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Decreased Secretion of ApoB Follows Inhibition of ApoB–MTP Binding by a Novel Antagonist[†]

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ABSTRACT: Apolipoprotein B (apoB) and microsomal triglyceride transfer protein (MTP) are essential for the efficient assembly of triglyceride-rich lipoproteins. Evidence has been presented for physical interactions between these proteins. To study the importance of apoB–MTP binding in apoB secretion, we have identified a compound, AGI-S17, that inhibited (60–70% at 40 μM) the binding of various apoB peptides to MTP but not to an anti-apoB monoclonal antibody, 1D1, whose epitope overlaps with an MTP binding site in apoB. AGI-S17 had no significant effect on the lipid transfer activity of the purified MTP. In contrast, another antagonist, BMS-200150, did not affect apoB–MTP binding but inhibited MTP's lipid transfer activity. The differential effects of these inhibitors suggest two functionally independent, apoB binding and lipid transfer, domains in MTP. AGI-S17 was then used to study its effect on the lipid transfer and apoB binding activities of MTP in HepG2 cells. AGI-S17 had no effect on cellular lipid transfer activities, but it inhibited coimmunoprecipitation of apoB with MTP. These studies indicate that AGI-S17 inhibits apoB–MTP binding but has no effect on MTP's lipid transfer activity. Experiments were then performed to study the effect of inhibition of apoB–MTP binding on apoB secretion in HepG2 cells. AGI-S17 (40 μM) did not affect cell protein levels but decreased the total mass of apoB secreted by 70–85%. Similarly, AGI-S17 inhibited the secretion of nascent apoB by 60–80%, but did not affect albumin secretion. These studies indicate that AGI-S17 decreases apoB secretion most likely by inhibiting apoB–MTP interactions. Thus, the binding of MTP to apoB may be important for the assembly and secretion of apoB-containing lipoproteins and can be a potential target for the development of lipid-lowering drugs. It is proposed that the apoB binding may represent MTP's chaperone activity that assists in the transfer from the membrane to the lumen of the endoplasmic reticulum and in the net lipidation of nascent apoB, and may be essential for lipoprotein assembly and secretion.

The presence of a neutral lipid transfer activity in the lumen of the endoplasmic reticulum was identified based on in vitro lipid transfer assays using lumenal contents of the endoplasmic reticulum (*1, 2*). Subsequently, the protein responsible for the lipid transfer activity, the microsomal triglyceride transfer protein (MTP),[1] was purified to homogeneity and was shown to consist of two polypeptides of 97 and 55 kDa subunits. The 55 kDa polypeptide was shown to be a ubiquitous enzyme, protein disulfide isomerase, that is present in the endoplasmic reticulum. The 97 kDa polypeptide is unique and has been shown to be responsible for the lipid transfer activity [for reviews, see (*3, 4*)]. The importance of the 97 kDa subunit in the assembly and secretion of apoB-containing lipoproteins was established using three independent approaches. First, mutations in the 97 kDa polypeptide were shown to be responsible for the lack of apoB-containing lipoproteins in the plasma of individuals with abetalipoproteinemia [for reviews, see (*3, 4*)]. Second, coexpression of the 97 kDa polypeptide with apoB in cells that do not secrete lipoproteins has been shown to be sufficient for lipoprotein assembly and secretion (*5–7*). Third, compounds that inhibit MTP's lipid transfer activity in vitro and decrease apoB secretion in cell cultures have been identified (*8–13*). Recently, second-generation inhibitors have been synthesized that decrease triglyceride secretion in rats, and lower plasma lipid levels in hamsters (*14*). More interestingly, they lower lipid levels (*14*) in Watanabe heritable hyperlipidemic rabbits (*15, 16*) that express low levels of LDL receptors due to a genetic defect.

In addition to its lipid transfer activity, MTP has been shown to interact physically with apoB (*17–23*). ApoB and MTP bind with high affinity, and these interactions are affected by the length and degree of lipidation of apoB (*19*). Lysine and arginine residues in the N-terminal 18% of apoB

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[1] Abbreviations: apoB, apolipoprotein B; LDL, low-density lipoprotein(s); MTP, microsomal triglyceride transfer protein; PBS, phosphate-buffered saline; PBS-Tween, PBS containing 0.05% Tween-20.

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are critical for its binding to MTP (*20*). Within this region, apoB and MTP can interact at multiple sites (*22, 23*). One of the MTP binding sites has been recently localized to amino acids 270–570 (*21*). In the present investigations, we have attempted to understand the importance of apoB–MTP binding. For this purpose, we identified a novel antagonist, AGI-S17, that inhibits apoB–MTP binding without affecting MTP's lipid transfer activity in vitro and in cells. AGI-S17 decreased apoB secretion in hepatoma cells, indicating that these interactions play an important role in the assembly and secretion of apoB-containing lipoproteins.

MATERIALS AND METHODS

Materials. AGI-S17 (602 Da) and AGI-3 (616 Da) are synthetic organic compounds of proprietary nature from Atherogenics Inc. Other compounds, BMS-200150 and CP-10447, were kindly provided by Bristol-Myers Squibb and Pfizer, respectively. Stocks (10 mM) of these compounds were prepared in dimethyl sulfoxide. Antibodies against MTP were kindly provided by Dr. Haris Jamil (Bristol-Myers Squibb). Egg phosphatidylcholine, cardiolipin, and triglycerides; and [^{14}C]triolein and [^{3}H]phosphatidylcholine used for vesicle preparations were purchased from Avanti Polar-Lipids (Alabaster, AL) and New England Nuclear (Boston, MA), respectively. Other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cells. HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD), and cultured in minimal essential medium containing 10% fetal bovine serum (Biofluids, Maryland, MD) and 1% antibiotic–antimycotic (Life Technologies, Grand Islands, NY). McA-RH7777 cells stably transfected with human apoB18 or apoB28 cDNAs (*24, 25*) were grown in Dulbecco's modified medium (DMEM) containing 10% fetal bovine serum, 10% horse serum, and 1% antibiotic–antimycotic.

MTP's Lipid Transfer Activity and ApoB–MTP Binding. Heterodimeric MTP was purified and assayed using synthetic unilamellar donor and acceptor vesicles as described (*1, 2, 9, 19, 26*). Various concentrations of different compounds were included during assays, and inhibition of the transfer of radiolabeled triglycerides and phospholipids was determined by scintillation counting. For apoB–MTP binding, ELISA plates were coated with purified MTP (1 μg/well) and incubated in triplicate with human LDL (1 μg/well) in the presence of the indicated concentrations of various compounds as described before (*19–21*). Microtiter wells were washed (3×) with PBS-Tween, and apoB bound was quantified by a sandwich ELISA (*25, 27*).

Cellular Lipid Transfer Activities and ApoB–MTP Binding. HepG2 cells (25 cm^2 flasks) were incubated with different concentrations of AGI-S17 for 6 h. Cells were collected in PBS, pelleted, homogenized in 1 mL of 50 mM KCl, 5 mM EDTA, leupeptin (100 μg/mL), 1 mM phenylmethylsulfonyl fluoride, 50 mM Tris-HCl buffer, pH 7.4, and incubated on ice for 30 min. Cell lysates were adjusted to 1 mg of protein/mL, 0.054% sodium deoxycholate, pH 7.4, incubated on ice for 1 h, and centrifuged to remove cellular debris. The supernatant was dialyzed extensively against 15 mM Tris-HCl buffer, pH 7.4, containing 40 mM of [^{3}H]triolein from the donor to acceptor small unilamellar vesicles (*9, 26, 28*).

The effect of AGI-S17 on intracellular apoB–MTP binding was evaluated by coimmunoprecipitation (*17*). First, we determined the effect of different concentrations of AGI-S17 (0–40 μM) on the binding of polyclonal antibodies to MTP using Western analysis and the chemiluminescence detection system. AGI-S17 did not inhibit the binding of polyclonal antibodies to the 97 kDa subunit (data not shown). We also studied the effect of AGI-S17 on the binding between apoB and its polyclonal antibody using ELISA. Again, AGI-S17 did not inhibit the recognition of apoB by its polyclonal antibodies (data not shown). These experiments indicate that AGI-S17 does not inhibit antigen–antibody interactions. Next, cells were incubated with different concentrations of AGI-S17 for 6 h, collected in a nondenaturing buffer (62.5 mM sucrose, 0.5% sodium deoxycholate, 0.5% Triton X-102, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, and protease inhibitors), and immunoprecipitated with anti-97 kDa MTP subunit antibody. Immunoprecipitates were separated on SDS–polyacrylamide gels, transferred to nitrocellulose membranes, and blocked with PBS-Tween containing 5% nonfat dried milk. Membranes were probed with sheep anti-human apoB antibodies (1:1000 dilution) and peroxidase-conjugated protein A (1:5000 dilution). The presence of apoB100 bands was visualized using enhanced chemiluminescence reagents (Amersham, Piscataway, NJ) and fluorography.

Effect of AGI-S17 on the Secretion of ApoB by Cultured Cells. To determine the effect of AGI-S17 on the secretion of nascent apoB, HepG2 cells (25 cm^2 flasks) were preincubated in serum-, cysteine-, and methionine-free medium for 2 h and radiolabeled with a mixture of radiolabeled amino acids (Expre^{35}S^{35}S, New England Nuclear) in the presence of various concentrations of AGI-S17 for 6 h. ApoB or albumin was immunoprecipitated from cells and media using sheep anti-human apoB or anti-human albumin antibodies (Boehringer Mannheim), separated on polyacrylamide gels, exposed to Phosphorimager screens, and visualized using Phosphorimager 445SI (Molecular Dynamics, Sunnyvale, CA). Individual bands were quantitated, and the local average background was subtracted using Imagequant Program.

To determine the effect of AGI-S17 on the total mass of apoB secreted, HepG2 cells (24-well plates) were incubated with various concentrations of AGI-S17 for 6 h, and the amount of apoB secreted was quantitated by ELISA (*25, 27*). In addition, the conditioned medium was subjected to density gradient ultracentrifugation (*29*), and apoB in different fractions was quantitated.

Other Analyses. Protein was determined using the Coomassie Plus reagent (Pierce Chemical Co., Rockford, IL) with BSA as a standard (*30*). Optical density in ELISA plates was measured at 405 nm using a Dynatech MRX microplate reader (Dynatech Labs, Chantilly, VA). The data were plotted as the mean ± the standard deviation.

RESULTS

Identification of an Antagonist That Inhibits ApoB–MTP Binding and Does Not Affect the Lipid Transfer Activity of

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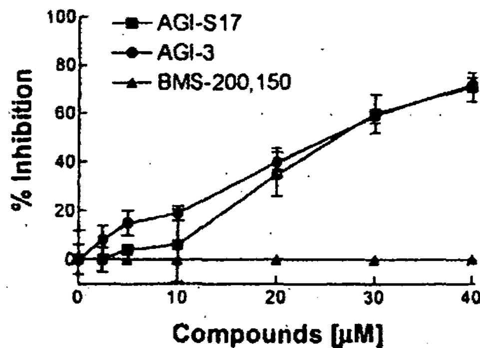


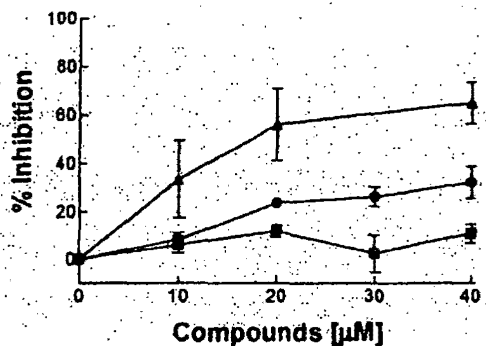
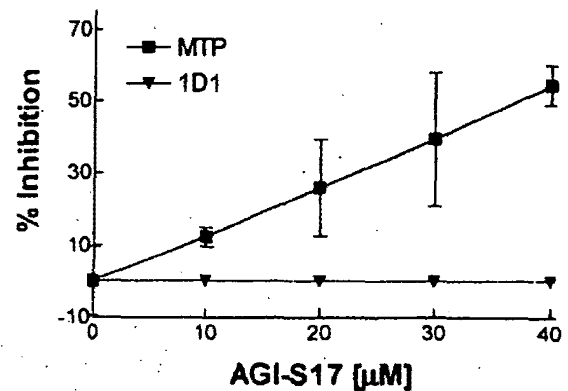


FIGURE 1: Effect of different compounds on LDL−MTP interactions and MTP's lipid transfer activity. (A) Effect on LDL−MTP binding. Heterodimeric MTP (1 μg/well) was immobilized and incubated in triplicate with LDL (1 μg/well) in the presence and absence of various concentrations of different compounds for 2 h as described under Materials and Methods. The LDL bound to MTP was quantitated by ELISA. Controls did not contain any drug but did contain dimethyl sulfoxide. The data are representative of six independent experiments. (B) Effect on MTP's lipid transfer activity. MTP's triglyceride transfer activity was determined in triplicate using purified MTP (1 μg/assay) and synthetic lipid vesicles in the presence and absence of the indicated concentrations of different compounds as described under Materials and Methods. The data are representative of three independent experiments. Mean ± standard deviations are plotted.



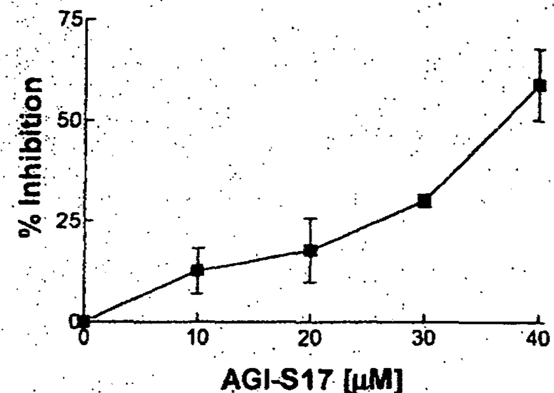


FIGURE 2: Effect of AGI-S17 on the binding of different apoB polypeptides to MTP. (A) Effect on apoB100 binding. Serum-free conditioned medium (24 h) obtained from HepG2 cells that secrete only apoB100 was incubated in triplicate with immobilized 1D1 or MTP for 2 h in the presence of the indicated concentrations of AGI-S17. ApoB bound was quantitated by ELISA, and the percent inhibition was plotted. (B) Effect on apoB18 binding. MTP was immobilized and incubated with conditioned media obtained from McA-RH7777 cells stably transfected with apoB18 in the presence of various concentrations of AGI-S17 for 2 h as described under Materials and Methods. The apoB bound was quantitated by ELISA. Each point represents a mean ± standard deviation.

we screened several compounds that might inhibit these interactions without affecting the lipid transfer activity of MTP. Representative data for three compounds are presented (Figure 1). In the absence of any compound, 15.5 ± 0.8 fmol of LDL was bound to immobilized MTP, which was similar to that reported previously (*19*). AGI-3 and AGI-S17 inhibited 72 and 71%, respectively, of the binding at 40 μM (Figure 1A). In contrast, BMS-200150, an inhibitor of MTP's lipid transfer activity, did not affect LDL−MTP binding. Similarly, other MTP inhibitors, CP-10447, BMS-192951, and BMS-197636, did not affect LDL−MTP binding (data not shown). Next, we evaluated the effect of these compounds on MTP's lipid transfer activity. As expected, BMS-200150 inhibited the triglyceride transfer activity (Figure 1B). AGI-3 (40 μM) inhibited 32% of the activity at this concentration. In contrast, AGI-S17 inhibited neither the triglyceride (Figure 1B) nor the phospholipid (data not

activity, whereas AGI-S17 inhibits LDL−MTP binding without affecting MTP's lipid transfer activity.

To determine the specificity of the effect of AGI-S17 on the binding of apoB to MTP, we studied the effect of AGI-S17 on the binding of apoB polypeptides to MTP and a monoclonal antibody, 1D1 (Figure 2A). 1D1 recognizes amino acids 474−539 in apoB (*31, 32*), and this epitope overlaps with an MTP binding site, amino acids 270−570, in apoB (*21*). AGI-S17 (40 μM) inhibited the binding of apoB100 to MTP by about 60% but had no inhibitory effect on its binding to 1D1 (Figure 2A). A likely explanation is that AGI-S17 interacts with MTP, but not with 1D1, and inhibits its binding to apoB.

To examine whether the continuous presence of AGI-S17 is required for the inhibition of LDL−MTP binding, the immobilized MTP was preincubated with AGI-S17, washed,

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that the presence of AGI-S17 is necessary for the inhibition of LDL–MTP binding.

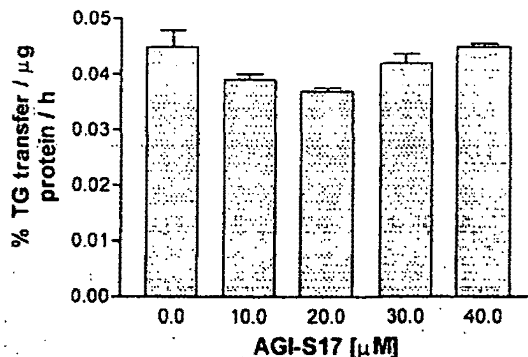
Next, we studied the effect of AGI-S17 on the binding of apoB18 to MTP (Figure 2B). Among the several truncated forms of apoB tested, maximal binding to MTP was observed for apoB18 (*19, 20*). A progressive increase in the concentration of AGI-S17 resulted in increased inhibition of apoB18–MTP binding. At 40 μM, it inhibited 60% of the binding between apoB18 and immobilized MTP. We have previously shown that AGI-S17 inhibits the binding of a FLAG/apoB chimera expressing amino acids B:270–570 to MTP but not to M2, a monoclonal antibody that recognizes the FLAG epitope (*21*). These studies showed that AGI-S17 specifically inhibits the binding of different apoB polypeptides to MTP but does not affect their binding to 1D1. Since the binding epitopes of 1D1 and MTP are overlapping (*21, 32*), it is likely that AGI-S17 interacts with MTP and prevents the binding of different apoB polypeptides to MTP.

In Vivo Effect of AGI-S17 on the Lipid Transfer and ApoB Binding Activities of MTP. To study the effect of AGI-S17 on MTP's lipid transfer activity, HepG2 cells were incubated with different concentrations of AGI-S17 for 6 h (Figure 3A). Cell lysates were used to assay for the lipid transfer activity (*9, 26*). Incubation of cells with different concentrations of AGI-S17 had no effect on the triglyceride transfer activity of microsomal MTP (Figure 3A). Similarly, phospholipid transfer activity in cell lysates was not affected (data not shown). As expected, incubation of cell lysates with BMS-200150 resulted in significant inhibition of the lipid transfer activities (data not shown). These studies show that AGI-S17 has no effect on cellular lipid transfer activity.

Next, we studied the effect of different concentrations of AGI-S17 on intracellular apoB–MTP binding (Figure 3B). Cells were incubated with different concentrations of AGI-S17 for 6 h, and MTP was immunoprecipitated under nondenaturing conditions (*17, 18*). Immunoprecipitates were separated on polyacrylamide gels and transferred to nitrocellulose, and the presence of apoB100 was visualized using polyclonal antibodies (Figure 3B). Treatment of HepG2 cells with 30 and 40 μM AGI-S17 resulted in a marked decrease (50–80% inhibition) in the coimmunoprecipitation of apoB with MTP. These studies indicate that AGI-S17 inhibits intracellular apoB–MTP binding.

Effect of AGI-S17 on the Secretion of ApoB by HepG2 Cells. We used two independent approaches to study the effect of AGI-S17 on the secretion of apoB. In the first approach, the effect of AGI-S17 on the secretion of newly synthesized apoB was determined. For this purpose, HepG2 cells were labeled with [^{35}S]methionine for 6 h in the presence and absence of AGI-S17, and the secretion of apoB100 and albumin was studied (Figure 4A). Albumin has been used as a control for apoB secretion in several studies under similar conditions (*11, 33–36*). Different concentrations of AGI-S17 (0–40 μM) did not affect the incorporation of radiolabeled amino acids into newly synthesized or secreted proteins as determined by trichloroacetic acid precipitation (data not shown). Next, we studied the effect of AGI-S17 on the secretion of apoB and albumin. In this experiment, the amount of apoB or albumin secreted by HepG2 cells was not inhibited by treatment of cells with 20

A. Microsomal Triglyceride Transfer Activity



B. Coimmunoprecipitation of ApoB100 with MTP

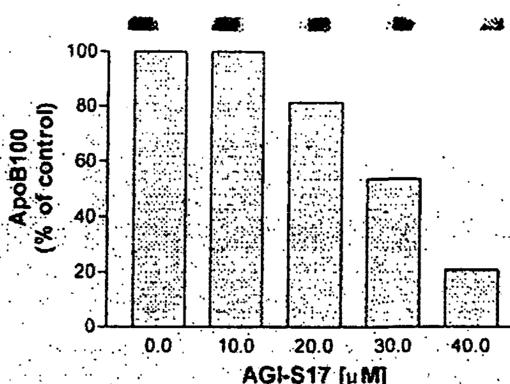


FIGURE 3: Effect of AGI-S17 on the lipid transfer and apoB binding activities of MTP in HepG2 cells. HepG2 cells were incubated with the indicated amounts of AGI-S17 in serum-free media containing 1% bovine serum albumin for 6 h as described under Materials and Methods. (A) Cells were lysed as described under Materials and Methods, and 100 μg of protein was used for in vitro lipid transfer activity using [^{3}H]triolein. Mean ± standard deviations, $n = 3$, are plotted as bar graphs and error bars, respectively. (B) Cells were lysed under nondenaturing conditions, and MTP was immunoprecipitated with 10 μL of anti-MTP antibodies. The immunoprecipitates were washed, eluted in sample buffers, separated on a 5% polyacrylamide gel, transferred to nitrocellulose, and reacted with anti-human apoB antibodies, and bands were visualized using chemiluminescence detection reagents and fluorography. The bands were scanned, quantified using a densitometer, and plotted as bar graphs. The data are a representative of two independent experiments.

apoB100 without affecting the secretion of albumin (Figure 4A). The experiment was repeated using triplicate wells. AGI-S17 (40 μM) inhibited apoB secretion by 61% ± 9%, but had no effect on albumin secretion. These studies indicate that AGI-S17 inhibits nascent apoB secretion.

In the second approach, we quantified the effect of AGI-S17 on the secretion of total mass of apoB by ELISA (Figure 4B). Total protein content in HepG2 cells was not affected by incubation with AGI-S17, but apoB secretion was inhibited by 73% at 40 μM (Figure 4B). Taken together, these studies show that AGI-S17 specifically inhibits apoB secretion.

To investigate further the effect of inhibition of apoB–MTP binding on the flotation properties of secreted apoB, conditioned medium obtained from cells incubated either with or without AGI-S17 was subjected to a density gradient

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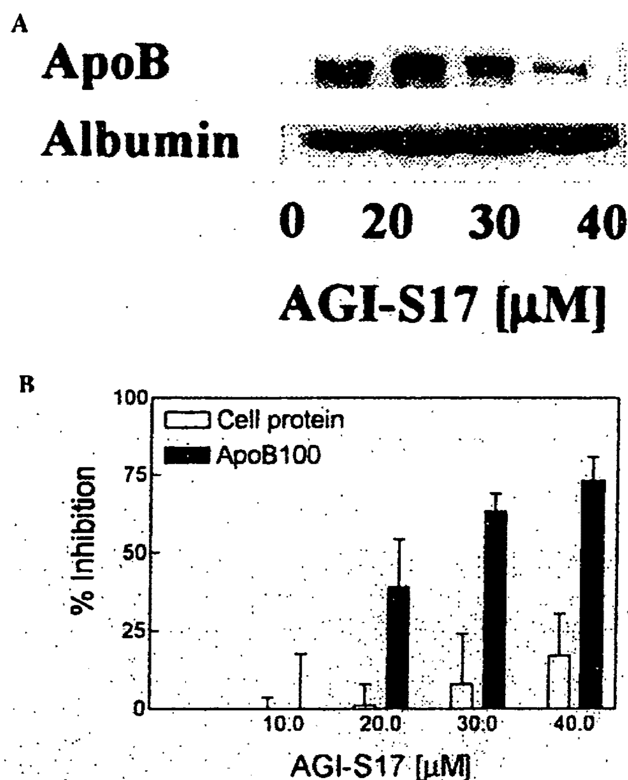

FIGURE 4: Effect of AGI-S17 on the secretion of apoB polypeptides by HepG2 cells. (A) Effect on the secretion of nascent apoB. Cells were incubated with different concentrations of AGI-S17 in serum-, methionine-, and cysteine-free medium containing 1% bovine serum albumin and 100 μCi/mL Expre^{35}S^{35}S for 6 h. ApoB and albumin were immunoprecipitated sequentially using polyclonal antibodies, separated on polyacrylamide gels, and exposed to Phosphorimager screens. (B) Effect on the secretion of total apoB mass. HepG2 cells were incubated in triplicate with different concentrations of AGI-S17 in serum-free medium containing 1% bovine serum albumin for 6 h. Control cells received dimethyl sulfoxide only. ApoB in the conditioned media was measured by ELISA. The data are representative of five independent experiments.

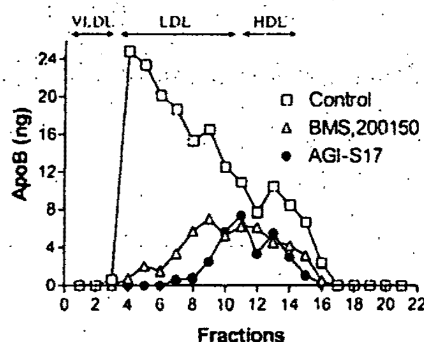

FIGURE 5: Effect of inhibition of MTP activities on the secretion of apoB-containing lipoproteins. HepG2 cells were incubated with either dimethyl sulfoxide (control), AGI-S17 (40 μM), or BMS-200150 (10 μM) in DMEM containing 1% bovine serum albumin for 6 h. KBr (1 g) was added to the conditioned media (2 mL), sequentially overlayed with 2, 2, 2, and 3 mL of 1.21, 1.063, 1.019, and 1.006 g/mL density solutions, and centrifuged (SW41 rotor, 40 000 rpm, 12 °C, 24 h), and different fractions (0.5 mL) were collected from the top of the tube. ApoB was measured in each fraction by ELISA. The distribution of apoB in different fractions is presented.

inhibited by 84%. Under these conditions, no LDL-size particles were observed. However, reduced amounts of HDL-size particles were secreted in the presence of AGI-S17. We also studied the effect of BMS-200150 on the flotation properties of secreted lipoproteins. As expected, BMS-200150 significantly decreased (73% inhibition) apoB secretion. More important, the major effect was on the secretion of LDL-size particles. These studies indicated that inhibition of either apoB−MTP binding or MTP's lipid transfer activity results in decreased secretion of apoB-containing lipoproteins. Thus, the phenotypic effect of inhibiting two independent functions of MTP is very similar.

DISCUSSION

We have identified a novel antagonist, AGI-S17, that inhibited apoB−MTP binding but did not affect MTP's lipid transfer activity (Figure 1). Treatment of HepG2 cells with AGI-S17 had no effect on intracellular lipid transfer activities (Figure 3A). However, it decreased the amounts of apoB coimmunoprecipitated with MTP (Figure 3B). Furthermore, it decreased apoB secretion in hepatoma cells (Figure 4). The extent of inhibition by AGI-S17 (40 μM) on the coimmunoprecipitation of apoB with MTP (Figure 3B) and apoB secretion (Figure 4B) by HepG2 cells was similar. Thus, AGI-S17 most likely inhibits apoB secretion by inhibiting intracellular apoB−MTP binding and may represent a novel class of compounds that can be useful in decreasing LDL levels in certain hyperlipidemias independent of their etiology.

Different experimental conditions and inhibitors have different effects on lipid transfer and apoB binding activities of MTP. Previously, we had demonstrated that immobilization of MTP results in ≈80% loss of its lipid transfer activity (*19*), whereas the binding of immobilized and soluble MTP to apoB peptides was similar (*21*). These studies suggest that immobilization of MTP results in partial loss of the lipid transfer activity but has little effect on apoB binding. In the present study, we have demonstrated that BMS-200150, an inhibitor of MTP's lipid transfer activity, has no effect on apoB−MTP interactions (Figure 1A). Similarly AGI-S17 inhibits apoB−MTP binding, but has no effect on the lipid transfer activity of MTP (Figures 1 and 3). Thus, the heterodimeric MTP contains at least two independent functional domains that are involved in apoB binding and lipid transfer activity, respectively.

The present study shows that AGI-S17 decreases apoB secretion, indicating that apoB−MTP binding is physiologically important for its secretion. In another study, mutation of an arginine residue that disrupts a salt bridge within apoB has been shown to decrease the binding of apoB to MTP and its secretion (*22, 23*). In both these studies, the lipid transfer activity of MTP was not affected. Thus, the assembly and secretion of apoB-containing lipoproteins may require both apoB binding and lipid transfer activities of MTP. Both of these activities may function independent of each other.

It is well-known that MTP's lipid transfer activity is necessary for the lipidation of nascent apoB and lipoprotein assembly. What would then be the role of protein−protein interactions between apoB and MTP? It has been suggested

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