SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell culture – RAW 264.7 and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), HEK293-ABCA1-GFP cells were previously described[1], BHK-ABCA1 cells were a kind gift of the late Dr. Oram.[2] All cells were routinely grown in DMEM containing 10% heat-inactivated fetal bovine serum (FBS) and microbial antibiotics (5 U/ml penicillin and 5 µg/ml streptomycin or 50 µg/ml gentamicin) at 37°C with 5% CO_2. Media for HEK293-ABCA1-GFP cells was also supplemented with geneticin (Invitrogen) to 100 µg/ml to select against loss of the transgene.

Reagents – Compound 1 and 2 were synthesized as previously described[3-6] and dissolved in dimethyl sulfoxide (DMSO) to 30 and 10 mmol/L, respectively. High-density lipoprotein (HDL) was isolated from human serum by sequential density gradient ultracentrifugation. Apolipoprotein AI (apoAI) was isolated from human dilipidated HDL preparations by fast-protein liquid chromatography on a Q-Sepharose Fast Flow column (Amersham), stored in 6 mol/L guanidine chloride and extensively dialyzed before use against an appropriate buffer. Acetylated low-density lipoprotein (AcLDL) was prepared as described in Basu et al.[7] ApoAI V93C was prepared as described in Gross et al.[8] and labeled with Alexa Fluor 568 (Invitrogen) as recommended by the manufacturer. 18A peptide (also called 2F) (DWLKAFYDKVAEKLKEAF)[9] was custom-synthesized, stored in 6 mol/L guanidine chloride and dialyzed against an appropriate buffer. Transferrin-Alexa Fluor 488 and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Invitrogen. Dimyristoylphosphatidylcholine (DMPC) was from Avanti Polar Lipids. [1α,2α(n)-^{3}H]Cholesterol (35-50 Ci/mmol) acquired from Amersham in toluene was dried down and re-dissolved in ethanol to 1 mCi/ml. [*methyl*-^{3}H]Choline chloride (60-85 Ci/mmol) was from Amersham. Probumin bovine serum albumin (BSA) was from Millipore. Calpain inhibitor I was from Sigma, and protease inhibitor cocktail set III was from Calbiochem. Dithiobis[succinimidylpropionate] (DSP) was from Thermo Scientific. The following antibodies were employed: mouse monoclonal anti-*β*-COP (clone maD; Sigma), rabbit polyclonal anti-ABCA1 (400-105; Novus Biologicals), goat polyclonal anti-apoAI (400-147; Novus Biologicals). Protein A-sepharose 4B, RIPA buffer and all other chemicals were from Sigma.

rHDL formation assay – DMPC dissolved in chloroform-methanol (2:1 v/v) was dried in a stream of nitrogen onto the sides of a glass culture tube and kept in vacuum overnight. Dry DMPC was rehydrated in either TBS-EDTA (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4) or glycine-HCl (10 mmol/L glycine, pH 3.0)[10] buffer by five cycles of freeze-thaw (dry ice/ethanol, 37°C water bath) and extensive vortexing after each thaw, to derive multilamellar vesicles (MLVs) at 5 mg/ml. DMSO stocks of compound 1 and 2 were diluted with TBS-EDTA to 1 mmol/L afresh on each day of experimentation. The DMPC MLV stock was diluted to 62.5-500 µg/ml with TBS-EDTA or glycine-HCl and supplemented with the 1 mmol/L compound 1 or 2 stocks or a DMSO solution in TBS-EDTA (3.3% as the vehicle control for compound 1 and 10% as the vehicle control for compound 2) to concentrations 12.5 µmol/L compound 1 or 2, or 0.04% or 0.13% DMSO. The DMPC MLV-compound 1/2/DMSO

preparations were vortexed briefly, incubated at ambient temperature (24.3-25.6°C) for 20-30 min, aliquoted into a clear flat-bottom 96-well plate (200 µl of the DMPC:compound 1/2/DMSO preparation or 100-12.5 µg of DMPC per well) and incubated for 10 more minutes. 6.25 to 50 µg of apoAI in 100 µl of TBS-EDTA or glycine-HCl was added per well (always at the 2:1 w/w ratio of DMPC to apoAI, which is equivalent to the 84:1 DMPC:apoAI molar ratio, unless specified otherwise; the final concentration of compound 1 and 2 in the reaction was 8.3 µmol/L), followed immediately by a time-course measurement of absorbance at 325 nm in a plate reader. Absorbance at the time point zero was set to one, and all subsequent readings were expressed as a fraction of the zero time value.

Circular dichroism – ApoAI was dialyzed against 50 mmol/L sodium phosphate buffer (pH 7.4) and diluted to 375 µg/ml or 250 µg/ml. 266.6 µl of the phosphate buffer were combined with 3.33 µl of 1 mmol/L compound 1 and 133.3 µl of the 375 µg/ml or 250 µg/ml apoAI stock (final compound 1 concentration 8.3 µmol/L, compound 1:apoAI molar ratios 1:0.53 and 1:0.35, respectively). Three spectra (180 to 260 nm in a continuous scanning mode, 50 nm/min, 0.1 nm increments) for each of the two compound 1:apoAI molar ratio samples were collected using a quartz cell (0.2 cm path length) under a constant stream of nitrogen at an ambient temperature in a Jasco J1810 Spectropolarimeter (Jasco Incorporated, Easton, MD). The spectra were analyzed using the CDSSTR program (CDPro software package) with reference SMP56.[11]

DPH fluorescence anisotropy – DPH dissolved in chloroform was added to a chloroform-methanol DMPC solution to 0.2 mole%. DPH-spiked DMPC was used to prepare MLVs as described above. DMPC-DPH MLVs were warmed up to 37°C and extruded 19 times through a polycarbonate membrane (Whatman) with 100 nm pores using a mini-extruder (Avanti Polar Lipids) to derive large unilamellar vesicles (LUVs).[12] DMPC-DPH LUVs were incubated with compound 1 or 2 at the DMPC:compound molar ratio 8:1 for 30 min, followed by anisotropy measurements with excitation at 357 nm and emission at 427 nm over a temperature range in a water jacketed spectrofluorimeter with a circulating heating/cooling waterbath.

Cellular cholesterol efflux assay – Cellular cholesterol efflux assays were conducted as previously described[13] with some modifications. RAW 264.7 cells were seeded at 1.5 x 10^5 cells per well in 24-well plates in regular medium, allowed to attach overnight and grown in DMEM + 1% or 2.5% FBS + 0.5-1.0 µCi/ml [^{3}H]cholesterol over the following night to label cellular pools of cholesterol. On day three, the medium was replaced with DGGB (DMEM, 50 mmol/L glucose, 2 mmol/L glutamine and 0.2% BSA) +/- 0.3 mmol/L 8Br-cAMP, or DMEM + 0.2% probumin BSA +/- 0.3 mmol/L 8-(4-chlorophenylthio) (CPT)-cAMP (these two media are equivalent) to induce ABCA1 expression. 14-19 hours later, on day four, the medium was replaced with a pretreatment medium, which was one of the following: DGGB +/- 8Br-cAMP + compound 1 or 2 or DMSO (0.1-0.3%), or DMEM +/- 0.3 mmol/L CPT-cAMP + compound 1 or DMSO (0.1%) or wheat germ agglutinin (WGA)/DMSO. Cells were pretreated for 1 (compound 1) or 3 (compound 2) hours, washed once with DGGB or DMEM and incubated with the cholesterol efflux medium, which was the same as the pretreatment medium +/- cholesterol acceptors: DGGB +/- 8Br-cAMP + compound 1 or 2 or DMSO (0.1-0.3%) +/- 5 µg/ml apoAI or 8-10 µg/ml HDL, or DMEM +/- 0.3 mmol/L CPT-cAMP + compound 1 or DMSO or WGA/DMSO +/- 5 µg/ml apoAI or 5 µg/ml 18A or 4 mmol/L sodium taurocholate. Following a

4 hour cholesterol efflux period, cell medium was collected, centrifuged or filtered through a 0.45 μm syringe filter, and radioactivity in an aliquot of it was measured in a scintillation counter; cell lipids were extracted with hexane-isopropanol (3:2 v/v) and, after evaporation of the solvent, radioactivity in the extract was measured in a scintillation counter. Cellular cholesterol efflux was expressed as the percentage of [^{3}H] counts in the medium out of the total [^{3}H] counts in cells and medium. Stably-transfected ABCA1-GFP-expressing HEK293 cells[1] were seeded at 1 x 10^5 cells per well in 24-well plates coated with poly-D-lysine, grown in regular medium for two days, followed by growth in DMEM + 1% FBS + 0.5 μCi/ml [^{3}H]cholesterol for one day. On day four, cells were pretreated with 10 μmol/L compound 1 or DMSO (0.1%) for 1 hour and assayed for the ability to release [^{3}H]cholesterol to apoAI as with RAW 264.7 cells. BHK-ABCA1 cells were treated the same way as RAW 264.7 cells, except instead of a cAMP analog, 10 nmol/L mifepristone[2] was used to induce ABCA1 expression.

Microparticle production assay – RAW 264.7 cells were handled as in a regular cholesterol efflux assay on day one through three. On day four, cells were pretreated with compound 1, 10 μg/ml WGA/DMSO or DMSO for 1 hour. After the pretreatment, the medium was replaced with identical fresh medium without cholesterol acceptors, and cells were allowed to produce microparticles for 8 hours. Percentage cellular cholesterol efflux in microparticles was calculated as in regular cholesterol efflux assays.

Phospholipid efflux assay – Cellular phospholipid efflux assays were identical to cellular cholesterol efflux assays, except cells were labeled with 2 μCi/ml [^{3}H]choline and, after the 4-h efflux period, lipids were extracted from the conditioned cell medium with methanol-chloroform (1:2 v/v); the solvent was evaporated and [^{3}H] radioactivity in the extracted lipids was measured in a scintillation counter.

Total phospholipid phosphorus assay – RAW 264.7 cells were plated in T75 flasks and put through the same sequence of medium changes as in the cholesterol efflux assay ([^{3}H]cholesterol was not added, but ABCA1 expression was induced with the cAMP treatment). On day four, after an incubation with an cAMP analog for 16-18 hours, the cells were washed twice with 150 mmol/L NaCl, scraped in 150 mmol/L NaCl, transferred to a glass tube with a Teflon cap and pelleted by centrifugation (10 min at 1000 x g). The supernatant was removed, leaving about 100 μl of wet cell pellet per T75 flask. The pellet was resuspended in 1.5 ml of methanol; 3 ml of chloroform was added, and the mixture was stirred on an orbital shaker for 45 min, followed by addition of 0.9 ml of distilled water and further incubation for one hour without stirring to ensure complete phase separation.[14] The mixtures were then centrifuged for 10 min at 1000 x g. 200 μl fractions of the estimated 2.7 ml lower phase were collected into fresh 13x100 mm glass tubes and evaporated. The amount of inorganic phosphorus in the collected lower phase fractions was determined as described in Rouser et al.[15]

Endocytic uptake of AcLDL – AcLDL was centrifuged to remove precipitate and incubated with [^{3}H]cholesterol (7.8-15.7 μCi/mg of AcLDL) at 37°C for 2-4 hours on the day of experiment. DGGB was supplemented with [^{3}H]AcLDL to 0.15 mg/ml and either warmed up to 37°C or cooled down to 20°C. RAW 264.7 cells were seeded at 2 x 10^5 cells per well in 24-well plates, allowed to attach overnight and pretreated with 10 μmol/L compound 1, 160 μmol/L dynasore[16]

or DMSO (0.1%) for 1 hour at 37°C. After the pretreatment, the medium was replaced, and cells were grown in the same medium as during the pretreatment + [^{3}H]AcLDL for 15-20 min at either 37°C or 20°C. To remove loosely bound AcLDL, cells were soaked in ice cold acidic solution (0.15 mol/L NaCl, pH 3.0) for 5 min. Cell lipids were extracted, and radioactivity in the extract was measured in a scintillation counter. Endocytic uptake of AcLDL was calculated by subtracting the average of [^{3}H] cpm in cells exposed to AcLDL at 20°C from individual values of [^{3}H] cpm in cells exposed to AcLDL at 37°C.

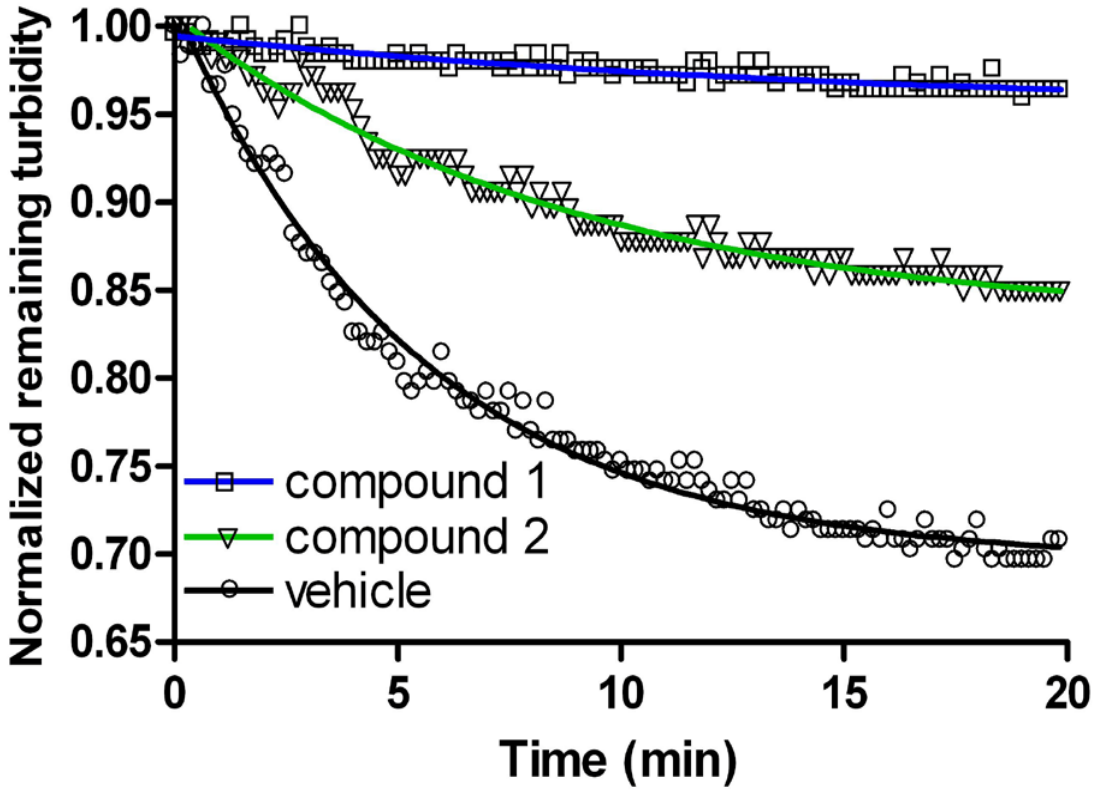
Effects of compound 1 on the ABCA1 expression level - RAW 264.7 cells were seeded in 12-well culture plates at 1/10 dilution of a confluent T75 flask and put through the same sequence of medium changes as in the cholesterol efflux assay, except [^{3}H]cholesterol was not added. On day four, after a treatment with +/- cAMP analog for 18 hours, the cells were incubated with +/- 10 μmol/L compound 1 +/- cAMP analog in DMEM for 5 hours. At the end of the incubation period, the cells were washed with phosphate buffered saline (PBS) once, scraped in PBS and pelleted by centrifugation for 5 min at 1000 x g. The supernatant was removed, and the pellet was resuspended in RIPA buffer supplemented with protease inhibitors. Cell proteins were separated using Tris-glycine SDS-PAGE, blotted and probed with an ABCA1 polyclonal antibody and a *β*-COP monoclonal anti-body.

Fluorescence microscopy – RAW 264.7 cells were seeded at 2 x 10^5 cells per chamber into chambered microscope slides and allowed to attach overnight. For endocytosis of transferrin, cells were pretreated with 10 μmol/L compound 1 or DMSO (0.1%) in DGGB for 1 hour and exposed to transferrin-Alexa Fluor 488 in the pretreatment medium for 20 min at 37°C, soaked in ice cold acidic solution for 5 min and fixed with formalin. For apoAI binding, cells were incubated in DGGB +/- 8Br-cAMP for 16-18 hours, pretreated with 10 μmol/L compound 1 or DMSO for one hour, exposed to apoAI V93C-Alexa Fluor 568 in the pretreatment medium for 15 or 45 min at 37°C, washed twice with DGGB and fixed with formalin. A Leica DMR epifluorescence microscope equipped with a Retiga-EXi camera was used to capture images.

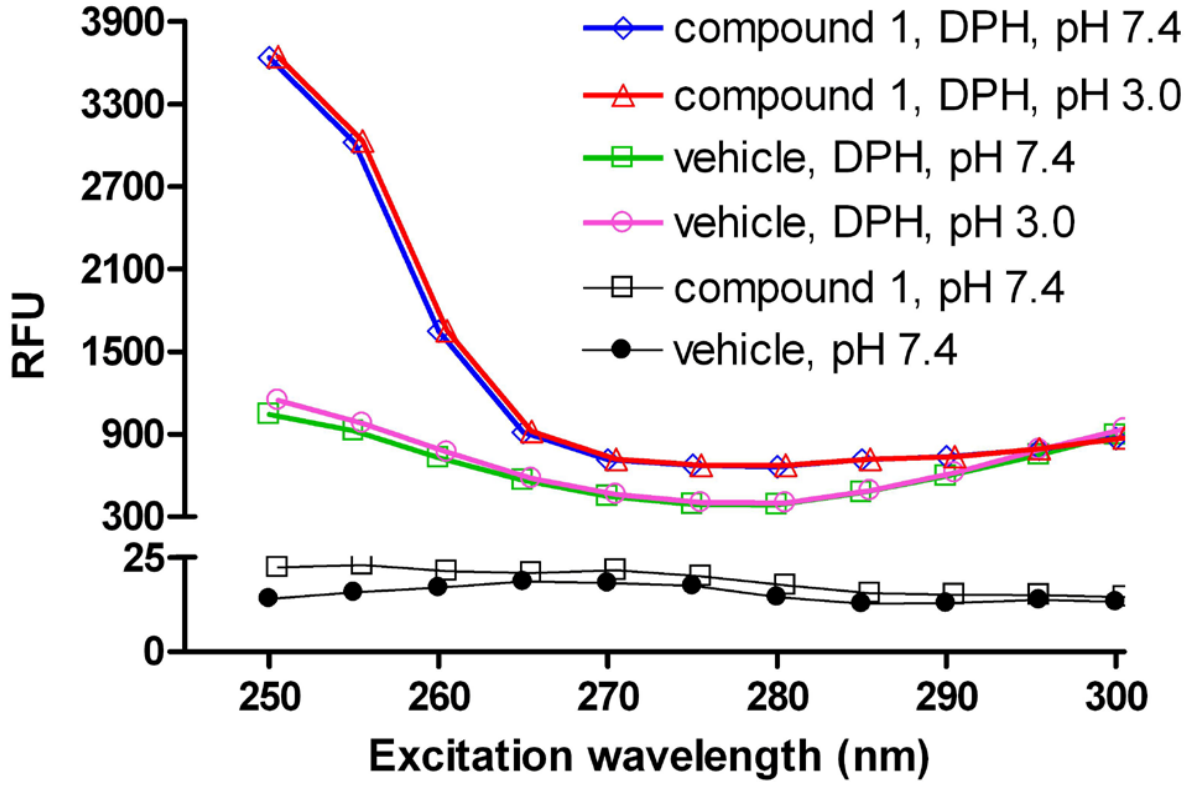
ABCA1-apoAI cross-linking – RAW 264.7 cells were seeded in T75 flasks (three flasks per treatment) at 1/10 dilution from a confluent T75 flask and put through the same sequence of medium changes as in the cholesterol efflux assay, except [^{3}H]cholesterol was not added. On day four, after a treatment with +/- cAMP analog for 18 hours, the cells were pretreated with 10 μmol/L compound 1 +/- cAMP analog in DMEM for 1 hour and exposed to 5 μg/ml apoAI in the presence of 10 μmol/L compound 1 +/- cAMP analog in DMEM for 2 hours. Thereafter, the cells were washed with cold PBS trice and incubated in 10 ml/flask 500 μmol/L DSP in PBS for 30 min at room temperature.[17] DSP was prepared immediately before use according to the manufacturer's instructions. Subsequently, the cells were washed with cold PBS twice, scraped in cold PBS (cells from the three flasks for the same treatment were combined together), pelleted by centrifugation (10 min at 1000 x g) and lysed in 5 ml of RIPA buffer supplemented with protease inhibitors. The cell lysates were centrifuged for 10 min at 8000 x g; the supernatants were collected, pre-cleared with protein A-sepharose 4B, incubated overnight at 4ºC with 10 μl of anti-ABCA1 polyclonal antibody (1:500 dilution), incubated with 25 mg of protein A-sepharose 4B for 2 hours at 4ºC and centrifuged. The supernatant was discarded; the pellet was washed twice with RIPA buffer supplemented with protease inhibitors and incubated in a low pH

buffer to elute primary antibodies off the sepharose beads according to the manufacturer's instructions. The eluates were brought to neutral pH with 0.1 mol/L NaOH, resolved using SDS-PAGE +/-reducing reagent, blotted and probed with an apoAI-specific polyclonal antibody.

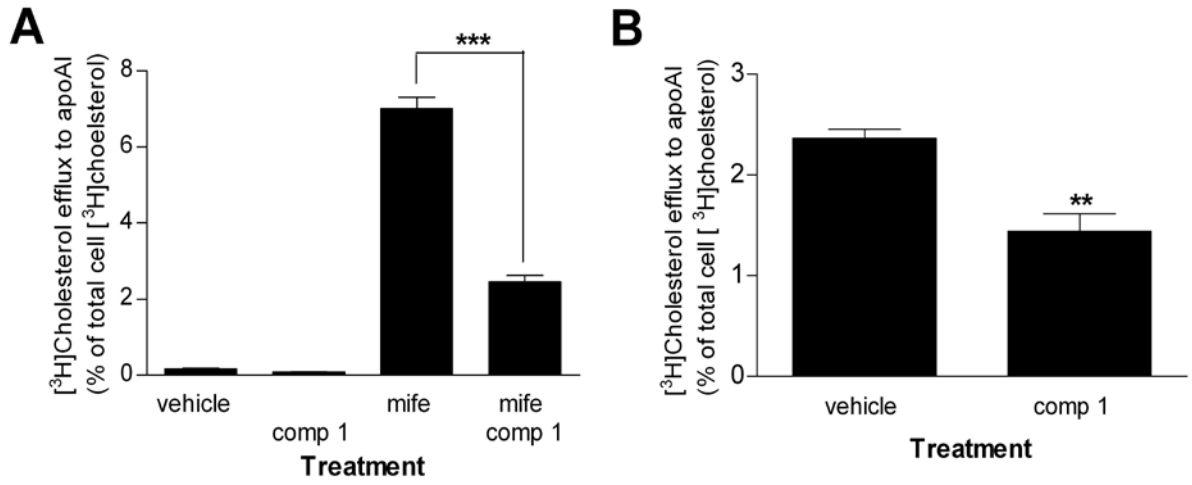
Supplemental Figures



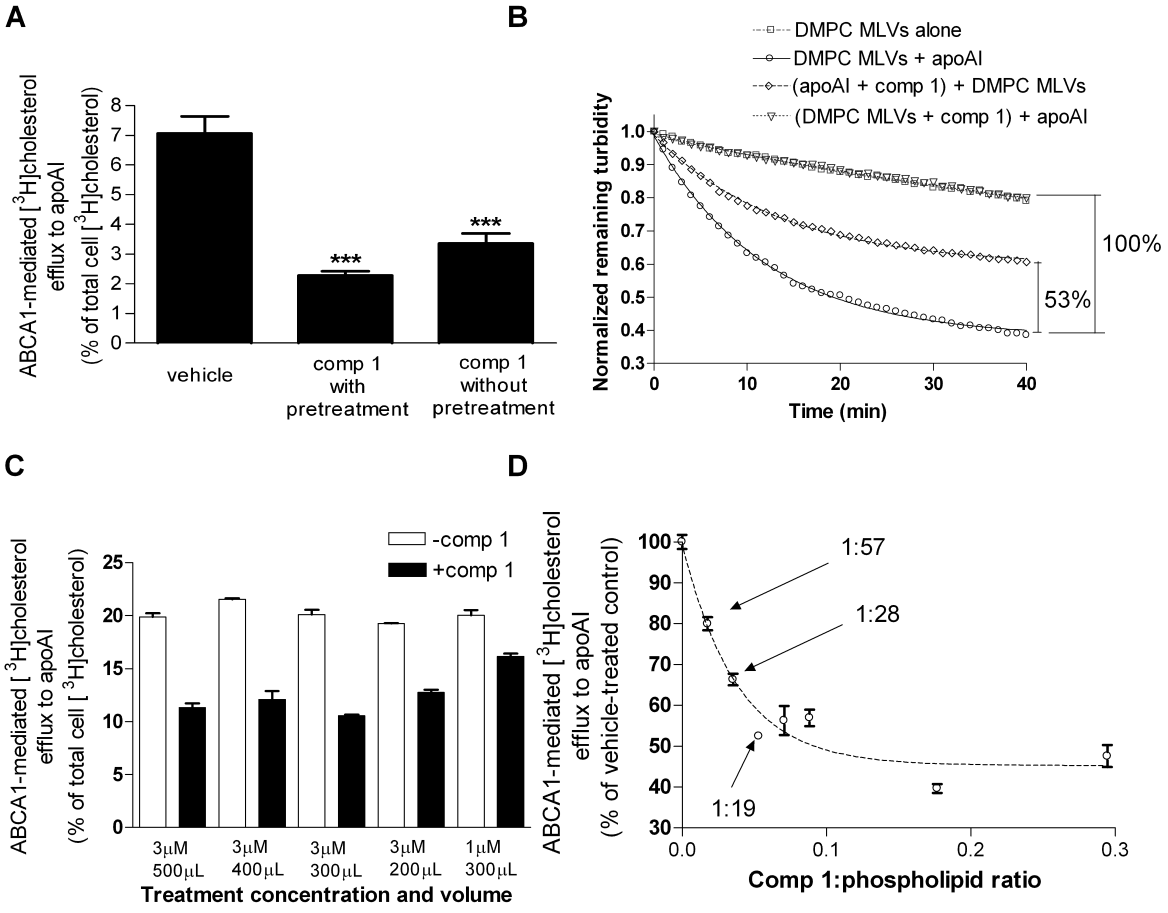
Supplemental Figure I. Inhibitory effect of compound 2 on the rHDL formation reaction in comparison with the inhibitory effect of compound 1. The DMPC:compound 1 or 2 ratio was 8:1.



Supplemental Figure II. Monitoring compound 1 partitioning into DMPC MLVs by tracing changes in DPH excitation spectrum. DMPC MLVs with 0.2 mole% DPH were incubated with the vehicle or 8.3 μmol/L compound 1 at the DMPC:compound 1 molar ratio 15:1 for 30 min at pH 7.4 or 3.0, followed by measurements of excitation spectrum in the 250-300 nm range (emission 427 nm). Compound 1 did not fluoresce, but notably altered excitation spectrum of DPH in the measured range. Partitioning of the compound into DMPC MLVs was not affected by the low pH. RFU – relative fluorescence units.

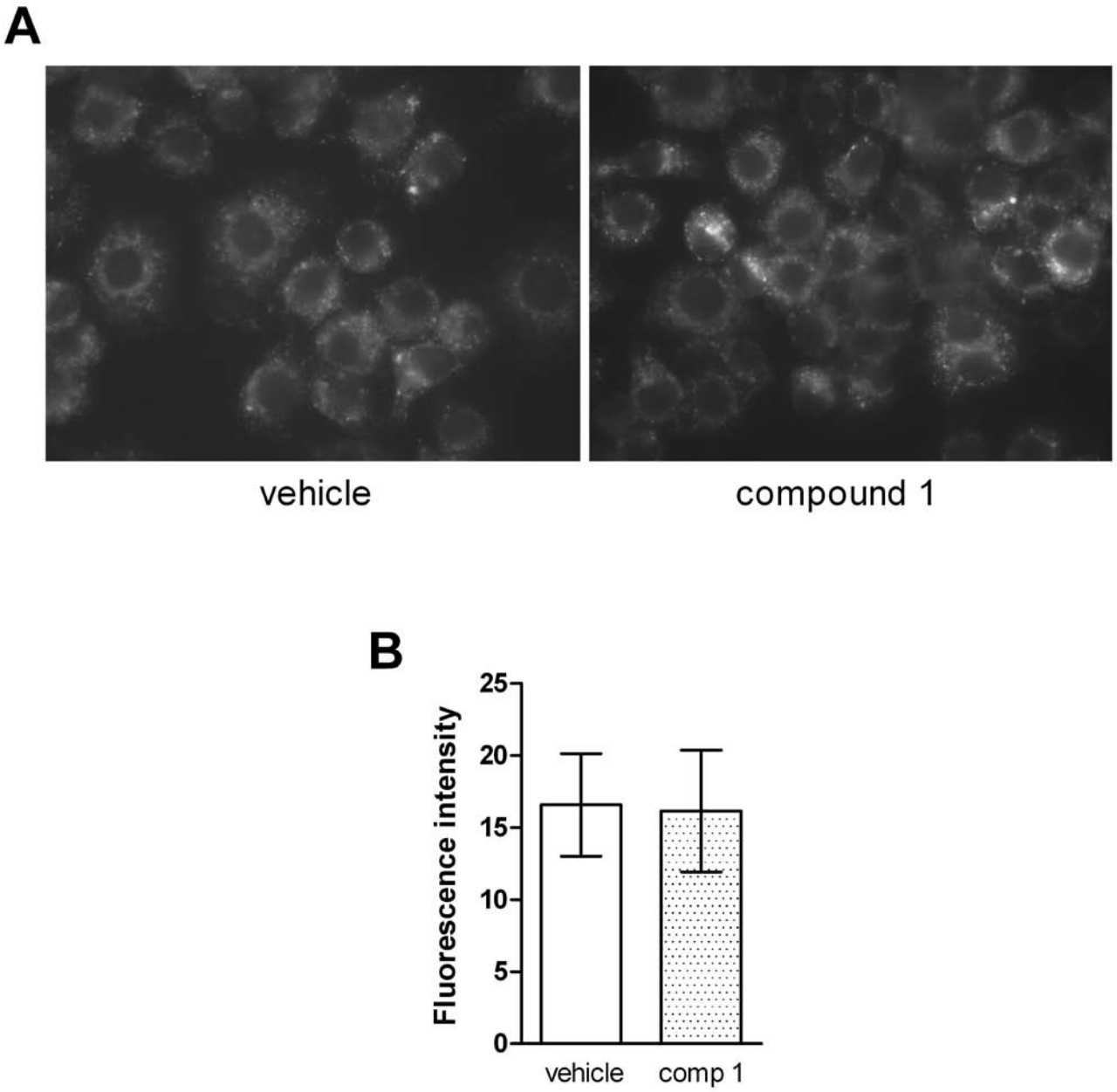


Supplemental Figure III. Compound 1 inhibits ABCA1-mediated cholesterol efflux to apoAI in BHK-ABCA1 (A) and HEK293-ABCA1-GFP (B) cells. ABCA1 expression in BHK cells was induced with mifepristone. HEK293 cells express ABCA1 constitutively at a low level. Cells were pretreated with 10 µmol/L compound 1 for 1 hour before addition of apoAI. The percentage of [^{3}H]cholesterol efflux to apoAI was calculated by subtracting the average percentage of [^{3}H]cholesterol efflux to medium without apoAI under a particular treatment (+/- mifepristone, +/- compound 1) from individual values of percentage of [^{3}H]cholesterol efflux to medium containing apoAI under the same treatment. Mean $\pm$ S.D.; *** - $p<0.001$, ** - $p<0.01$ by t-test.

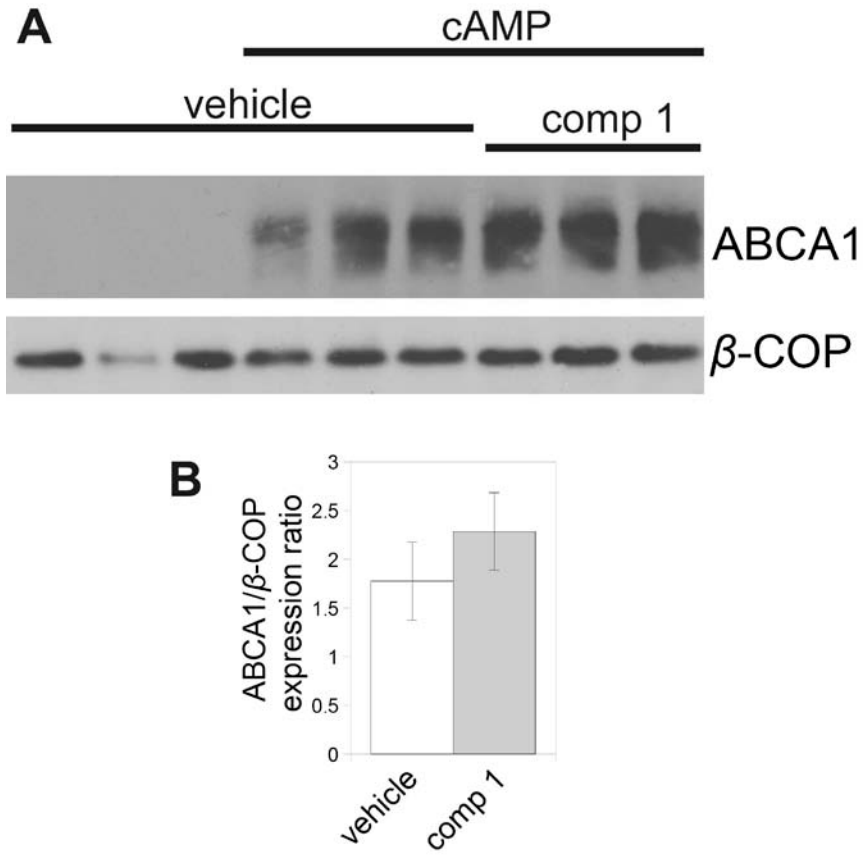


Supplemental Figure IV. Construction of the compound 1 dose-response curve. (A) When compound 1 (at 10 μmol/L) and apoAI were added to ABCA1-expressing RAW 264.7 cells simultaneously – without first pretreating the cells with the inhibitor, the compound suppressed ABCA1-mediated cholesterol efflux to apoAI by 50%, which is somewhat less than when the pretreatment step was included. (B) Similar behavior of compound 1 was observed in the rHDL assembly assay as well: when the compound and DMPC MVLs were incubated together for 30 min and then added to apoAI (compound 1:DMPC ratio 1:30), no formation of rHDL occurred; however, when the compound and apoAI were incubated together for 30 min and then added to DMPC MLVs (compound 1:DMPC ratio same as above), rHDL assembly proceeded to a significant extent. (C) ABCA1-mediated cholesterol efflux to apoAI as a function of the amount of added compound 1. The cholesterol efflux assay was modified in order to derive a reliable compound 1:cell phospholipid molar ratio at which the compound loses its activity. First, the inhibitor was added to RAW 264.7 cells only once – without pretreatment – together with apoAI. This blunted the magnitude of cholesterol efflux suppression relative the treatment scheme with a pretreatment (see panel A), but eliminated the need to estimate the amount of compound 1 that associated with cells during a pretreatment period. Second, the amount of medium added per well of a tissue culture plate was varied 500 to 200 μl. Compound 1 molecules residing in the

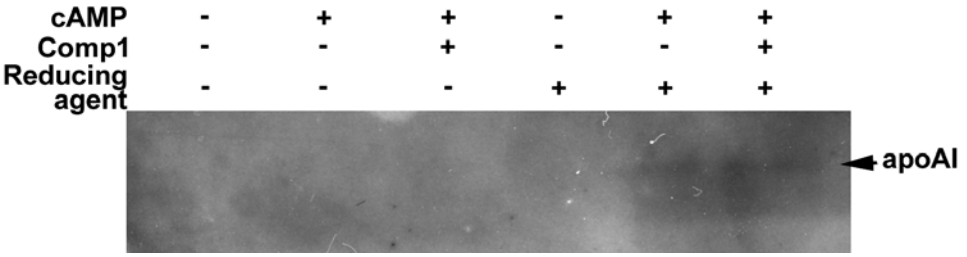
upper layers of the medium column may never reach cells growing on the well bottom and thus, never participate in suppression of the ABCA1-mediated cholesterol efflux and only distort the actual compound 1:cell phospholipid ratio. (D) The compound 1 dose-response curve. It was determined that a confluent population of RAW 264.7 cells (put through the same medium changes as in the cholesterol efflux assay and expressing ABCA1; see Supplemental Methods) in a well of a 24-well cell culture plate contains 0.526 ± 0.02 μg or 1.7×10^{-8} moles of inorganic phosphorus in the cellular lipid. Assuming that a molecule of phospholipid contains a single phosphate residue, the total amount of phospholipid in cells in a well of a 24-well plate would be 1.7×10^{-8} moles. (This assumption ignores such phospholipids as phosphatidylinositol phosphate that carry phosphate residues inaccessible to compound 1, but these species comprise a minor portion of the total phospholipid). The dosage of compound 1 was expressed as the ratio of moles of compound 1 added to a well of a 24-well plate divided by 1.7×10^{-8}, the number of moles of phospholipid in the same well, and the data in panels A and C were plotted as a composite dose-response curve. Mean $\pm$ S.D.; *** - $p<0.001$ by t-test.



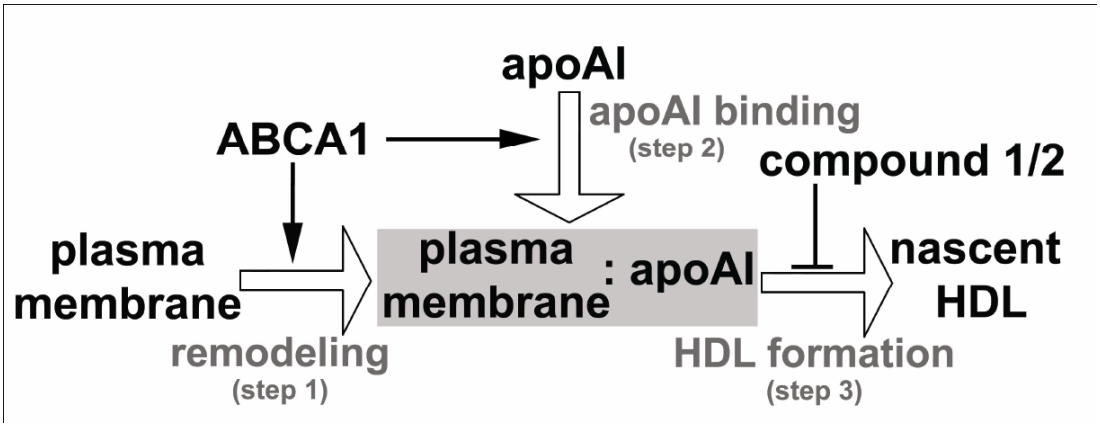
Online Figure V. Compound 1 does not inhibit transferrin endocytosis. (A) Representative images of RAW 264.7 cells pretreated with vehicle or 10 μmol/L compound 1 for 1 hour and exposed to fluorescently-labeled transferrin. (B) Mean pixel fluorescence intensity in the cell area (ROI) calculated from the images represented in A (30-40 cells per treatment). Mean ± S.D., $p>0.05$ by t-test.



Supplemental Figure VI. Compound 1 does not affect ABCA1 expression levels. (A) ABCA1 expression with and without compound 1 in RAW 264.7 cells. Cells were treated with 10 μmol/L compound 1 for 5 hours. With this treatment scheme, the inhibitor suppresses ABCA1-mediated cholesterol efflux to apoAI by 50% (see Supplemental Figure IVC). (B) Quantification of ABCA1 expression levels relative expression of *β*-COP. Band intensities in panel A were measured and expressed as the ratio of ABCA1 band intensity divided by *β*-COP band intensity. Mean $\pm$ S.D.



Supplemental Figure VII. Compound 1 does not affect ABCA1-apoAI cross-linking. RAW 264.7 cells were pretreated with 10 μmol/L compound 1 for 1 hour and incubated with 5 μg/ml apoAI in the presence of 10 μmol/L compound 1 for 2 hours. ABCA1-apoAI complexes were cross-linked with DSP, immunoprecipitated with an ABCA1 polyclonal antibody, resolved using SDS-PAGE, blotted and probed with an anti-apoAI antibody.



Supplemental Figure VIII. A three-step model of nascent HDL formation.

Supplemental References

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