Influence of HDL-cholesterol-elevating drugs on the in vitro activity of the HDL receptor SR-BI

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Abstract Treatment of atherosclerotic disease often focuses on reducing plasma LDL-cholesterol or increasing plasma HDL-cholesterol. We examined in vitro the effects on HDL receptor [scavenger receptor class B type I (SR-BI)] activity of three classes of clinical and experimental plasma HDL-cholesterol-elevating compounds: niacin, fibrates, and HDL376. Fenofibrate (FF) and HDL376 were potent (IC_{50} $\sim$ 1 μM), direct inhibitors of SR-BI-mediated lipid transport in cells and in liposomes reconstituted with purified SR-BI. FF, a prodrug, was a more potent inhibitor of SR-BI than an activator of peroxisome proliferator-activated receptor α, a target of its active fenofibric acid (FFA) derivative. Nevertheless, FFA, four other fibrates (clofibrate, gemfibrozil, ciprofibrate, and bezafibrate), and niacin had little, if any, effect on SR-BI, suggesting that they do not directly target SR-BI in vivo. However, similarities of HDL376 treatment and SR-BI gene knockout on HDL metabolism in vivo (increased HDL-cholesterol and HDL particle sizes) and structure-activity relationship analysis suggest that SR-BI may be a target of HDL376 in vivo. HDL376 and other inhibitors may help elucidate SR-BI function in diverse mammalian models and determine the therapeutic potential of SR-BI-directed pharmaceuticals.—Nieland, T. J. F., J. T. Shaw, F. A. Jaipuri, Z. Maliga, J. L. Duffner, A. N. Koehler, and M. Krieger. **Influence of HDL-cholesterol-elevating drugs on the in vitro activity of the HDL receptor SR-BI. *J. Lipid Res.* 2007.** 48: **1832–1845.**

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Cardiovascular disease is the principal killer in Western industrialized societies. In the United States, ~76% of cardiovascular disease is attributed to coronary heart disease, stroke, and heart failure (1). Occlusive and unstable atherosclerosis is a major cause of cardiovascular disease. Epidemiologic and interventional studies have clearly established the critical role of lipoprotein metabolism in atherosclerotic disease, in which risk increases with plasma levels of LDL-cholesterol and is inversely proportional to HDL-cholesterol (2). Understanding receptor-mediated LDL metabolism (3) has led to profound insights into basic biological mechanisms and key clinical advances, such as the introduction of HMG-CoA reductase inhibitor (statin) therapy (4). The success of LDL-cholesterol-lowering therapy has stimulated increased interest in HDL-cholesterol-directed approaches. Several HDL-cholesterol-elevating drugs are currently available or under development (5, 6). Some of the most widely used HDL-cholesterol-elevating drugs in the clinic include statins (~5–10% increase), fibrates [peroxisome proliferator-activated receptor α (PPARα) agonists; ~5–20% increase], and nicotinic acid or niacin (uncertain mechanism; ~15–35% increase). Unfortunately, results in recent clinical trials of another HDL-cholesterol-elevating agent, the cholesteryl ester transfer protein (CETP) inhibitor torcetrapib, were disappointing (7–9), casting uncertainty on the future of CETP as a target for pharmacotherapy.

The identification of the scavenger receptor class B type I (SR-BI) as an HDL receptor (10, 11) has raised the possibility that agents modulating SR-BI activity or expression levels might represent attractive candidates for HDL-cholesterol-directed therapies (11–13). In addition to its HDL receptor activity, SR-BI can function as a receptor for other lipoproteins, including LDL (14) and chylomicrons

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Abbreviations: ACTH, adrenocorticotropic hormone; BLT-1, blocker of lipid transport-1; CE, cholesteryl oleyl ether; CETP, cholesteryl ester transfer protein; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FAF, fatty acid-free; FF, fenofibrate; FFA, fenofibric acid; PBS+, PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$; PPARα, peroxisome proliferator-activated receptor α; SEM, standard error of the mean; SR-BI, scavenger receptor class B type I.

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(15, 16). SR-BI binds HDL primarily via HDL's main protein component, apolipoprotein A-I (17). SR-BI-mediated delivery to cells of HDL-cholesterol occurs via selective lipid uptake, a two-step process involving HDL binding followed by a lipid transfer step (18–21) that differs from classic LDL receptor-mediated endocytosis via clathrin-coated pits (3). After HDL binds to SR-BI, HDL-cholesterol (primarily cholesteryl ester but also unesterified cholesterol) is transferred to the cells, and then the lipid-depleted HDL particles dissociate and reenter the extracellular space. SR-BI can also mediate unesterified cholesterol efflux from cells to HDL (22). We have previously identified a set of small molecules, called blockers of lipid transport (BLTs), that inhibit both cellular SR-BI-mediated selective lipid uptake from HDL and cholesterol efflux to HDL yet increase the affinity of HDL binding to SR-BI (12, 13). At a fixed HDL concentration below the dissociation constant (K_d) the increase in affinity is readily observed as an increase in net HDL binding. The molecular target(s) of the BLTs has not yet been reported. BLTs are currently being used to explore the detailed mechanism underlying SR-BI's multiple activities (12, 13, 23–40).

Numerous genetic studies in mice have established that SR-BI plays a key role in murine HDL metabolism (11). For example, adenovirus-mediated hepatic overexpression of SR-BI dramatically decreases plasma HDL-cholesterol (41). Complete loss of SR-BI expression by homozygous gene inactivation increases both plasma HDL-cholesterol level (~2-fold) and HDL particle size (42). A tissue-specific, ~95% reduction in hepatic SR-BI protein level attributable to targeted disruption of the gene encoding a cytoplasmic adaptor protein, PDZK1, which binds to the C terminus of SR-BI (43), increases both HDL-cholesterol level (~1.5- to 1.7-fold) and HDL particle size (44). The influence of SR-BI on murine atherosclerosis has been examined in both the LDL receptor and apoE knockout models. Hepatic overexpression of SR-BI suppresses and loss of SR-BI expression enhances atherosclerosis in these models (45–55). The atheroprotective activity of SR-BI, even though its expression decreases plasma HDL-cholesterol levels, suggests that SR-BI's role in reverse cholesterol transport (the movement of cholesterol from peripheral tissues to the liver and then excretion in the bile) may contribute to its protective activity in mice. The physiologic role of SR-BI in HDL metabolism is probably conserved among mammals; however, supporting data in species less genetically tractable than mice are very limited. Thus, it is not known whether the atheroprotective effects of SR-BI in mice, associated with reduced HDL-cholesterol levels, would be exhibited in humans, in which HDL metabolism differs and reduced HDL-cholesterol levels are associated with increased risk (56).

In this study, we explored in cultured cells and in an in vitro system of highly purified SR-BI reconstituted in liposomes the possibility that some of the in vivo plasma HDL-cholesterol-elevating activities of three classes of drugs might be attributable to their ability to inhibit SR-BI directly. These drugs included niacin (**Fig. 1A**) and members of the fibrate family (Fig. 1B–G) such as gemfibrozil, drugs currently in use in humans as indicated above. Our results suggest that SR-BI is not a direct target of these drugs in vivo. Although the prodrug fenofibrate (FF) does inhibit SR-BI in vitro, it is rapidly hydrolyzed to fenofibric acid (FFA) in vivo (reviewed in Ref. 57). Because we show here that FFA does not inhibit SR-BI in vitro, it is highly unlikely that the in vivo HDL-cholesterol-elevating activity of FF involves the inhibition of SR-BI.

We also studied an experimental compound called HDL376 [*N*-(5-chloro-2-methyphenyl)-*N*′-(1-methylethyl)-thiourea] (58, 59) (Fig. 1H). HDL376 was discovered in an in vivo screen designed to identify novel drugs that increase plasma HDL-cholesterol levels in rats (58, 59). It also increases plasma HDL-cholesterol levels in hamsters and dogs, two species not sensitive to gemfibrozil, and in rhesus and cynomolgus monkeys (58, 59). The HDL376-induced percentage increases in HDL-cholesterol varied from 15% (chow-fed hamsters at 64 mg/kg/day) to 100% (chow-fed cynomolgus monkeys at 12 mg/kg/day) (58). Furthermore, it increases HDL particle size in rhesus monkeys (59), and a similar compound (Fig. 1J, compound **2**) has the same effect in rats (58). Importantly, HDL376 has been reported to increase serum HDL-cholesterol levels in healthy humans [~13% after 7 days of treatment at 100 mg/day (59)] to about the same extent as seen with statins and the fibrates (see above). These data, together with the report that HDL376 does not appear to act as a PPARα or PPARδ agonist (58), indicate that the mechanism by which HDL376 increases plasma HDL-cholesterol, which has been undefined to date, differs from that of the fibrates.

The similarities of the effects on plasma HDL of either HDL376 treatment or dramatically decreasing hepatic SR-BI activity by genetic manipulation (SR-BI or PDZK1 knockout mice) led us to hypothesize that it might be an SR-BI inhibitor; thus, we included HDL376 in this study. We found that HDL376 does inhibit SR-BI-mediated lipid transport in cells and in liposomes containing purified SR-BI and that HDL376 behaves as a BLT-like drug. This raises the possibility that some of its in vivo HDL-cholesterol-elevating activity may be attributable to inhibition of SR-BI. Our results underscore the potential usefulness of HDL376 for the in vivo study of SR-BI function in animals that are not easily amenable to genetic studies, such as targeted gene inactivation, and raise the possibility that modulators of SR-BI activity in vivo may lead to the development of novel HDL-elevating therapeutics.

MATERIALS AND METHODS

Compounds

FF, clofibrate, ciprofibrate, gemfibrozil, bezafibrate, and niacin were obtained from Sigma. FFA was obtained from Biofine International (Vancouver, Canada). HDL376 and HDL376U were synthesized as described below. Stock solutions of all compounds were dissolved in DMSO at concentrations ranging from 25 to 500 mM.

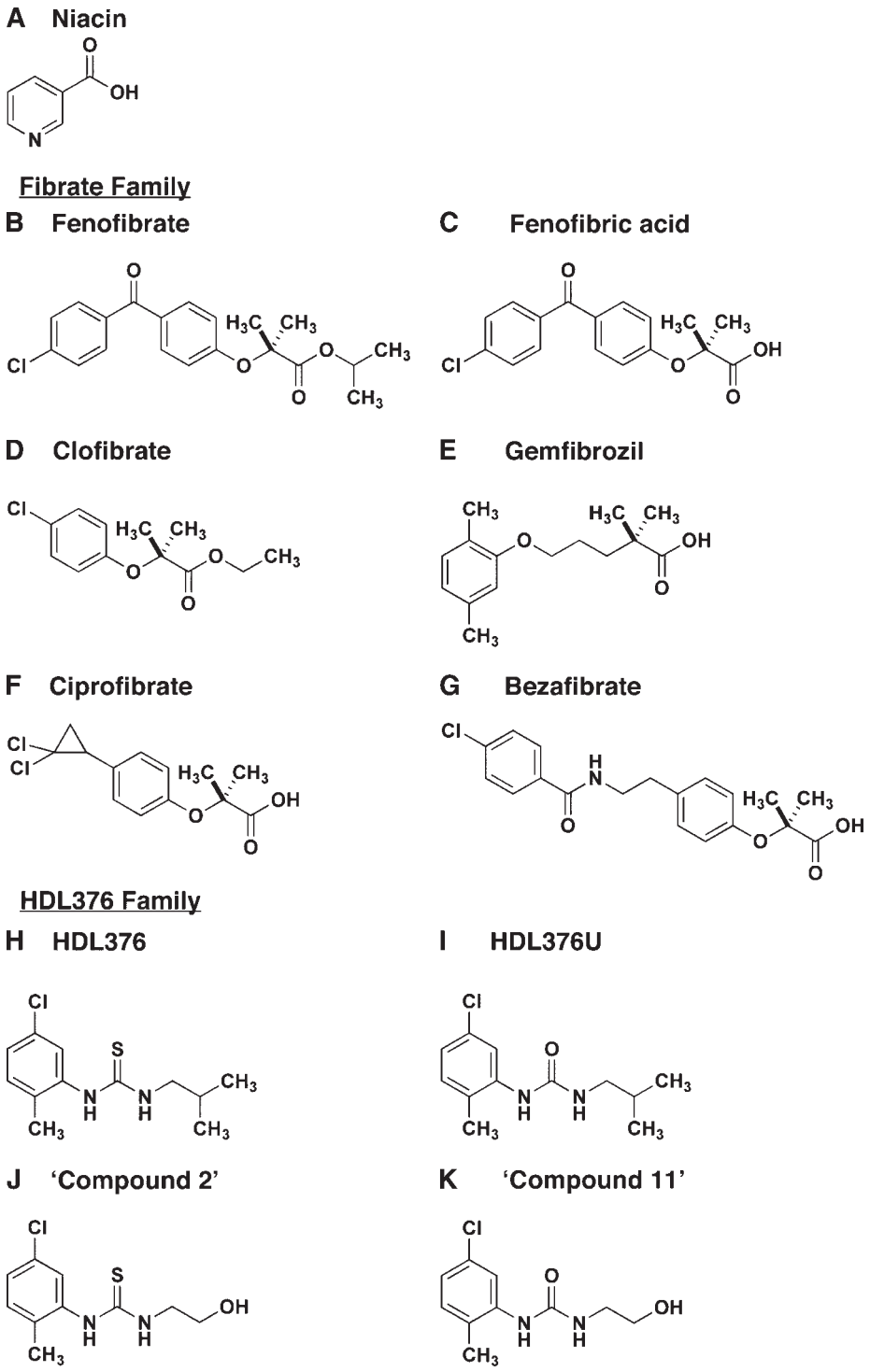

Fig. 1. Structures of drugs that increase plasma HDL-cholesterol levels and related compounds. A: Niacin. B–G: Fibrates. H–K: HDL376, a thiourea (58, 59, 75, 79); HDL376U, urea derivative of HDL376; and two related compounds, compound **2** (58, 59, 75, 79) [also called SDZ 45-904 (58, 59)] and compound **11** (75). The esters fenofibrate (FF) (B) and clofibrate (D) are considered to be prodrugs that are hydrolyzed in the body to form carboxylic acid, active, peroxisome proliferator-activated receptor α agonists. As is the case for HDL376, compound **2** (J) increases plasma HDL-cholesterol levels and HDL particle sizes in rat, whereas its urea derivative compound **11** (K) does not exhibit this activity (75).

Synthesis and chemical characterization of HDL376 and HDL376U

HDL376: N-(5-chloro-2-methyphenyl)-N′-(1-methylethyl)-thiourea. Isobutylamine (0.110 ml, 1.09 mmol) was added to a solution of 5-chloro-2-methylphenyl isothiocyanate (0.200 g, 1.09 mmol) in dichloromethane (6 ml) and stirred for 1 h, all at room temperature. The mixture was filtered to yield the product as a white solid (0.263 g, 94%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (br s, 1H), 7.24–7.28 (m, 3H), 5.70 (br s, 1H), 3.41–3.45 (m, 2H), 2.27 (s, 3H), 1.84–1.93 (m, 1H), 0.88 (d, J = 6.6, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.1, 135.5, 134.2, 132.7, 132.5, 128.5, 127.4, 52.8, 28.1, 20.1, 17.3; IR (thin film) 3,320, 2,975, 2,870, 1,632, 1,566, 1,479, 1,407, 1,238, 1,082, 814 cm^{-1}; reverse-phase LC-MS *m/z* for $C_{12}H_{17}ClN_2S$ $(M + H)^+$ 258.9, retention time = 1.76 min. LC-MS (Waters 2795/Micromass LCT) data for HDL376 and HDL376U were collected using a 5:95 to 95:5 acetonitrile-water (0.01% formic acid) solvent gradient on an XTerra column using electrospray ionization.

HDL376U: N-(5-chloro-2-methyphenyl)-N′-(1-methylethyl)-urea. Isobutylamine (0.600 ml, 5.95 mmol) was added to a solution of 5-chloro-2-methylphenyl isocyanate (1.00 g, 5.95 mmol) in *n*-hexane (50 ml) and stirred for 1 h, all at room temperature. The mixture was filtered to yield the product as a white solid (1.33 g, 93%): ^{1}H NMR (300 MHz, acetone-d_6) δ 8.26 (d, J = 2.1, 1H), 7.33 (br s, 1H), 7.11 (d, J = 8.1, 1H), 6.87 (dd, J = 2.4, 8.1, 1H), 6.27 (br s, 1H), 3.06 (dd, J = 6, 6.6, 2H), 2.19 (s, 3H), 1.71–1.84 (m, 1H), 0.92 (d, J = 6.6, 6H); ^{13}C NMR (75 MHz, acetone-d_6) δ 155.8, 140.7, 132.1, 131.9, 125.3, 122.0, 120.2, 47.9, 29.7, 20.3, 17.5; IR (thin film) 3,222, 2,956, 2,869, 1,530, 1,483, 1,407, 1,363, 1,270, 1,206, 1,149, 1,124, 1,084, 1,036, 995, 912, 809, 733 cm^{-1}; reverse-phase LC-MS *m/z* for $C_{12}H_{17}ClN_2O$ $(M + H)^+$ 242.9, retention time = 1.76 min.

Lipoproteins and cells

Human HDL (density of ~1.09–1.16 g/ml) was isolated and labeled either with ^{125}I to label its protein constituents (^{125}I-HDL) or with [^{3}H]cholesteryl oleyl ether (CE; [^{3}H]CE-HDL) as described previously (10, 14, 18, 60). Most experiments using intact cells were conducted using two stable cell lines, ldlA-7 and ldlA[mSR-BI] cells. The ldlA-7 cells are LDL receptor-deficient Chinese hamster ovary cells that express very low levels of endogenous SR-BI (61), and ldlA[mSR-BI] cells are ldlA-7 cells stably transfected with an expression vector in which murine SR-BI (mSR-BI) cDNA is under the control of a cytomegalovirus promoter, which results in the expression of high levels of mSR-BI (10). These cells were maintained in tissue culture medium (Ham's F12 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and, for ldlA[mSR-BI] cells only, 0.25 mg/ml G418) (10).

In a few control experiments, we examined the effects of FF and HDL376 on the HDL lipid uptake activity using Y1-BS1 cells, in which endogenous, rather than recombinant, SR-BI is expressed. Y1-BS1 cells are cultured murine adenocortical cells that express high levels of endogenous SR-BI on stimulation with adrenocorticotropic hormone (ACTH) (62, 63). All experiments with cultured cells were conducted at 37°C.

Cellular lipid transport and ^{125}I-HDL binding assays

Assays for the uptake of [^{3}H]CE from [^{3}H]CE-HDL, efflux of [^{3}H]cholesterol from labeled cells, and ^{125}I-HDL binding were performed using ldlA[mSR-BI] cells and control untransfected ldlA-7 cells as described previously (10, 13, 14, 18, 60). For uptake and binding assays, cells were seeded on 24-well plates on day 0 in tissue culture medium at a density of 75,000 cells per well. Assays were performed on day 2. For efflux assays, cells were seeded on day 0 on 24-well plates at a density of 50,000 cells per well in the same medium. On day 1, the medium was replaced with Ham's F12 medium supplemented with 10% bovine lipoprotein-deficient serum, 1 μCi/ml [1,2-^{3}H]cholesterol (40–60 Ci/mmol; NEN Life Science), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and, for ldlA[mSR-BI] cells only, 0.25 mg/ml G418. On day 3, the cells were washed twice in Ham's F12 medium without any supplements and then cultured for another 24 h in tissue culture medium in which fetal bovine serum was replaced with 1% fatty acid-free (FAF) BSA (Sigma catalog No. A6003). The efflux assay was performed on day 4. On the day of the uptake, binding, or efflux assay, 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, and 25 mM HEPES, pH 7.4) containing 0.5% (w/v) of either albumin (hereafter referred to as BSA; Sigma catalog No. A3059) derived from whole bovine serum (medium A; standard conditions) or γ-globulin (Sigma catalog No. G9887) from sheep (medium B; data not shown) for selective uptake and binding experiments or 0.5% FAF BSA for efflux assays (medium C). Subsequently, the cells were incubated for an additional 2 h with the same concentrations of small molecules and with the indicated concentrations of ^{125}I-HDL (binding), [^{3}H]CE-HDL (uptake), or unlabeled HDL (efflux).

For all experiments, the values presented were normalized so that 100% of control represents receptor-specific activity in ldlA[mSR-BI] cells in the presence of 0.5% DMSO without compounds. For selective uptake, binding, and efflux assays, 0% activity was defined as the activity determined in ldlA-7 cells. The amount of cell-associated [^{3}H]CE is expressed as the equivalent amount of [^{3}H]CE-HDL protein (ng) to permit direct comparison of the relative amounts of ^{125}I-HDL binding and [^{3}H]CE uptake (64). Data analysis was performed using GraphPad Prism4 software from GraphPad Software, Inc. (San Diego, CA). IC_{50} values are those concentrations of the compounds that caused a half-maximal inhibition of SR-BI-mediated lipid transport. EC_{50} values are those concentrations of the compounds that caused a half-maximal increase in ^{125}I-HDL binding. All selective uptake and binding data presented are representative of results from three or more independent experiments, and the values presented are means of duplicate determinations. For cholesterol efflux assays, the average results from two or three independent experiments are shown.

To determine whether the HDL or the cells were the target of FF or HDL376, in some experiments we preincubated either the cells or ^{125}I-HDL with the compounds and then the compounds were subsequently removed by washing or dilution before performing the binding assays in the absence of the compounds. In this modified protocol, we tested whether cells are the targets by preincubating ldlA[mSR-BI] or ldlA7 cells at 37°C for 1 h with the indicated concentrations of compound in medium D (medium B containing 0.5% DMSO). In the case of HDL376 and BLT-1, the cells where then quickly rinsed (three times). In the case of FF, cells were washed three times every 5 min, for a total of nine washes. Cells were then incubated for 1 h in assay medium D (without compound) and 10 μg protein/ml ^{125}I-HDL. To test whether HDL is the target, ^{125}I-HDL was preincubated at 37°C at a concentration of 3.7 mg protein/ml with the indicated concentrations of compounds in HDL buffer (150 mmol/l NaCl, 0.24 mmol/l EDTA, pH 7.4, 0.5% DMSO, and 0.5% globulin), after which the mixture was diluted in assay medium D to a final concentration of 10 μg protein/ml ^{125}I-HDL and added to cells not exposed previously to compound for an additional 1 h. At the

end of the 1 h incubation, cellular ^{125}I-HDL binding was measured as described above.

In some experiments, uptake of the fluorescent lipid 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) from DiI-HDL was measured to assess the ability of FF and HDL376 to inhibit endogenous cellular SR-BI activity in Y1-BS1 cells. In these experiments on day 0, murine adrenal cortical Y1-BS1 cells were seeded at 100,000 cells per well in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. On day 1, ldlA[mSR-BI] and ldlA-7 cells were seeded at 50,000 cells per well as described above. On day 2, Y1-BS1 cells were treated with 1 μM ACTH (Sigma-Aldrich) to induce the expression of endogenous SR-BI. On day 3, all cells were washed twice with RPMI and 25 mM HEPES, pH 7.4, and preincubated for 2 h at 37°C in assay medium E (RPMI, 25 mM HEPES, pH 7.4, 0.5% sheep γ-globulin, and 0.5% DMSO) with no additions, 25 μM HDL376, 25 μM FF, or 1 μM BLT-1 in the presence or absence 400 μg protein/ml unlabeled HDL to determine nonspecific background (unsaturable) DiI uptake. DiI-HDL was then added for 2 h at 37°C at a final concentration of 10 μg/ml in the presence of the same amounts of compound. Unincorporated DiI-HDL was removed by washing the cells twice with ice-cold assay medium E without DMSO and twice with ice-cold PBS. Cell-associated DiI fluorescence was measured using a Spectramax GeminiXS plate reader from Molecular Devices (excitation, 514 nm; emission, 575 nm). The ldlA[mSR-BI] and ldlA-7 cells and inclusion of BLT-1 (13) were used as controls, and the results were as expected: DiI uptake by ldlA[mSR-BI] cells was high and that by ldlA-7 cells was low; BLT-1 inhibited the uptake of DiI by all of the cells (data not shown).

Lipid transport and ^{125}I-HDL binding assays in liposomes

For the purification of C-terminally epitope-tagged murine SR-BI (mSR-BI-t1) with uniform, truncated *N*-linked oligosaccharide chains, we previously described the overexpression of mSR-BI-t1 in HEK293S cells, its single-step immunoaffinity purification to virtual homogeneity, the reconstitution of the detergent-solubilized receptor into liposomes, and the in vitro demonstration of SR-BI-mediated ^{125}I-HDL binding and selective uptake of [^{3}H]CE from [^{3}H]CE-HDL in the reconstituted liposomes (65). We used a similar approach in this study to examine the potential direct effect 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, unpublished data). The mSR-BI-t1 was expressed in an *N*-acetylglucosaminyltransferase I (GnTI)-defective HEK293S derivative, HEK293S GnTI(−), which generates a glycoprotein with uniform, truncated *N*-linked oligosaccharide chains under the control of a tetracycline-inducible promoter (66, 67).

The mSR-BI-t1 with truncated *N*-linked chains was purified and reconstituted into liposomes as described previously (65). Briefly, 20 μg of SR-BI (or an equivalent volume of protein-free buffer to generate control liposomes that are devoid of SR-BI) was reconstituted into liposomes by acetone precipitation. SR-BI liposomes were washed once by resuspension of the acetone precipitate in protein-free assay medium followed by a centrifugation step for 25 min and 48,000 *g* at 4°C. The pellet was first reconstituted in assay medium without protein, and then an equal volume of assay medium with 1% FAF BSA was added to yield liposomes at a nominal final concentration of ~18 ng SR-BI/ml. In each reaction, 30 μl were preincubated together with 30 μl of assay medium containing 0.5% FAF BSA, 1% DMSO, and the indicated compounds 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) were added to a final concentration of 10 μg 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 (65). SR-BI-specific activity was determined by subtracting the uptake and binding values in control liposomes from those in SR-BI-t1-containing liposomes, and selective uptake values were determined as described above for cellular assays. The 100% of control value represents receptor-specific activity in SR-BI-t1-containing liposomes in the presence of 0.5% DMSO without compounds, and the 0% of control value represents background selective uptake in control liposomes devoid of SR-BI-t1. For HDL376, HDL376U, FF, and FFA, representative results from two or three independent experiments are shown. For all other compounds, average results of three independent experiments are shown.

Fluorescence microscopic analysis of intracellular trafficking of transferrin

The effect on intracellular traffic of the two most potent compounds found in this study, HDL376 and FF, was determined at compound concentrations of 50 μM (~50 times their IC_{50} values) as described previously (13). Cells were seeded on 24-well plates on day 0 in tissue culture medium at a density of 75,000 cells/well. On day 2, ldlA[mSR-BI] cells were incubated for a total of 3 h with the drug in assay medium containing 0.5% (w/v) BSA, similar to experiments examining selective uptake, binding, and efflux. During the last 30 min or 5 min, Alexa-488-labeled transferrin (Molecular Probes; 50 μg/ml final concentration) was added to the medium. Cells were chilled on ice for 10 min, washed three times with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS+), and then fixed for 1 h on ice with 4% (w/v) paraformaldehyde dissolved in PBS+. The paraformaldehyde was quenched by the addition of 50 mM NH_4Cl for 5 min at room temperature. Cells were imaged by epifluorescence microscopy on a Nikon Eclipse TE200 inverted microscope using a 60×, 1.4 numerical aperture oil-immersion objective.

Flow cytometric analysis of SR-BI cell surface expression

Cells were seeded on 24-well plates on day 0 in tissue culture medium at a density of 75,000 cells per well. On day 2, cells were incubated for 3 h in assay medium containing 0.5% (w/v) BSA with or without compounds at the indicated concentrations. Cells were then placed on ice and washed twice with PBS+, and the levels of SR-BI surface expression in unfixed cells were determined by flow cytometry (13) using the SR-BI-specific polyclonal antibody KKB-1 [a kind gift of Karen Kozarsky (18)] and an Alexa-488-labeled secondary goat anti-rabbit fluorescent antibody (Molecular Probes). The 0% of control values represent the background binding of KKB-1 to ldlA-7 cells, which typically was <5% of the levels of SR-BI in ldlA[mSR-BI] cells. In some cases (data not shown), the flow cytometric results were confirmed using compounds at concentrations up to 500 μM and a fluorescence plate reader assay of antibody binding to determine surface expression. Data presented are representative of results from two independent experiments, and the values shown are means of duplicate determinations.

Surface plasmon resonance analysis of compound binding to BSA, FAF BSA, and γ-globulin

The affinity of the binding of compounds to bovine BSA, bovine FAF BSA, and sheep γ-globulin was assessed by surface plasmon resonance analysis on a BiaCore S51 instrument as described previously for binding studies using human serum albu-

min (68–70). BSA, FAF BSA, and γ-globulin were 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-dimethylaminopropyl]carbodiimide hydrochloride/*N*-hydroxysuccinimide coupling chemistry (71). A total of 5,000–9,000 response units (corresponding to ~1 pg protein/mm^2 sensor area) of BSA and 5,000–9,000 response units of γ-globulin were immobilized on each sensor chip.

The binding assay was conducted twice in buffer TBS-P, pH 7.4 [25 mM Tris-HCl, 137 mM NaCl, 3 mM KCl, and 0.005% (v/v) P20 surfactant (BiaCore; catalog No. BR-100-54)] containing either 2% (v/v) DMSO or 5% (v/v) DMSO. The only differences observed with 2% (v/v) versus 5% (v/v) DMSO were seen with HDL376 (no substantial binding at 2% to any of the proteins, low binding with a K_d of 150 μM at 5%, perhaps attributable to increased HDL376 solubility). 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 at 200 μM (to cover the 50–200 μM range). 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 (both from Sigma) were used as positive controls for serum albumin binding and tested in the range of 381 nM to 200 μM. Data were analyzed using Scrubber software (available from the 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 response units for each compound. Equilibrium binding was evaluated and affinity was determined using GraphPad Prism with a nonlinear regression one-site binding model.

Statistical analysis

The statistical significance of differences was determined by a two-tailed unpaired Student's *t*-test. Differences were considered significant at $P < 0.05$. Average values are presented ± standard error of the mean (SEM). Error bars in the figures represent the SEM.

RESULTS

To determine the ability of HDL-cholesterol-elevating drugs and their derivatives (structures in Fig. 1) to acutely influence SR-BI activity in cultured cells, we used a transfected cell line that stably expresses high levels of mSR-BI under the control of a cytomegalovirus promoter, ldlA[mSR-BI] (10), and the control, untransfected parental cell line, ldlA-7, which expresses very little SR-BI (61). We pretreated cells with varying concentrations of the compounds. We then used standard assays conducted in the presence of the same concentrations of compounds to measure SR-BI-mediated *i*) binding of ^{125}I-HDL (10), *ii*) selective uptake of [^{3}H]CE from [^{3}H]CE-HDL (10), and *iii*) efflux of cellular [^{3}H]cholesterol to unlabeled HDL (18, 22). We also used anti-mSR-BI antibody staining and flow cytometry to determine whether the drug treatments altered the surface expression of SR-BI (13). In some experiments, we also included as a positive control the SR-BI lipid transport inhibitor BLT-1 (13) to ensure that any negative findings did not represent unanticipated aberrant behavior of the cells (data not shown).

The effects of niacin on SR-BI activity

Figure 2 shows that niacin at concentrations up to 500 μM had no effect on binding (Fig. 2A, open circles), selective uptake (Fig. 2A, closed circles), efflux (Fig. 2B), or the level of cell surface-expressed SR-BI (Fig. 2C). Thus, it appears unlikely that niacin's ability to increase HDL-cholesterol in vivo is the result of its direct influence on SR-BI.

The effects of FF on SR-BI activity

Figure 3 shows the effects on SR-BI of six members of the fibrate family of PPARα agonists. Strikingly, FF (Fig. 1B), the isopropyl ester prodrug of FFA (Fig. 1C), altered SR-BI's activities in a manner similar to that described previously for BLTs (12, 13). FF inhibited SR-BI-mediated cellular selective uptake of [^{3}H]CE from [^{3}H]CE-HDL with an IC_{50} of ~1.2 ± 1.5 μM while inducing increased ^{125}I-HDL binding (EC_{50} ~ 4.1 μM at a ^{125}I-HDL concentration of 10 μg protein/ml) (Fig. 3A, top). FF also inhibited SR-BI-mediated efflux of [^{3}H]cholesterol to HDL (Fig. 3B, FF). Notably, the IC_{50} for SR-BI inhibition of selective uptake is as much as 100-fold lower than the reported EC_{50} values for the activation of PPARα-dependent transcription in cells (72, 73), suggesting that FF's effect on SR-BI is uncoupled from PPARα-mediated transcriptional regulation.

We also tested FF inhibition of HDL lipid uptake in a distinct cell system in which endogenous, rather than recombinant, SR-BI is expressed. We examined the effects of fenfibrate on uptake of the fluorescent lipid DiI from DiI-HDL in ACTH-treated Y1-BS1 cells, cultured murine adenocortical cells that express high levels of endogenous SR-BI on stimulation with ACTH (62, 63). We found that FF (25 μM) inhibited receptor-dependent DiI uptake (data not shown); thus, its inhibitory activity is not restricted to SR-BI encoded by a transgene in Chinese hamster ovary-derived cells.

To rule out the remote possibility that FF inhibited SR-BI-mediated lipid transport by altering the surface expression of SR-BI, for example by altering rates of SR-BI synthesis or degradation or its intracellular localization, we performed flow cytometry experiments using antibodies recognizing an extracellular epitope in SR-BI. Indeed, FF did not reduce SR-BI-mediated lipid transport by altering the level of cell surface-expressed SR-BI protein (Fig. 3C). Furthermore, FF did not block the endocytosis of Alexa-labeled transferrin, as determined by epifluorescence microscopy (data not shown; see Materials and Methods) (13). Thus, the inhibitory effects of FF on SR-BI-mediated lipid transport were specific, in that they were not attributable to a global inhibition of cell surface receptor activity and intracellular membrane transport. These data raised the possibility that FF may inhibit SR-BI-mediated lipid transport directly, rather than indirectly (e.g., by altering other features of cellular cholesterol metabolism). Indeed, studies of highly purified, epitope-tagged SR-BI (mSR-BI-t1) incorporated into phospholipid/cholesterol liposomes (65) showed that this is the case (Fig. 2D). The IC_{50} of FF for

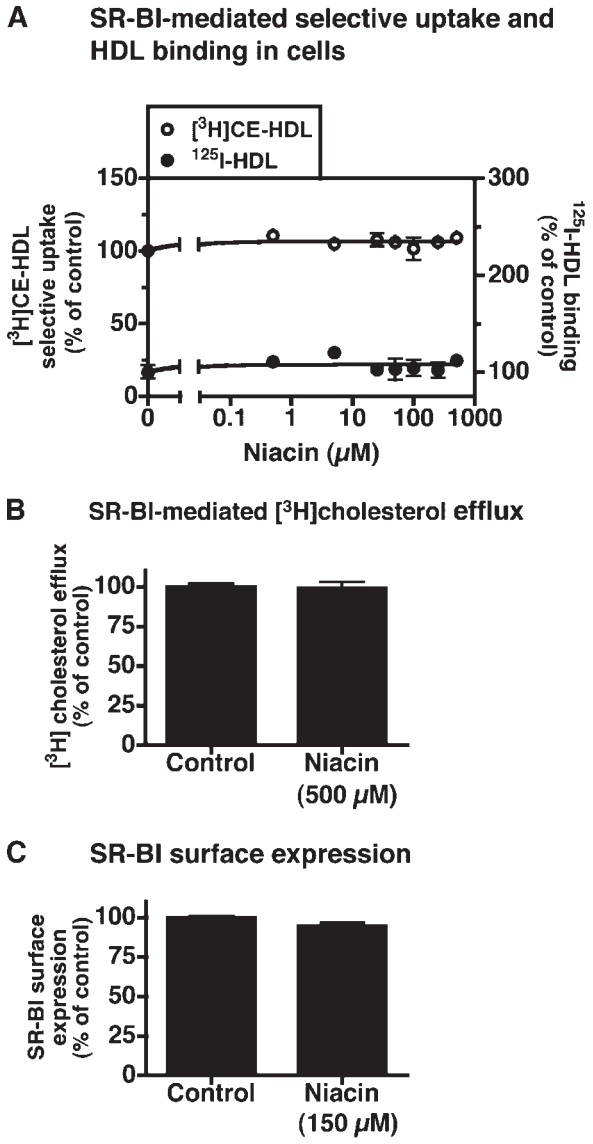

Fig. 2. Effects of niacin on scavenger receptor class B type I (SR-BI) activity in cells. Cells (ldlA[mSR-BI] and ldlA-7) were preincubated for 1 h at 37°C in medium containing the indicated concentrations of niacin. Subsequently, standard assays of SR-BI-mediated selective uptake of [^{3}H]cholesteryl oleyl ether (CE) from [^{3}H]CE-HDL (A), binding of ^{125}I-HDL (A), efflux of cellular unesterified [^{3}H]cholesterol to unlabeled HDL (B; average of two experiments), and the relative level of cell surface SR-BI determined by flow cytometry (C) were performed in the presence of the same concentration of niacin. The values were normalized so that 100% of control represents receptor-specific activity in ldlA[mSR-BI] cells in the presence of 0.5% DMSO without compounds. For selective uptake, binding, and efflux assays, 0% activity was defined as the activity determined in ldlA-7 cells, and for flow cytometry, the 0% of control values represent the background binding of KKB-1 to ldlA-7 cells. Values determined in the absence of niacin of [^{3}H]CE-HDL uptake, ^{125}I-HDL binding, and [^{3}H]cholesterol efflux were as follows: for ldlA[mSR-BI] cells, 6,766 ng protein (equivalent)/mg cell protein, 167 ng protein/mg, and 32% of the initial cellular [^{3}H]cholesterol level; for ldlA-7 cells: 968 ng/mg, 39 ng/mg, and 10%. Selective uptake was calculated as the difference between [^{3}H]CE-HDL uptake and ^{125}I-HDL binding.

SR-BI-mediated selective uptake ($IC_{50} \sim 0.96 \pm 0.17$ μM) in receptor-containing liposomes free of any other detectable proteins (data not shown) was virtually identical to that in SR-BI-expressing cells. Thus, SR-BI incorporated into phospholipid/cholesterol liposomes is a molecular target for FF. In vivo, FF, which is considered to be a prodrug, is rapidly hydrolyzed to the unesterified form, FFA, a PPARα agonist (reviewed in Ref. 57). Unlike FF, FFA exhibited virtually no SR-BI-inhibitory activity (Figs. 1C, 3A, B, D). These results suggest that the charged carboxylate group on FFA might interfere with BLT-like activity.

To determine whether the target of FF was SR-BI-containing membranes or the HDL particles themselves, we performed preincubation-washout/dilution experiments. FF was preincubated with either the cells or the ^{125}I-HDL and removed, and then ^{125}I-HDL binding to the cells in medium virtually free of FF was measured. To test whether cells are the target, we preincubated ldlA[mSR-BI] and ldlA-7 cells for 1 h with FF (50 μM) and then washed the cells to remove all FF from the medium. Subsequently, ^{125}I-HDL binding was determined in medium in the absence of FF using ^{125}I-HDL not previously exposed to this compound. For comparison, we also performed the binding assay using culture medium that contained FF during both the preincubation with cells and the subsequent incubation with ^{125}I-HDL (standard assay conditions) or that did not contain FF at any stage (control). The cell preincubation-washout procedure resulted in ^{125}I-HDL binding (**Fig. 4A**, white bar) that was increased compared with that for cells that did not receive compound during the experiment (hatched bar control). It appears that at least some of the FF was incorporated into the cells during the preincubation, and this was sufficient to influence SR-BI activity, suggesting that the SR-BI-expressing cells were a target of FF. The increase in ^{125}I-HDL binding seen under cell preincubation-only conditions was not as great as in the standard assay conditions, in which FF was present in both incubations (Fig. 4A, black bar), suggesting that some of the FF that associated with the cells during the preincubation probably dissociated during the washing or afterward. Using a complementary protocol, we preincubated ^{125}I-HDL, rather than the cells, for 1 h with the same concentration of FF (50 μM), followed by "removal" of the FF by a 370-fold dilution. This diluted and preincubated ^{125}I-HDL (final concentration of 10 μg protein/ml) was then used in a standard binding assay in the absence of additional FF. Figure 4A (gray bar) shows that there was no significant effect of this preincubation on ^{125}I-HDL binding to the cells compared with the control (hatched bar; $P = 0.43$). Thus, it is unlikely that FF functions by interacting directly with HDL; rather, it apparently interacts directly with the SR-BI-expressing cells. As reported previously (13), the results were similar for BLT-1 (Fig. 4C).

The effects of other fibrates on SR-BI activity

To explore whether SR-BI inhibitory activity is a common feature of the diverse family of fibrates, we examined the activities of four other fibrates on SR-BI in cells and

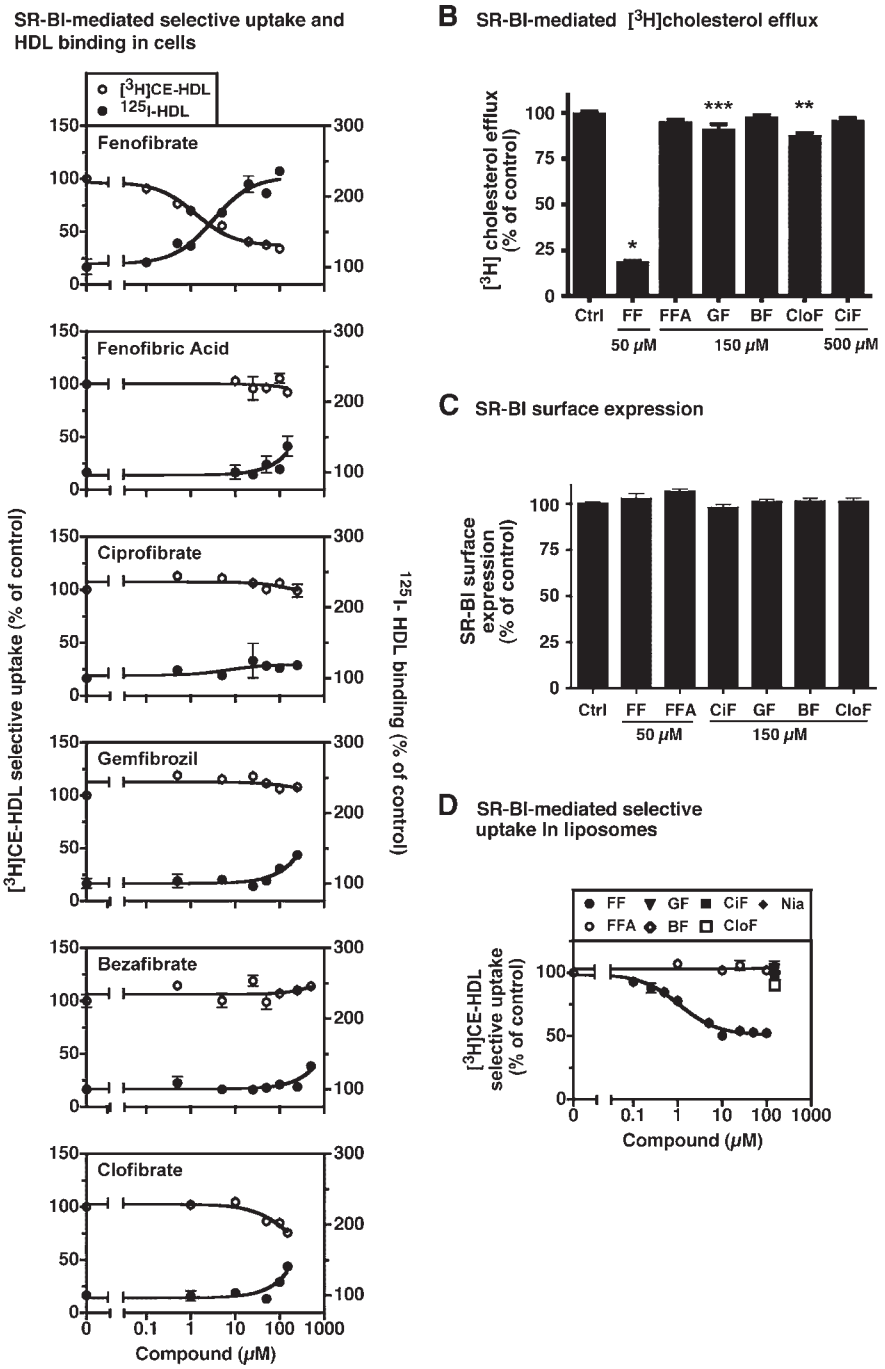

Fig. 3. Effects of fibrates on SR-BI activity in cells and liposomes. SR-BI-mediated selective uptake of [^{3}H]CE from [^{3}H]CE-HDL (A), binding of ^{125}I-HDL (A), efflux of cellular unesterified [^{3}H]cholesterol to unlabeled HDL [B; average of two experiments (* $P < 0.0001$, ** $P < 0.0005$, *** $P < 0.05$)], and the relative level of cell surface SR-BI (C) were determined as described for Fig. 2 in the presence of the indicated concentrations of the following fibrates: FF, fenofibric acid (FFA), ciprofibrate (CiF), gemfibrozil (GF), bezafibrate (BF), and clofibrate (CloF). D represents selective uptake of [^{3}H]CE from [^{3}H]CE-HDL mediated by purified SR-BI-t1 reconstituted in liposomes. At the concentrations tested, clofibrate had very little, but statistically significant ($P < 0.005$), influence on SR-BI-dependent selective uptake by liposomes, whereas FFA, ciprofibrate, gemfibrozil, and bezafibrate had no significant effects. For liposome assays, the 100% of control value represents receptor-specific activity in SR-BI-t1-containing liposomes in the presence of 0.5% DMSO without compounds, and 0% of control value represents background selective uptake in control liposomes devoid of SR-BI-t1. Values for intact cells (A–C) determined in the absence of drugs of [^{3}H]CE-HDL uptake, ^{125}I-HDL binding, and [^{3}H]cholesterol efflux were as follows: for ldlA[mSR-BI] cells: 4,826 ng protein (equivalent)/mg cell protein, 155 ng protein/mg, and 40% of the initial cellular [^{3}H]cholesterol level; for ldlA-7 cells: 909 ng/mg, 58 ng/mg, and 9.9%. For liposomes, the values of selective uptake and binding were as follows: for mSR-BI-t1-containing liposomes, 235 ng (equivalent)/sample and 65 ng protein/sample; for control, protein-free liposomes, 19 ng (equivalent)/sample and 6 ng protein/sample.

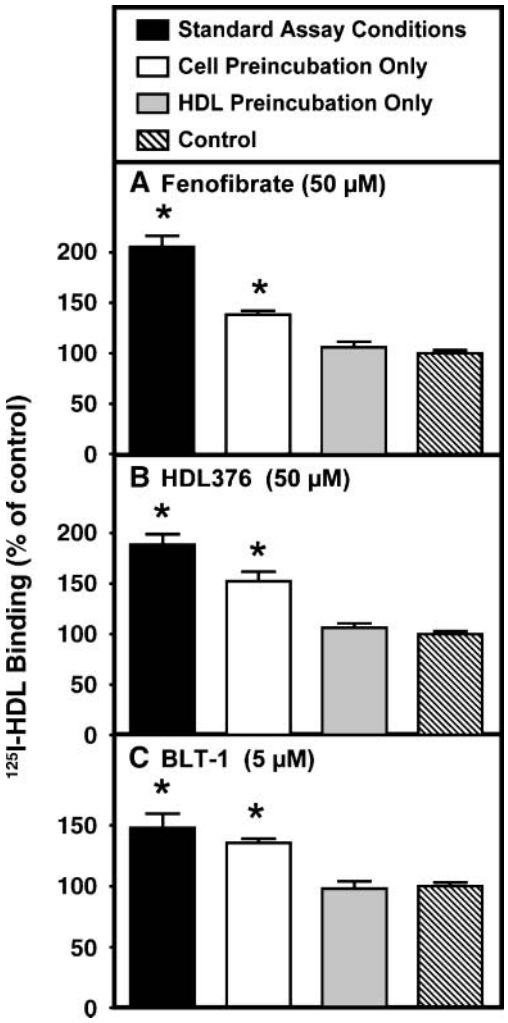

Fig. 4. Effects on ^{125}I-HDL binding of preincubating either cells or ^{125}I-HDL with compounds. The effects of compounds on ^{125}I-HDL (10 μg protein/ml) binding to ldlA[mSR-BI] or ldlA-7 cells were measured using either standard assay conditions as described in Materials and Methods (standard assay conditions; black bars) or variations involving preincubations (white and gray bars). In standard assay conditions, compounds [50 μM FF (A), 50 μM HDL376 (B), and 5 μM blocker of lipid transport-1 (BLT-1) (C)] were present during both the preincubation period and the binding incubation period. Controls in which all steps were performed in the absence of compounds are shown by hatched bars. Alternatively (cell preincubation only; white bars), cells were preincubated for 1 h with the compounds at the indicated concentrations, the unincorporated compounds were subsequently removed by washing, and then ^{125}I-HDL (10 μg protein/ml) binding was determined in medium to which no compound was added. HDL preincubation only (gray bars) indicates assays in which ^{125}I-HDL (3.7 mg protein/ml), but not the cells, was preincubated for 1 h with 50 μM FF or HDL376 or 5 μM BLT-1, then diluted and subsequently added to cells at a final ^{125}I-HDL concentration of 10 μg protein/ml and compound concentration of 135 nM (FF or HDL376) or 13.5 nM (BLT-1) (370-fold dilution). Values of receptor-specific binding (nonspecific binding to untransfected ldlA-7 cells subtracted) are means from three or more replicate determinations per experiment (averages from two independent experiments). The values for each experiment were normalized to 100% for the controls. Asterisks indicate binding significantly different from the control value ($P < 0.0001$). In all cases, the P values for comparisons of HDL preincubation-only results with those of controls were >0.4.

liposomes. The fibrates were used at concentrations comparable to and exceeding those used previously to study their activities as PPARα agonists in cultured cells (72, 73). Clofibrate (Fig. 1D) was far less potent (Fig. 3A, B, D) than FF in inhibiting SR-BI-mediated selective uptake in cells. Indeed, in some experiments, essentially no inhibition of selective uptake could be detected at clofibrate concentrations up to 500 μM. Three other fibrates, gemfibrozil, ciprofibrate and bezafibrate, all with unesterified carboxylate groups (Fig. 1E–G, respectively), exhibited very little, if any, inhibition of SR-BI-mediated lipid transport (Fig. 3), even when used at 500 μM (data not shown). These data highlight the unique, dual inhibitory activities of the prodrug FF. It is an SR-BI inhibitor at low doses and a PPARα agonist at high doses. This duality is not shared with its structurally related derivative FFA or with the other fibrates or niacin, all drugs that share the ability to increase HDL levels in humans.

Unesterified fibrates, including FFA, have been reported to readily bind to albumin in the circulation (57). The BSA included in the medium of our standard SR-BI activity assays to prevent nonspecific binding can bind organic anions (reviewed in Ref. 74) and thus might influence the bioavailability of the fibrates. This might lead either to an underestimation of the potency of the drugs or to possible misinterpretation of the inactivity of the drugs. Therefore, we directly measured BSA binding to fibrates using surface plasmon resonance (BiaCore). The ester clofibrate (Fig. 1D) showed no appreciable binding at concentrations up to 200 μM. As expected, all four of the drugs with free carboxylic acids bound to BSA to similar extents, with the following affinities (K_d): FFA, ~22 μM; ciprofibrate, ~44 μM; gemfibrozil, ~134 μM; bezafibrate, ~164 μM. However, similar analyses of binding to γ-globulin in place of BSA at fibrate concentrations up to 200 μM showed no substantial binding of any of these fibrates to γ-globulin [FF binding to BSA or γ-globulin could not be determined in the protein-free buffer used in the BiaCore assay because of its limited solubility in that buffer (visible precipitation at ~50 μM)]. Therefore, we replaced the BSA in the SR-BI activity assay medium with γ-globulin (0.5%, w/v) and retested these compounds. We observed no substantial differences in the relative potencies of the fibrates when assayed with γ-globulin in place of BSA (data not shown). We observed some low-potency inhibition of selective uptake at high concentrations of FFA in assays performed with γ-globulin ($IC_{50} \sim 300$ μM). This suggests that the reduced bioavailability of FFA in the presence of BSA may have made a very minor contribution to its low inhibitory activity toward SR-BI, but this effect is unlikely to be responsible for the very large differences observed in the activities of FF and FFA. Thus, differences in bioavailability attributable to albumin binding are not responsible for the different SR-BI-inhibitory activities of the distinct fibrates. (In some experiments in which concentrations of the fibrates were >150 μM, high concentrations of these compounds altered somewhat the activities of untransfected ldlA-7 control cells as well as ldlA[mSR-BI] cells. Thus, it was not possible to determine with certainty whether these

compounds were BLTs that simply are not very potent or whether SR-BI-independent effects of the compounds at high concentrations influenced the observations. At concentrations of <150 μM, these compounds did not affect the low levels of background binding and uptake in ldlA-7 control cells.)

The effect of HDL376 on SR-BI activity

Figure 5 shows the effects on SR-BI activity of the compound HDL376, a phenylthiourea (Fig. 1H) previously shown to increase HDL-cholesterol levels and increase HDL particle size in a variety of mammals (58, 59). HDL376 was a potent BLT in cultured cells, inhibiting SR-BI-mediated selective uptake of [^{3}H]CE from [^{3}H]CE-HDL ($IC_{50} \sim 1.4 \pm 1.3$ μM) and cholesterol efflux while increasing ^{125}I-HDL (10 μg protein/ml) binding ($EC_{50} \sim 0.9 \pm 0.8$ μM) (Fig. 5A, B) without altering the surface expression of SR-BI (Fig. 5C). This compound did not affect the low levels of background binding and uptake in ldlA7 control cells. HDL376 did not block the endocytosis of Alexa-labeled transferrin (data not shown; see Materials and Methods) (13). Thus, its inhibitory activity was specific in that it was not attributable to a global inhibition of cell surface receptor activity and intracellular membrane transport.

We also tested HDL376 inhibition of HDL lipid uptake in ACTH-treated Y1-BS1 cells. HDL376 (25 μM) inhibited receptor-dependent DiI uptake (data not shown); thus, its inhibitory activity is not restricted to SR-BI encoded by a transgene in Chinese hamster ovary-derived cells.

A previously published structure-activity relationship study showed that a urea analog of HDL376, compound **11** (Fig. 1K), was incapable of increasing HDL-cholesterol levels in vivo. These results suggested that the thiourea moiety of HDL376 substantially contributes to its HDL-cholesterol-elevating activity in vivo. To determine the importance of this thiourea moiety for the BLT-like activity of HDL376, we synthesized and tested the activity of HDL376U, a urea analog of HDL376 (Fig. 1I). Although HDL376U exhibited some BLT-like activity (inhibiting selective uptake and efflux, increasing binding), it was substantially less potent than HDL376 (Fig. 4A–C). Its IC_{50} was ~36 μM, and the maximum extent of inhibition of selective uptake was substantially less than that of HDL376. Substitution of BSA in the assay medium with γ-globulin had virtually no effect on the activities of either HDL376 or HDL376U, and we did not detect substantial binding of either HDL376 or HDL376U to either BSA or γ-globulin using surface plasmon resonance. [The dissociation constants for binding to BSA ($K_{d\ BSA}$) or γ-globulin ($K_{d\ Glob}$) were greater than the highest concentrations tested, 150 μM for HDL376 and 200 μM for HDL376U.] Thus, HDL376 depends on its thiourea moiety for its high potency, and the difference in activity between HDL376 and HDL376U cannot be explained by binding to albumin.

Similar to the results with FF and BLT-1, we found that preincubation of cells, but not ^{125}I-HDL, with HDL376 (50 μM) followed by a washout increased the binding of ^{125}I-HDL to cells (Fig. 4B), suggesting that the HDL376 is also likely to alter SR-BI activity by interacting directly

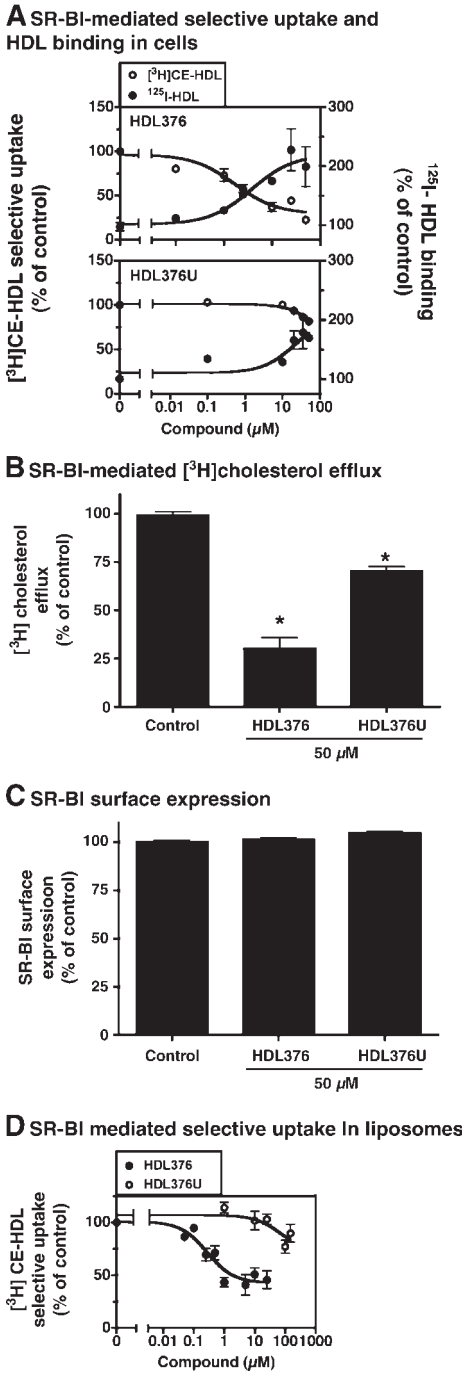

Fig. 5. Effects of HDL376 and its derivative HDL376U on SR-BI activity in cells and liposomes. SR-BI-mediated selective uptake of [^{3}H]CE from 10 μg protein/ml [^{3}H]CE-HDL (A), binding of ^{125}I-HDL (A), efflux of cellular unesterified [^{3}H]cholesterol to unlabeled HDL [B; average of two experiments (* $P < 0.0001$)], and the relative level of cell surface SR-BI (C) were determined as described for Fig. 2 in the presence of the indicated concentrations of HDL376 or HDL376U. Values for intact cells (A–C) determined in the absence of drugs of [^{3}H]CE-HDL uptake, ^{125}I-HDL binding, and [^{3}H]cholesterol efflux were the same as in Fig. 3. D represents selective uptake of [^{3}H]CE from [^{3}H]CE-HDL mediated by purified SR-BI-t1 reconstituted in liposomes, as described for Fig. 3. For liposomes, the values of selective uptake and binding were as follows: for SR-BI-t1-containing liposomes, 184 ng (equivalent)/sample and 56 ng protein/sample; for control liposomes, 79 ng (equivalent)/sample and 8 ng protein/sample.

with SR-BI-expressing cells and not with HDL particles. Studies of purified SR-BI incorporated into phospholipid/cholesterol liposomes showed that HDL376 ($IC_{50} \sim 0.22 \pm 0.007$ μM) and, albeit at greatly reduced activity, HDL376U ($IC_{50} \sim 84.5 \pm 16.2$ μM) directly inhibited SR-BI-mediated lipid transport (Fig. 5D), demonstrating that SR-BI in liposomes is a direct target of HDL376.

DISCUSSION

Our results suggest that the active, unesterified forms of fibrates, which are thought to influence HDL-cholesterol levels primarily as a consequence of their PPARα agonist activity, are not direct inhibitors or activators of SR-BI. We observed similar results for niacin. Thus, these drugs are not likely to influence plasma HDL levels by directly altering SR-BI activity in vivo. It is somewhat surprising, therefore, that the prodrug FF, which is usually thought to be inactive until rapidly hydrolyzed in vivo to the active unesterified form (57), was a potent BLT in cultured cells, inhibiting SR-BI-mediated lipid transport with an IC_{50} of ~1 μM. There are no obvious structural similarities between FF and the previously identified BLTs (13). Because FF is rapidly hydrolyzed in vivo, it is unlikely that this drug increases HDL-cholesterol in vivo by inhibiting SR-BI. However, these results raise the possibility that esterase-resistant, uncharged derivatives of FF, such as the isopropyl ether or analogous ketone, might be potent SR-BI lipid transport inhibitors that could be used to further study SR-BI structure and function in vitro and in vivo.

The mechanism by which HDL376 increases HDL-cholesterol in vivo has not been determined previously. Importantly, a previously published structure-activity relationship study suggested that the thiourea moiety of HDL376 substantially contributes to its HDL-cholesterol-elevating activity in vivo (75), as was the case for its BLT-like activity in vitro in this study. In that study, a thiourea analog of HDL376 (Fig. 1J, compound **2**) increased plasma HDL-cholesterol in rats, whereas its urea analog (Fig. 1K, compound **11**) was inactive (75). HDL376 in vitro behaves very much like previously described BLTs, inhibiting SR-BI-dependent lipid transport in intact cells. This effect of HDL376 on SR-BI may be responsible, at least in part, for its in vivo activity on HDL metabolism, observed as increased HDL-cholesterol levels and particle size. However, it is possible that SR-BI-independent targets of HDL376 play a role in its HDL-cholesterol-elevating activity. Importantly, these in vivo effects of HDL376 are similar to those observed in mice in which hepatic SR-BI activity is dramatically reduced or absent, as a result of homozygous null mutations in the SR-BI gene itself or in the gene encoding PDZK1, a cytoplasmic adaptor protein required to maintain normal hepatic SR-BI protein expression (42, 44). These similarities raise the possibility that HDL376 may function as an inhibitor of SR-BI in vivo. Previous reports have characterized the effects of other BLTs on SR-BI activity in vitro (12, 13); however, unlike HDL376, their influence on HDL metabolism in vivo has not been described.

Although numerous studies have clearly directly established the importance of SR-BI in murine HDL metabolism (reviewed in Ref. 11), there is only limited and mainly indirect evidence for a similar role in other species, including humans. There are important differences between human and murine HDL metabolism [e.g., the presence of CETP in humans but not mice and their relatively low LDL- to HDL-cholesterol ratios compared with humans (76, 77)]. CETP mediates the transfer of cholesteryl esters from HDL to LDL and other lipoproteins and thus facilitates a distinctive pathway for lipoprotein metabolism. Thus, caution must be exercised in drawing conclusions about human HDL metabolism and associated pathology based on murine studies. For example, genetic ablation and transgenic hepatic overexpression studies have clearly established that SR-BI expression protects against atherosclerosis in standard murine models (11), even though reduced SR-BI activity results in increased steady-state levels of plasma HDL-cholesterol (42) and increased hepatic SR-BI expression reduces plasma HDL-cholesterol (41). The participation of SR-BI in the hepatic removal of cholesterol from the body, a process called reverse cholesterol transport (78), apparently contributes to SR-BI's antiatherogenic activity in mice (11). Therefore, pharmacologic inhibition of SR-BI activity, for example by HDL376, would be expected to promote atherosclerosis in mice. However, the effects of the inhibition of SR-BI activity on atherosclerosis in species that do express CETP (e.g., rabbits, dogs, primates, and humans) are currently uncertain. Potent and specific pharmacologic modulators of SR-BI activity should prove invaluable in extending the analysis of SR-BI function to other mammals, particularly CETP-expressing animals, less readily amenable to genetic manipulation (e.g., rabbits, dogs, and nonhuman primates). Such pharmacologic modulators can substitute for the very powerful gene knockout and transgenic overexpression technologies commonly used in murine systems.

It is noteworthy that HDL376 appears to have similar effects on HDL metabolism in vivo in a wide variety of species from rodents to primates (58, 59, 75, 79), including demonstration of its ability to increase HDL-cholesterol in humans (59), possibly targeting SR-BI in both. Indeed, the in vitro studies reported here and the in vivo activities of HDL376 reported previously are consistent with the proposal that SR-BI is a functional HDL receptor in humans, and therefore HDL376 might be useful in characterizing the role of this receptor in humans and in animal models. Future animal and clinical studies will determine the therapeutic potential of the pharmacologic manipulation of SR-BI (either inhibition or enhancement) for treating pathologies for which SR-BI has been shown to play a role in mice, including atherosclerotic coronary artery disease and female infertility (reviewed in Ref. 11).

Note added in proof

After submission of this work, Nishizawa and colleagues (80, 81) reported the identification of *N*-[4-(4-tert-butoxycarbonyl-piperazin-1-yl)phenyl]-(2-chloro-5-nitrophenyl)carboxamide

(R-138329) as a small molecule that can increase plasma HDL-cholesterol in hamsters and mice, decrease murine hepatic selective uptake of [^{3}H]CE from HDL in vivo, exacerbate atherosclerotic lesion formation in apolipoprotein E-deficient mice, and inhibit rodent SR-BI-mediated lipid uptake activity in cultured cells.

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