

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart
Association®



Learn and Live SM

A Novel Compound Inhibits Reconstituted High-Density Lipoprotein Assembly and Blocks Nascent High-Density Lipoprotein Biogenesis Downstream of Apolipoprotein AI Binding to ATP-Binding Cassette Transporter A1-Expressing Cells

Nicholas N. Lyssenko, Gregory Brubaker, Bradley D. Smith and Jonathan D. Smith

Arterioscler Thromb Vasc Biol published online August 11, 2011

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association.
7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2011 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/content/early/2011/08/11/ATVBAHA.111.234906>

Data Supplement (unedited) at:

<http://atvb.ahajournals.org/content/suppl/2011/08/11/ATVBAHA.111.234906.DC1.html>

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at

<http://atvb.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:

journalpermissions@lww.com

Reprints: Information about reprints can be found online at

<http://www.lww.com/reprints>

A Novel Compound Inhibits Reconstituted High-Density Lipoprotein Assembly and Blocks Nascent High-Density Lipoprotein Biogenesis Downstream of Apolipoprotein AI Binding to ATP-Binding Cassette Transporter A1-Expressing Cells

Nicholas N. Lyssenko, Gregory Brubaker, Bradley D. Smith, Jonathan D. Smith

Objective—Nascent high-density lipoprotein (HDL) particles form from cellular lipids and extracellular lipid-free apolipoprotein AI (apoAI) in a process mediated by ATP-binding cassette transporter A1 (ABCA1). We have sought out compounds that inhibit nascent HDL biogenesis without affecting ABCA1 activity.

Methods and Results—Reconstituted HDL (rHDL) formation and cellular cholesterol efflux assays were used to show that 2 compounds that bond via hydrogen with phospholipids inhibit rHDL and nascent HDL production. In rHDL formation assays, the inhibitory effect of compound 1 (methyl 3 α -acetoxy-7 α ,12 α -di[(phenylaminocarbonyl)amino]-5 β -cholan-24-oate), the more active of the 2, depended on its ability to associate with phospholipids. In cell assays, compound 1 suppressed ABCA1-mediated cholesterol efflux to apoAI, the 18A peptide, and taurocholate with high specificity, without affecting ABCA1-independent cellular cholesterol efflux to HDL and endocytosis of acetylated low-density lipoprotein and transferrin. Furthermore, compound 1 did not affect ABCA1 activity adversely, as ABCA1-mediated shedding of microparticles proceeded unabated and apoAI binding to ABCA1-expressing cells increased in its presence.

Conclusion—The inhibitory effects of compound 1 support a 3-step model of nascent HDL biogenesis: plasma membrane remodeling by ABCA1, apoAI binding to ABCA1, and lipoprotein particle assembly. The compound inhibits the final step, causing accumulation of apoAI in ABCA1-expressing cells. (*Arterioscler Thromb Vasc Biol.* 2011;31:00-00.)

Key Words: ABCA1 ■ apoAI ■ nascent HDL ■ rHDL ■ reverse cholesterol transport

Biogenesis of nascent high-density lipoprotein (HDL) particles marks the origins of the critically important antiatherogenic reverse cholesterol transport pathway, which removes excessive cholesterol from peripheral tissues, such as macrophages, and carries it to the liver.¹ Nascent HDL particles form from extracellular lipid-free apolipoprotein AI (apoAI) and cellular lipids in a process mediated by ATP-binding cassette transporter A1 (ABCA1).² In vitro studies suggest that the process of nascent HDL biogenesis consists of at least 3 steps. The first step is apparent when cells express ABCA1 but apoAI is not present in the medium. Even without the apolipoprotein, the transporter creates significant changes in the plasma membrane organization. In particular, it induces redistribution of phosphatidylserine to the cell surface and drives production of apoAI-free microparticles.^{3–5} On addition of apoAI to ABCA1-expressing cells, the apolipoprotein rapidly binds to the plasma membrane, and newly formed nascent HDL particles appear in the

medium at a detectable level in 15 minutes.⁶ At 21°C, apoAI still binds to ABCA1-expressing cells, but formation of nascent HDL particles completely ceases.⁶ The difference in sensitivity to temperature suggests that apoAI binding to the ABCA1-remodeled plasma membrane and apoAI and lipid assembly into lipoprotein particles are distinct—second and third, respectively—steps of nascent HDL biogenesis.

In addition to nascent HDL, apoAI can form reconstituted HDL (rHDL) particles in the absence of ABCA1 from liposomes made of synthetic short-chain phospholipids or certain physiologically relevant lipid mixtures.^{7,8} rHDL particles are similar in size and shape to nascent HDL. For a large group of apoAI mutants with widely divergent abilities to form rHDL and nascent HDL, the efficiency of rHDL formation positively correlates with the efficiency of nascent HDL biogenesis.⁸ This suggests that the 2 processes—ie, apoAI and synthetic lipid assembly into rHDL and apoAI and cell lipid assembly into nascent HDL (step 3 in nascent HDL biogenesis)—share substantial mechanistic similarities.

Received on: July 14, 2011; final version accepted on: July 29, 2011.

From the Department of Cell Biology, Cleveland Clinic, Cleveland, OH (N.N.L., G.B., J.D.S.); Lipid Research Group, Gastroenterology, Hepatology and Nutrition Division, Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA (N.N.L.); Department of Chemistry and Biochemistry, University of Notre Dame, South Bend, IN (B.D.S.); Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University School of Medicine, Cleveland, OH (J.D.S.).

Correspondence to Jonathan D. Smith, Box NC10, Cleveland Clinic, 9500 Euclid Ave, Cleveland, OH 44195. E-mail smithj4@ccf.org

© 2011 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.111.234906

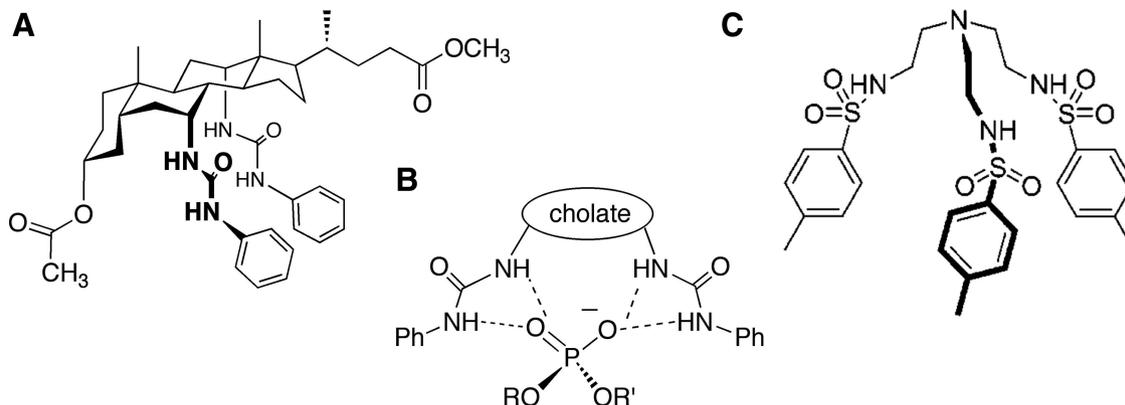


Figure 1. Chemical structures of compounds 1 and 2. A, Chemical structure of compound 1. B, Hydrogen bonding between compound 1 and the phosphate residue of the phospholipid. C, Chemical structure of compound 2.

To unambiguously show the sequential nature of nascent HDL biogenesis, we have sought out chemicals that inhibit rHDL and nascent HDL formation without affecting ABCA1 activity. Our attention was drawn to a group of compounds called synthetic chemical phospholipid translocases/scramblases, which had been designed to form hydrogen bonds with the phosphate residue and carboxyl group of phospholipids.^{9,10} The original purpose of these chemicals was to facilitate phospholipid transbilayer flip-flop by concealing the negative charges inside a hydrophilic pocket.¹¹ As phospholipid translocases, these compounds turned out to be ineffective in nucleated cells.¹² Nonetheless, we hypothesized that large hydrogen-bonded translocase-phospholipid complexes could interfere with the apoAI-lipid assembly into lipoprotein particles. Here, we show that a representative member of the translocase group, methyl 3 α -acetoxy-7 α ,12 α -di[(phenylaminocarbonyl)amino]-5 β -cholan-24-oate (referred to in the following as compound 1), inhibits rHDL and nascent HDL formation, causes accumulation of apoAI in ABCA1-expressing cells, and thus resolves the final stages of nascent HDL biogenesis into individual steps in cultured cells under normal physiological conditions.

Methods

Compound 1 and *N*-[2-((4-nitrophenylaminocarbonyl)amino)ethyl]-*N,N*-di[2-((4-methylphenylsulfonyl)amino)ethyl]amine (compound 2) were synthesized in-house as previously described.^{9,10,13} rHDL formation assays were performed by reacting dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (MLVs) and human apoAI in either Tris-buffered saline-EDTA (pH 7.4)⁷ or glycine-HCl (pH 3.0)¹⁴ buffer at ambient instrument temperature (24.3 to 25.6°C) in the presence of the vehicle (dimethyl sulfoxide [DMSO]) or one of the compounds. DMPC MLV solubilization by apoAI was monitored by measuring sample turbidity (absorbance) at 325 nm. For steady-state 1,6-diphenyl-1,3,5-hexatriene (DPH) anisotropy measurements, DMPC MLVs spiked with DPH to 0.2 mol% were extruded 19 times through a polycarbonate membrane with 100-nm pores (Whatman) using a mini-extruder (Avanti Polar Lipids) to derive large unilamellar vesicles; the large unilamellar vesicles were incubated with DMSO or one of the compounds for 30 minutes, followed by measurements of DPH anisotropy at 357 nm excitation and 427 nm emission wavelengths at a range of temperatures in a water-jacketed spectrofluorometer. A previously described⁶ 4-day protocol was followed to assess effects of the compounds on cellular cholesterol and phospholipid efflux to apoAI from cells either uninduced or induced to express ABCA1. The endocytosis assay was

performed as the following: cells pretreated with DMSO, compound 1, or dynasore¹⁵ were exposed to [³H]cholesterol-labeled acetylated low-density lipoprotein (AcLDL) for 15 to 20 minutes at either 37°C or 20°C in the presence of the same compounds, washed with an acidic buffer (0.15 mol/L NaCl, pH 3.0), and extracted with hexane/isopropanol. AcLDL endocytosis was calculated by subtracting the ³H cell-associated counts at 20°C from those at 37°C. For the microparticle production assay, cells were labeled with [³H]cholesterol; treated with or without cAMP to induce ABCA1; pretreated with DMSO, compound 1, or wheat germ agglutinin/DMSO⁴ for 1 hour; and further exposed to the same treatment for 8 hours. Then, cells and media were collected and analyzed for ³H counts as in the cholesterol efflux assay, and microparticle production was expressed as the percentage of cellular [³H]cholesterol released to media without any lipid acceptors. Fluorescent microscopy has been previously described.⁶ Detailed protocols of all methods are included in the Supplemental Material, available online at <http://atvb.ahajournals.org>.

Results

Compounds 1 and 2 Inhibit rHDL Formation From DMPC Liposomes

Compound 1 is a derivative of cholate in which the hydroxyl groups at positions C-7 and C-12 are replaced with phenylurea groups for hydrogen bonding with the phosphate residue of phospholipids (Figure 1A and 1B).¹⁰ At the DMPC phase transition temperature (T_m), DMPC MLVs and apoAI spontaneously and rapidly form rHDL particles.⁷ Because MLVs are turbid, whereas rHDL particles are pellucid, rHDL assembly from MLVs can be monitored by measuring the remaining MLV turbidity.^{7,16} To determine whether compound 1 affects rHDL formation, DMPC MLVs were incubated with the compound for 30 minutes to allow compound 1 to enter the bilayer, followed by addition of apoAI and immediate commencement of turbidity measurements. The concentration of compound 1 was kept constant at a level (8.3 μ mol/L) below its critical micelle concentration to ensure that compound 1 micelles did not contribute to sample turbidity. Whereas the DMPC:apoAI molar ratio was also kept constant at a value (84:1) at which rHDL formation proceeds to completion,¹⁶ the absolute concentrations of DMPC and apoAI were varied to achieve different compound 1:DMPC and compound 1:apoAI molar ratios. At compound 1:DMPC molar ratios of 1:60 and 1:44 (compound 1:apoAI molar ratios of 1:0.71 and 1:0.53, respectively), compound 1 did not affect rHDL formation (Figure 2A and data not shown); however, at compound 1:DMPC molar ratios of 1:30, 1:15,

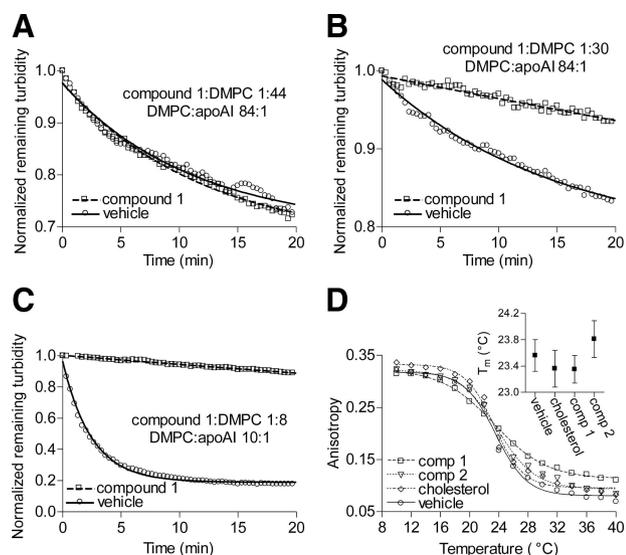


Figure 2. Inhibition of reconstituted high-density lipoprotein (rHDL) formation by compound 1. A, At a molar ratio of 1:44 for compound 1:dimyristoylphosphatidylcholine (DMPC), compound 1 did not have an effect on rHDL formation. apoAI indicates apolipoprotein AI. B, However, at a compound 1:DMPC molar ratio of 1:30, the chemical suppressed the reaction. C, Compound 1 also vigorously suppressed rHDL formation when the compound 1:DMPC molar ratio was high but the compound 1:apoAI ratio was low. D and inset, Addition of compound (comp) 1 or 2 to DMPC did not significantly change the T_m of the phospholipid. 1,6-Diphenyl-1,3,5-hexatriene anisotropy data points were fitted with sigmoidal curves to derive phase transition temperatures. Error bars represent 95% confidence limits.

and 1:7.4 (compound 1:apoAI molar ratios of 1:0.35, 1:0.18, and 1:0.09, respectively), the chemical markedly inhibited the process (Figure 2B and data not shown). Circular dichroism measurements showed that there were only minor differences in the structure of apoAI in the presence of compound 1 at the ineffective compound 1:apoAI molar ratio of 1:44 and the inhibitory compound 1:apoAI molar ratio of 1:30 (apoAI was 50.6% and 53.7% helix, respectively), suggesting that the compound 1 did not alter the apoAI secondary structure. Furthermore, when the compound 1:DMPC molar ratio was 1:8 (ie, within the range in which compound 1 inhibited rHDL formation) and the compound 1:apoAI molar ratio was changed to 1:0.8 (ie, outside the effective range of the compound), the compound still suppressed rHDL formation (Figure 2C). The latter observation suggests that the inhibitory potency of compound 1 depends on its stoichiometric ratio with DMPC and not with apoAI and thus that the compound likely impairs the ability of the lipid and not the apolipoprotein to form rHDL.

Compound 2 (Figure 1C), a derivative of tris(2-aminoethyl)amine, carries 2 arylsulfonamide groups and 1 nitrophenylurea group that can bond via hydrogen with the phosphate residue of phospholipids.⁹ This compound partitions less readily to the lipid bilayer in comparison with compound 1.¹⁰ At a compound 2:DMPC molar ratio of 1:8 (DMPC:apoAI molar ratio of 84:1), compound 2 also inhibited rHDL formation, but less effectively than compound 1 (Supplemental Figure D).

The rate of rHDL formation declines precipitously as the reaction temperature deviates above or below the phospholipid

T_m .⁷ Temperature-dependent changes in steady-state anisotropy of DPH were measured to determine whether addition of compound 1 or 2 affects the T_m of DMPC. DMPC large unilamellar vesicles spiked with DPH were incubated with one of the compounds, cholesterol (inactive control), or the vehicle for 30 minutes at ambient temperature to allow the compounds and cholesterol to enter the bilayer, followed by DPH anisotropy measurements. The compound/cholesterol:DMPC molar ratio was 1:7.4. The calculated T_m for pure DMPC was 23.6°C, a value similar to those previously published.¹⁷ Neither compound significantly shifted the T_m of DMPC toward higher or lower temperatures (Figure 2D). The above observations suggest that compounds 1 and 2 suppress rHDL formation by acting on the phospholipid without affecting its T_m .

Hydrogen Bonding Between Compound 1 and DMPC Is Essential for Inhibition of rHDL Formation

The finding that compounds 1 and 2, which are structurally very divergent, both suppress rHDL formation suggests that hydrogen-bonding with phospholipids—ie, the intended activity of these chemicals¹¹—in all probability is responsible for the compounds' inhibitory action. To further test this conjecture, rHDL formation assays were carried out at pH 3.0, which is below the apparent pKa of 3.5 for the phosphate group of phosphatidylcholine.¹⁸ DMPC MLVs and apoAI form rHDL particles just as readily at a low as at a neutral pH.¹⁴ However, at pH 3.0, most DMPC molecules would be protonated at the phosphate residue and unable to form hydrogen bonds. Compound 2 is not suitable for this experiment, because it undergoes protonation at the tertiary amine with a pKa of 5.0 and assumes a conformation that binds phospholipids poorly.¹⁹ Compound 1 can undergo protonation at the carbonyl oxygen of the urea groups; however, the pKa of protonated urea is close to zero, ie, much lower than the pH of 3.0.²⁰ Furthermore, it was fortuitously discovered that compound 1 alters the excitation spectrum of DPH without altering its anisotropic behavior (Supplemental Figure II). DPH is a small cylindrical molecule that resides in the hydrophobic middle of the bilayer.²¹ To interact with DPH, compound 1 must penetrate into the hydrophobic middle as well. At pH 7.4 and pH 3.0, compound 1 affected DPH excitation spectrum identically, indicating that the low pH did not hinder partitioning of the compound to the lipid bilayer (Supplemental Figure II). At a compound 1:DMPC molar ratio of 1:15 (a ratio within the effective range of the compound) and pH 3.0, compound 1 failed to suppress rHDL formation (Figure 3). This suggests that hydrogen bonding between compound 1 and DMPC is essential for suppression of the reaction.

Compounds 1 and 2 Specifically Inhibit Nascent HDL Formation

RAW 264.7 murine macrophage cells generate nascent HDL in the presence of exogenous lipid-free apoAI after a treatment with a cAMP analog to induce ABCA1 expression.⁶ Because cholesterol and phospholipids are incorporated into the lipoprotein concurrently, nascent HDL biogenesis can be readily measured as the percentage of total cell cholesterol or phospholipid that is released to apoAI when ABCA1 is expressed.⁶ As is evident from the lack of effect on receptor-mediated endocytosis (see below), compound 1 is not toxic to cells at the concentra-

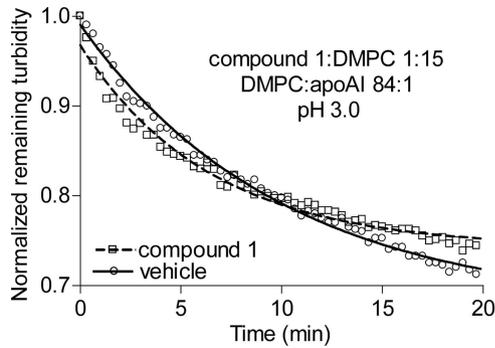


Figure 3. Compound 1 did not inhibit reconstituted high-density lipoprotein (rHDL) formation at pH 3.0. At a compound 1:dimyristoylphosphatidylcholine (DMPC) molar ratio of 1:15 and pH 3.0, compound 1 did not have an effect on rHDL formation.

tions used in this study. Compound 2 also does not exhibit cell toxicity at the doses used ($LD_{50} > 100 \mu\text{mol/L}$).¹² To determine whether compounds 1 and 2 inhibit nascent HDL biogenesis, RAW 264.7 cells labeled with [³H]cholesterol and induced to express ABCA1 were pretreated with 10 $\mu\text{mol/L}$ compound 1 for 1 hour or 30 $\mu\text{mol/L}$ compound 2 for 3 hours; thereafter, the pretreatment medium was replaced with fresh medium that contained lipid-free apoAI and the same compound at the same concentration as during the pretreatment, and the cells were allowed to produce nascent HDL for 4 hours. Compound 1 suppressed ABCA1-mediated cholesterol efflux to apoAI by more than 70% (Figure 4A). Even through applied at a higher concentration during a longer pretreatment, compound 2 was less effective and suppressed the process by only $\approx 50\%$ (Figure 4B). The lower efficacy of compound 2 in this assay is in line with its lower efficacy in the rHDL formation assays (Supplemental Figure I). In addition to RAW 264.7 cells, compound 1 applied at the same concentration with the same treatment scheme significantly inhibited ABCA1-mediated cholesterol efflux to apoAI in BHK-ABCA1 and HEK293-ABCA1 cells (Supplemental Figure III). The 1-hour, 10 $\mu\text{mol/L}$ treatment with compound 1 suppressed ABCA1-mediated phospholipid efflux to apoAI in RAW 264.7 cells by approximately 37% (Figure 4C). Nascent HDL particles arise as several species with different lipid compositions.²² The greater effect of compound 1 on cholesterol than phospholipid efflux suggests that this inhibitor may restrict production of cholesterol-rich species to a greater degree.

A dose-response curve for the suppression of ABCA1-mediated cholesterol efflux to apoAI by compound 1 was derived to identify the threshold compound 1:cellular phospholipid molar ratio at which the inhibitor loses its efficacy (Supplemental Figure IV). Based on the previous observations that compound 1 readily enters the cell and freely disperses among the subcellular membranes,¹² it was assumed that all cellular phospholipid was available for bonding with the inhibitor. The dosage of compound 1 was expressed as the ratio of moles of this inhibitor added per cell culture well to moles of cell phospholipid in the same well. Compound 1's efficacy remained essentially unchanged at the compound 1:cellular phospholipid molar ratios from 1:3.4 (10 $\mu\text{mol/L}$ compound 1 in 500 μL of medium per well) to 1:19 (3 $\mu\text{mol/L}$ compound 1 in 300 μL of medium per well) but

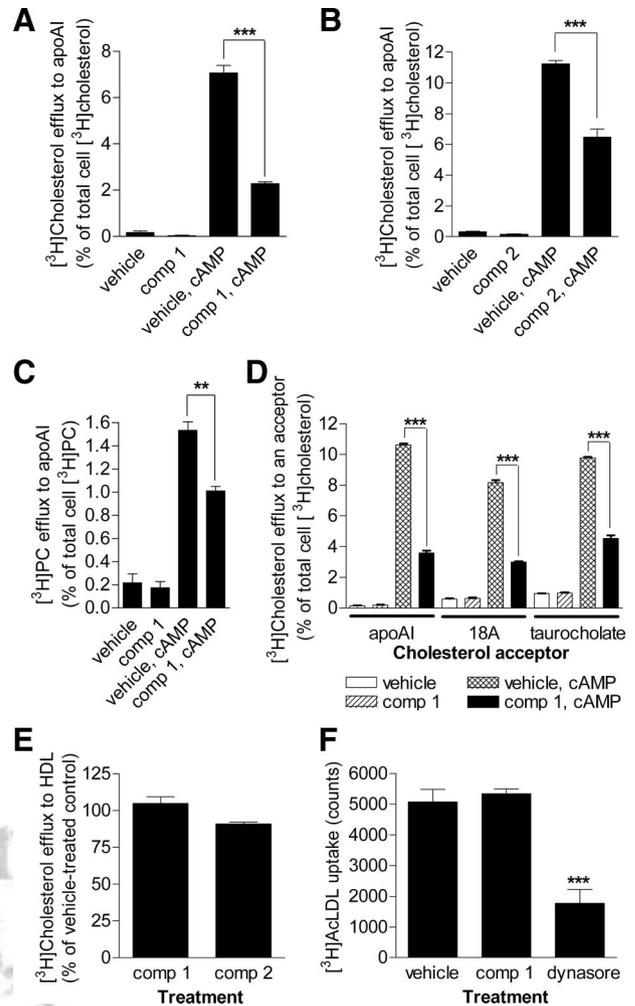


Figure 4. Compounds 1 and 2 suppress nascent high-density lipoprotein (HDL) formation with high specificity in RAW 264.7 cells. A and B, Inhibition of ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux to apolipoprotein AI (apoAI) by compounds (comp) 1 (A) and 2 (B). C, Inhibition of ABCA1-mediated phospholipid efflux to apoAI by compound 1. D, Compound 1 inhibited ABCA1-mediated cholesterol efflux irrespective of cholesterol acceptor. Concentrations of apoAI and 18A were 5 $\mu\text{g/mL}$; sodium taurocholate was applied at 4 mmol/L . Compounds 1 and 2 were applied at 10 and 30 $\mu\text{mol/L}$, respectively, with pretreatments of 1 and 3 hours, respectively. 8Br-cAMP (0.3 mmol/L) was used to induce ABCA1 expression. The percentage of [³H]cholesterol efflux to an acceptor was calculated by subtracting the percentage of [³H]cholesterol efflux to medium without this acceptor from the percentage of [³H]cholesterol efflux to medium with the acceptor. Data are mean \pm SD; *** $P < 0.001$ by *t* test. E, Compounds 1 and 2 applied to RAW 264.7 cells at 10 and 30 $\mu\text{mol/L}$, respectively, with pretreatments of 1 and 3 hours, respectively, did not have an effect on cellular cholesterol efflux to HDL. The percentage of ABCA1-independent [³H]cholesterol efflux to HDL was calculated by subtracting the percentage of efflux to medium from the percentage of efflux to HDL. The percentage of [³H]cholesterol efflux to HDL under the vehicle treatment was set to 100%. Data are mean \pm SD; $P > 0.5$ by *t* test. F, Compound 1 at 10 $\mu\text{mol/L}$ with a pretreatment did not alter AcLDL endocytosis in RAW 264.7 cells. Dynasore (160 $\mu\text{mol/L}$), an inhibitor of dynamin,¹⁵ was used as a positive control. Data are mean \pm SD; *** $P < 0.001$ vs other treatments by ANOVA with the Dunnett multiple comparison post test.

began declining at the ratios lower than 1:19 (Supplemental Figure IVD). Given the drastic differences between the systems, the threshold compound 1:phospholipid molar ratios at which compound 1 loses its efficacy in DMPC liposomes (1:30) and in cells (1:19) are remarkably similar.

In addition to apoAI, ABCA1 mediates cholesterol efflux to peptide 18A (also called 2F), a small amphipathic peptide,²³ and sodium taurocholate.²⁴ At a concentration of 10 $\mu\text{mol/L}$ with a pretreatment, compound 1 suppressed ABCA1-mediated cholesterol efflux to 5 $\mu\text{g/mL}$ 18A, 4 mmol/L taurocholate, and 5 $\mu\text{g/mL}$ apoAI to a similar extent (Figure 4D). This shows that the nature of cholesterol acceptor is irrelevant to the inhibitory action of the compound.

RAW 264.7 cells in the absence of ABCA1 induction release cholesterol to HDL.²⁵ The treatment schemes with compounds 1 and 2 that dramatically suppressed ABCA1-mediated cholesterol efflux to apoAI did not significantly affect ABCA1-independent efflux of this lipid to HDL (Figure 4E). Effects of compound 1 on receptor-mediated endocytosis of AcLDL and transferrin were also assessed. At a concentration of 10 $\mu\text{mol/L}$ with a pretreatment, compound 1 did not block uptake of either ligand, whereas the endocytosis inhibitor dynasore¹⁵ markedly suppressed AcLDL uptake (Figure 4F and Supplemental Figure V). Finally, compound 1 did not affect ABCA1 expression levels at concentrations sufficient to suppress ABCA1-mediated cholesterol efflux to apoAI (Supplemental Figure VI). Cumulatively, the above observations suggest that compound 1 specifically inhibits nascent HDL biogenesis by acting on cell membranes that are remodeled by ABCA1.

Compound 1 Does Not Affect ABCA1-Driven Generation of Microparticles

ABCA1 renders the plasma membrane less rigid and induces exposure on the cell surface of plasma membrane phosphatidylserine, which is normally sequestered in the inner leaflet of the organelle.^{3,4} These ABCA1-driven changes in the plasma membrane organization promote budding of a certain species of microparticles irrespective of presence or absence of apoAI.⁴ At a concentration of 10 $\mu\text{mol/L}$ with a pretreatment, compound 1 did not affect microparticle formation (Figure 5). In contrast, in line with previous observations,⁴ wheat germ agglutinin significantly inhibited microparticle biogenesis. Using ABCA1-driven production of microparticles as a proxy for ABCA1 activity, this observation suggests that compound 1 does not upset the proper functioning of the transporter.

Compound 1 Promotes Retention of Bound ApoAI in ABCA1-Expressing Cells

Lipid-free apoAI binds to the plasma membrane of ABCA1-expressing cells before the appearance of nascent HDL in the medium.⁶ A fraction of cell-bound apoAI is further taken up into subcellular compartments.²⁶ To determine whether compound 1 affects the apoAI binding step, RAW 264.7 cells induced to express ABCA1 were pretreated with the compound at 10 $\mu\text{mol/L}$ for 1 hour and thereafter exposed to fluorescently labeled apoAI and simultaneously treated with compound 1 for 15 or 45 minutes. A detectable level of nascent HDL first appears in the medium of ABCA1-expressing cells \approx 15 minutes

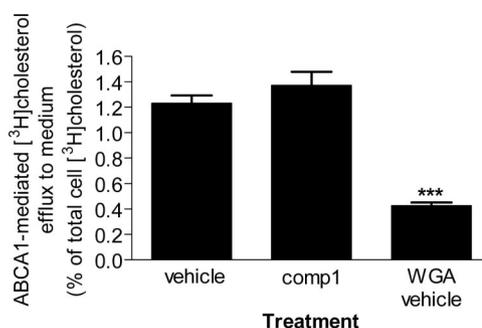


Figure 5. Compound (comp) 1 does not affect ATP-binding cassette transporter A1 (ABCA1)-driven production of microparticles. ABCA1-mediated efflux of cellular [³H]cholesterol to the medium in the absence of apolipoprotein AI (apoAI) was taken as a measure of microparticle production. To calculate ABCA1-specific efflux, the percentage of [³H]cholesterol efflux from cells not induced to express ABCA1 was subtracted from the percentage of [³H]cholesterol efflux from ABCA1-expressing cells. Wheat germ agglutinin (WGA) was used as a positive control known to reduce microparticle production.⁴ Data are mean \pm SD; *** $P < 0.001$ by *t* test.

after addition of the apolipoprotein.⁶ Compound 1 and vehicle-treated ABCA1-expressing cells bound and internalized similar amounts of the labeled apoAI after a 15-minute incubation period (Figure 6C, 6D, and 6G). However, after a 45-minute incubation with the labeled apoAI, compound 1-treated cells bound and internalized significantly more of the label than vehicle-treated cells (Figure 6E, 6F, and 6H). Furthermore, after the 15-minute incubation, the amount of punctate fluorescence in subcellular compartments appeared to be similar in the compound treated and untreated cells (Figure 6C and 6D). But after the 45-minute incubation—30 minutes after the onset of apoAI release—cells treated with compound 1 appeared to contain more and brighter subcellular fluorescent foci (Figure 6E and 6F). Complex formation between ABCA1 and apoAI is required for nascent HDL biogenesis and for partial protection of the transporter from degradation in the intracellular compartment.^{27,28} At 10 $\mu\text{mol/L}$ with a pretreatment, compound 1 did not suppress cross-linking between ABCA1 and apoAI, suggesting that it did not interfere with the complex formation (Supplemental Figure VII). The above observations indicate that compound 1 does not affect apoAI binding to ABCA1-expressing cells or to ABCA1 but, by shutting down formation of nascent HDL and thus halting release of cell-bound apoAI, promotes accumulation of the apolipoprotein in subcellular compartments.

Discussion

The effects of compound 1 on nascent HDL biogenesis are consistent with a 3-step model of this process (Supplemental Figure VIII). The first step entails remodeling of the plasma membrane by ABCA1.³ In the second step, apoAI specifically binds to ABCA1, as is evident from the apoAI-ABCA1 cross-linking studies,²⁷ and is then brought in contact with the remodeled membrane regions. In vivo, these 2 steps likely occur simultaneously. The third step is the spontaneous assembly of the remodeled-region lipids and apoAI into the nascent HDL lipoprotein particle.

ABCA1-Mediated Membrane Remodeling

One feature of ABCA1 remodeling of the plasma membrane is increased cell surface phosphatidylserine exposure, al-

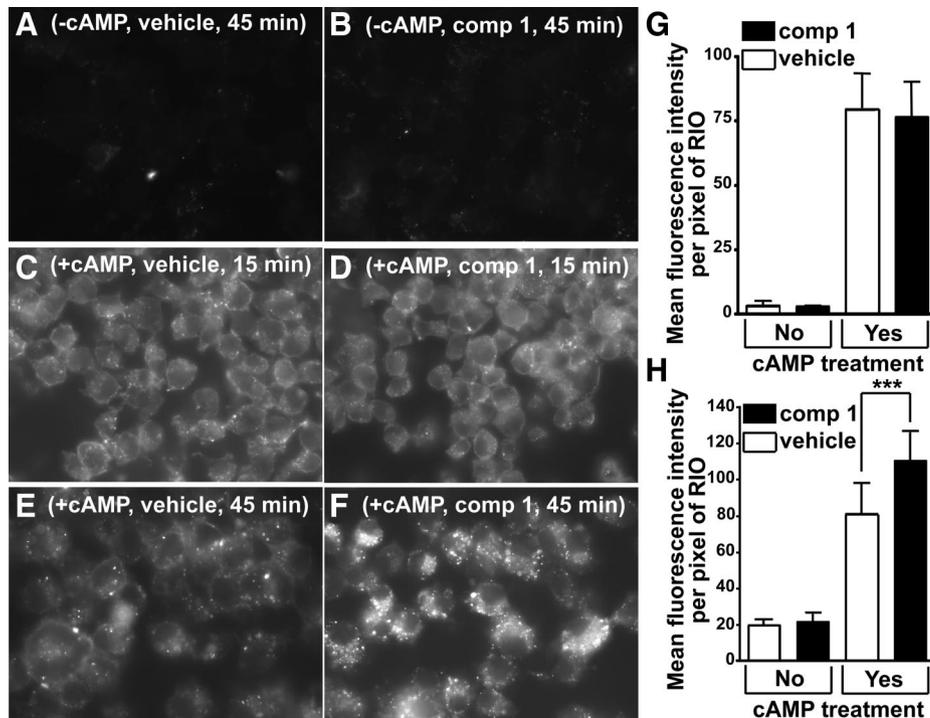


Figure 6. Compound (comp) 1 promotes retention of apolipoprotein AI (apoAI) bound to ABCA1-expressing cells. A and B, RAW 264.7 cells not induced to express ABCA1 did not appreciably bind Alexa Fluor 568–labeled apoAI. C and D, Cells induced to express ABCA1 and incubated with Alexa Fluor 568–labeled apoAI for 15 minutes bound and internalized the apolipoprotein robustly regardless of the treatment with compound 1. E and F, Compound 1–treated cells incubated with labeled apoAI for 45 minutes bound and internalized more of the apolipoprotein than vehicle-treated cells exposed to apoAI for the same duration. G and H, Mean pixel fluorescence intensity within a cell/region of interest calculated from the images shown in A to F (30 to 40 cells per treatment). G, 15-minute incubation; H, 45-minute incubation. Data are mean \pm SD; *** P < 0.001 by t test.

though cell surface phosphatidylserine by itself does not support lipid efflux to apoAI.⁵ A consequence of ABCA1 remodeling of the plasma membrane is the apolipoprotein-independent release of microparticles.⁴ Our data show that compound 1 does not impair this function and thus does not alter the first step of our 3-step model. Some particulars of ABCA1-mediated cholesterol efflux to taurocholate suggest that only certain membrane regions—likely those in the vicinity of the transporter—are remodeled. The ability of taurocholate to solubilize bilayers depends on its concentration and on bilayer stability.²⁹ Taurocholate at 4 mmol/L can induce cholesterol efflux (a proxy for membrane solubilization) only when ABCA1 is expressed (Figure 4D); however, 10 mmol/L taurocholate promotes robust ABCA1-independent cholesterol efflux (data not shown). This suggests that ABCA1 renders some regions of the plasma membrane less stable and more susceptible to the bile acid, relative the bulk of the organelle. Compound 1 may accumulate in these remodeled regions and protect the bilayer from low but not high concentrations of taurocholate.

ApoAI Binding to ABCA1-Expressing Cells

ApoAI is readily cross-linked to ABCA1.² Furthermore, those apoAI mutants that exhibit high affinity for the transporter in the cross-linking assay are also very efficient at formation of nascent HDL.²⁷ A recent study reported that a large portion of cell-bound apoAI cannot be cross-linked to the transporter.³⁰ This observation is consistent with the

mechanism in which apoAI first binds to ABCA1 and thereafter associates with ABCA1-modified lipid sites, where it briefly resides before forming nascent HDL. Unimpaired binding of apoAI to ABCA1-expressing cells and to ABCA1 in the presence of compound 1 suggests that the compound does not interfere with this step of nascent HDL biogenesis.

Assembly of ApoAI and Cellular Membrane Lipids Into Nascent HDL

The final step in HDL biogenesis is the assembly of cell-bound apoAI and membrane lipids into nascent HDL particles and particle release from the cell. Several lines of evidence suggest that compound 1 inhibits this final step. First, as outlined above, the first 2 steps are not impaired by the compound. Second, the bilayer stabilizing activity of compound 1 that protects membranes against solubilization by taurocholate would also likely protect the same membranes against solubilization by apoAI. Third, accumulation of cell-bound apoAI in the presence of the compound indicates that the apolipoprotein fails to form nascent HDL. Fourth, compound 1 inhibits rHDL formation, demonstrating that it can interfere with apoAI and lipid assembly into lipoprotein particles. Solubilization of phosphatidylcholine liposomes by taurocholate begins with adsorption of the bile acid to the bilayer surface via interaction with the charged lipid headgroups.³¹ Compound 1 likely blocks taurocholate solubilizing activity at this first step by forming hydrogen bonds with the phosphate residue of glycerophospholipids

and preventing the bile acid from electrostatically interacting with the lipid charges. Charge interactions are also critical for adsorption to the bilayer surface of cationic amphipathic peptides, such as 18A, and cationic amphipathic helix-containing proteins, such as apoA1.^{32,33} It is likely that hydrogen bonding of compound 1 to glycerophospholipids forestalls electrostatic interactions between ABCA1-recruited apoA1 and ABCA1-remodeled plasma membrane regions.

To the best of our knowledge, compound 1 is the first known chemical that inhibits both rHDL formation and the lipoprotein assembly step of nascent HDL biogenesis. The finding that the 2 are susceptible to the same compound (as well as to compound 2) strongly suggests they are mechanistically similar. This implies that after ABCA1 prepares a region of the plasma membrane and brings apoA1 in close apposition to it, the apolipoprotein associates with the bilayer without further assistance from the transporter. Thus, rHDL formation is a faithful model of the assembly step of nascent HDL biogenesis. Compound 1 is a very useful tool for investigations of rHDL and nascent HDL assembly. Future research with this compound may aid in further elucidation of the molecular mechanism of apoA1-driven lipoprotein production.

Sources of Funding

This work was supported by National Institutes of Health Grants HL66082 and HL098055 (to J.D.S.) and American Heart Association Postdoctoral Fellowship 10POST3630047 (to N.N.L.).

Disclosures

None.

References

- Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? *Circulation*. 2006;113:2548–2555.
- Yvan-Charvet L, Wang N, Tall AR. Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. *Arterioscler Thromb Vasc Biol*. 2010;30:139–143.
- Hamon Y, Brocardo C, Chambenoit O, Luciani MF, Toti F, Chaslin S, Freyssinet JM, Devaux PF, McNeish J, Marguet D, Chimini G. ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. *Nat Cell Biol*. 2000;2:399–406.
- Nandi S, Ma L, Denis M, Karwatsky J, Li Z, Jiang XC, Zha X. ABCA1-mediated cholesterol efflux generates microparticles in addition to HDL through processes governed by membrane rigidity. *J Lipid Res*. 2009;50:456–466.
- Smith JD, Waelde C, Horwitz A, Zheng P. Evaluation of the role of phosphatidylserine translocase activity in ABCA1-mediated lipid efflux. *J Biol Chem*. 2002;277:17797–17803.
- Smith JD, Le Goff W, Settle M, Brubaker G, Waelde C, Horwitz A, Oda MN. ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein A-I. *J Lipid Res*. 2004;45:635–644.
- Jonas A. Reconstitution of high-density lipoproteins. *Methods Enzymol*. 1986;128:553–582.
- Vedhachalam C, Duong PT, Nickel M, Nguyen D, Dhanasekaran P, Saito H, Rothblat GH, Lund-Katz S, Phillips MC. Mechanism of ATP-binding cassette transporter A1-mediated cellular lipid efflux to apolipoprotein A-I and formation of high density lipoprotein particles. *J Biol Chem*. 2007;282:25123–25130.
- Sasaki Y, Shukla R, Smith BD. Facilitated phosphatidylserine flip-flop across vesicle and cell membranes using urea-derived synthetic translocases. *Org Biomol Chem*. 2004;2:214–219.
- Lambert TN, Boon JM, Smith BD, Pérez-Payán MN, Davis AP. Facilitated phospholipid flip-flop using synthetic steroid-derived translocases. *J Am Chem Soc*. 2002;124:5276–5277.
- Boon JM, Smith BD. Synthetic membrane transporters. *Curr Opin Chem Biol*. 2002;6:749–756.
- DiVittorio KM, Hofmann FT, Johnson JR, Abu-Esba L, Smith BD. Facilitated phospholipid translocation in vesicles and nucleated cells using synthetic small molecule scramblases. *Bioorg Med Chem*. 2009;17:141–148.
- Boon JM, Lambert TN, Sisson AL, Davis AP, Smith BD. Facilitated phosphatidylserine (PS) flip-flop and thrombin activation using a synthetic PS scramblase. *J Am Chem Soc*. 2003;125:8195–8201.
- Fukuda M, Nakano M, Miyazaki M, Tanaka M, Saito H, Kobayashi S, Ueno M, Handa T. Conformational change of apolipoprotein A-I and HDL formation from model membranes under intracellular acidic conditions. *J Lipid Res*. 2008;49:2419–2426.
- Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. Dynasore, a cell-permeable inhibitor of dynamin. *Dev Cell*. 2006;10:839–850.
- Massey JB, Pownall HJ. Cholesterol is a determinant of the structures of discoidal high density lipoproteins formed by the solubilization of phospholipid membranes by apolipoprotein A-I. *Biochim Biophys Acta*. 2008;1781:245–253.
- Jonas A, Mason WR. Interactions of dipalmitoyl- and dimyristoylphosphatidylcholines and their mixtures with apolipoprotein A-I. *Biochemistry*. 1981;20:3801–3805.
- Tatulian SA. Ionization and ion binding. In: Cevc G, ed. *Phospholipids Handbook*. New York, NY: Marcel Dekker; 1993:511–552.
- Boon JM, Lambert TN, Smith BD, Beatty AM, Ugrinova V, Brown SN. Structure/activity study of tris(2-aminoethyl)amine-derived translocases for phosphatidylcholine. *J Org Chem*. 2002;67:2168–2174.
- Wen N, Brooker MH. Urea protonation: Raman and theoretical study. *J Phys Chem*. 1993;97:8608–8616.
- Lentz BR. Use of fluorescent probes to monitor molecular order and motions within liposome bilayers. *Chem Phys Lipids*. 1993;64:99–116.
- Duong PT, Collins HL, Nickel M, Lund-Katz S, Rothblat GH, Phillips MC. Characterization of nascent HDL particles and microparticles formed by ABCA1-mediated efflux of cellular lipids to apoA-I. *J Lipid Res*. 2006;47:832–843.
- Tang C, Vaughan AM, Anantharamaiah GM, Oram JF. Janus kinase 2 modulates the lipid-removing but not protein-stabilizing interactions of amphipathic helices with ABCA1. *J Lipid Res*. 2006;47:107–114.
- Nagao K, Zhao Y, Takahashi K, Kimura Y, Ueda K. Sodium taurocholate-dependent lipid efflux by ABCA1: effects of W590S mutation on lipid translocation and apolipoprotein A-I dissociation. *J Lipid Res*. 2009;50:1165–1172.
- Oram JF, Lawn RM, Garvin MR, Wade DP. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J Biol Chem*. 2000;275:34508–34511.
- Faulkner LE, Panagotopoulos SE, Johnson JD, Woollett LA, Hui DY, Witting SR, Maiorano JN, Davidson WS. An analysis of the role of a retroendocytosis pathway in ABCA1-mediated cholesterol efflux from macrophages. *J Lipid Res*. 2008;49:1322–1332.
- Chroni A, Liu T, Fitzgerald ML, Freeman MW, Zannis VI. Cross-linking and lipid efflux properties of apoA-I mutants suggest direct association between apoA-I helices and ABCA1. *Biochemistry*. 2004;43:2126–2139.
- Lu R, Arakawa R, Ito-Osumi C, Iwamoto N, Yokoyama S. ApoA-I facilitates ABCA1 recycle/accumulation to cell surface by inhibiting its intracellular degradation and increases HDL generation. *Arterioscler Thromb Vasc Biol*. 2008;28:1820–1824.
- Schubert R, Schmidt KH. Structural changes in vesicle membranes and mixed micelles of various lipid compositions after binding of different bile salts. *Biochemistry*. 1988;27:8787–8794.
- Vedhachalam C, Ghering AB, Davidson WS, Lund-Katz S, Rothblat GH, Phillips MC. ABCA1-induced cell surface binding sites for ApoA-I. *Arterioscler Thromb Vasc Biol*. 2007;27:1603–1609.
- Andrieux K, Forte L, Lesieur S, Paternostre M, Ollivon M, Grabielle-Madrelmont C. Solubilisation of dipalmitoylphosphatidylcholine bilayers by sodium taurocholate: a model to study the stability of liposomes in the gastrointestinal tract and their mechanism of interaction with a model bile salt. *Eur J Pharm Biopharm*. 2009;71:346–355.
- Dathe M, Schumann M, Wieprecht T, Winkler A, Beyermann M, Krause E, Matsuzaki K, Murase O, Bienert M. Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry*. 1996;35:12612–12622.
- Drin G, Antony B. Amphipathic helices and membrane curvature. *FEBS Lett*. 2010;584:1840–1847.

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 described¹, 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% CO₂. 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 described³⁻⁶ 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 delipidated 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. [$1\alpha,2\alpha(n)$ -³H]Cholesterol (35-50 Ci/mmol) acquired from Amersham in toluene was dried down and re-dissolved in ethanol to 1 mCi/ml. [*methyl*-³H]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 described¹³ with some modifications. RAW 264.7 cells were seeded at 1.5×10^5 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 [³H]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 [^3H] counts in the medium out of the total [^3H] counts in cells and medium. Stably-transfected ABCA1-GFP-expressing HEK293 cells¹ were seeded at 1×10^5 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 $\mu\text{Ci/ml}$ [^3H]cholesterol for one day. On day four, cells were pretreated with 10 $\mu\text{mol/L}$ compound 1 or DMSO (0.1%) for 1 hour and assayed for the ability to release [^3H]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 mifepristone² 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 $\mu\text{g/ml}$ 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 $\mu\text{Ci/ml}$ [^3H]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 [^3H] 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 ([^3H]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 [^3H]cholesterol (7.8-15.7 $\mu\text{Ci/mg}$ of AcLDL) at 37°C for 2-4 hours on the day of experiment. DGGB was supplemented with [^3H]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 2×10^5 cells per well in 24-well plates, allowed to attach overnight and pretreated with 10 $\mu\text{mol/L}$ compound 1, 160 $\mu\text{mol/L}$ dynasore¹⁶

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 + [³H]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 [³H] cpm in cells exposed to AcLDL at 20°C from individual values of [³H] cpm in cells exposed to AcLDL at 37°C.

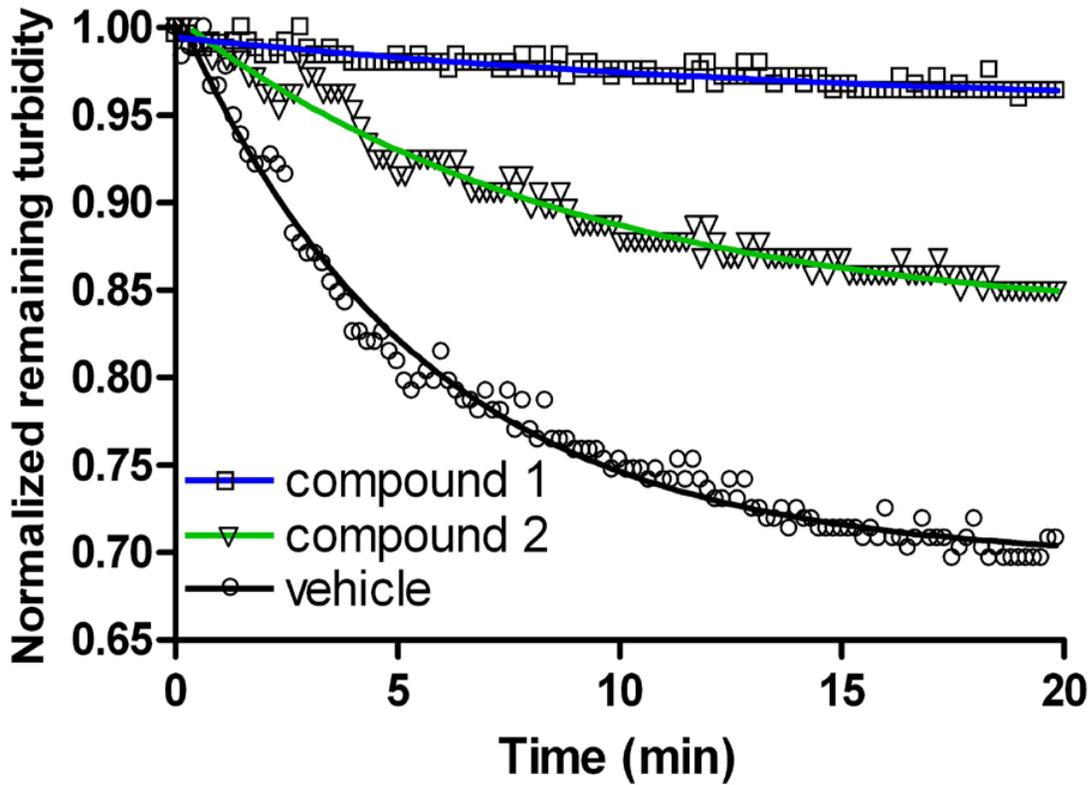
Effects of compound 1 on the ABCA1 expression level - RAW 264.7 cells were seeded in 12-well culture plates at 1/10 dilution of a confluent T75 flask and put through the same sequence of medium changes as in the cholesterol efflux assay, except [³H]cholesterol was not added. On day four, after a treatment 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 10⁵ 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.

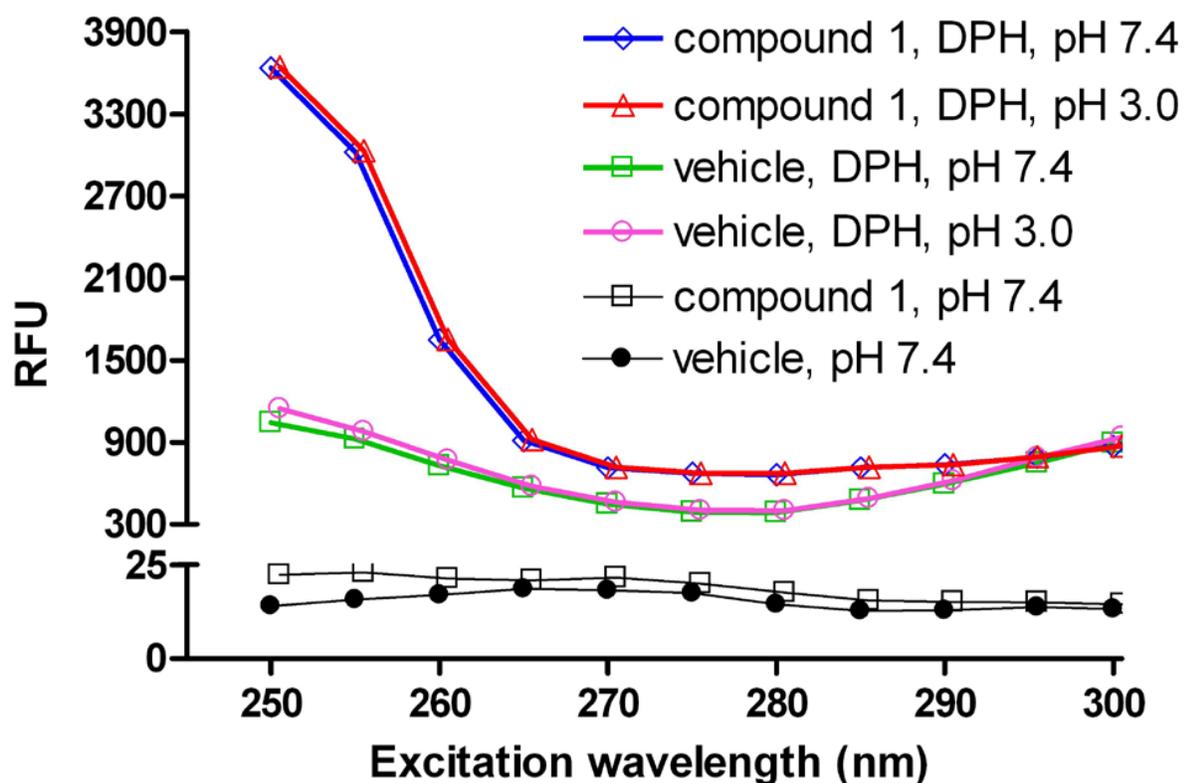
ABCA1-apoAI cross-linking – RAW 264.7 cells were seeded in T75 flasks (three flasks per treatment) at 1/10 dilution from a confluent T75 flask and put through the same sequence of medium changes as in the cholesterol efflux assay, except [³H]cholesterol was not added. On day four, after a treatment with +/- 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 4°C with 10 µl of anti-ABCA1 polyclonal antibody (1:500 dilution), incubated with 25 mg of protein A-sepharose 4B for 2 hours at 4°C 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.

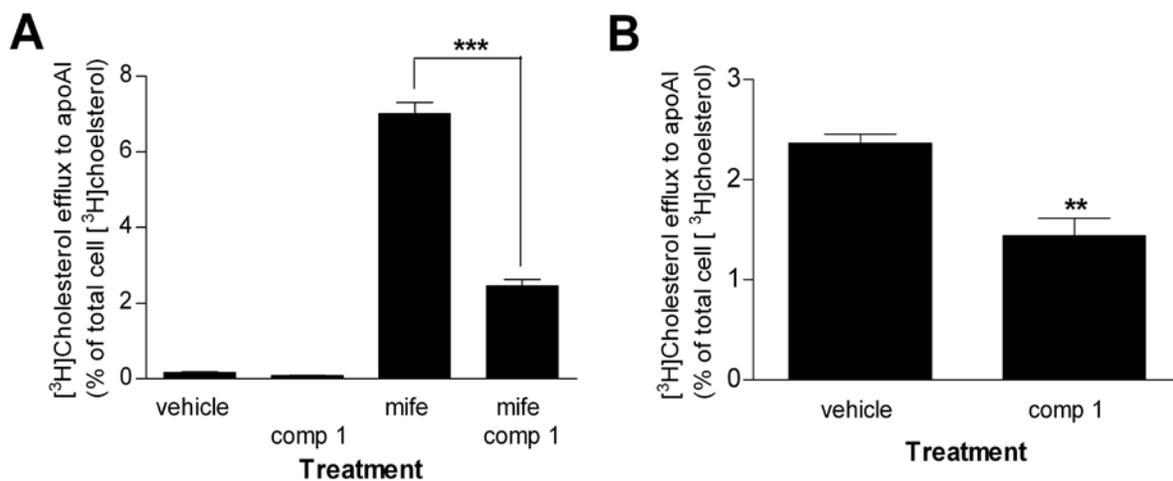
Supplemental Figures



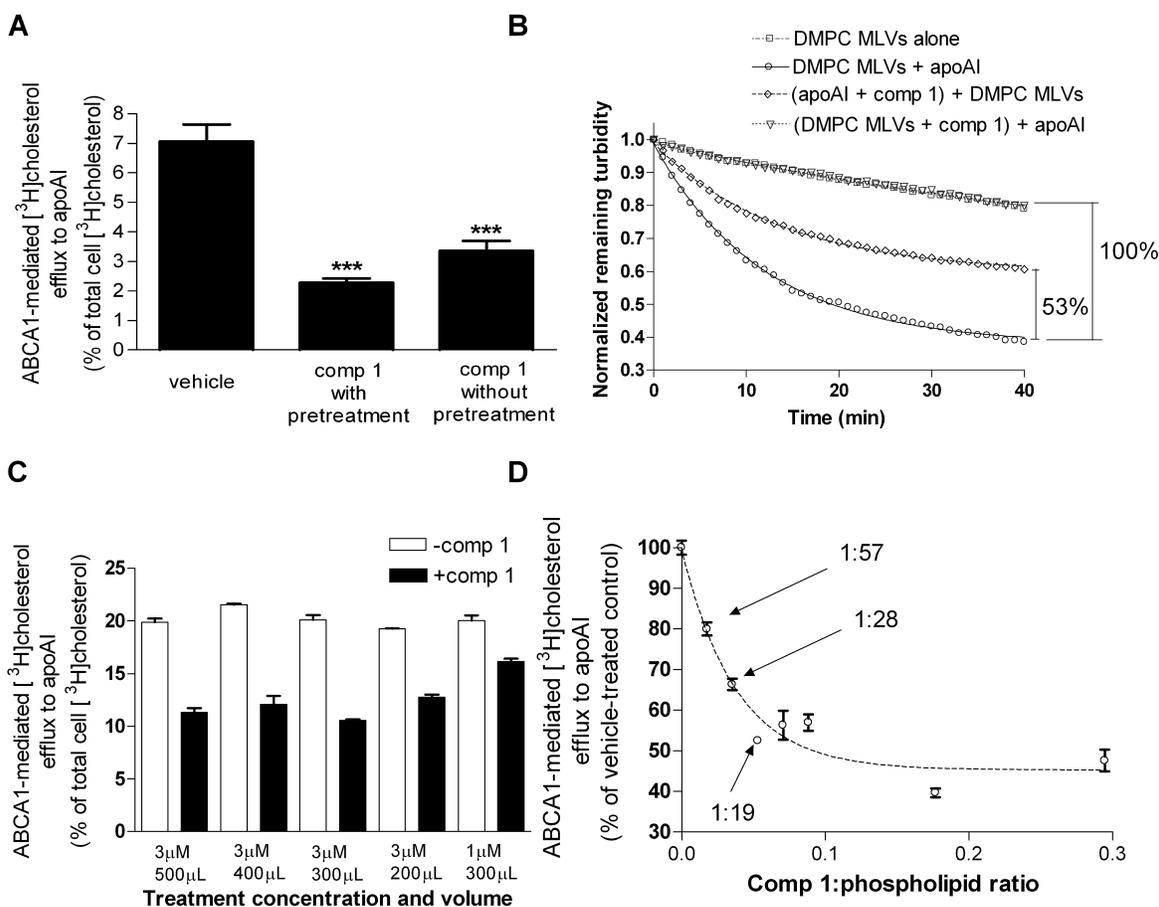
Supplemental Figure I. Inhibitory effect of compound 2 on the rHDL formation reaction in comparison with the inhibitory effect of compound 1. The DMPC:compound 1 or 2 ratio was 8:1.



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 $\mu\text{mol/L}$ 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.

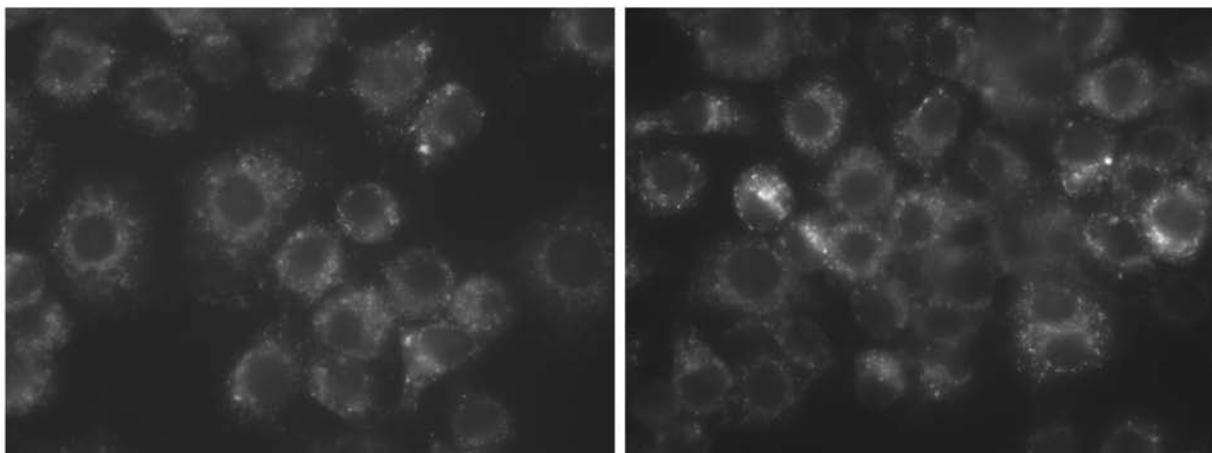


Supplemental Figure III. Compound 1 inhibits ABCA1-mediated cholesterol efflux to apoAI in BHK-ABCA1 (A) and HEK293-ABCA1-GFP (B) cells. ABCA1 expression in BHK cells was induced with mifepristone. HEK293 cells express ABCA1 constitutively at a low level. Cells were pretreated with 10 $\mu\text{mol/L}$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



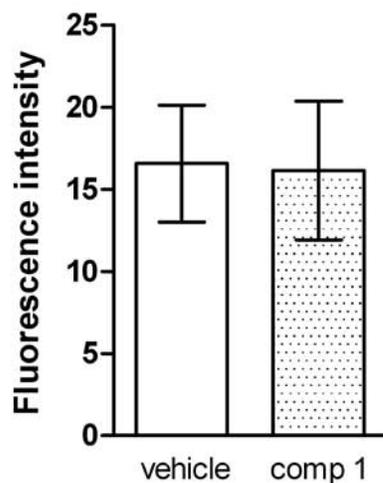
Supplemental Figure IV. Construction of the compound 1 dose-response curve. (A) When compound 1 (at 10 μmol/L) and apoAI were added to ABCA1-expressing RAW 264.7 cells simultaneously – without first pretreating the cells with the inhibitor, the compound suppressed ABCA1-mediated cholesterol efflux to 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 MLVs 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 $0.526 \pm 0.02 \mu\text{g}$ 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.

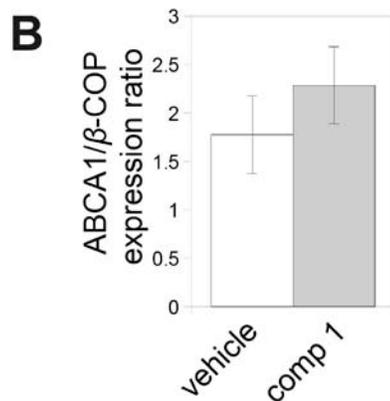
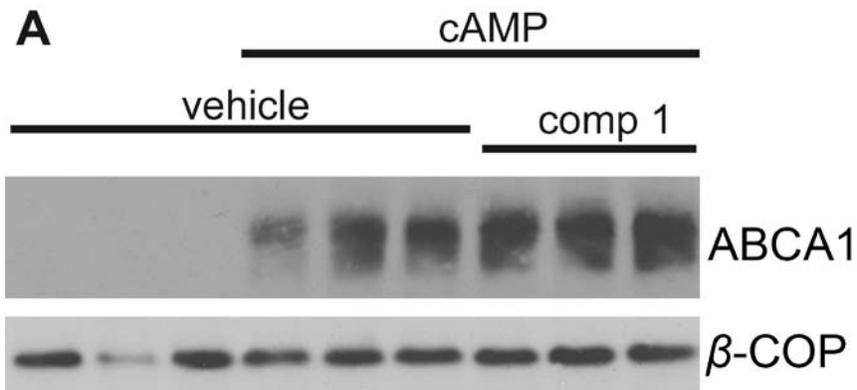
A

vehicle

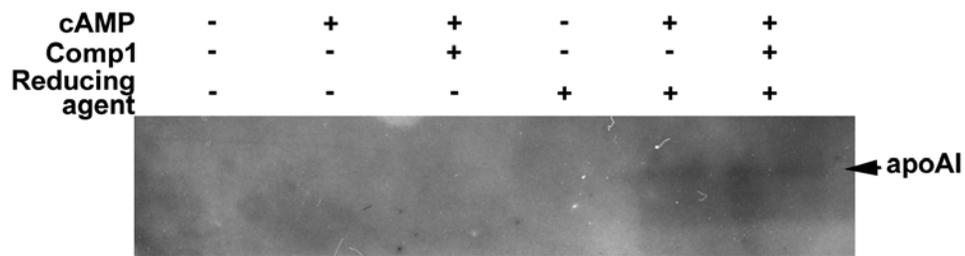
compound 1

B

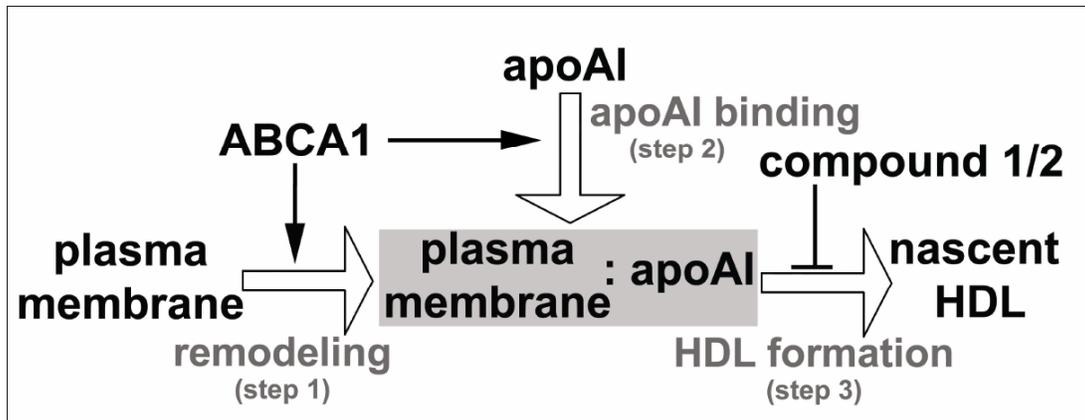
Online Figure V. Compound 1 does not inhibit transferrin endocytosis. (A) Representative images of RAW 264.7 cells pretreated with vehicle or 10 $\mu\text{mol/L}$ compound 1 for 1 hour and exposed to fluorescently-labeled transferrin. (B) Mean pixel fluorescence intensity in the cell area (ROI) calculated from the images represented in A (30-40 cells per treatment). Mean \pm 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 μ mol/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 $\mu\text{mol/L}$ compound 1 for 1 hour and incubated with 5 $\mu\text{g/ml}$ apoAI in the presence of 10 $\mu\text{mol/L}$ 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

1. Smith JD, Waelde C, Horwitz A, Zheng P. Evaluation of the role of phosphatidylserine translocase activity in ABCA1-mediated lipid efflux. *J Biol Chem.* 2002;277:17797-803.
2. Oram JF, Vaughan AM, Stocker R. ATP-binding cassette transporter A1 mediates cellular secretion of alpha-tocopherol. *J Biol Chem.* 2001;276:39898-902.
3. Boon JM, Lambert TN, Sisson AL, Davis AP, Smith BD. Facilitated phosphatidylserine (PS) flip-flop and thrombin activation using a synthetic PS scramblase. *J Am Chem Soc.* 2003;125:8195-201.
4. Lambert TN, Boon JM, Smith BD, Pérez-Payán MN, Davis AP. Facilitated phospholipid flip-flop using synthetic steroid-derived translocases. *J Am Chem Soc.* 2002;124:5276-7.
5. Sasaki Y, Shukla R, Smith BD. Facilitated phosphatidylserine flip-flop across vesicle and cell membranes using urea-derived synthetic translocases. *Org Biomol Chem.* 2004;2:214-9.
6. DiVittorio KM, Hofmann FT, Johnson JR, Abu-Esba L, Smith BD. Facilitated phospholipid translocation in vesicles and nucleated cells using synthetic small molecule scramblases. *Bioorg Med Chem.* 2009;17:141-8.
7. Basu SK, Goldstein JL, Anderson GW, Brown MS. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci USA.* 1976;73:3178-82.
8. Gross E, Peng DQ, Hazen SL, Smith JD. A novel folding intermediate state for apolipoprotein A-I: role of the amino and carboxy termini. *Biophys J.* 2006;90:1362-70.
9. Anantharamaiah GM, Jones JL, Brouillette CG, Schmidt CF, Chung BH, Hughes TA, Bhowm AS, Segrest JP. Studies of synthetic peptide analogs of the amphipathic helix. Structure of complexes with dimyristoyl phosphatidylcholine. *J Biol Chem.* 1985;260:10248-55.
10. Fukuda M, Nakano M, Miyazaki M, Tanaka M, Saito H, Kobayashi S, Ueno M, Handa T. Conformational change of apolipoprotein A-I and HDL formation from model membranes under intracellular acidic conditions. *J Lipid Res.* 2008;49:2419-26.
11. Sreerama N, Woody RW. Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal Biochem.* 2000;287:252-260.
12. Zhu K, Brubaker G, Smith JD. Large disk intermediate precedes formation of apolipoprotein A-I-dimyristoylphosphatidylcholine small disks. *Biochemistry.* 2007;46:6299-307.

13. Brubaker G, Peng DQ, Somerlot B, Abdollahian DJ, Smith JD. Apolipoprotein A-I lysine modification: effects on helical content, lipid binding and cholesterol acceptor activity. *Biochim Biophys Acta*. 2006;1761:64-72.
14. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957;226:497-509.
15. Rouser G, Fkeischer S, Yamamoto A. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids*. 1970;5:494-496.
16. Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. Dynasore, a cell-permeable inhibitor of dynamin. *Dev Cell*. 2006;10:839-50.
17. Vedhachalam C, Ghering AB, Davidson WS, Lund-Katz S, Rothblat GH, Phillips MC. ABCA1-induced cell surface binding sites for ApoA-I. *Arterioscler Thromb Vasc Biol*. 2007;27:1603-1609.