

## MIT Open Access Articles

*Exoplasmic cysteine Cys384 of the HDL receptor SR-BI is critical for its sensitivity to a small-molecule inhibitor and normal lipid transport activity*

The MIT Faculty has made this article openly available. **Please share** how this access benefits you. Your story matters.

**Citation:** Yu, M. et al. "Exoplasmic cysteine Cys384 of the HDL receptor SR-BI is critical for its sensitivity to a small-molecule inhibitor and normal lipid transport activity." Proceedings of the National Academy of Sciences 108.30 (2011): 12243-12248. Web. 1 Feb. 2012.

**As Published:** <http://dx.doi.org/10.1073/pnas.1109078108>

**Publisher:** Proceedings of the National Academy of Sciences (PNAS)

**Persistent URL:** <http://hdl.handle.net/1721.1/69003>

**Version:** Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

**Terms of Use:** Article is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.



# Exoplasmic cysteine Cys384 of the HDL receptor SR-BI is critical for its sensitivity to a small-molecule inhibitor and normal lipid transport activity

Miao Yu<sup>a,1</sup>, Katherine A. Romer<sup>a,1</sup>, Thomas J. F. Nieland<sup>a,b</sup>, Shangzhe Xu<sup>a</sup>, Veronica Saenz-Vash<sup>b,2</sup>, Marsha Penman<sup>a</sup>, Ayce Yesilaltay<sup>a</sup>, Steven A. Carr<sup>b</sup>, and Monty Krieger<sup>a,3</sup>

<sup>a</sup>Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139; and <sup>b</sup>Broad Institute of Harvard and MIT, 7 Cambridge Center, Cambridge, MA 02142

Contributed by Monty Krieger, June 8, 2011 (sent for review April 4, 2011)

The HDL receptor, scavenger receptor, class B, type I (SR-BI), is a homooligomeric cell surface glycoprotein that controls HDL structure and metabolism by mediating the cellular selective uptake of lipids, mainly cholesteryl esters, from HDL. The mechanism underlying SR-BI-mediated lipid transfer, which differs from classic receptor-mediated endocytosis, involves a two-step process (binding followed by lipid transport) that is poorly understood. Our previous structure/activity analysis of the small-molecule inhibitor blocker of lipid transport 1 (BLT-1), which potently ( $IC_{50} \sim 50$  nM) blocks SR-BI-mediated lipid transport, established that the sulfur in BLT-1's thiosemicarbazone moiety was essential for activity. Here we show that BLT-1 is an irreversible inhibitor of SR-BI, raising the possibility that cysteine(s) in SR-BI interact with BLT-1. Mass spectrometric analysis of purified SR-BI showed two of its six exoplasmic cysteines have free thiol groups (Cys251 and Cys384). Converting Cys384 (but not Cys251) to serine resulted in complete BLT-1 insensitivity, establishing that the unique molecular target of BLT-1 inhibition of cellular SR-BI dependent lipid transport is SR-BI itself. The C384S substitution reduced the receptor's intrinsic lipid uptake activity by approximately 60% without dramatically altering its surface expression, homooligomerization, or HDL binding. Thus, a small-molecule screening approach identified a key residue in SR-BI involved in lipid transport, providing a powerful springboard into the analyses of the structure and mechanism of SR-BI, and highlighting the power of this approach for such analyses.

cholesterol | chemical biology | lipoprotein | disulfide

High-throughput screening in intact cells of chemically diverse small-molecule compound libraries can identify powerful reagents useful for analyses of a wide range of biological systems (1). For example, we (2, 3) and others (4–6) have identified small-molecule inhibitors of the activity of the HDL receptor [scavenger receptor, class B, type I (SR-BI)]. SR-BI (reviewed in refs. 7, 8) plays an important role in controlling the structure and metabolism of HDL in mice and humans (7–9), in whom plasma levels of HDL cholesterol are inversely proportional to the risk of atherosclerotic disease (10, 11). SR-BI is expressed most highly in the liver and steroidogenic tissues, and is found in other types of cells. Studies, primarily in mice, have shown that SR-BI's expression can profoundly influence lipoprotein metabolism and gastrointestinal, endocrine, reproductive, and cardiovascular physiology, as well as development and susceptibility to pathogens [e.g., hepatitis C virus (HCV); ref. 12] (reviewed in refs. 7, 8). SR-BI protects against female infertility, red blood cell and platelet pathophysiology, and atherosclerosis/coronary heart disease (reviewed in refs. 7, 8).

SR-BI controls HDL metabolism by mediating the cellular uptake of its lipids via a mechanism called selective lipid uptake (13–15). Selective uptake differs fundamentally from coated-pit receptor-mediated endocytosis employed, for example, by LDL receptors (16). During selective uptake, HDL binds to

the receptor, which then selectively transfers HDL's cholesterol, primarily in the form of cholesteryl esters (CE), into the cells. After lipid transfer, the cholesteryl ester-depleted HDL dissociates and reenters the circulation. Cellular internalization of the SR-BI/HDL complex is not required for selective lipid uptake (17). SR-BI also facilitates bidirectional transfer of unesterified cholesterol between lipoproteins and cells (18) and can bind other lipoproteins, such as LDL (reviewed in ref. 7).

SR-BI is a member of the CD36 superfamily of proteins that share a common topology: a large, glycosylated exoplasmic loop connected to the plasma membrane by N- and C-terminal transmembrane domains, each with short cytoplasmic extensions (19). SR-BI's homooligomerization (20–24) is mediated by a glycine dimerization motif in its N-terminal transmembrane domain. Mutagenesis and genetics studies have identified a few residues in SR-BI that play roles in lipoprotein binding and lipid transport (24–28) and in interactions with HCV (29). For example, disruption of the glycine dimerization motif interferes with oligomerization and efficient lipid transfer into cells (24). However, the molecular mechanisms underlying SR-BI's activities are not well defined (7).

To better understand the mechanism and function of SR-BI, we previously performed a high-throughput screen for small-molecule inhibitors of SR-BI-mediated cellular uptake of the fluorescent lipid 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) from DiI-HDL (2). Five compounds identified in the screen (BLT 1–5), as well as fenofibrate and HDL376 (3), are potent blockers of lipid transport (BLTs). BLTs have been used to study a plethora of SR-BI related activities, including caveolar microheterogeneity (30), lutein and vitamin E transport (31, 32), phagocytosis of apoptotic cells (33), and cellular entry of HCV (29). Blocker of lipid transport 1 (BLT-1) (Fig. 1A), the most potent SR-BI inhibitor known, blocks SR-BI-mediated selective lipid uptake and bidirectional cholesterol flux with an  $IC_{50}$  of approximately 50 nM, while simultaneously increasing the affinity of HDL binding to SR-BI (2). BLT-1's effects on SR-BI suggested that it might be a useful tool for examining SR-BI's mechanism.

Structure activity relationship (SAR) analysis of BLT-1 established that its alkyl chain significantly contributes to potency and that the sulfur in the thiosemicarbazone moiety is essential

Author contributions: M.Y., K.A.R., T.J.F.N., S.X., M.P., A.Y., S.A.C., and M.K. designed research; M.Y., K.A.R., T.J.F.N., S.X., V.S.-V., and M.P. performed research; M.Y., K.A.R., T.J.F.N., S.X., V.S.-V., M.P., S.A.C., and M.K. analyzed data; and M.Y. and M.K. wrote the paper.

The authors declare no conflict of interest.

<sup>1</sup>M.Y. and K.A.R. contributed equally to this work.

<sup>2</sup>Present address: Novartis Institutes for BioMedical Research, Inc., 250 Massachusetts Avenue, Cambridge, MA 02139.

<sup>3</sup>To whom correspondence should be addressed. E-mail: krieger@mit.edu.

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109078108/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109078108/-DCSupplemental).

for inhibitory activity—replacement of the sulfur by oxygen completely inactivates BLT-1 (34). A similar substitution in the thiourea moiety of HDL376 is also inactivating (3). This dependence on sulfur raised the possibility that free thiol groups (reduced cysteines) in SR-BI might be required for BLT-1's and HDL376's activities (e.g., by nucleophilic attack of the thiol at the inhibitor's thiocarbonyl carbon) (34) and result in essentially irreversible inhibition. Here we show that inhibition by BLT-1 is essentially irreversible and that there are two reduced cysteines in native SR-BI's exoplasmic loop (Cys251 and Cys384), one of which, Cys384, is necessary for inhibition by BLT-1 and significantly contributes to the intrinsic lipid uptake activity of SR-BI.

## Results and Discussion

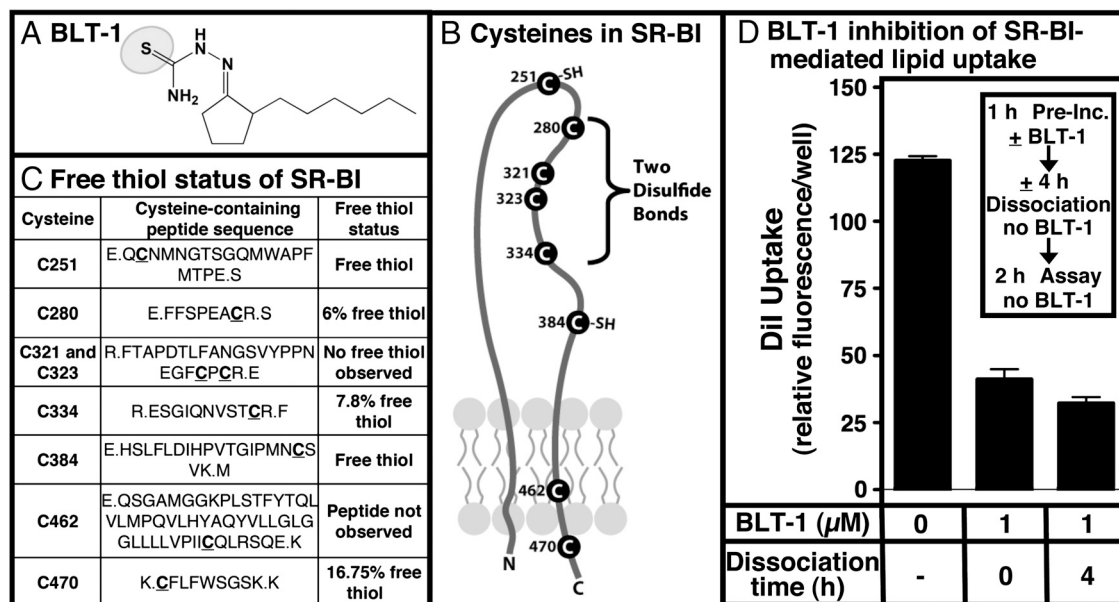
**Mass Spectrometric Analysis Reveals Two Reduced Cysteines with Free Thiol Groups in SR-BI.** Murine SR-BI has eight cysteines: six exoplasmic, one transmembrane, and one cytoplasmic (Fig. 1*B*). A close homologue, bovine CD36 has six exoplasmic cysteines, all of which participate in disulfide bonds (35) between cysteines 242–310, 271–332, and 312–321. A comparison of the SR-BI and CD36 sequences suggests that the following exoplasmic cysteines in the two proteins are likely to be in equivalent positions (SR-BI: CD36): 251:242; 280:271; 321:312; 323:321; and 334:332. There is no corresponding cysteine for SR-BI's Cys384 (C384) in CD36 or for CD36's Cys310 in SR-BI.

We used differential alkylation before and after reduction and liquid chromatography (LC)-MS/MS to identify free thiols in a homogeneously pure, epitope-tagged, and biologically active form of SR-BI, SR-BI-t1 (see *Experimental Procedures*) (34, 36–38). Fig. 1*B* and *C* shows that there were no free thiols at Cys321 and Cys323, and only 6–8% at Cys280 and Cys334, suggesting that these cysteines participate in disulfide bonds. The

formation of two disulfide bonds involving these four cysteines is consistent with disulfides at equivalent residues in CD36 (312–321 and 271–332). Comparisons of the electrophoretic mobilities of unreduced and reduced SR-BI (both unmodified and chemically cross-linked; Fig. S1) suggest that stable disulfide bonds in SR-BI are intramolecular. Exoplasmic Cys251 and Cys384 in SR-BI were fully reduced in the native protein, and thus potentially available to interact with the thiosemicarbazone in BLT-1, possibly covalently, and thus mediate its activity. Indeed, Fig. 1*D* shows that in cells stably expressing wild-type murine SR-BI (IdIA[SR-BI]) inhibition of receptor-mediated uptake of the fluorescent lipid DiI from DiI-HDL by BLT-1 was essentially irreversible (slow inhibitor off rate). The cells were preincubated with or without 1  $\mu$ M BLT-1 for 1 h, then bound BLT-1 was allowed to dissociate (0 or 4 h), and then SR-BI-mediated DiI uptake from DiI-HDL was assessed during a subsequent 2-h incubation without BLT-1. After the 4-h dissociation, the extent of inhibition ( $26 \pm 2\%$  of the control with no BLT-1 treatment) was similar to that observed with no dissociation period ( $34 \pm 3\%$ ) or when BLT-1 was included in both the preincubation and DiI uptake steps ( $43 \pm 2\%$ ). Similar results were observed in another two independent experiments. (In contrast, see Fig. S2 for a control with the apparently reversible SR-BI inhibitor BLT-4; ref. 2.) Reduction in the apparent  $IC_{50}$  (increased potency) of BLT-1 accompanied increases in the time of preincubation with BLT-1 (see Fig. S3), supporting the suggestion that BLT-1 is probably an irreversible inhibitor.

## Roles of Cys251 and Cys384 in the Inhibition of SR-BI Activity by BLT-1.

We generated cDNA expression vectors encoding mutant murine SR-BIs with single (C251S, C384S) or double (C251/384S) Cys-to-Ser substitutions. Together with a plasmid expressing wild-type



**Fig. 1.** Structure of BLT-1 (*A*), determination of free thiols in murine SR-BI by mass spectrometry (*B* and *C*), and apparent irreversibility of SR-BI inhibition by BLT-1 (*D*). (*A*) Structure of BLT-1. The sulfur in the thiosemicarbazone ( $R_2CH_3N_3S$ ) moiety of BLT-1 is essential for BLT-1's effects on SR-BI. All activity is lost when the sulfur (shaded) is replaced with oxygen (semicarbazone). (*B*) Model of murine SR-BI illustrating the approximate locations of the cysteines. Cysteines 251 and 384 are fully reduced (free thiols) whereas there is little or no free thiol in cysteines 280, 321, 323, and 334 suggesting their participation in two intramolecular disulfide bonds. (*C*) Determination of the free thiols in murine SR-BI by mass spectrometry. Purified SR-BI-t1 was subjected to differential alkylation with NEM before and after reduction, deglycosylated, and proteolytically digested (trypsin and Glu C), and peptides were subjected to LC-MS/MS analysis as described in *Experimental Procedures*. Cytoplasmic Cys470 is at least partially fatty acylated (52), which presumably accounts for its low level of free thiol. (*D*) Cells expressing wild-type murine SR-BI (IdIA[SR-BI]) were preincubated with (septuplet determinations) or without (sextuplet determinations) 1  $\mu$ M BLT-1 for 1 h, and then incubated without BLT-1 for 0 or 4 h to permit dissociation of any reversibly bound BLT-1. The cells were then incubated for 2 h with DiI-HDL (20  $\mu$ g of protein per mL) without BLT-1. The cells were then washed and SR-BI specific DiI uptake (relative fluorescence units per well) was determined as described in *Experimental Procedures*. Nonspecific uptake was measured in the presence of a 50-fold excess of unlabeled HDL (duplicate determinations). All incubations were at 37  $^{\circ}$ C. Similar results were observed in two additional independent experiments.

murine SR-BI, these vectors were used to transiently transfect COS cells and to generate stably transfected cell lines (IdIA [C251S], IdIA[C384S], IdIA[C251/384S]) with cell surface receptor levels similar to those of IdIA[SR-BI] cells (13). The effects of the mutations on receptor activities were similar in stably and transiently transfected cells.

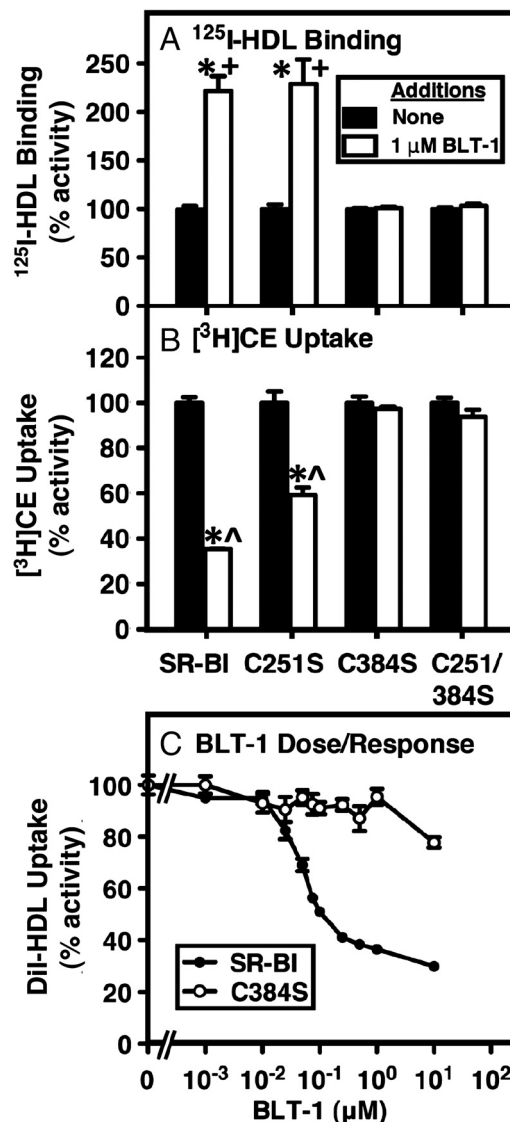
We compared the ability of BLT-1 to alter three receptor-specific activities:  $^{125}\text{I}$ -HDL binding, and uptake of either [ $^3\text{H}$ ]CE from [ $^3\text{H}$ ]CE-HDL or uptake of DiI from DiI-HDL. All measurements were performed at 37°C and a subsaturating HDL concentration (10  $\mu\text{g}$  of protein per mL). Under these conditions, BLT-1 increases the amount of bound  $^{125}\text{I}$ -HDL due to increased binding affinity of wild-type SR-BI and decreases the receptor-mediated lipid uptake (2).

Fig. 2 *A* and *B* shows that, as previously reported (2), 1  $\mu\text{M}$  BLT-1 increased  $^{125}\text{I}$ -HDL binding to and inhibited uptake of [ $^3\text{H}$ ]CE by wild-type SR-BI in IdIA[SR-BI] cells. Similar results were observed for the C251S mutant. Thus, the free thiol in Cys251 is not required for BLT-1's activity. We also showed that cysteines in SR-BI's transmembrane (Cys462) and cytoplasmic (Cys470) domains are not required for BLT-1's activity (see Fig. S4). However, the binding and lipid uptake activities of the C384S mutant and the C251/384S double mutant were essentially completely resistant to BLT-1. Fig. 2*C* shows that, unlike wild-type SR-BI (black circles,  $\text{IC}_{50} < 100$  nM; ref. 2), C384S was resistant to BLT-1 inhibition of uptake of DiI over a wide range of concentrations (white circles). Thus, the free thiol on Cys384 is critical for BLT-1 inhibition of SR-BI. The insensitivity of C384S to BLT-1 provides strong evidence that there is a unique molecular target of BLT-1 responsible for its inhibition of SR-BI in cells—SR-BI itself. This finding agrees with previous analysis of the effects of BLT-1 on the activity of purified SR-BI-t1 incorporated into liposomes (34).

Potential mechanisms by which the thiosemicarbazone of BLT-1 (Fig. 1) might interact with Cys384's free thiol (34) include (i) thiol nucleophilic attack on the thiocarbonyl as proposed for thiosemicarbazone inhibition of cysteine proteases (39), (ii) transition metal mediated interaction (40, 41), and (iii) disulfide bond formation between the thiosemicarbazone's sulfur and Cys384's free thiol (42, 43). These mechanisms could contribute to BLT-1's high potency. The importance of Cys384 for BLT-1's activity raised the question: Does this residue contribute to the intrinsic activity of SR-BI?

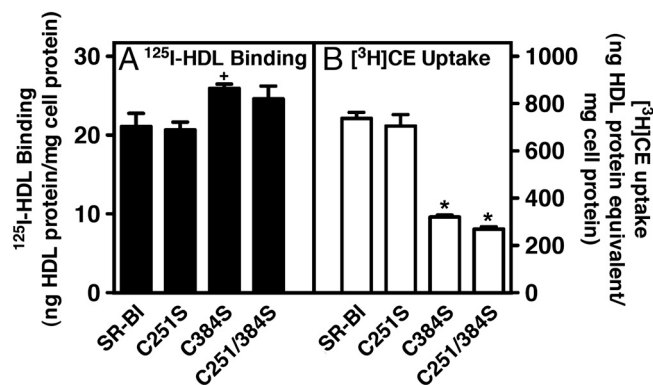
#### Contribution of Cys 384 to the Intrinsic Activity and Structure of SR-BI.

Fig. 3 shows the absolute amounts of binding of  $^{125}\text{I}$ -HDL and uptake of [ $^3\text{H}$ ]CE from [ $^3\text{H}$ ]CE-HDL (10  $\mu\text{g}$  of protein per mL) at 37°C by SR-BI and the cysteine mutants when transiently expressed in COS cells. The values are corrected for differences in relative cell surface expression levels of the receptors, which were as follows: SR-BI, 1.0; C251S, 1.1; C384S, 1.3; and C251/384S, 1.5 (determined by flow cytometry in an independent experiment). The similar levels of surface expression—assuming the mutations did not alter antibody binding (see *Experimental Procedures*)—suggest that the mutations do not dramatically interfere with receptor folding, intracellular transport to the cell surface or stability. Fig. 3*A* shows that there were no significant differences in  $^{125}\text{I}$ -HDL binding based on ANOVA analysis with Tukey posttesting [there was a barely significant difference ( $P = 0.0336$ ) between SR-BI and C384S based on the Student *t* test]. There was no difference in lipid uptake mediated by SR-BI and C251S (Fig. 3*B*). In contrast, the C384S and C251/384S mutants exhibited significantly reduced (*ca.* 60%) lipid uptake ( $P < 0.0001$  for ANOVA analysis with Tukey post test or Student's *t* test) compared to SR-BI and C251S. Similar results were observed using stably transfected cells (see Fig. S5). Thus the effects of the C384S mutation were similar to the effects of BLT-1 on wild-type SR-BI-mediated lipid uptake, but not



**Fig. 2.** Influence of BLT-1 on the HDL binding (*A*) and lipid uptake (*B* and *C*) activities of Cys-to-Ser mutants of SR-BI.  $^{125}\text{I}$ -HDL binding (*A*) and either [ $^3\text{H}$ ]CE uptake from [ $^3\text{H}$ ]CE-HDL (*B*) or DiI uptake from DiI-HDL (*C*) activities of wild-type SR-BI and the indicated Cys-to-Ser mutants in stably transfected IdIA cells. Experiments were performed at 37°C with a subsaturating concentration of HDL (10  $\mu\text{g}$  of protein per mL) in the absence (black bars) or presence (white bars) of the indicated concentrations of BLT-1 as described in *Experimental Procedures*. All values represent receptor-specific activities calculated as the differences between activity in the absence (quadruplicate determinations) and presence (duplicate determinations) of a 40-fold excess of unlabeled HDL. The values (not corrected for cell surface receptor expression) were normalized so that 100% activity represents receptor-specific activity in the absence of BLT-1. These values in *A* (ng bound/mg cell protein) were as follows: SR-BI,  $22 \pm 2$ ; C251S,  $23 \pm 2$ ; C384S,  $64 \pm 1$ ; and C251/384S,  $27 \pm 1$ . The 100% of control values in *B* (nanogram HDL protein equivalent per milligram cell protein) were as follows: SR-BI,  $2616 \pm 131$ ; C251S,  $1899 \pm 196$ ; C384S,  $1721 \pm 98$ ; and C251/384S,  $949 \pm 42$ . The 100% of control values in *C* (relative fluorescence units) were SR-BI,  $85 \pm 6$  and C384S,  $49 \pm 4$ . Statistical analyses comparing without or with BLT-1 were performed using either one-way ANOVA with Tukey posttesting ( $*P < 0.0001$ ) or the unpaired two-tailed *t* test at 95% confidence intervals ( $^{\dagger}P < 0.0025$  or  $^{\wedge}P < 0.001$ ).

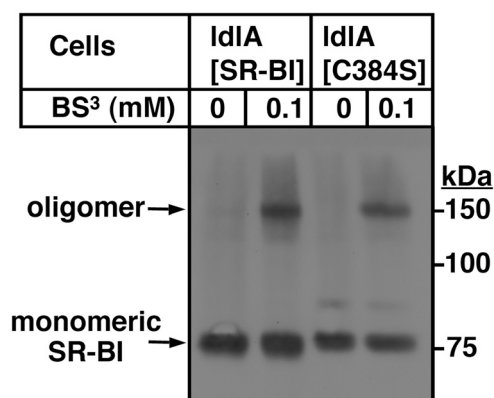
HDL binding. Thus, it appears that Cys384, presumably through its free thiol group, significantly contributes to the intrinsic ability of SR-BI to mediate lipid uptake in a manner that is inhibited by BLT-1. A similar approach (Cys-to-Ala mutation) has been used to show that exoplasmic Cys97 in the ADP receptor P2Y12



**Fig. 3.** <sup>125</sup>I-HDL binding (A) and [<sup>3</sup>H]CE uptake from [<sup>3</sup>H]CE-HDL (B) activities of Cys-to-Ser mutants of SR-BI. Receptor-specific <sup>125</sup>I-HDL binding (A) and [<sup>3</sup>H]CE uptake from [<sup>3</sup>H]CE-HDL (B) mediated by wild-type SR-BI and the indicated Cys-to-Ser mutants in transiently transfected COS cells were measured at 37 °C with a subsaturating concentration of HDL (10 μg of protein per mL) as described in *Experimental Procedures*. Receptor-specific values were calculated as the differences between the total binding and uptake values (triplicate determinations) and the nonspecific values measured in the presence of a 40-fold excess of unlabeled HDL (single determination). All values were normalized to correct for differences in receptor surface expression relative to wild-type SR-BI based on flow cytometry (see text). Statistical analyses comparing wild-type SR-BI and the individual mutants were performed using either one-way ANOVA with Tukey posttesting (\**P* < 0.0001) or the unpaired two-tailed *t* test at 95% confidence intervals (+*P* = 0.0336 or \**P* < 0.0001).

is necessary for its normal activity and sensitivity to the free-thiol-containing active metabolite of its inhibitor clopidogrel (44).

The phenotype of the C384S mutant is reminiscent of that of SR-BI with a mutagenic disruption of the glycine dimerization motif (GX<sub>3</sub>AX<sub>2</sub>G) in its N-terminal transmembrane domain: essentially normal surface expression and HDL binding, but a twofold reduction in lipid uptake (24). There is almost a complete loss of receptor homooligomerization when the dimerization motif is disrupted, as detected by cross-linking with the water-soluble, membrane-impermeable cross-linker bis(sulfosuccinimidyl)suberate (BS<sup>3</sup>) or by coimmunoprecipitation. We therefore used cross-linking with BS<sup>3</sup> to determine if the C384S substitution



**Fig. 4.** Cell surface cross-linking of SR-BI and C384S in stable transfectants. Stably transfected IdIA[SR-BI] and IdIA[C384S] cells were treated with the indicated concentrations of the water-soluble, membrane impermeable chemical cross-linker BS<sup>3</sup> for 60 min at 4 °C. Monomers (ca. 82 kDa) and oligomers (>150 kDa) were separated by SDS-PAGE and visualized by immunoblotting with an anti-C-terminus SR-BI polyclonal antibody (see *Experimental Procedures*). Analysis of the same filter with an antibody to ε-COP (Fig. S6) showed that this intracellular protein that is part of a multiprotein complex was not cross-linked by BS<sup>3</sup> to itself or other proteins. This result suggests that BS<sup>3</sup>-mediated cross-linking was restricted to exoplasmic domains of surface proteins.

disrupted receptor homooligomerization (Fig. 4). The ε-subunit of coatomer protein I (ε-COP) was used as a loading control and control for artifactual intracellular cross-linking (see Fig. S6). BS<sup>3</sup> treatment converted 49 ± 3% of wild-type and 50 ± 5% of C384S receptors into covalently cross-linked oligomers (averages from four experiments). Thus, the effects of C384S on lipid uptake cannot be attributed to loss of receptor oligomerization.

In summary, using a chemical biology approach we identified a highly potent, thiosemicarbazone inhibitor of SR-BI-mediated lipid uptake, BLT-1 (2). SAR analysis showed that the sulfur of BLT-1's thiosemicarbazone was essential for activity (34). These analyses led us to investigate the cysteines of SR-BI and identify one cysteine, Cys384, that plays a critical role in the mechanism by which BLT-1 inhibits SR-BI-mediated lipid uptake and that contributes to its intrinsic lipid uptake activity. It is noteworthy that human CD36, which does not have a cysteine equivalent to SR-BI's Cys384, binds to, and yet does not mediate efficient lipid uptake from, HDL. Our findings suggest that identification and characterization of other small-molecule modulators of SR-BI's activity might provide additional insight into this receptor's mechanism of action.

### Experimental Procedures

**Materials. Lipoproteins and inhibitors.** Human HDL was isolated and labeled with <sup>125</sup>I (<sup>125</sup>I-HDL), the fluorescent lipid DiI (Invitrogen; DiI-HDL) or [<sup>3</sup>H]cholesteryl ester ([<sup>3</sup>H]CE, [<sup>3</sup>H]CE-HDL) as described previously (8, 45–48) (see *SI Experimental Procedures*). The BLT-1 and BLT-4 were obtained as described (2, 34) and stored in 5 mM stock solutions in DMSO at –20 °C.

### Mass spectrometric analysis of the free thiols in recombinant SR-BI-t1.

We isolated essentially homogenous SR-BI-t1 using immunoaffinity chromatography as described previously (34, 36). SR-BI-t1 is a C-terminally epitope-tagged murine SR-BI with uniform, truncated N-linked oligosaccharide chains (34, 49). Purified SR-BI-t1 (10 μg) and bovine RNase A (10 μg, as a reaction control, from Sigma-Aldrich) were alkylated prior to reduction with undeuterated *N*-ethylmaleimide (NEM) to modify free thiols, and then alkylated after reduction with deuterated NEM d<sub>5</sub> to modify those cysteines that had been in disulfide bonds in the native structure (37, 38). The products were then deglycosylated (peptide:N-glycosidase F) and proteolytically digested (trypsin and Glu C), and the product peptides subjected to LC-MS/MS analysis on an Orbitrap mass spectrometer (Thermo) to determine relative amounts of cysteines in SR-BI that were or were not in the free thiol state (see *SI Experimental Procedures* for details). Results with NEM alkylation were confirmed using iodoacetamide (nonreductive) and 4-vinylpyridine (reductive) alkylation.

### Generation of mutant cDNAs.

Murine SR-BI cDNA incorporated into the pcDNA1 vector (Invitrogen) (13) was used as a template for site-directed mutagenesis (QuickChange II site directed mutagenesis kit, Stratagene). Cys-to-Ser mutations were engineered at positions 251 and 384 (C251S, C384S, and C251/384S) and introduced into a CMV promoter-driven expression vector for wild-type SR-BI (pmSR-BI ex68; ref. 25) (see *SI Experimental Procedures*).

**Cells and Cell Culture.** Unless otherwise noted, cells were maintained and assay incubations were performed at 37 °C in a humidified 5% CO<sub>2</sub>, 95% air incubator. Cellular protein levels were determined by the method of Lowry et al. (50).

**Stable transfectants.** LDL receptor-deficient Chinese hamster ovary cells (IdIA-7) (51), which express low levels of endogenous SR-BI (13), were stably transfected with the SR-BI mutant

cDNAs described above and transfectants with similar levels of surface receptor expression (determined by flow cytometry using anti-SR-BI antibody KKB-1; ref. 52) were isolated (see *SI Experimental Procedures*) and maintained in Ham's F12 medium containing 2 mM L-glutamine, 50 units/mL penicillin, and 50  $\mu$ g/mL streptomycin (medium A) supplemented with 5% (vol/vol) FBS and 0.25  $\mu$ g/mL G418 (medium B).

**Transient expression of cDNAs in COS cells.** COS cells were grown in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 50 units/mL penicillin, and 50  $\mu$ g/mL streptomycin (medium D) supplemented with 10% (vol/vol) FBS (medium E) and transfected with wild-type (25) and mutant SR-BI cDNAs (see *SI Experimental Procedures*).

**Assays. [ $^3$ H]CE uptake from [ $^3$ H]CE-HDL and [ $^{125}$ I]-HDL binding.** Specific [ $^{125}$ I]-HDL binding to transiently or stably transfected cells (4 or 37  $^{\circ}$ C) and cellular uptake of [ $^3$ H]CE from [ $^3$ H]CE-HDL (37  $^{\circ}$ C) were measured for 2 h in the absence (total activity) or presence (nonspecific activity) of a 40-fold excess of unlabeled HDL (8, 13). Specific binding or uptake is the difference between total and nonspecific activities. The amounts of cell-associated [ $^3$ H]CE are expressed as the equivalent amount of [ $^3$ H]CE-HDL protein (nanogram) to permit direct comparison of the relative amounts of [ $^{125}$ I]-HDL binding and [ $^3$ H]CE uptake (53). All calculated errors represent standard errors of the mean. In assays using BLT-1 [37  $^{\circ}$ C, medium A plus 0.5% (wt/vol) BSA (medium C) plus 0.5% (vol/vol) DMSO (medium F)], cells were preincubated with the indicated concentrations of BLT-1 for 1 h and then assayed in the presence of BLT-1 as described above. (See *SI Experimental Procedures* for details.)

**Lipid uptake from DiI-labeled HDL.** Cellular uptake of DiI from DiI-HDL (all incubations at 37  $^{\circ}$ C) was measured on day 2 after

plating stably transfected cells (50,000 cells per well) in 96-well plates (Costar, black with clear, flat bottom). Cells were preincubated in assay medium F with the indicated concentrations of BLT-1 for the indicated times prior to incubations with DiI-HDL (2 h). Cell-associated DiI (relative fluorescence units per well) was measured using a fluorescence plate reader and represents SR-BI specific uptake [differences between total uptake and nonspecific uptake (determined in presence of excess HDL)]. [See *SI Experimental Procedures* for details.]

**Flow cytometric analysis of SR-BI cell surface expression.** Cell surface levels of receptors were determined using anti-SR-BI antibody KKB-1 and flow cytometry (26) (see *SI Experimental Procedures*). Comparisons of relative surface expression of wild-type and mutant receptors assumed that the mutations did not interfere with KKB-1 binding. The major conclusions of this study are independent of potential differences in affinities of KKB-1 binding.

**Determination of receptor oligomerization by cross-linking and immunoblotting.** In four independent experiments, surface proteins of IdIA[SR-BI] and IdIA[C384S] cells were cross-linked with the indicated amounts of the water-soluble, membrane-impermeable cross-linker BS<sup>3</sup> (Thermo Scientific) and monomeric and oligomeric forms of SR-BI and C384S were detected by immunoblotting with anti-SR-BI antibody 495 (13, 54) (see *SI Experimental Procedures*). The same samples were analyzed by immunoblotting with a polyclonal rabbit anti- $\epsilon$ -COP antibody (55) as a loading and intracellular cross-linking control.

**ACKNOWLEDGMENTS.** We thank Drs. Leonid Gaidukov, Kosuke Tsukamoto, Stephen Lippard, Barbara Imperiali, Robert Sauer, Jon Clardy, and Jack Taunton for helpful discussions and Dr. Soriri Banakos for help with generating SR-BI-t1. This work was supported by the US National Institutes of Health (M.K.) and the Proteomics Platform of the Broad Institute (S.A.C.).

- Tochtrop GP, King RW (2004) Target identification strategies in chemical genetics. *Comb Chem High Throughput Screen* 7:677–688.
- Nieland TJF, Penman M, Dori L, Krieger M, Kirchhausen T (2002) Discovery of chemical inhibitors of the selective transfer of lipids mediated by the HDL receptor SR-BI. *Proc Natl Acad Sci USA* 99:15422–15427.
- Nieland TJF, et al. (2007) Influence of HDL-cholesterol-elevating drugs on the in vitro activity of the HDL receptor SR-BI. *J Lipid Res* 48:1832–1845.
- Altmann SW, et al. (2002) The identification of intestinal scavenger receptor class B, type I (SR-BI) by expression cloning and its role in cholesterol absorption. *Biochim Biophys Acta* 1580:77–93.
- Nishizawa T, et al. (2007) A novel compound, R-138329, increases plasma HDL cholesterol via inhibition of scavenger receptor BI-mediated selective lipid uptake. *Atherosclerosis* 194:300–308.
- Masson D, et al. (2009) Increased HDL cholesterol and ApoA-I in humans and mice treated with a novel SR-BI inhibitor. *Arterioscler Thromb Vasc Biol* 29:2054–U2170.
- Rigotti A, Miettinen HE, Krieger M (2003) The role of the high-density lipoprotein receptor SR-BI in the lipid metabolism of endocrine and other tissues. *Endocr Rev* 24:357–387.
- Nieland TJF, Xu S, Penman M, Krieger M (2011) Negatively cooperative binding of high density lipoprotein to the HDL receptor SR-BI. *Biochemistry* 50:1818–1830.
- Teslovich TM, et al. (2010) Biological, clinical and population relevance of 95 loci for blood lipids. *Nature* 466:707–713.
- Gordon T, Castelli W, Hjortland M, Kannel W, Dawber T (1977) High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am J Med* 62:707–714.
- Miller GJ, Miller NE (1975) Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. *Lancet* 305:16–19.
- Dreux M, et al. (2009) Receptor complementation and mutagenesis reveal SR-BI as an essential HCV entry factor and functionally imply its intra- and extra-cellular domains. *PLoS Pathog* 5:e1000310.
- Acton S, et al. (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* 271:518–520.
- Glass C, Pittman RC, Weinstein DB, Steinberg D (1983) Dissociation of tissue uptake of cholesterol ester from that of apoprotein A-I of rat plasma high density lipoprotein: selective delivery of cholesterol ester to liver, adrenal, and gonad. *Proc Natl Acad Sci USA* 80:5435–5439.
- Stein Y, Dabach Y, Hollander G, Halperin G, Stein O (1983) Metabolism of HDL-cholesterol ester in the rat, studied with a nonhydrolyzable analog, cholesteryl linoleyl ether. *Biochim Biophys Acta* 752:98–105.
- Goldstein JL, Brown MS (2009) The LDL receptor. *Arterioscler Thromb Vasc Biol* 29:431–438.
- Nieland TJF, Ehrlich M, Krieger M, Kirchhausen T (2005) Endocytosis is not required for the selective lipid uptake mediated by murine SR-BI. *Biochim Biophys Acta* 1734:44–51.
- Ji Y, et al. (1997) Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J Biol Chem* 272:20982–20985.
- Greenwalt DE, et al. (1992) Membrane glycoprotein CD36: A review of its roles in adherence, signal transduction, and transfusion medicine. *Blood* 80:1105–1115.
- Landschulz KT, Pathak RK, Rigotti A, Krieger M, Hobbs HH (1996) Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. *J Clin Invest* 98:984–995.
- Williams DL, et al. (2000) Binding and cross-linking studies show that scavenger receptor BI interacts with multiple sites in apolipoprotein A-I and identify the class A amphipathic alpha-helix as a recognition motif. *J Biol Chem* 275:18897–18904.
- Reaven E, Cortez Y, Leers-Sucheta S, Nomoto A, Azhar S (2004) Dimerization of the scavenger receptor class B type I: Formation, function, and localization in diverse cells and tissues. *J Lipid Res* 45:513–528.
- Sahoo D, Darlington YF, Pop D, Williams DL, Connelly MA (2007) Scavenger receptor class B type I (SR-BI) assembles into detergent-sensitive dimers and tetramers. *Biochim Biophys Acta* 1771:807–817.
- Gaidukov L, Nager AR, Xu S, Penman M, Krieger M (2011) Glycine dimerization motif in the N-terminal transmembrane domain of the HDL receptor SRBI required for normal receptor oligomerization and lipid transport. *J Biol Chem* 286:18452–18464.
- Gu X, Lawrence R, Krieger M (2000) Dissociation of the high density lipoprotein and low density lipoprotein binding activities of murine scavenger receptor class B type I (mSR-BI) using retrovirus library-based activity dissection. *J Biol Chem* 275:9120–9130.
- Gu X, Kozarsky K, Krieger M (2000) Scavenger receptor class B, type I-mediated [ $^3$ H] cholesterol efflux to high and low density lipoproteins is dependent on lipoprotein binding to the receptor. *J Biol Chem* 275:29993–30001.
- Vergeer M, et al. (2011) Genetic variant of the scavenger receptor BI in humans. *N Engl J Med* 364:136–145.
- Parathath S (2007) Effects of amino acid substitutions at glycine 420 on SR-BI cholesterol transport function. *J Lipid Res* 48:1386–1395.
- Dreux M, et al. (2009) Receptor complementation and mutagenesis reveal SR-BI as an essential HCV entry factor and functionally imply its intra- and extra-cellular domains. *PLoS Pathog* 5:e1000310.
- Ortegren U, et al. (2006) Separation and characterization of caveolae subclasses in the plasma membrane of primary adipocytes; segregation of specific proteins and functions. *FEBS J* 273:3381–3392.
- Reboul E, et al. (2005) Lutein transport by Caco-2 TC-7 cells occurs partly by a facilitated process involving the scavenger receptor class B type I (SR-BI). *Biochem J* 387:455–461.
- Reboul E, et al. (2006) Scavenger receptor class B type I (SR-BI) is involved in vitamin E transport across the enterocyte. *J Biol Chem* 281:4739–4745.

33. Sada Y, Shiratsuchi A, Nakanishi Y (2006) Involvement of mitogen-activated protein kinases in class B scavenger receptor type I-induced phagocytosis of apoptotic cells. *Exp Cell Res* 312:1820–1830.
34. Nieland TJJ, et al. (2008) Identification of the molecular target of small molecule inhibitors of HDL receptor SR-BI activity. *Biochemistry* 47:460–472.
35. Rasmussen JT, Berglund L, Rasmussen MS, Petersen TE (1998) Assignment of disulfide bridges in bovine CD36. *Eur J Biochem* 257:488–494.
36. Liu B, Krieger M (2002) Highly purified scavenger receptor class B type I reconstituted into phosphatidylcholine/cholesterol liposomes mediates high affinity high density lipoprotein binding and selective lipid uptake. *J Biol Chem* 277:34125–34135.
37. Held JM, et al. (2010) Targeted quantitation of site-specific cysteine oxidation in endogenous proteins using a differential alkylation and multiple reaction monitoring mass spectrometry approach. *Mol Cell Proteomics* 9:1400–1410.
38. Nair SS, et al. (2006) De novo sequencing and disulfide mapping of a bromotryptophan-containing conotoxin by Fourier transform ion cyclotron resonance mass spectrometry. *Anal Chem* 78:8082–8088.
39. Kumar GDK, et al. (2010) Functionalized benzophenone, thiophene, pyridine, and fluorene thiosemicarbazone derivatives as inhibitors of cathepsin L. *Bioorg Med Chem Lett* 20:6610–6615.
40. Katz BA, et al. (1998) Design of potent selective zinc-mediated serine protease inhibitors. *Nature* 391:608–612.
41. Rosenblum G, et al. (2007) Molecular structures and dynamics of the stepwise activation mechanism of a matrix metalloproteinase zymogen: Challenging the cysteine switch dogma. *J Am Chem Soc* 129:13566–13574.
42. Pedrido R, et al. (2008) Metal-catalysed oxidation processes in thiosemicarbazones: New complexes with the ligand N-{2-[(4-N-ethylthiosemicarbazone)methyl]-phenyl}-p-toluenesulfonamide. *Chem Eur J* 14:500–512.
43. Killian DM, Hermeling S, Chikhale PJ (2007) Targeting the cerebrovascular large neutral amino acid transporter (LAT1) isoform using a novel disulfide-based brain drug delivery system. *Drug Deliv* 14:25–31.
44. Savi P, et al. (2006) The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts. *Proc Natl Acad Sci USA* 103:11069–11074.
45. Chung BH, Wilkinson T, Geer JC, Segrest JP (1980) Preparative and quantitative isolation of plasma lipoproteins: Rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J Lipid Res* 21:284–291.
46. Patsch JR, Patsch W (1986) Zonal ultracentrifugation. *Methods Enzymol* 129:3–26.
47. Goldstein JL, Basu SK, Brown MS (1983) Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol* 98:241–260.
48. Gwynne JT, Mahaffee DD (1989) Rat adrenal uptake and metabolism of high density lipoprotein cholesteryl ester. *J Biol Chem* 264:8141–8150.
49. Reeves PJ, Kim JM, Khorana HG (2002) Structure and function in rhodopsin: A tetracycline-inducible system in stable mammalian cell lines for high-level expression of opsin mutants. *Proc Natl Acad Sci USA* 99:13413–13418.
50. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275.
51. Kingsley DM, Krieger M (1984) Receptor-mediated endocytosis of low density lipoprotein: Somatic cell mutants define multiple genes required for expression of surface-receptor activity. *Proc Natl Acad Sci USA* 81:5454–5458.
52. Gu X, et al. (1998) The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain. *J Biol Chem* 273:26338–26348.
53. Pittman RC, Knecht TP, Rosenbaum MS, Taylor CA (1987) A nonendocytotic mechanism for the selective uptake of high-density lipoprotein-associated cholesterol esters. *J Biol Chem* 262:2443–2450.
54. Rigotti A, et al. (1997) A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc Natl Acad Sci USA* 94:12610–12615.
55. Guo Q, Penman M, Trigatti BL, Krieger M (1996) A single point mutation in epsilon-COP results in temperature-sensitive, lethal defects in membrane transport in a Chinese hamster ovary cell mutant. *J Biol Chem* 271:11191–11196.