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Identification and Characterization of Dual Inhibitors for Phospholipid Transfer Protein and Microsomal Triglyceride Transfer Protein

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ABSTRACT

Phospholipid transfer protein (PLTP) plays an important role in atherogenesis and lipoprotein metabolism. PLTP exerts its functions intracellularly and extracellularly. Both PLTP and microsomal triglyceride transfer protein (MTP) have been shown to regulate the secretion of apolipoprotein B (apoB) in hepatocytes. We have previously reported the characterization of inhibitors that selectively inhibit PLTP activity and reduce apoB secretion in hepatocytes. In the present study, we identified more compounds that inhibit both PLTP and MTP activity to various extents. These dual inhibitors are structurally different from the PLTP-selective inhibitors. In human hepatoma cell lines, dual inhibitors seem to be more effective in reducing apoB secretion than selective PLTP or MTP inhibitors. Furthermore, the dual inhibitors markedly reduced triglyceride secretion from hepatocytes. In the absence of PLTP, the dual inhibitors can further reduce apoB secretion, whereas selective PLTP inhibitors had no effect. We conclude that MTP and PLTP may work coordinately in the process of hepatic apoB assembly and secretion. To avoid liver toxicity mediated by MTP inhibition, selective PLTP inhibitors should be pursued.

Introduction

Phospholipid transfer protein (PLTP) plays an important role in the metabolism of lipoproteins (Tall and Lalanne, 2003) and belongs to the family of lipid transfer/lipopolysaccharide binding proteins, including cholesteryl ester transfer protein (CETP), lipopolysaccharide binding protein, and bactericidal permeability increasing protein (Tollefson et al., 1988; Day et al., 1994). It has been shown that PLTP facilitates the transfer and exchange of phospholipids between very low-density lipoprotein (LDL) and high-density lipoprotein (Tall et al., 1985). Several clinical studies suggest that high plasma PLTP activity is a risk factor for coronary artery disease and a determinant of carotid intima-media thickness in type 2 diabetes mellitus (Schlitt et al., 2003; de Vries et al., 2006). Studies using genetically modified mice strongly suggest that PLTP functions as a proatherogenic factor (Jiang et al., 2001; van Haperen et al., 2002; Yang et al., 2003). Deletion of PLTP in hyperlipidemic apolipoprotein E-deficient and human apoB transgenic mouse strains results in reduced LDL and atherosclerotic lesion areas (Jiang et al., 2001). Overexpression of PLTP in hyperlipidemic mouse models increased susceptibility to atherosclerosis (van Haperen et al., 2002, 2008; Yang et al., 2003; Samyn et al., 2008).

In addition to its function in circulation, intracellular PLTP has been shown to regulate apoB-containing lipoprotein secretion in murine hepatocytes (Jiang et al., 2001). PLTP deficiency reduces apoB secretion from mouse primary hepatocytes. Microsomal triglyceride transfer protein (MTP) is required for the assembly of apoB lipoproteins and secretion (Hussain et al., 2003). Inhibition of MTP nearly abolished apoB secretion and apoB-containing lipoprotein production (Jamil et al., 1996, 1998; Chandler et al., 2003). MTP has been reported to transfer not only triglyceride, but also phospholipids between membranes (Athar et al., 2004; Rava et al., 2005). However, there is no homology between MTP and PLTP at gene or protein sequence levels. MTP and apoB belong to the vitellogenin family of lipid transfer proteins. Read et al. (2000) predicted the three-dimensional structure of the C-terminal lipid binding cavity of MTP based on the

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ABBREVIATIONS: PLTP, phospholipid transfer protein; CETP, cholesteryl ester transfer protein; LDL, low-density lipoprotein; MTP, microsomal triglyceride transfer protein; apoB, apolipoprotein B; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin.

crystal structure of lipoviellin. It has been implied that these binding sites may be responsible for triglyceride and phospholipid transport in MTP (Jamil et al., 1996; Read et al., 2000). PLTP and MTP may work sequentially to regulate the assembly and secretion of apoB-containing lipoproteins (Jiang et al., 2005).

We have reported the identification of small-molecule inhibitors that selectively inhibit phospholipid transfer activity of PLTP (Luo et al., 2010). We found that specific inhibition of PLTP activity reduces the secretion of apoB from human hepatoma cells and mouse primary hepatocytes. Here, we report the identification of compounds that inhibit both MTP and PLTP. These compounds markedly reduced apoB secretion from hepatocytes.

Materials and Methods

PLTP Activity Assay. PLTP activity was measured as described previously (Luo et al., 2010). In brief, phosphatidylcholine liposomes containing [^{3}H]phosphatidylcholine were used as donors. Transfer of radiolabeled phospholipid was measured by incubating purified recombinant PLTP with radiolabeled phospholipid vesicles and high-density lipoprotein 3 in the presence of 1% DMSO (vehicle) or compounds in room temperature for 15 min. Vesicles were subsequently precipitated with a $MnCl_2$/heparin solution, and the radioactivity of the supernatant was measured on a Wallac Microbeta scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Nonspecific transfer wells (−PLTP) were included for background subtraction. Transfer rate was calculated as [(total dpm − background dpm) × 3.5]/specific activity (dpm/nmol)/assay time (hours).

MTP Activity Assay. MTP activity was measured as described previously (Chandler et al., 2003) with minor modification. Human microsomes purchased from Sigma-Aldrich (St. Louis, MO) were extracted as described by Haghpassand et al. (1996) to obtain soluble MTP protein. Solubilized MTP protein was dialyzed and used as the source for MTP activity. Donor and acceptor liposomes were prepared as described previously (Haghpassand et al., 1996). Donor liposomes were prepared by bath sonication of a mixture containing 447 μM egg phosphatidylcholine, 83 μM bovine heart cardiolipin, and 0.91 μM [^{14}C]triolein (110 Ci/mol). Acceptor liposomes were prepared by bath sonication of a dispersion containing 1.3 mM egg phosphatidylcholine, 2.6 μM triolein, and 0.5 nM [^{3}H] egg phosphatidylcholine in assay buffer. The donor and acceptor liposomes were centrifuged at 160,000*g* for 2 h at 7°C. MTP activity was determined by adding 200 μl of a buffer containing 5% bovine serum albumin (BSA) with either DMSO or compounds to a mixture containing 50 μl of donor liposomes, 100 μl of acceptor liposomes, and 150 μl of MTP protein. After incubation at 37°C for 45 min, triglyceride transfer was terminated by addition of 300 μl of a 50% (w/v) DEAE cellulose suspension in assay buffer. After thorough mixing, the donor liposomes, bound to DEAE cellulose, were selectively precipitated by centrifugation at 3000*g* for 5 min. An aliquot of the supernatant containing the acceptor liposomes was collected to determine ^{14}C and ^{3}H counts. The obtained radioactivities were used to calculate the percentage of triglyceride transfer by using first-order kinetics.

ApoB and Triglyceride Secretion. HepG2 or Huh7 cells were maintained in DMEM with 10% fetal bovine serum. A total of 200,000 cells were seeded and cultured for 24 h in each well of 24-well plates. Cells were treated with DMEM containing 1% BSA and 0.4 mM oleic acid in the presence or absence of compounds for 24 h. The compounds were dissolved in DMSO and added in culture medium at 0.5% DMSO. Vehicle control contained 0.5% DMSO. Secreted apoB and transferrin were measured by ELISA. ApoB ELISA was performed as described previously (Chandler et al., 2003). Transferrin levels were measured as suggested by the manufacturer (Bethyl Laboratories, Montgomery, TX). Triglyceride levels in medium were measured by using the L-Type Triglyceride M assay with 180 μl of Color A (Wako Diagnostics, Richmond, VA), 60 μl of Color B (Wako Diagnostics), and the Multi-Calibrator Lipid standard (Wako Diagnostics). One hundred microliters of medium was used for the assay. Absorbance was measured at 600 nm, with a reference wavelength of 700 nm.

MTT Cytotoxicity. Cytotoxicity was monitored by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). The assay was performed as described in the manufacturer's manual (Sigma-Aldrich;, in vitro toxicity assay kit, MTT based).

ApoB Secretion in Mouse Primary Hepatocytes. Hepatocytes were incubated for 1 h in methionine/cysteine-free DMEM supplemented with 0.2 mM oleic acid/BSA. [^{35}S]methionine/cysteine (100 μCi) was added, the cells were incubated in the presence of DMSO or compound for 4 h, and media were collected. Cells were then washed once with DMEM and twice with PBS. ApoB in medium and cell lysate was then immunoprecipitated by antibody against ApoB (Abcam Inc., Cambridge, MA) plus protein A/G agarose, and precipitates were analyzed by 4% SDS-polyacrylamide gel electrophoresis. Incorporation of ^{35}S into ApoB48 and ApoB100 was assessed with a Fuji (Tokyo, Japan) Bio-Imaging Analyzer.

Results

Identification and Characterization of Dual Inhibitors for PLTP and MTP. High-throughput screening of the Pfizer compound collection led to the identification of several inhibitors for PLTP (Luo et al., 2010) (Fig. 1). Compound A was reported previously to selectively inhibit PLTP (Luo et al., 2010). Compounds F and G are structurally different from compound A and belong to the piperazinedione series. Compounds F and G dose-dependently inhibited the phospholipid transfer activity of PLTP (Fig. 2). The IC_{50}s of the compounds are summarized in Table 1. Compound G, which had similar potency to compound A (IC_{50} = 0.3 μM), is a more potent PLTP inhibitor than compound F (Fig. 2). Compound H is an enantiomer of compound G and is essentially inactive in inhibiting PLTP activity (Fig. 2), suggesting the effect of compound G on PLTP activity is specific.

MTP is an important intracellular lipid transfer protein that regulates apoB secretion (Athar et al., 2004, Jamil et al., 1996; Rava et al., 2005). Even though MTP has no homology with PLTP at the protein sequence level, we analyzed the selectivity of the PLTP inhibitors against MTP activity be-

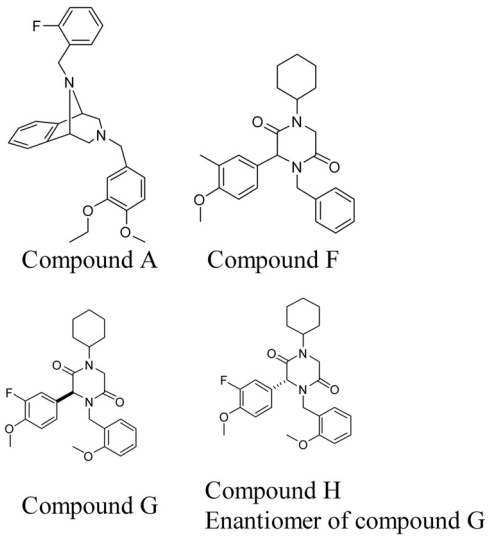

Fig. 1. Structures of the compounds that inhibit PLTP activity.

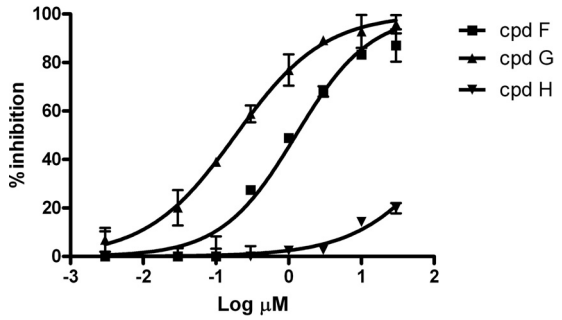

Fig. 2. Inhibition of human PLTP-mediated phospholipid transfer activity by the compounds. The phospholipid transfer activity of human PLTP was measured in the presence of 0 to 30 μM compound (cpd) as described under *Materials and Methods*. The data are expressed as percentage of inhibition.

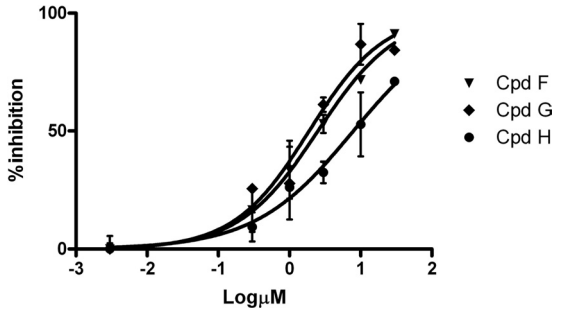

Fig. 3. Inhibition of human MTP-mediated triglyceride transfer activity by the compounds. Triglyceride transfer activity of human MTP was measured in the presence of 0 to 30 μM compound (Cpd) as described under *Materials and Methods*. The data are expressed as percentage of inhibition.

TABLE 1
Inhibition of PLTP and MTP activity by PLTP inhibitors

Compound	IC_{50}	
	PLTP Activity	MTP Activity
	μM	
A	0.3	101
F	1.0	2.5
G	0.2	1.9
H	183	7.9

cause both proteins regulate apoB secretion. We tested these compounds for their effects on MTP-mediated triglyceride transfer activity. The PLTP inhibitors demonstrated differential effects on MTP activity (Luo et al., 2010) (Fig. 3; Table 1). Unlike compound A, which is selective for PLTP (Luo et al., 2010), compounds F and G inhibited MTP triglyceride transfer activity with IC_{50} approximately 2 μM. Compound F had no selectivity and equally inhibited PLTP and MTP with a similar IC_{50}. Compound G was 10 times less potent in inhibiting MTP activity than inhibiting PLTP activity. Compound H, an enantiomer of compound G, is active in inhibiting MTP activity with IC_{50} approximately 8 μM, but it had little effect on PLTP activity, suggesting the specificity of the compound.

Effects of PLTP Inhibitors on ApoB Secretion in Human Hepatoma Cells. The PLTP gene is highly expressed in the human hepatoma cell lines HepG2 and Huh7 (Luo et al., 2010). We analyzed the effects of the dual inhibitors on apoB secretion in HepG2 and Huh7 cells (Fig. 4, A and C). These compounds had no significant effect on transferrin secretion, which was used to normalize the secretion of apoB (Fig. 4B), except compound G at 30 μM decreased transferrin ($P < 0.05$). None of the compounds at 30 μM altered cellular PLTP protein expression (data not shown), confirming that the effects of these compounds on apoB secretion were mediated through reducing PLTP activity not PLTP protein. ApoB secretion was normalized to secreted transferrin to rule out any variations, including compound-mediated effects on protein secretion and cell number caused by cytotoxicity or seeding. HepG2 and Huh7 cells showed a similar response to compound treatment. Both compound F and G at 30 μM suppressed apoB secretion by 60 and 80%, respectively. Compound G is a more potent PLTP inhibitor than compound F, whereas they both equally inhibit MTP activity. Compound G at 3 μM reduced apoB secretion to a similar level (−50%) as compound F at 30 μM in HepG2 cells (Fig. 4A), implying that PLTP inhibition may contribute to the difference. Similar effects were observed in Huh7 cells (Fig. 4B). Furthermore, compound H, which is selective for MTP with an in vitro IC_{50} of 8 μM, had no effect on apoB secretion at 10 μM (Fig. 4, A and C). The data suggest that treatment of cells with compound H at a concentration similar to the MTP IC_{50} is not enough to reduce apoB secretion, probably because of the metabolism of the compound in cells that could reduce the efficacious concentration. However, compound G at 3 μM, which is the IC_{50} concentration for MTP and 15× the IC_{50} concentration for PLTP, reduced apoB secretion, confirming that PLTP inhibitory activity of compound G contributed to the potent effect of this compound in reducing apoB secretion. Compounds A and G had similar activity on PLTP, whereas compound A is essentially inactive on MTP. The inhibitory effect of compound G on MTP led to more marked reduction of apoB secretion than compound A (Fig. 4A). These data suggest that dual inhibition of both PLTP and MTP had an additive effect on the reduction of apoB secretion.

Inhibition of MTP reduces both apoB and triglyceride secretion (Jamil et al., 1996; Chandler et al., 2003). We have previously shown that a PLTP-selective inhibitor did not reduce triglyceride secretion in HepG2 cells (Luo et al., 2010). Here, we confirmed that PLTP-selective inhibitor (compound A) did not affect triglyceride secretion; however, PLTP/MTP dual inhibitor (compound G) markedly reduced triglyceride secretion from HepG2 cells (Fig. 4D). In this study, the compounds did not show overt cytotoxicity measured by MTT methods (Fig. 4E).

Furthermore, we analyzed the effects of these compounds on apoB secretion in primary hepatocytes isolated from wild-type or PLTP-deficient mice. Newly synthesized protein was labeled by incubation with [^{35}S]Met for 4 h. Secreted apoB was immunoprecipitated from culture media, run on SDS-polyacrylamide gel electrophoresis, and quantified by PhosphorImager. All of the compounds at 30 μM concentration reduced apoB100 and apoB48 secretion by approximately 50%, but not cellular apoB levels, in hepatocytes from wild-type mice (Fig. 5A). However, in PLTP-deficient hepatocytes isolated from PLTP-deficient mice, only MTP/PLTP dual inhibitors (compounds F and G) reduced apoB secretion. Compound A, which is relatively selective for PLTP, had no effect on apoB secretion (Fig. 5B). All of the compounds did not significantly change cellular apoB levels (Fig. 5B, inset).

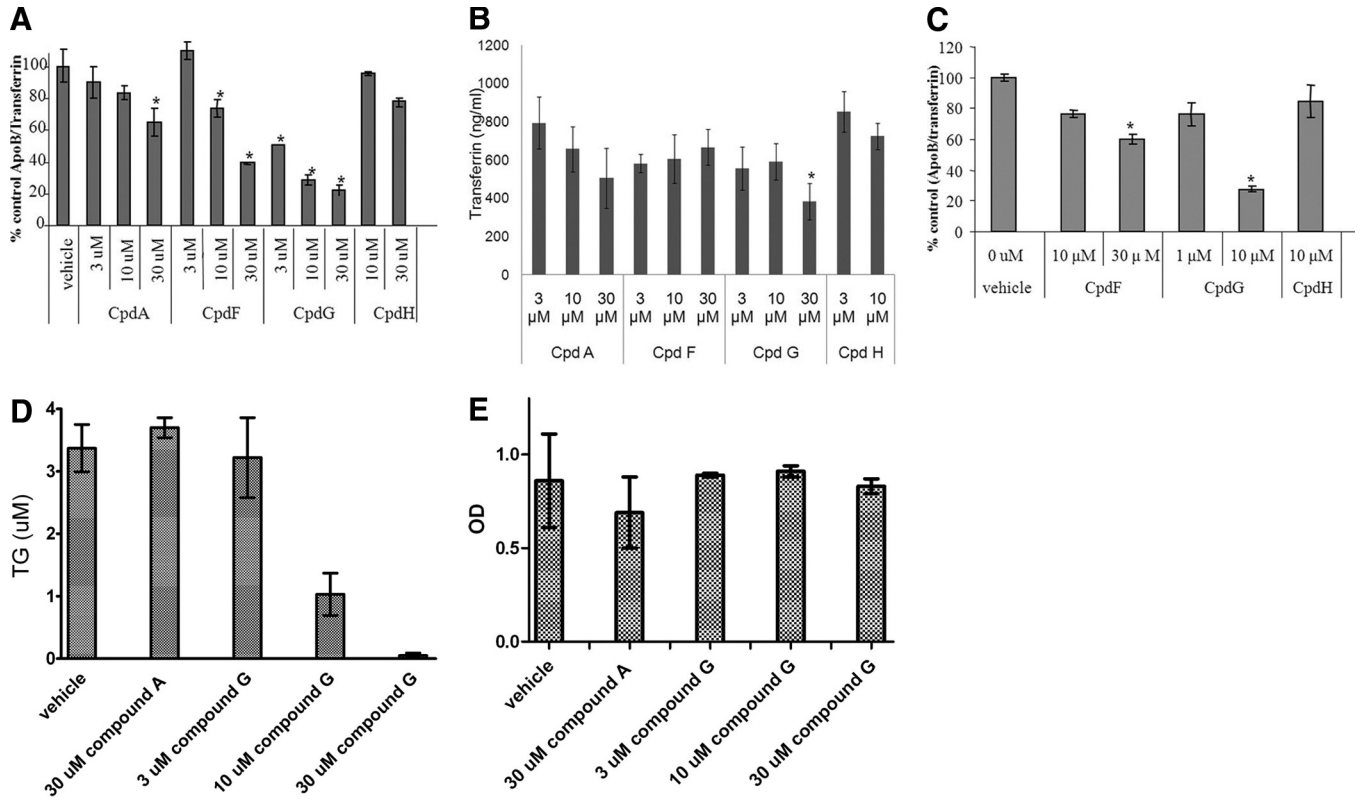

Fig. 4. A and C, effects of the compounds on ApoB secretion in HepG2 cells (A) and Huh7 cells (C). A–C, HepG2 or Huh7 cells were treated with the compounds (Cpd) for 24 h, and medium was collected for apoB (A and C) and transferrin (B) measurement by ELISA. ApoB levels normalized to transferrin are presented. B, transferrin levels from experiments shown in A. $*$, $P < 0.05$. D, effects of compounds on triglyceride secretion in HepG2 cells. Cells were treated with compounds for 24 h, and medium was harvested to measure triglyceride levels as described under *Materials and Methods*. E, MTT cytotoxicity for experiment shown in C. Cells were incubated with MTT reagent, and cytotoxicity was measured as described under *Materials and Methods*. Data are the average of triplicates ± standard deviation.

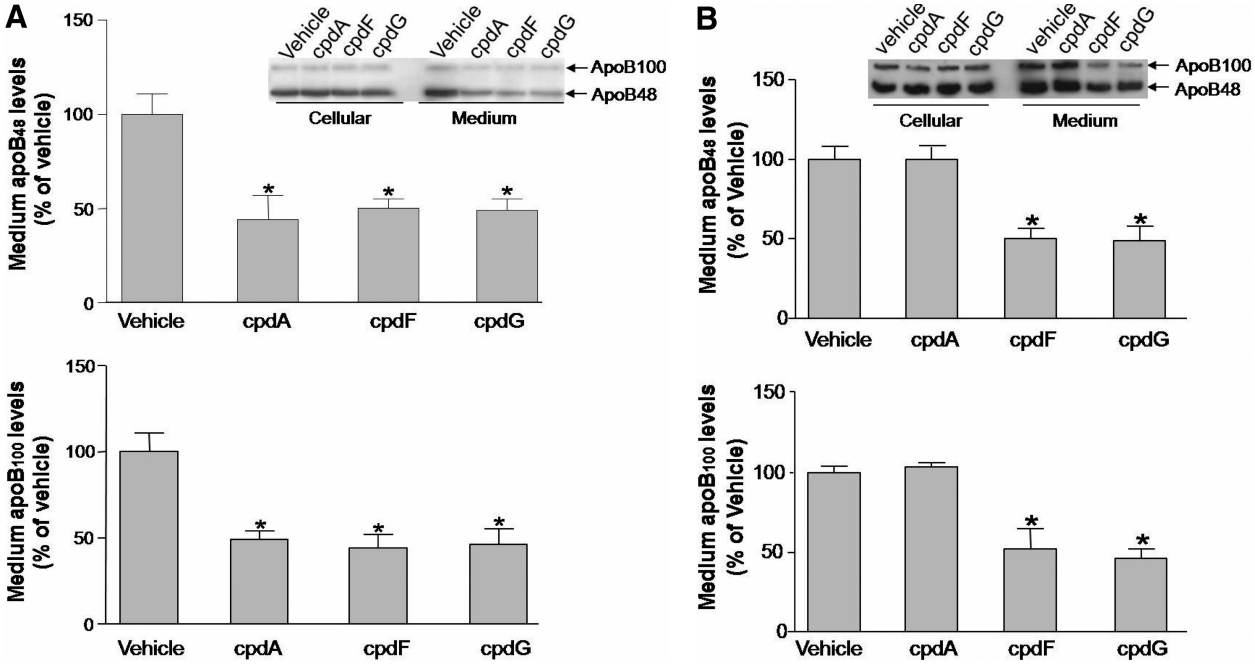

Fig. 5. Effect of selective PLTP and dual inhibitors on apoB secretion in mouse primary hepatocytes isolated from wild-type mice (A) and PLTP-deficient mice (B). Cells were labeled with [^{35}S]methionine/cysteine in the presence of vehicle (DMSO) or 30 μM compound (cpd) for 4 h. Medium and cells were collected to detect labeled apoB as described under *Materials and Methods*. ApoB levels are the average of triplicate experiments. Representative images are shown in the insets.

Discussion

We have reported studies characterizing small-molecule inhibitors that selectively inhibit PLTP activity and concomitantly reduce apoB secretion. In the present study, we identified small molecules that inhibit both PLTP and MTP activities, which are known to regulate apoB secretion. This is the first report to identify dual inhibitors for PLTP and MTP activities. The discovery was not expected based on the lack of homology of PLTP and MTP at protein sequence levels. Although CETP and PLTP have 40% homology and belong to the family of lipid transfer/lipopolysaccharide-binding proteins (Tollefson et al., 1988; Day et al., 1994), none of these compounds inhibit CETP activity (Luo et al., 2010). MTP and apoB belong to the vitellogenin family of lipid transfer proteins. Read et al. (2000) predicted the three-dimensional structure of the C-terminal lipid binding cavity of MTP based on the crystal structure of lipoviellin. The lipid cavity in MTP bears a resemblance to the lipid binding domain of bactericidal permeability increasing protein, suggesting that PLTP and MTP have similar lipid binding cavities where the dual inhibitors could potentially bind. It has been implied that these binding sites may be responsible for triglyceride and phospholipid transport in MTP (Jamil et al., 1996; Read et al., 2000). Compound G is 10 times more potent in inhibiting PLTP than inhibiting MTP; however, its enantiomer, compound H, is selective for MTP and is essentially inactive in inhibiting PLTP, suggesting that the inhibitory effect is specific and is unlikely caused by nonspecific interference in lipid binding. The inhibitory mechanisms of these compounds are not clear and warrant further investigation. The compounds could bind to the lipid binding domain and inhibit the transfer of the substrates (triglyceride or phospholipids), or they could block the binding of the protein to lipoproteins and subsequently interfere with the activities.

Dual inhibitors seem to have additive effects in reducing apoB secretion in hepatoma cells, implying that MTP and PLTP contribute to apoB-containing lipoprotein assembly at different steps. MTP transfers triglyceride to nascent apoB in endoplasmic reticulum and stabilize apoB-containing particles for secretion (Jamil et al., 1996, 1998). A number of MTP inhibitors have been reported and characterized. The MTP inhibitors are very potent in inhibiting triglyceride transfer activity of MTP; however, they have little effect on the phospholipid transfer activity of MTP (Jamil et al., 1996, 1998; Chandler et al., 2003). It has been shown that phospholipid transfer activity of MTP plays a role in the assembly and secretion of apoB-containing lipoproteins (Rava et al., 2005, 2006). Although the mechanisms of PLTP regulation of apoB secretion are not well understood, we speculate that PLTP may participate in the second step of apoB-containing particle formation, i.e., the addition of more phospholipids to the nascent very low-density lipoprotein particle in Golgi (Hamilton et al., 1998), where significant PLTP activity is present and considerable phospholipids are synthesized (Fang et al., 1998). Detailed understanding of the mechanism of PLTP deserves further investigation.

Compounds F and G are structurally similar and had similar activity on MTP, but compound G is five times more active in inhibiting PLTP than compound F and is more potent in reducing apoB secretion (Table 1; Fig. 4A). These results indicate that the more potent PLTP inhibitory activity of compound G may contribute the difference. However, one could argue that the differential effect could be caused by the potential difference in the metabolic fate of compounds F and G because the two compounds are different in structure, although they belong to the same series. Compounds G and H are enantiomers, and compound H is a selective MTP inhibitor. At IC_{50} concentration for MTP, compound H (at 10 μM) had no effect on apoB secretion, but dual inhibitor compound G (at 3 μM) reduced apoB secretion by 50% (Fig. 4A), suggesting that inhibition of PLTP may contribute to the difference. When compound A (selective PLTP inhibitor) and compound G are compared, MTP inhibitory activity of compound G may contribute to the marked suppression of apoB secretion in HepG2 cells. However, when PLTP-selective inhibitor compound A and dual-inhibitors compound F/G were compared, no differential suppression of apoB secretion was observed in mouse primary hepatocytes treated with 30 μM compounds (Fig. 5A). We speculate that differential metabolic disposition of the compounds in primary hepatocytes may explain the lack of additive effects of the dual inhibitors (compounds F and G), because primary hepatocytes usually have higher metabolic activity than hepatoma cells. Thus, it is difficult to compare the activities of the compounds with different structures in primary hepatocytes.

MTP inhibitors have been developed to reduce LDL, and the major hurdle is the fatty liver side effect. Inhibition of PLTP can potentially reduce LDL and may not cause fatty liver (Luo et al., 2010). This makes a PLTP inhibitor an enticing therapeutic agent for dyslipidemia and atherosclerosis. This study suggests that to avoid the liver toxicity caused by MTP inhibition all PLTP inhibitors should be tested in an MTP activity assay to identify selective PLTP inhibitors.

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