Cross-inhibition of SR-BI- and ABCA1-mediated cholesterol transport by the small molecules BLT-4 and glyburide

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Abstract **Scavenger receptor class B type I (SR-BI) and ABCA1 are structurally dissimilar cell surface proteins that play key roles in HDL metabolism. SR-BI is a receptor that binds HDL with high affinity and mediates both the selective lipid uptake of cholesteryl esters from lipid-rich HDL to cells and the efflux of unesterified cholesterol from cells to HDL. ABCA1 mediates the efflux of unesterified cholesterol and phospholipids from cells to lipid-poor apolipoprotein A-I (apoA-I). The activities of ABCA1 and other ATP binding cassette superfamily members are inhibited by the drug glyburide, and SR-BI-mediated lipid transport is blocked by small molecule inhibitors called BLTs . Here, we show that one BLT, [1-(2-methoxy-phenyl)-3-naphthalen-2-yl-urea] (BLT-4), blocked ABCA1-mediated cholesterol efflux to lipid-poor apoA-I at a potency similar to that for its inhibition of SR-BI ($IC_{50} \sim 55–60$ μM). Reciprocally, glyburide blocked SR-BI-mediated selective lipid uptake and efflux at a potency similar to that for its inhibition of ABCA1 ($IC_{50} \sim 275–300$ μM). As is the case with BLTs, glyburide increased the apparent affinity of HDL binding to SR-BI. The reciprocal inhibition of SR-BI and ABCA1 by BLT-4 and glyburide raises the possibility that these proteins may share similar or common steps in their mechanisms of lipid transport.**—Nieland, T. J. F., A. Chroni, M. L. Fitzgerald, Z. Maliga, V. I. Zannis, T. Kirchhausen, and M. Krieger. **Cross-inhibition of SR-BI- and ABCA1-mediated cholesterol transport by the small molecules BLT-4 and glyburide** ***J. Lipid Res.*** **2004.** 45: **1256–1265.**

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Cholesterol and cholesteryl ester movement into and out of cells mediated by lipoproteins and cell surface proteins such as scavenger receptor class B type I (SR-BI) and ABCA1 plays an important role in cellular and whole body lipid metabolism and can profoundly influence the risk of atherosclerosis and coronary heart disease (1–8). The pathologic consequences of naturally arising or experimentally induced disruptions in the SR-BI and ABCA1 genes clearly demonstrate that these lipid transport proteins serve physiologically important, yet dramatically distinct, purposes in HDL metabolism (1, 2).

SR-BI, a member of the CD36 superfamily of proteins, is a 509 residue glycoprotein (apparent mass of 82 kDa) containing two predicted transmembrane helices adjacent to very short N- and C-terminal cytoplasmic domains, with the bulk of the protein consisting of a large extracellular loop (3, 4). Alterations in SR-BI expression can influence profoundly several physiologic processes, including biliary cholesterol secretion, female fertility, red blood cell maturation, and the development of atherosclerosis and coronary heart disease (5–7, 9–14). SR-BI tightly binds large, spherical, cholesteryl ester-rich HDL particles, primarily via its major apolipoprotein, apolipoprotein A-I (apoA-I), but lipid-free apoA-I is a poor ligand (15). SR-BI mediates the selective uptake of HDL cholesteryl esters (16–19) and other lipids (3, 20, 21) into cells, after which the lipid-depleted particles dissociate from the cells. The mechanism of selective lipid uptake differs markedly from that of the classic coated pit-mediated endocytic uptake of LDL receptors (22). The binding of native spherical HDL par-

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Abbreviations: ABC, ATP binding cassette; apoA-I, apolipoprotein A-I; BLT, small molecules that block lipid transport; COE, cholesteryl oleyl ether; DSP, dithiobis (succinimidyl propionate); K_d, dissociation constant; SR-BI, scavenger receptor class B type I; SUR, sulfonylurea receptor.

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ticles or reconstituted apoA-I/phospholipid/cholesterol disks to SR-BI also results in the efflux of unesterified cholesterol from cells to the particles (23–25), but the physiological significance of this activity has not yet been determined.

ABCA1, a member of the ATP binding cassette (ABC) superfamily of proteins, has a structure strikingly different from that of SR-BI. ABCA1 is a much larger protein (~200 kDa) that contains 12 membrane-spanning helices, two cytoplasmic ATP binding domains, and two large extracellular loops (2, 26, 27). It mediates the transfer of unesterified cholesterol and phospholipids from cells to lipid-poor apoA-I (28–31), which can subsequently mature into spherical, cholesteryl ester-rich HDL particles (32–35). These mature HDLs are poor acceptors for ABCA1-mediated cholesterol efflux (36). Mutations in ABCA1 are the underlying cause of Tangier disease, which is characterized by the virtual absence of spherical, cholesteryl ester-rich HDL particles in the plasma and an accumulation of cholesterol in peripheral macrophages (28, 37–39). Mice with targeted homozygous inactivating mutations in the ABCA1 gene exhibit phenotypes similar to those of human Tangier patients (40–42). In contrast, SR-BI-deficient mice (homozygous null mutations) have increased levels of abnormally large lipid-rich HDL particles in their plasma (43). The differences in the structures of SR-BI and ABCA1 suggest that they use different mechanisms to transfer lipids between cells and HDL. This is supported by experiments showing that the structural or conformational determinants on apoA-I that are crucial for its interaction with ABCA1 (e.g., apoA-I's C terminus) appear to differ from those necessary for productive interactions with SR-BI (15, 24, 44). However, their common abilities to mediate cholesterol efflux from cells led us to probe their activities using small molecules that had previously been shown to inhibit their lipid transport activities. These included small molecules that block lipid transport (BLTs) mediated by SR-BI (45) and the drug glyburide (29, 31, 46–51), which blocks ABCA1-mediated cholesterol efflux.

Glyburide (also called glybenclamide; **Fig. 1A**) is a sulfonylurea that has received wide attention in clinical settings as a potent drug for the treatment of non-insulin-dependent diabetes mellitus (52). Glyburide binds tightly to and inhibits sulfonylurea receptors 1 and 2 (SUR1 or SUR2), members of the ABC superfamily of proteins that form the SUR subunit of the ATP-sensitive potassium channel. SUR1 and SUR2 are responsible for the binding and hydrolysis of ATP and effectively control the closing of the channel and the regulation of insulin release. The dissociation constant (K_d) for glyburide binding to SUR1, which apparently involves association with its transmembrane domains, is in the low nanomolar range [reviewed in ref. (53)]. Glyburide also inhibits the activities of other ABC proteins (54–56), including ABCA1. Inhibition of ABCA1 by glyburide has been reported to occur in the concentration range of 100–1,000 μM (29, 31, 46–51).

By using a high-throughput chemical library screen, we recently identified five small molecules (BLT-1 to BLT-5)

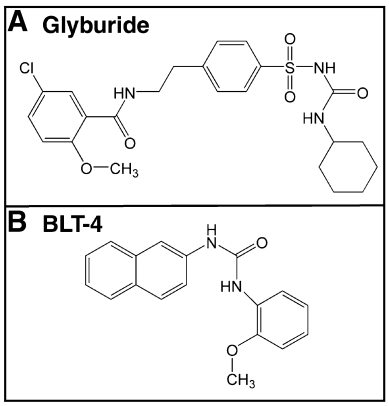

Fig. 1. Chemical structures of glyburide (A) and 1-(2-methoxy-phenyl)-3-naphthalen-2-yl-urea (BLT-4) (B).

that in the low nanomolar to micromolar range block lipid transport (selective uptake from and efflux to HDL) mediated by SR-BI (45). Unexpectedly, they enhance rather than decrease the apparent affinity of HDL binding to SR-BI.

Here, we report that 1-(2-methoxy-phenyl)-3-naphthalen-2-yl-urea (BLT-4) (Fig. 1B) blocks ABCA1-mediated cholesterol efflux to lipid-free apoA-I. The other BLTs did not inhibit ABCA1 activity. Conversely, glyburide prevented SR-BI-mediated selective lipid uptake from and cholesterol efflux to HDL. Similar to the BLTs, glyburide enhanced the affinity of HDL binding to SR-BI. The concentrations at which glyburide inhibited SR-BI- and ABCA1-mediated lipid efflux were similar (IC_{50} ~ 275–300 μM), as were those for BLT-4, but BLT-4 was the more potent inhibitor (IC_{50} ~ 55–60 μM). The reciprocal or cross-inhibition of these two lipid transport proteins by BLT-4 and glyburide raises the possibility that there may be similarities in at least one step of the mechanisms of SR-BI- and ABCA1-mediated lipid transport

EXPERIMENTAL PROCEDURES

Stock solutions of BLTs (Chembridge Corp.) and glyburide (Sigma) were prepared in 100% DMSO and diluted into the assay medium immediately before use. Dithiobis (succinimidyl propionate) (DSP; from Pierce Biotechnology, Inc.), which contains thiol-sensitive bonds, was dissolved in dimethyl sulfoxide and then diluted to 250 μM with PBS immediately before use.

Lipoproteins and cells

Human HDL (density of ~1.09–1.16 g/ml) and recombinant lipid-free human apoA-I were isolated and labeled with either ^{125}I (^{125}I-HDL or ^{125}I-apoA-I) or [^3H]cholesteryl oleyl ether ([^3H]COE, [^3H]COE-HDL) (3, 19, 25, 44, 57, 58). LDL receptor-deficient Chinese hamster ovary cells that express low levels of endogenous SR-BI (ldlA-7) (59), ldlA-7 cells stably transfected to express high levels of murine SR-BI (ldlA[mSR-BI]) (19), and HEK293-EBNA-T human embryonic kidney (HEK) cells (27) were maintained in culture as previously described. All assay media contained 0.5% or 0.29% (v/v) DMSO and 0.5% (w/v) BSA (fatty acid-free form in efflux assays), and all experiments were conducted at 37°C.

Lipid transport and binding assays

All data presented are representative of results from two or more independent experiments.

SR-BI. Assays for the uptake of [^{3}H]COE from [^{3}H]COE-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 previously described (19, 23, 25). We also determined the SR-BI-mediated efflux of [^{3}H]cholesterol from HEK293-EBNA-T cells transiently transfected with an SR-BI expression plasmid (19), as described for ABCA1 below. On the day of the assay, cells were preincubated for 1 h at 37°C in assay medium (Ham's F12, 0.5% BSA, 0.5% DMSO, and 25 mM HEPES, pH 7.4) containing compounds at the indicated concentrations. Subsequently, the cells were incubated for an additional 2 h with the same concentration of small molecules and with the indicated concentrations of ^{125}I-HDL (binding), [^{3}H]COE-HDL (uptake), or unlabeled HDL (efflux). ^{125}I-HDL saturation binding assays were performed as previously described, in which a 40-fold excess of unlabeled HDL was included in some of the incubations to permit the correction for nonspecific binding (19, 45). Analysis of saturation binding curves was performed using GraphPad Prism3 software from GraphPad Software, Inc. (San Diego, CA).

For some experiments, the values presented were normalized so that 100% of control represents activity in the absence of compounds. In Figs. 2 and 3B, 0% activity was defined as the activity determined in the presence of a 1:800 dilution of the anti-SR-BI blocking antibody KKB-1 (25), a generous gift from Karen Kozarsky. In other experiments, the 0% values were defined as the amount of activity either in ldlA-7 control cells in the presence of drugs (Fig. 4C) or in ldlA[mSR-BI] cells in the presence of a 40-fold excess of unlabeled HDL (Fig. 5). The amount of cell-associated [^{3}H]COE is expressed as the equivalent amount of [^{3}H]COE-HDL protein (nanograms) to permit direct comparison of the relative amounts of ^{125}I-HDL binding and [^{3}H]COE uptake (60).

ABCA1. ABCA1-dependent efflux of [^{3}H]cholesterol was measured using HEK293-EBNA-T cells transiently transfected with either an ABCA1 pcDNA1 expression vector or the empty pcDNA1 vector control, as previously described (44, 61). On day 0, HEK293-EBNA-T cells were plated at 200,000 cells/well on 24-well poly-D-lysine-coated plates (Becton Dickinson) in medium A [DMEM containing high glucose and 10% (v/v) fetal calf serum without antibiotics]. On day 1, cells were transfected with either the ABCA1 vector or an "empty" vector control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's suggestions. The media were removed 16 h later, and cells were incubated for 24 h in medium A supplemented with 0.5 μCi/ml 1,2[^{3}H]cholesterol (Perkin-Elmer). The cells were then washed twice with DMEM (high glucose) and incubated for 1 h in assay medium [DMEM (high glucose) supplemented with 0.2% fatty acid-free BSA, 0.5% DMSO, and 25 mM HEPES, pH 7.4]. The cells were then pretreated with the indicated concentrations of small molecules in the assay medium for 1 h at 37°C and then incubated with the same concentrations of compounds in the presence or absence of 10 μg/ml lipid-free recombinant human apoA-I for an additional 4 h to permit the efflux of cellular cholesterol. The supernatant was harvested and clarified by centrifugation (5 min, 6,000 g), the cells were lysed in 0.1 M NaOH, and radioactivity in both fractions was measured by liquid scintillation counting.

Cholesterol efflux was defined as the amount of radioactivity in the extracellular media at the end of the 4 h incubation divided by the total amount of cellular radioactivity in the media plus cells (the percentage efflux). The apoA-I-dependent efflux is defined as the difference between the efflux values determined for incubations in medium that did or did not contain lipid-free apoA-I. The ABCA1- and apoA-I-dependent efflux is defined as the difference between the apoA-I-dependent efflux values determined using cells that express the ABCA1 transgene (HEK[ABCA1]) and the empty vector controls (HEK[control]). Efflux values are presented as the percentage of total cellular 1,2[^{3}H]cholesterol present in the cells at the beginning of the 4 h incubation.

The IC_{50} values are those concentrations of the compounds that caused a half-maximal inhibition of lipid transport mediated by either ABCA1 or SR-BI.

Cross-linking assay of apoA-I binding to ABCA1

The binding assays were conducted as described by Wang et al. (62) with minor modifications. HEK293-EBNA-T cells were plated as described above on 12-well poly-D-lysine-coated plates and 3 days later were transfected with plasmids when the cells were ~95% confluent, as described above. On day 4, the cells were preincubated in DMEM (high glucose) and 0.2% (w/v) fatty acid-free BSA with the indicated amounts of compounds at 37°C for 1 h before incubation at 37°C for 1 h with 2 μg/ml ^{125}I-apoA-I and the indicated compounds in the presence or absence of a 30-fold excess of unlabeled apoA-I. Cells were then washed, reversibly cross-linked with DSP (2 ml/well), and washed again as previously described (62). For immunoprecipitation, cell lysates prepared in buffer RI [50 mM Tris, pH 7.6, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40] containing a protease inhibitors mixture (Roche Molecular Biochemicals GmbH), and 1 mM phenylmethylsulfonyl fluoride were subjected to centrifugation at 1,000 *g* in a microcentrifuge for 10 min. The supernatant was collected and precleared with 10 μl of GammaBind G Sepharose beads for 1 h at room temperature with rotation. Polyclonal anti-ABCA1 antibody (50 μg) directed against the C terminus of ABCA1 (61) was added to the preabsorbed cell lysates and incubated with rotation at room temperature for 2 h. Twenty microliters of GammaBind G Sepharose beads was then added, and the incubation with rotation was continued at room temperature for 1 h. The samples were subjected to a brief centrifugation, and the pellets were washed three times with cold buffer RI at room temperature. The bound proteins were eluted from the beads by incubation and boiling in Laemmli sample buffer in the presence of 5% β-mercaptoethanol, which also serves to break the DSP-mediated cross-links between apoA-I and ABCA1. The eluted proteins were fractionated by 12% SDS-polyacrylamide gel electrophoresis, and the radioactivity in the dried gels was analyzed using a PhosphorImaging STORM860 system (Molecular Dynamics, Inc., Sunnyvale, CA) and ImageQuant software.

Determination of ABCA1 expression levels

To determine the effect of the compounds on the cell surface expression of ABCA1, we used previously described quantitative assays for cell surface ABCA1 that detects a FLAG tag inserted in the first large extracellular loop of ABCA1 (27). In brief, HEK293-EBNA-T cells on 24-well plates were transfected with a FLAG-ABCA1 cDNA or empty vectors as described above. The media containing DNA and lipofectamine were removed 1 day after transfection, and 1 day later the cells were exposed for 5 h at 37°C to assay medium [DMEM (high glucose) supplemented with 0.2% fatty acid-free BSA, 0.5% DMSO, and 25 mM HEPES, pH 7.4] with or without 150 μM BLT-4 or 500 μM glyburide. The cells were then chilled on ice for 10 min, and cell surface ABCA1-FLAG expression was measured as described by Fitzgerald et al. (27).

Total cellular ABCA1 expression was measured by immunoblot analysis using a polyclonal antibody as previously described (61). Transfected cells were treated for 5 h at 37°C with 500 μM glyburide or 150 μM BLT-4 in assay medium [DMEM (high glucose) supplemented with 0.2% fatty acid-free BSA, 0.5% DMSO, and 25 mM HEPES, pH 7.4] and subsequently lysed in a hypotonic buffer (250 mM sucrose, 10 mM HEPES, pH 7.4, and 1 mM EDTA) supplemented with a protease inhibitor mini-cocktail containing EDTA (Roche). Nuclei and cell debris were removed by centrifugation (800 *g* for 10 min). The amount of protein was measured in the postnuclear supernatants by the method of Bradford (63) and 15 μg of each sample were separated by 6% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were processed for antibody staining, and ABCA1 was detected using an enhanced chemiluminescence system (Pierce) as previously described (61).

Measurement of total cellular cholesterol and cholesteryl ester levels by TLC

HEK, ldlA7, and ldlA[mSR-BI] cells were labeled with [^{3}H]cholesterol as described above for cholesterol efflux assays. After incubation for 5 h with or without 500 μM glyburide or 150 μM BLT-4, the cells were lysed (lysis buffer: 50 mM Tris, pH 7.6, 150 mM NaCl, 0.25% sodium deoxycholate, and 1% Nonidet P-40) for 30 min at room temperature. A mixture of 2:1 chloroform-methanol (v/v) containing unlabeled lipids (200 μg/μl cholesterol and 200 μg/μl cholesteryl oleate) was added to the lysate as an internal standard. The organic phase of the resulting mixture was evaporated under nitrogen, dissolved in 75 μl of chloroform, and fractionated by TLC using ITLC SA plates (Pal Corporation) using petroleum ether-ether-acetic acid (85:15:1). Spots containing cholesteryl ester, cholesterol, and origin spots were cut out, and radioactivity was measured by liquid scintillation counting.

Synthesis of BLT-4

Naphthyl isocyanate (Sigma-Aldrich) was dissolved in ethyl acetate and *N*-methyl-pyrrolidinone (Sigma-Aldrich) and then cooled to 0°C. 2-Anisidine was added drop-wise by syringe over 10 min. Solid naphthyl isocyanate (1.00 g, 5.92 mmol) was dissolved in 45 ml of anhydrous 1:2 *N*-methyl pyrrolidinone-ethyl acetate (Acros Organics) under a nitrogen atmosphere and cooled to 0°C. 2-Anisidine (900 μl, 7.32 mmol) was added drop-wise by syringe over 10 min and the reaction was stirred, first at 0°C for 2 h and then for an additional 2 h at room temperature. The reaction was quenched by the addition of 100 ml of 0.1 N HCl and extracted three times with 50 ml of dichloromethane. The pooled organic fractions were dried over anhydrous potassium carbonate, concentrated by rotary evaporation, and purified by flash chromatography using silica gel 60 (EMD Pharmaceuticals) equilibrated in 2:1 (v/v) hexane-ethyl acetate. BLT-4 was eluted with 1:1 (v/v) hexane-ethyl acetate and lyophilized with a final yield of 22%. The product was more than 95% pure as determined by liquid chromatography-mass spectrometry and NMR spectroscopy. The ^{1}H-NMR resonance frequencies for BLT-4 are as follows: (d_6-Me_2SO): δ 3.89 (3H, s), 6.90 (1H, td, J = 1.5 Hz, 1.5 Hz), 6.96 (1H, td, J = 1.5, 5.5 Hz), 7.02 (1H, dd, J = 7, 1.5 Hz), 7.34 (1H, T, J = 8 Hz), 7.44 (3H, m), 7.77 (1H, s), 7.79 (1H, s), 7.81 (1H, d, J = 4 Hz), 7.84 (1H, s), 8.12 (1H, d, J = 2 Hz), 8.17 (1H, dd, J = 1.5, 6.5 Hz), 8.30 (1H, s), 9.52 (1H, s).

RESULTS AND DISCUSSION

Effects of BLTs on ABCA1-mediated [^{3}H]cholesterol efflux to apoA-I

To determine if SR-BI- and ABCA1-mediated cholesterol efflux share common features, we examined the effects of BLTs on ABCA1-mediated cholesterol efflux from HEK293 cells transiently transfected with either an ABCA1-expression vector (HEK[ABCA1]) or an empty vector control (HEK[control]).

Cholesterol efflux was measured from cells labeled with unesterified [^{3}H]cholesterol for 24 h, followed by a 1 h preincubation with the BLT. This was followed by a 4 h incubation with the BLT in the presence or absence of 10 μg of protein per milliliter of lipid-free apoA-I, which served as an acceptor for cholesterol efflux. The amounts of [^{3}H]cholesterol in the incubation media and the amounts remaining associated with the cells were measured, and efflux was expressed as the percentage of cellular [^{3}H]cholesterol released into the medium during the 4 h incubation. **Figure 2** shows that the ABCA1-mediated [^{3}H]cholesterol efflux to apoA-I (closed bars) was not in-

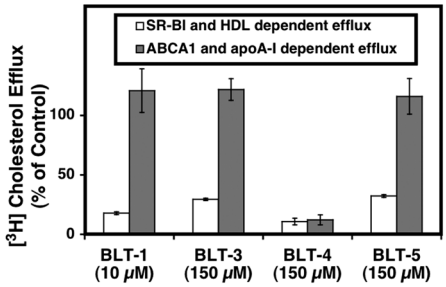

Fig. 2. Effects of BLTs on ABCA1- and scavenger receptor class B type I (SR-BI)-dependent efflux of [^{3}H]cholesterol to extracellular acceptors. HEK293-EBNA-T cells were transiently transfected with either an ABCA1 expression vector or the control pcDNA1 vector. One day after transfection, the cells were labeled for 24 h with [^{3}H]cholesterol and then preincubated for 1 h at 37°C in assay medium containing 0.5% (v/v) DMSO with or without the indicated concentrations of BLTs. After the preincubation, the cells were incubated for an additional 4 h at 37°C with the same concentrations of BLTs in the presence or absence of 10 μg/ml lipid-free recombinant human apolipoprotein A-I (apoA-I) to measure cholesterol efflux. The amounts of [^{3}H]cholesterol transferred from the cells to the medium and the values for ABCA1- and apoA-I-dependent efflux were determined as described in Experimental Procedures (closed bars). The control efflux values (percentage of cellular cholesterol released into the medium) measured in the absence of BLTs were as follows: HEK[ABCA1] with apoA-I, 7.4%; HEK [ABCA1] without apoA-I, 4%; HEK[control] with apoA-I, 1.7%; HEK[control] without apoA-I, 1%. The 100% control value determined in the absence of BLTs for ABCA1- and apoA-I-dependent efflux was 2.7% [calculated as (7.4-4) − (1.7-1)%]. Error bars represent the range of duplicate determinations. In independent experiments, SR-BI-mediated and HDL-dependent [^{3}H]cholesterol efflux was determined in [^{3}H]cholesterol-labeled ldlA[mSR-BI] cells (open bars). Cells were preincubated with the indicated amounts of BLTs for 1 h and then incubated for 2 h at 37°C with 500 μg of protein per milliliter of HDL and the same concentrations of BLTs in the presence or absence of a 1:800 dilution of the anti-SR-BI blocking antibody KKB-1 (25). The 100% of control value represents efflux in the absence of compound (50% of initial cellular radiolabeled cholesterol for efflux to HDL), and the 0% of control value represents efflux in the presence of HDL and the KKB-1 antibody (1:800 dilution) (12% of initial cellular radiolabeled cholesterol). There was no effect of the compounds on efflux to HDL in the presence of KKB-1. Error bars represent standard deviations from triplicate measurements.

hibited by BLT-1 (10 μM), BLT-3 (150 μM), or BLT-5 (150 μM), at concentrations that inhibit SR-BI-mediated [^{3}H] cholesterol efflux to HDL from ldlA[mSR-BI] cells, a cell line stably transfected with murine SR-BI (open bars) (45). At these concentrations, all three BLTs inhibited cholesterol efflux to HDL from HEK cells transfected with a cDNA construct encoding murine SR-BI, indicating that HEK cells are not intrinsically resistant to BLT-1, BLT-3, and BLT-5 (data not shown). Because the structures of BLT-1 and BLT-2 are very similar (45), we did not test BLT-2 in these studies. In contrast to the other BLTs, BLT-4 (Fig. 1B) at a concentration of 150 μM inhibited ABCA1- and apoA-I-dependent efflux by ~90%, approximately the same extent of its inhibition of cholesterol efflux observed for SR-BI-mediated efflux to HDL (Fig. 2) from transfected ldlA-7 (45) or HEK293 (data not shown) cells.

Figure 3A shows the concentration dependence of BLT-4-mediated inhibition of [^{3}H]cholesterol efflux. HEK [ABCA1] and HEK[control] cells were labeled with [^{3}H] cholesterol and incubated with or without lipid-free apoA-I and the indicated concentrations of BLT-4. As described previously (28, 29, 44, 61, 64), we found that [^{3}H]cholesterol efflux depended on the expression of ABCA1 and

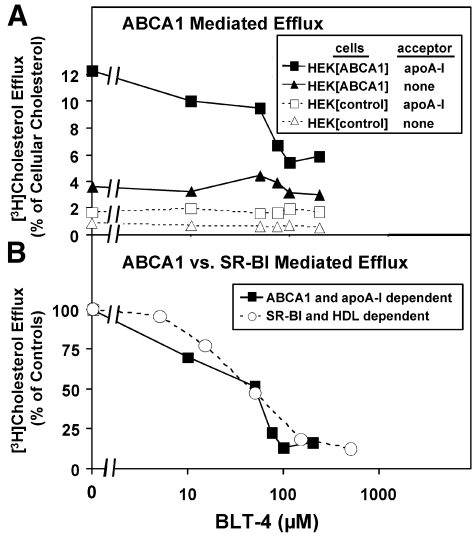

Fig. 3. Concentration dependence of the inhibition of ABCA1- and SR-BI-mediated [^{3}H]cholesterol efflux by BLT-4. A: [^{3}H]cholesterol efflux from HEK[ABCA1] (closed symbols) or HEK[control] (open symbols) cells was determined in the presence (squares) or absence (triangles) of 10 μg/ml lipid-free recombinant human apoA-I and the indicated concentrations of BLT-4 as described in the legend to Fig. 2 and Experimental Procedures. Values are expressed as the percentage of cellular [^{3}H]cholesterol released into the medium after a 4 h incubation at 37°C. B: The effect of BLT-4 on ABCA1-mediated and apoA-I-dependent [^{3}H]cholesterol efflux (closed squares) was calculated from the data in A as described in the legend to Fig. 2 and Experimental Procedures. In an independent experiment, SR-BI-mediated and HDL (500 μg protein/ml)-dependent [^{3}H]cholesterol efflux (open circles) was determined as described in the legend to Fig. 2 and Experimental Procedures. The maximum efflux in the absence of BLT-4 (60% of total cellular cholesterol) was set to 100% (the 0% of control was 19%).

the presence of apoA-I as an acceptor in the incubation medium. As the concentration of BLT-4 increased, there was a substantial inhibition of efflux from HEK[ABCA1] cells incubated with apoA-I, but there was little or no inhibition of the relatively low background efflux from HEK[ABCA1] cells in the absence of apoA-I or from HEK[control] cells either with or without apoA-I. Figure 3B shows that the ABCA1- and apoA-I-dependent [^{3}H] cholesterol efflux, calculated as described in Experimental Procedures, was inhibited by BLT-4 with an IC_{50} of ~60 μM. This value is similar to that for its inhibition of SR-BI-dependent [^{3}H]cholesterol efflux to HDL (IC_{50} ~ 55 μM; Fig. 3B) (45).

The shared sensitivities to BLT-4 suggest that there may be similarities in the mechanisms by which BLT-4 inhibited ABCA1- and SR-BI-mediated cellular cholesterol efflux. The inability of BLT-1, BLT-3, and BLT-5 to inhibit ABCA1 raises the possibility that the mechanisms by which they block SR-BI activity may differ from that of BLT-4, although additional studies will be required to directly address this question.

Effects of the ABCA1 inhibitor glyburide on SR-BI-mediated [^{3}H]cholesterol efflux to HDL

The inhibition of ABCA1-mediated cholesterol efflux by BLT-4 prompted us to determine if the ABCA1 inhibitor glyburide (29, 31, 46–51) (Fig. 1A) could block SR-BI-mediated [^{3}H]cholesterol efflux to HDL. **Figure 4** compares the concentration dependence of glyburide's inhibition of ABCA1-mediated [^{3}H]cholesterol efflux to apoA-I (A, C) with its effects on SR-BI-mediated [^{3}H]cholesterol efflux to HDL (B, C).

As expected from previous studies (29, 31, 48–50), glyburide inhibited ABCA1-mediated [^{3}H]cholesterol efflux from HEK[ABCA1] cells to apoA-I (10 μg protein/ml) with an IC_{50} of ~300 μM (Fig. 4A), and it had little effect on the low background efflux in the absence of ABCA1 transgene expression, in the absence of apoA-I, or both. This IC_{50} value is substantially greater than that for BLT-4 (~60 μM). Thus, BLT-4 was a more potent inhibitor of ABCA1 than was glyburide.

Strikingly, glyburide's effects on SR-BI-mediated efflux were virtually identical to those on ABCA1-mediated efflux. Figure 4B shows that glyburide inhibited SR-BI-mediated [^{3}H]cholesterol efflux from ldlA[mSR-BI] cells to HDL (500 μg protein/ml) with a IC_{50} of ~275 μM, and it had little effect on the low background efflux in the absence of SR-BI transgene expression (ldlA-7 control cells), in the absence of HDL, or both. Figure 4C, which compares directly glyburide's inhibition of ABCA1- and apoA-I-dependent cholesterol efflux and its inhibition of SR-BI- and HDL-dependent cholesterol efflux, shows that the drug is equally potent in inhibiting these cell surface transport proteins.

We also found that lipid-free apoA-I (10 μg protein/ml) was a poor acceptor of SR-BI-mediated [^{3}H]cholesterol efflux compared with HDL (500 μg protein/ml) [2% vs. 61%, respectively; also see ref. (36)]. The low level of lipid-free apoA-I-dependent [^{3}H]cholesterol efflux from

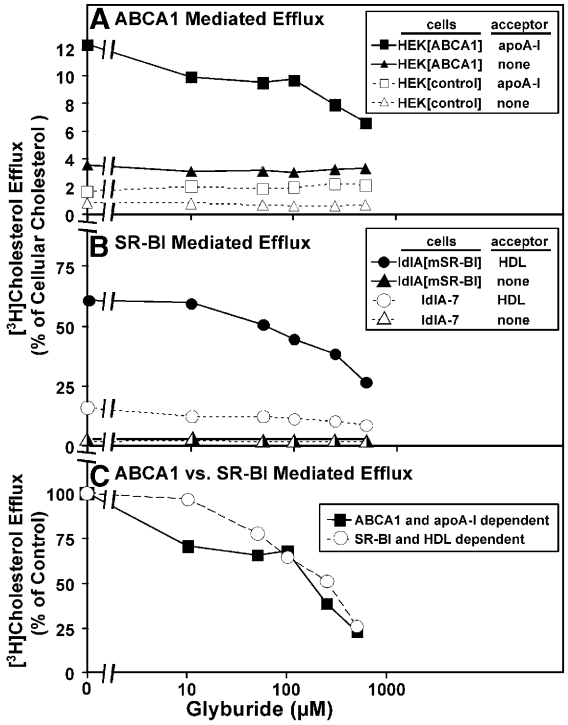

Fig. 4. Concentration dependence of the inhibition of ABCA1- and SR-BI-mediated [^{3}H]cholesterol efflux by glyburide. A: [^{3}H]cholesterol efflux from HEK[ABCA1] (closed symbols) or HEK[control] (open symbols) cells was determined in the presence (squares) or absence (triangles) of 10 μg/ml lipid-free recombinant human apoA-I and the indicated concentrations of glyburide as described in Experimental Procedures. B: [^{3}H]cholesterol efflux from SR-BI-expressing ldlA[mSR-BI] (closed symbols) or control ldlA-7 (open symbols) cells was determined in the presence (circles) or absence (triangles) of 500 μg of protein per milliliter of HDL and the indicated concentrations of glyburide as described in the legend to Fig. 3B. C: ABCA1-mediated and apoA-I-dependent [^{3}H]cholesterol efflux (closed squares) was calculated from the data in A as described in Experimental Procedures. SR-BI-mediated and HDL-dependent [^{3}H]cholesterol efflux (open circles) was calculated from the data in B. Efflux to HDL in ldlA[mSR-BI] cells in the absence of compound, and in ldlA-7 cells in the presence of compound, was used to set the 100% and 0% of control values, respectively. There was a small, KKB-1-inhibitable effect of glyburide on cholesterol efflux to HDL in ldlA-7 cells, which represents the efflux mediated by the low levels of endogenous SR-BI that is expressed in these cells.

ldlA[mSR-BI] and untransfected ldlA-7 control cells (~2%) was insensitive to glyburide (data not shown). It is thus unlikely that glyburide's ability to affect SR-BI's activity has complicated the interpretation of previous studies of ABCA1 activity, because those studies used lipid-free apoA-I as an acceptor of glyburide-sensitive cholesterol efflux.

We and others have found that the concentration of glyburide required to inhibit ABCA1 is substantially higher than its reported in vitro Ki for other ABC proteins, such as SUR1/2 [reviewed in ref. (53)]. It is possible that the inhibition of ABCA1-mediated cholesterol efflux by glyburide is a secondary consequence of high concentrations of glyburide interfering with other cellular processes, including features of cellular cholesterol metabolism. We were unable to detect glyburide inhibition of cholesterol esterification (thin layer chromatographic assay) or of the total cellular cholesterol levels in either HEK cells or ldlA[mSR-BI] cells loaded with [^{3}H]cholesterol (data not shown). Thus, at least some aspects of cellular cholesterol metabolism appear to be unaffected by high-dose glyburide treatment under the conditions used in these experiments. We previously have shown that BLT-4 is a relatively specific inhibitor in that it does not affect multiple membrane transport processes, such as clathrin-dependent endocytosis, lipid-raft-dependent endocytosis, and the protein secretory pathway, nor does it have a detectable impact on the integrity of the actin and microtubular networks (45). As was the case with glyburide, BLT-4 did not appear to inhibit cholesterol esterification or to alter total cellular cholesterol levels in either HEK cells or ldlA[mSR-BI] cells loaded with [^{3}H]cholesterol (data not shown).

Effects of glyburide on SR-BI-mediated [^{3}H]COE uptake from [^{3}H]COE-HDL and ^{125}I-HDL binding

Previous studies have shown a close relationship between SR-BI-mediated binding to HDL with both SR-BI-mediated cholesterol efflux to HDL and selective lipid uptake from HDL (24, 57, 65–68). We tested the effects of glyburide on these two additional activities of SR-BI. **Figure 5** shows that glyburide suppressed SR-BI-mediated [^{3}H]COE uptake from [^{3}H]COE-HDL by ldlA[mSR-BI] cells ($IC_{50} \sim 150$ μM). Flow cytometric analysis of the surface expression of SR-BI established that the effects of glyburide (at concentrations of ⩽500 μM) on SR-BI's activities were not attributable to a reduction in the steady-state level of SR-BI on the cell surface (data not shown).[2] Thus, glyburide inhibited both lipid transport activities of SR-BI, selective uptake and cholesterol efflux, as is the case for the BLTs (45).[3]

BLTs 1–5 increase the affinity of ^{125}I-HDL binding to SR-BI at 37°C (lower apparent K_d) (45). The decreased apparent K_d values are attributable, at least in part, to decreased dissociation rates and are not accompanied by substantially altered maximal binding values. Figure 5 shows that increasing concentrations of glyburide in-

[2] When ldlA[mSR-BI] or ldlA7 cells were treated with a very high concentration of glyburide (1 mM) for a total of 3 h, we observed morphological changes (e.g., changes in the appearance of the nucleus) and occasionally signs of toxicity as measured by trypan blue exclusion.

[3] Glyburide at a concentration of 1 mM has been reported to prevent specific ABCA1- and apoA-I-dependent phospholipid (phosphatidylcholine) efflux from cells, determined as the difference in phospholipid efflux from ABCA1-expressing cells measured in the presence and absence of apoA-I in the extracellular medium (29). We also observed this inhibition at very high doses of glyburide (500 μM and higher). However, we found in our experiments that the glyburide-induced decrease in specific apoA-I-dependent phospholipid efflux was largely attributable to an increase in ABCA1-mediated efflux to the apoA-I-free medium rather than to a substantial decrease in efflux to the apoA-I containing medium (data not shown).

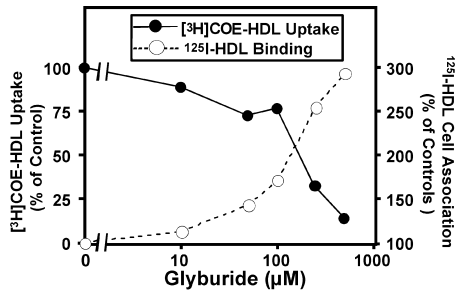

Fig. 5. Effects of glyburide on SR-BI-mediated [^{3}H]cholesteryl oleyl ether ([^{3}H]COE) uptake from [^{3}H]COE-HDL and ^{125}I-HDL binding. SR-BI-expressing ldlA[mSR-BI] or control ldlA-7 cells were preincubated with the indicated concentrations of glyburide, and then the uptake of [^{3}H]COE from [^{3}H]COE-HDL (10 μg protein/ml) (closed circles) or the binding of ^{125}I-HDL (10 μg protein/ml) (open circles) was determined in the presence or absence of a 40-fold excess of unlabeled HDL (duplicate incubations). The specific values (difference between the values in the absence and presence of excess HDL) were normalized such that the value in the absence of glyburide was set to 100%. The 100% of control values (nanograms of HDL protein per milligram of cell protein) for ldlA[mSR-BI] cells were as follows: [^{3}H]COE uptake, 1,962; ^{125}I-HDL binding, 198. The 100% of control values for ldlA-7 cells were as follows: [^{3}H]COE uptake, 289; ^{125}I-HDL binding, 21.

creased the binding of ^{125}I-HDL (10 μg protein/ml) to ldlA[mSR-BI] cells in a manner that was correlated inversely with its inhibition of lipid transport (Figs. 4 and 5). The increased binding of ^{125}I-HDL to SR-BI observed at a subsaturating concentration of ^{125}I-HDL appeared to be attributable, at least in part, to a glyburide-induced increase in the affinity of SR-BI for ^{125}I-HDL without a substantial change in the maximal binding values (**Fig. 6**). For the ^{125}I-HDL concentrations used in these experiments [a relatively broad density range (23)], the apparent K_d values were 26.2 ± 3.7 μg protein/ml in the absence of glyburide and 4.7 ± 0.7 μg protein/ml in the presence of 250 μM glyburide. Thus, the effects of the ABCA1 inhibitor glyburide on SR-BI's activities were similar to the activities of the BLTs: increased HDL binding and decreased lipid transport.

Effects of BLT-4 and glyburide on apoA-I binding to ABCA1

The increase in affinity of SR-BI for HDL induced by BLT-4 [see also ref. (45)] and glyburide prompted us to examine the binding of apoA-I to ABCA1. **Figure 7** shows a representative assay that involved incubating ABCA1-expressing cells with ^{125}I-apoA-I, treating the cells with a cleavable cross-linking agent (DSP), immunoprecipitating cross-linked complexes with anti-ABCA1 antiserum, and then detecting the coprecipitated ^{125}I-apoA-I after breaking the cross-links and fractionating the samples by SDS-PAGE (62). As expected, there was no detectable binding of ^{125}I-apoA-I to control cells transfected with empty vector (no ABCA1). Both glyburide [250–1,000 μM; see also ref. (62)] and BLT-4 (150 μM) effectively blocked the binding of ^{125}I-apoA-I to ABCA1 at concentrations that inhibit ABCA1-mediated cholesterol efflux to apoA-I (Figs.

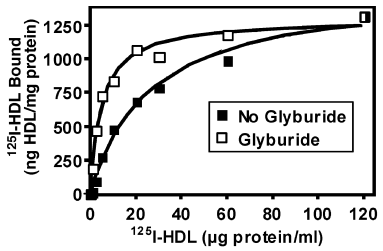

Fig. 6. Effects of glyburide on the concentration dependence of ^{125}I-HDL binding to SR-BI-expressing cells. SR-BI-expressing ldlA[mSR-BI] cells were preincubated in the absence (closed squares) or presence (open squares) of 250 μM glyburide and then incubated for 2 h at 37°C with the same amount of glyburide and the indicated concentrations of ^{125}I-HDL in the presence (single incubations) or absence (duplicate incubations) of a 40-fold excess of unlabeled HDL. The specific binding values are shown.

2–4). We did not detect a decrease in either the cell surface levels or the total protein levels of ABCA1 in cells treated under similar conditions with either compound (data not shown). Thus, glyburide and BLT-4 inhibition of ^{125}I-apoA-I binding to ABCA1 cannot be explained by decreases in either cell surface or total levels of ABCA1. The inhibition of apoA-I binding to ABCA1 by glyburide and BLT-4 contrasts their abilities to enhance the affinity of HDL binding to SR-BI.

It is possible that the reciprocal inhibition of SR-BI and ABCA1 by the structurally distinct small molecules BLT-4 and glyburide was simply a coincidence of multiple independent activities of these compounds. An alternative, more appealing explanation is that, despite the very different structures and physiologic functions of SR-BI and

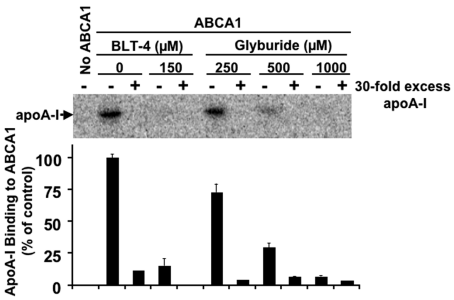

Fig. 7. Effects of BLT-4 and glyburide on ^{125}I-apoA-I binding to ABCA1. HEK293-EBNA-T cells were transiently transfected with either an ABCA1 expression vector (ABCA1) or a control pcDNA1 vector (No ABCA1). One day after transfection, the cells were preincubated for 1 h at 37°C in assay medium with the indicated concentrations of BLT-4 or glyburide. The binding of ^{125}I-apoA-I (2 μg/ml, 1 h, 37°C) in the presence or absence of a 30-fold excess of unlabeled apoA-I and the indicated concentrations of inhibitors was measured using a dithiobis (succinimidyl propionate) cross-linking assay as described in Experimental Methods. Bound ^{125}I-apoA-I was fractionated by SDS-PAGE and detected in the gels using a PhosphorImaging STORM860 system (top), and the relative amounts of ^{125}I-apoA-I in the lanes were determined using ImageQuant software (bottom). Values shown in the lower portion of the figure are representative of two independent experiments performed in duplicate. Error bars represent the ranges of duplicate determinations.

ABCA1, some features of their mechanisms of lipid transport may have similarities or even share common steps that are sensitive to BLT-4 and glyburide. This explanation is supported by the observation that the potencies (IC_{50}) for glyburide inhibition of SR-BI and ABCA1 are virtually identical, as they are for BLT-4.

The molecular targets of BLT-4 and glyburide responsible for their inhibition of SR-BI- and ABCA1-mediated lipid transport activities have not yet been identified, and they need not be the same. However, these lipid transport proteins themselves are obvious candidates. Glyburide and BLT-4 might bind to either the same or distinct sites on each protein. It is also possible that the direct targets of these inhibitors are not the transporters themselves but rather some other protein(s) or lipids (e.g., a specific membrane domain). It is noteworthy that, even though the structures of glyburide and BLT-4 (with the exception of the methoxyphenyl and the urea functionalities) differ (Fig. 1), they both can inhibit the activities of several ABC proteins. BLT-4 inhibits ethidium bromide efflux from *Staphylococcus aureus* mediated by NorA, a member of the ABC superfamily (69), as well as ABCA1 activity (this study). Glyburide, which was originally identified as a drug that binds to and inhibits the ABC subunit (SUR) of the ATP-sensitive K^+_{ATP} channels, can interfere with pathways involving the ABC proteins cystic fibrosis transmembrane regulator (54), bile salt export pump (56), and multidrug resistance P-glycoprotein (55), as well as ABCA1 (29, 31, 46–51). It is possible that the target of the inhibitory activities of both drugs could be a cell surface or intracellular ABC transporter(s) other than ABCA1 that may directly or indirectly influence lipid transport mechanisms mediated by both SR-BI and ABCA1.

Despite the similar abilities of either glyburide or BLT-4 to inhibit lipid transport mediated by ABCA1 and SR-BI, there are several striking differences in the mechanisms by which these transporters function. Lipid-free apoA-I binds preferentially to ABCA1, and along with preβ-apoA-I it is the preferred cholesterol acceptor from ABCA1 relative to spherical, lipid-rich HDL particles (32–36). In contrast, spherical, lipid-rich HDL particles are the preferred ligands for SR-BI (15). The effects of glyburide and BLT-4 on ligand binding also highlight the differences between these transporters. Both inhibitors block ligand binding to ABCA1, whereas they enhance the ligand binding affinity of SR-BI. Additional studies will be required to elucidate the mechanisms underlying the complex effects of these inhibitors on these key cholesterol transporters.

In summary, we have identified BLT-4 as a new and relatively potent inhibitor of ABCA1. We also show that glyburide is a heretofore unrecognized inhibitor of SR-BI. The reciprocal inhibition of these transport proteins by BLT-4 and glyburide raises the possibility that the mechanisms of lipid transport by SR-BI and ABCA1 might possibly involve another ABC transporter.

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