-1 has a chiral carbon and our synthetic method produces a racemic mixture; it is possible that only one of the two sterioisomers may be active. In contrast, BLT-1ncp7 does not have a chiral center. The cyclopentyl group is clearly not required for the inhibitory activity of BLT-1.

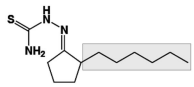
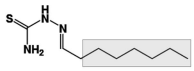
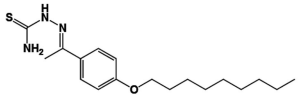
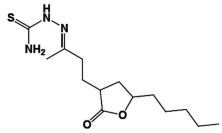
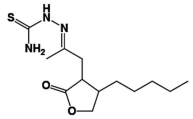
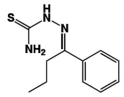
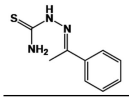
Table 1: SAR Analysis of the Alkyl Chain of BLT-1

Compound	IC_{50} (μM)[a]
BLT-1	0.021 ± 0.009
BLT-1ncp7	0.012 ± 0.001
BLT-1ncp9	0.011 ± 0.003
BLT-1ncp11	0.038 ± 0.006
BLT-2	0.35 ± 0.18
Compound 1	0.047 ± 0.026
BLT-1nac	36.2 ± 6.5
Compound 2	45.2 ± 23
Compound 3	0.33 ± 0.19
Compound 4	0.28 ± 0.1

[a] SAR analysis of BLT-1 derivatives for DiI-HDL uptake was performed in ldlA[mSR-BI] and ldlA-7 cells as described for Figure 1. IC_{50} values are derived from an average of four (BLT-1), three (compound **1**), or two (all other compounds) experiments using nonlinear regression analysis.

(B) Alkyl Chain. We next determined the influence of the length of the alkyl chains on the activities of BLT-1 and its BLT-1ncp7 derivative (no cyclopentane) using the DiI-HDL assay (Table 1). When the length of the alkyl chain of BLT-1ncp7 (IC_{50} = 12 ± 1 nM) was extended by two (BLT-1ncp9) or four (BLT-1ncp11) methylenes, there was little change in potency or extent of inhibition (IC_{50} = 11 ± 3 and 38 ± 6 nM, respectively). The addition of a methyl group on the imine carbon of BLT-1ncp9 also had very little effect (compound **1**; IC_{50} = 47 ± 26 nM), which was to be expected because there was little difference in activity between the compounds with (BLT-1) and without (BLT-1ncp7) the cyclopentane ring. In contrast, shortening the alkyl chain had a dramatic, negative effect on the potency of BLT-1 (Table 1). Substitution of the six-carbon-long alkyl chain of BLT-1 with a proton (no alkyl chain (nac), compound BLT-1nac) resulted in an approximately 1700-fold increase in the IC_{50} (36.2 ± 6.5 μM) but no substantial change in the extent of maximal inhibition. Another derivative that similarly lacks the six-carbon alkyl chain, in which the cyclopentane group is substituted by a cyclohexane group

Table 2: SAR Analysis of the Alkyl Chain of BLT-1

Compound	IC_{50} (μM)[a]
 BLT-1	0.021 ± 0.009
 BLT-1ncp7	0.012 ± 0.001
 Compound 5	0.013 ± 0.003
 Compound 6	0.11 ± 0.08
 Compound 7	0.29 ± 0.18
 Compound 8	0.75 ± 0.32
 Compound 9	3.6 ± 0.5

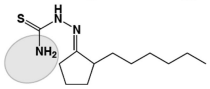
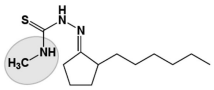
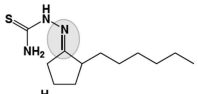
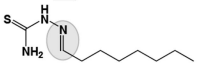
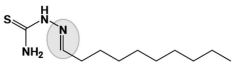
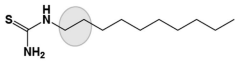
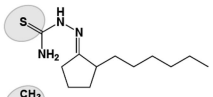
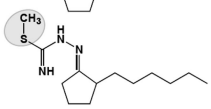
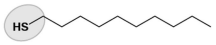
[a] IC_{50} values are based on results from an average of four (BLT-1), three (compound **5**), or two (all other compounds) experiments using DiI-HDL and ldlA[mSR-BI] and ldlA-7 cells as described in Figure 1.

(compound **2**; $IC_{50} = 45.2 \pm 23.0$ μM), was almost as potent as BLT-1nac. This reinforces the conclusion from Figure 1 that the cyclopentane group per se does not substantially influence activity. Addition of a C_4 alkyl chain to the cyclohexane ring of compound **2** enhanced its potency ~100-fold (compound **3**; $IC_{50} = 0.33 \pm 0.19$ μM). Addition of a cyclohexene to compound **2** had an effect (compound **4**; $IC_{50} = 0.28 \pm 0.1\ 9\mu$M) similar to that of adding a C_4 alkyl chain. Because the cyclopentane or cyclohexane rings do not appear to influence inhibitory activity, we conclude that the greater potencies (lower IC_{50} values) of BLT-1ncp7 and BLT-1 compared to compounds **3** and **4** can be attributed to their longer alkyl chains.

We have previously shown that introducing an oxygen atom (ether bond) into the middle of the alkyl chain of BLT-1 increased the IC_{50} by about 17-fold (BLT-2; $IC_{50} = 0.35 \pm 0.18$ μM, Table 1; see ref *30*). Table 2 shows the IC_{50} values for other derivatives of BLT-1 or BLT-1ncp7. In general, addition of bulky cycloester groups or replacement of flexible linear alkyl chains with rings reduced the potency of the inhibitors. Thus, a flexible alkyl chain substantially contributes to the remarkably high potency of BLT-1, with optimal chain lengths of ~6−8 carbons. Although compounds BLT-1nac and **2** were less potent than BLT-1 (Table 1), they were nevertheless moderately potent inhibitors, indicating that the thiosemicarbazone portion of BLT-1 plays a key role in determining its activity.

(C) Thiosemicarbazone. We next modified various features of the thiosemicarbazone of either BLT-1 or BLT-1ncp7 and examined the consequences on inhibition of SR-BI-mediated lipid transport from DiI-HDL or [^{3}H]CE-HDL (Table 3 and Figure 2).

Table 3: SAR Analysis of the Thiosemicarbazone Group of BLT-1

Compound	IC_{50} (μM)[a,c]
Primary amine ([^{3}H]CE-HDL assay)[b]	
 BLT-1	0.057 ± 0.015
 BLT-1NMe	0.16 ± 0.005
Imino group (DiI-HDL assay)	
 BLT-1	0.021 ± 0.009
 BLT-1ncp7	0.012 ± 0.001
 BLT-1ncp9	0.011 ± 0.003
 1-decyl-2-thiourea	1.9 ± 0.3
Thiocarbonyl group ([^{3}H]CE-HDL assay)[b]	
 BLT-1	0.057 ± 0.015
 BLT-1SMe	82 ± 42
 1-decanethiol	> 250

[a] IC_{50} values were determined using either the DiI-HDL or [^{3}H]CE-HDL assays as indicated. [b] SR-BI specific values determined in the absence of compound for the [^{3}H]CE-HDL assays were as follows: selective [^{3}H]CE uptake, 4480 ng of protein (equivalent)/mg of cell protein; ^{125}I-HDL binding, 153 ng of protein/mg of cell protein. [c] Values are averages from six (BLT-1), five (BLT-1NMe), four (BLT-1), three (compound **5**), or two (all other compounds) experiments.

(1) Primary Amine. Conversion of the primary amine on the thiosemicarbazone of BLT-1 into a secondary amine by the addition of a single methyl group decreased the potency only by about 3-fold (BLT-1NMe; $IC_{50} = 0.16 \pm 0.05$ μM, [^{3}H]CE-HDL assay (Table 3)). There was also a relatively small increase in IC_{50} caused by the analogous methylation of BLT-1ncp7 (BLT-1ncp7NMe; single experiment, data not shown). Thus, the primary amine is not essential for robust inhibitory activity.

(2) Imino Group. As described above, compounds BLT-1ncp7 and BLT-1ncp9, which do not have cyclopentanes, are potent inhibitors with activities comparable to that of BLT-1 (Figure 1, Table 1). Their thiourea analogue, 1-decyl-2-thiourea, in which the imino group is replaced with two methylenes (Table 3), is about 100-fold less potent ($IC_{50} = 1.9 \pm 0.3$ μM) in the DiI-HDL assay. This indicates that the imino group substantially contributes to the inhibitory activity, even though it is apparently not essential for activity

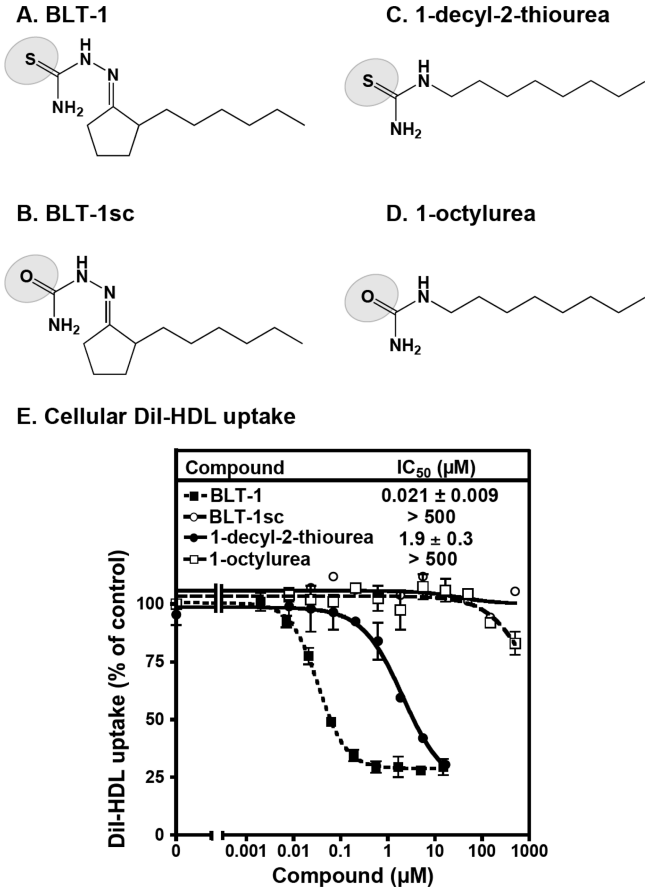


FIGURE 2: Effects of replacing the thiocarbonyl group's sulfur with oxygen on BLT-1's or 1-decyl-2-thiourea's inhibition of SR-BI-mediated lipid transport. The effects of BLT-1 (A, E, solid squares), BLT-1sc (B, E, open circles), 1-decyl-2-thiourea (C, E, solid circles), and 1-octylurea (D, E, open squares) on SR-BI-dependent uptake of DiI from DiI-HDL (10 μg of protein/mL) in ldlA[mSR-BI] and ldlA-7 cells were determined as described in Figure 1. IC_{50} values were derived from an average of four experiments with BLT-1 or two experiments with the other compounds.

(see below for examples of compounds with essentially complete loss of activity).

(3) Thiocarbonyl. The sulfur atom in the thiocarbonyl of thiosemicarbazones may be capable of forming disulfide bonds with the sulfhydryl groups in cysteines (*66*). At least two of the eight cysteines in murine SR-BI, Cys^{462} and Cys^{470}, are potentially available to form disulfide bonds with the sulfur in the thiocarbonyl group of BLT-1, because they do not appear to be engaged in inter- or intramolecular disulfide bonds. Indeed, they are at least partially fatty acylated (*53*). Thus, if SR-BI were the direct target of BLT-1 (see below), its ability to be inhibited by BLT-1 might depend on free sulfhydryl groups. We therefore examined the ability of BLT-1 to inhibit the selective uptake of [^{3}H]CE from [^{3}H]-CE-HDL by HEK293 cells transiently expressing either wild-type mSR-BI or a mutant mSR-BI in which both Cys^{462} and Cys^{470} were replaced by serines (mSR-BI (CC $\rightarrow$ SS); double cysteine mutant). Previous studies established that the mutant mSR-BI (CC $\rightarrow$ SS) exhibits normal HDL binding and selective lipid uptake activities (*53*). BLT-1 at a concentration of 1 μM was equally effective in inhibiting selective lipid uptake mediated by the wild-type (17.0 $\pm$ 3.5% of the control without BLT-1) and double cysteine mutant (26.0 $\pm$ 2.1% of the control, $P = 0.07$) receptors. Thus, formation of a disulfide bond between BLT-1 and the side chain of either Cys^{462} or Cys^{470} is not required for potent inhibition of mSR-BI by BLT-1. The disulfide bonding pattern of the additional six cysteine residues in the extracellular domain of mSR-BI has not yet been reported; however, by analogy with a report of the disulfide pattern for SR-BI's homologue CD36 (*67*), it is possible that all six of these cysteines participate in intramolecular disulfide bonds. Future studies will be required to determine whether any of these other cysteines in mSR-BI are available to form potential disulfide bonds with the sulfur atom in the thiosemicarbazone group of BLT-1.

When the thiocarbonyl of BLT-1 ($IC_{50} = 0.057 \pm 0.015$ μM in the [^{3}H]CE-HDL assay**)** was converted to a methyl thioether (BLT-1SMe; $IC_{50} = 82 \pm 42$ μM, [^{3}H]CE-HDL assay), there was a ~1440-fold increase in the IC_{50} (Table 3). The activity of the less potent inhibitor BLT-1nac (no alkyl chain, $IC_{50} = 38.6 \pm 9.1$ μM in the [^{3}H]CE-HDL assay) was also substantially reduced by this modification (data not shown). Not surprisingly, 1-decanethiol exhibited virtually no inhibitory activity. These results raise the possibility that either formation of a disulfide bond between the sulfur of BLT-1's thiosemicarbazone and its target or nucleophilic attack of the target on the thiocarbonyl carbon might, in part, contribute to BLT-1's tight binding to its target, and thus its high potency. Nevertheless, the significant residual inhibitory activity of BLT-1SMe relative to virtually inactive derivatives with $IC_{50} > 500$ μM (see below) indicates that neither of these two potential mechanisms is absolutely essential for at least some inhibitory activity.

When the sulfur atom in the thiocarbonyl of BLT-1 was replaced with an oxygen atom, forming the semicarbazone BLT-1sc (Figure 2A,B), there was a >20000-fold loss of potency ($IC_{50} > 500$ μM) in both the DiI-HDL (Figure 2E) and [^{3}H]CE-HDL (see below) assays. Similarly, 1-octylurea was also almost inactive (Figure 2D, $IC_{50} > 500$ μM, 20% of the maximal BLT-1 inhibition at 500 μM; compare to 1-decyl-2-thiourea (Figure 2C), $IC_{50} = 1.9 \pm 0.3$ μM). Because BLT-1sc has virtually no inhibitory activity yet its structure and hydrophobicity (cLogP = 4.48) are almost identical to that of BLT-1 (cLogP = 4.41), it seems unlikely that the principle mechanism by which BLT-1 inhibits SR-BI-mediated lipid transport is its nonspecific partition into membranes and consequent alteration of membrane biophysical properties. BLT-1sc can be used as an isostructural, negative control for further analysis of the mechanism by which BLT-1 inhibits SR-BI activity.

SR-BI is the Target of BLT-1. Because BLT-1 was identified in an intact cell-based screening assay (*30*), a critical unresolved issue regarding its mechanism was the identification of its molecular target. Candidate targets include SR-BI itself as well as other potential, but as yet unknown, cellular components that may be required for SR-BI activity in intact cells. To determine whether SR-BI is BLT-1's target, we examined the ability of BLT-1 and BLT-1sc to inhibit selective lipid uptake mediated by homogeneous, purified, epitope-tagged receptor (mSR-BI-t1) protein reconstituted into phosphatidylcholine/cholesterol (PC) liposomes (*57*). Figure 3 shows that BLT-1 can inhibit SR-BI-dependent selective uptake of [^{3}H]CE from [^{3}H]CE-HDL by mSR-BI-t1-containing liposomes and that the IC_{50} values for BLT-1 were essentially identical in cells (Figure 3A, 0.057

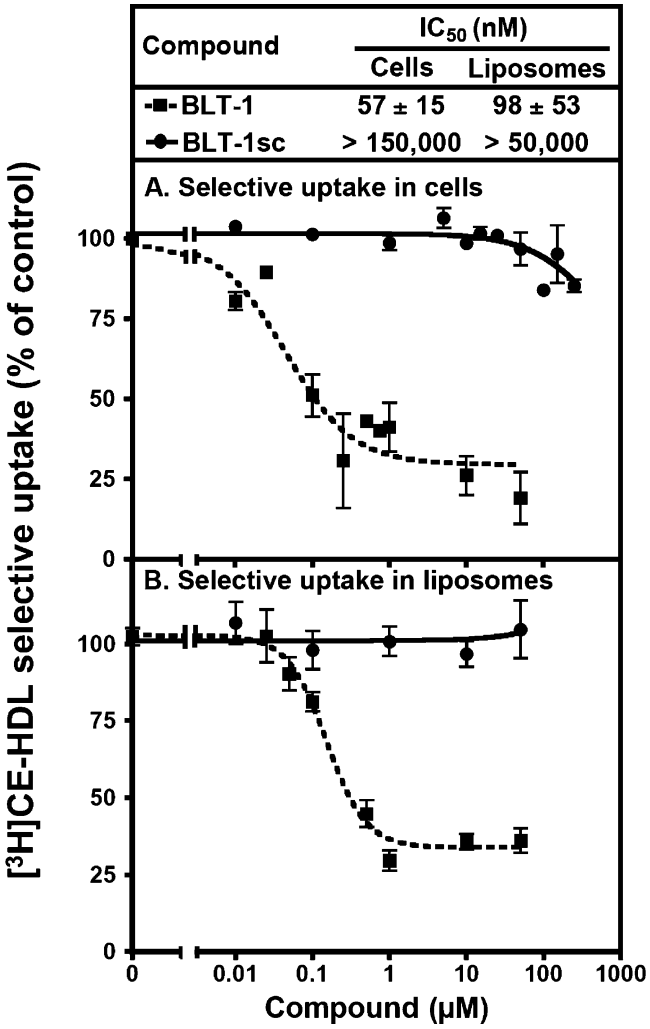
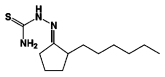
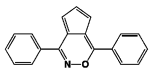
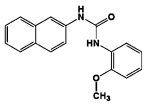
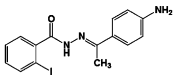


FIGURE 3: Effects of BLT-1 and BLT-1sc on SR-BI activity in cells and liposomes. (A) Cellular SR-BI-mediated selective uptake of [^{3}H]CE from [^{3}H]CE-HDL (10 μg of protein/mL) using ldlA-[mSR-BI] and ldlA7 cells measured in the presence of the indicated concentrations of BLT-1 (average of six independent experiments) or BLT-1sc (average of four independent experiments) was determined as described in Figure 2 for DiI-HDL. (B) SR-BI-t1-containing liposomes or control liposomes (devoid of SR-BI-t1) were preincubated for 1 h at 37 °C in medium containing the indicated concentrations of BLT-1 or BLT-1sc, followed by incubation with 10 μg of protein/mL of either [^{3}H]CE-HDL or ^{125}I-HDL for 4 h at 37 °C in the presence of the same concentrations of compounds. These incubation media with and without the compounds contained 0.5% (v/v) DMSO. For liposome assays, 100% of the control value represents receptor specific activity in SR-BI-t1 containing liposomes without compounds and 0% of the control value represents background selective uptake in control liposomes. SR-BI specific values (ng of protein/mg of cell protein) for cells determined in the absence of compounds were ([^{3}H]CE-HDL selective uptake) 4480 ng and (^{125}I-HDL binding) 153 ng. For liposomes the values (ng of protein/sample) of selective uptake and binding were (mSR-BI-t1 containing liposomes) 159 and 46 and (control, protein-free liposomes) 44 and 5.

$\pm$ 0.015 μM ($n = 6$)) and liposomes (Figure 3B, 0.098 $\pm$ 0.053 μM ($n = 2$)). BLT-1 did not affect low background [^{3}H]CE uptake observed in control liposomes prepared without mSR-BI-t1. The inhibitory effect is specific, because the negative control BLT-1sc did not inhibit SR-BI activity in the reconstituted system (Figure 3). Because the IC_{50} values for cells and liposomes were nearly identical, we conclude that the molecular target of BLT-1 for SR-BI-dependent selective uptake in cells is SR-BI itself in the context of a lipid environment.

Table 4: Effects of BLTs on SR-BI Activity in Cells and Liposomes

Compound	IC_{50} (μM)[a-c] Cells	Liposomes
 BLT-1	0.057 ± 0.015[d]	0.098 ± 0.053[e]
 BLT-3	2.3 ± 1.5[d]	2.0 ± 0.1[e]
 BLT-4	3.9 ± 0.8[d]	4.7 ± 3.3[f]
 BLT-5	13.8 ± 8.5[g]	25.9 ± 1.7[e]

[a] The effect of BLTs on SR-BI-mediated selective uptake of [^{3}H]CE from [^{3}H]CE-HDL in cells and liposomes was determined as described in Figure 3. [b] IC_{50} values for cells for BLT-3, BLT-4, and BLT-5 were from ref *30*. [c] No statistical differences were found beween IC_{50} values for selective uptake in cells compared to liposomes (*P* values: BLT-1, 0.0976; BLT-3, 0.7975; BLT-4, 0.5681; BLT-5, 0.1169). [d] $n = 6$. [e] $n = 2$. [f] $n = 3$. [g] $n = 5$.

Identification of the Target of BLT-3, BLT-4, and BLT-5. Table 4 shows that each of these BLTs can inhibit SR-BI-mediated selective uptake of [^{3}H]CE from [^{3}H]CE-HDL in reconstituted liposomes with IC_{50} values for inhibition essentially identical to those previously determined in intact cell assays (*30*). As was the case for BLT-1 and BLT-1sc, no effect of any of these three BLTs was seen in control liposomes lacking mSR-BI-t1 protein. Thus, we tentatively conclude that SR-BI in the context of a lipid environment is also the molecular target of these BLTs.

DISCUSSION

High-throughput screening in intact cells of chemically diverse small-molecule compound libraries to identify inhibitors and activators of biological systems provides powerful reagents for the analyses of the functions and mechanisms of action of these systems (*68*). We previously employed such a screen of intact cells to identify small-molecule inhibitors, called BLTs, of lipid transport mediated by the HDL receptor SR-BI (*30*). This receptor plays a key role in lipoprotein metabolism and is a potential target for the prevention and treatment of cardiovascular disease (*1*). Unfortunately, identification of the molecular target(s) of compounds identified in such screens remains problematic. Previously, we have shown that the target of BLTs resides in cells and is not in the HDL ligand (*30*). In the current study we identified SR-BI in the context of a lipid environment as a molecular target of BLT-1, BLT-3, BLT-4, and BLT-5 using an in vitro system of purified SR-BI reconstituted into liposomes and information derived from SAR analysis of the most potent of these BLTs, the thiosemicarbazone BLT-1.

We first used SAR analysis to characterize the features of BLT-1 that contribute to its high (nanomolar IC_{50}) potency.

BLT-1 comprises three distinct structural features: cyclopentyl, linear alkyl, and thiosemicarbazone moieties. The linear alkyl chain substantially contributes to the potency of BLT-1, with an optimal chain length of ~6−8 carbons and substantially reduced potency for shorter chains of reduced lipophilicity. Indeed, there was a linear relationship between the hydrophobicity (calculated partition coefficient between octanol and water, cLogP) and log(IC_{50}) (negative slope) for all 15 thiosemicarbazones tested (not shown), indicating that hydrophobicity played a significant role in determining a compound's potency. For example, the addition of a relatively hydrophilic oxygen in the middle of the alkyl chain of BLT-1 (cLogP = 4.41) to form BLT-2 (cLogP = 2.88 was accompanied by an approximately 17-fold increase in IC_{50} (reduced potency). It is possible that the alkyl chain facilitates interaction of BLT-1 with the plasma membrane, increasing its effective concentration near the receptor, or that it interacts with lipid binding sites on the receptor that normally participate in the lipid transport process. The cyclopentyl group has little influence on activity. The sulfur in the thiosemicarbazone is essential for activity. Because the isostructural semicarbazone derivative of BLT-1, called BLT-1sc (sulfur replaced by oxygen, Figures 2 and 3), is essentially completely inactive (IC_{50} >20000-fold higher than that of BLT-1) even though its hydrophobicity is almost identical to that of BLT-1 (cLogP of 4.48 vs 4.41), it seems unlikely that the principle mechanism by which BLT-1 inhibits SR-BI-mediated lipid transport is its nonspecific partition into membranes and consequent alteration of membrane biophysical properties.

The precise mechanism by which the thiosemicarbazone of BLT-1 mediates inhibition of SR-BI remains to be established. Thiosemicarbazone-based drugs are used for diverse molecular targets to treat a wide variety of unrelated diseases and targets (e.g., tuberculosis (*69*−*71*), tumor proliferation (*72*−*74*), ribonucleotide reductase (*75*, *76*), trypanosomal cystein proteases (*77*)). Mechanisms of actions ascribed to thiosemicarbazone-based drugs include (a) chelation of transition metals (*78*) (b) disulfide bond formation (thiosemicarbazone's sulfur and a sulfhydryl on the target) (*66*), and (c) covalent bond formation (nucleophilic attack of the target on the thiocarbonyl carbon of the thiosemicarbazone) (*77*). Each of these may contribute to BLT-1's high potency, because BLT-1 derivatives that cannot participate in such interactions were significantly less potent than BLT-1. Regardless of the detailed role of the thiosemicarbazone, BLT-1 and many of its derivatives inhibited SR-BI-mediated lipid uptake with Hill coefficients that were very close to unity, indicating that inhibitor cooperativity did not contribute to the activity of these compounds. Additional studies will be required to establish the mechanism of BLT-1 inhibition. Nevertheless, identification of BLT-1sc as an inactive, isostructural, semicarbazone derivative of BLT-1 permitted us to use it as a negative control in studies designed to identify the molecular target of BLT-1.

In previous studies we established that a C-terminally epitope-tagged variant of murine SR-BI, mSR-BI-t1, could be purified to homogeneity, could be reconstituted into phosphatidylcholine/cholesterol liposomes, and could mediate the binding of HDL and selective uptake of its lipids with an affinity similar to that of SR-BI in intact cells (*42*, *57*). In this study we showed that BLT-1 can inhibit selective lipid uptake mediated by purified mSR-BI-t1 in liposomes with a potency (IC_{50}) similar to that seen in intact cells. BLT-1sc, the semicarbazone derivative inactive in intact cell assays, was also inactive in the purified receptor lipid transport assay. Thus, we conclude that SR-BI in the context of a lipid environment is the molecular target of BLT-1, their interaction resulting in inhibition of receptor-mediated selective lipid uptake in cells. It is noteworthy that in the initial SAR studies reported here we were unable to identify a derivative or variant of BLT-1 that exhibited substantially greater potency than BLT-1 itself.

Three other blockers of SR-BI-mediated lipid transport identified by intact cell screening (*30*)—BLT-3, BLT-4, and BLT-5 (Table 4)—were also shown to inhibit SR-BI-mediated lipid uptake in reconstituted liposomes with potencies similar to those in intact cells. Thus, even though we have not conducted SAR analysis of these inhibitors to generate corresponding negative control compounds, it seems reasonable to conclude that SR-BI is also a molecular target of these compounds in the context of a lipid environment. In the case of BLT-4, this was somewhat unexpected. This compound can inhibit, with essentially identical potencies, cholesterol transport out of cells mediated by two distinct surface transporters, SR-BI and ABCA1 (*31*). BLT-4 appears to be a pan-ABC superfamily inhibitor (for example, it also inhibits efflux of toxic substances from *Staphylococcus aureus* by the ABC pump NorA (*79*)). Similar to BLT-4, another ABC superfamily inhibitor, glyburide (sometimes called glybenclamide), can inhibit SR-BI and ABCA1 with low, but similar, potencies (*31*). It thus seemed likely that these drugs would indirectly, perhaps through an unidentified ABC protein, rather than directly, inhibit SR-BI. The results reported here suggest that SR-BI is a molecular target of BLT-4 and this direct interaction is likely to account for some, if not all, of the inhibition of SR-BI selective uptake activity in cells by BLT-4.

In summary, we identified SR-BI in the context of a lipid environment as a direct molecular target of BLTs in studies aided by SAR analysis of BLT-1. The results of the SAR analysis can be used in the future to direct the synthesis of BLT-1 derivatives (and semicarbazone inactive controls) for additional structure/function analysis of SR-BI. For example, covalently cross-linkable derivatives of BLT-1 might be used to help map the binding site(s) of BLT-1 on SR-BI and identify amino acid residues or regions in the protein important for the activity of the receptor. Identification of such sites, together with ongoing mutational analysis of SR-BI and biophysical studies, may help elucidate the distinctive, but as yet very poorly defined, mechanism by which SR-BI mediates lipid transport between HDL and cells. Also, it is possible that BLT-1 or other BLTs may be adapted for in vivo studies of the role of SR-BI in HDL metabolism (*80*, *81*), especially in species in which genetic modification of SR-BI activity (e.g., loss or gain of function) is difficult or impossible (rabbit, rat, dog, nonhuman primate). Such studies could significantly contribute to our understanding of the in vivo function of SR-BI and possible help direct future development of pharmaceuticals that target SR-BI and its role in atherosclerosis, cardiovascular disease, and female infertility (*1*).

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