Microsomal triglyceride transfer protein (MTP) inhibitors: Discovery of clinically active inhibitors using high-throughput screening and parallel synthesis paradigms

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The inhibition of microsomal triglyceride transfer protein (MTP) blocks the hepatic secretion of very low density lipoproteins (VLDL) and the intestinal secretion of chylomicrons. Consequently, this mechanism provides a highly efficacious pharmacological target for the lowering of low density lipoprotein (LDL) cholesterol and reduction of postprandial lipemia. The combination of these effects could afford unprecedented benefit in the treatment of atherosclerosis and consequent cardiovascular disease. The promise of this therapeutic target has attracted widespread interest in the pharmaceutical industry. Independent efforts have yielded strikingly similar series of lipophilic amide inhibitors. The way in which the evolutionary paths of distinct inhibitor series have tended to converge through the course of robotics-assisted synthesis efforts is illustrated with candidates from Bristol-Myers Squibb and Pfizer. Hanging in the balance with the exceptional potency of the compounds presented are the potential adverse effects due to blockage of intestinal fat absorption and hepatic lipid secretion. Finding a degree of efficacy that can be safely tolerated defines the dilemma surrounding the advancement of these compounds to clinical practice.

Keywords Apolipoprotein A1, apolipoprotein B, cholesterol, HepG2 cells, LDL, lipid transfer inhibition, MTP, triglycerides, VLDL

Abbreviations

AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
apo	Apolioprotein
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
ER	Endoplasmic reticulum
FH	Familial hypercholesterolemia
HDL	High density lipoprotein
HTS	High-throughput screening
LDL	Low density lipoprotein
MTP	Microsomal triglyceride transfer protein
VLDL	Very low density lipoprotein
WHHL	Watanabe heritable hyperlipedemic

Introduction

Cardiovascular disease remains the leading cause of death in industrialized nations and, as such, accounted for 950,000 or 41% of all deaths in the US in 1998 [1•]. Coronary heart disease (CHD), as a consequence of atherosclerosis, is the most common cause of cardiovascular morbidity and mortality, with an estimated 12 million people suffering from CHD in the US alone [1•]. Elevated total and low density lipoprotein (LDL) cholesterol are both accepted primary risk factors for atherosclerosis [1•,2•,3]. An estimated 101 million US adults have elevated blood cholesterol (> 200 mg/dl) and are candidates for LDL cholesterol lowering through dietary intervention [1•,4,5•]. Of these, 41 million are considered high risk, having blood cholesterol > 240 mg/dl, and drug therapy is recommended [1•,4,5•].

Epidemiological studies have shown that elevated triglycerides and reduced high density lipoprotein (HDL) cholesterol are also contributing factors for the development of CHD [2•,3,6-8]. Among the adult US population, 19% have low HDL cholesterol (< 40 mg/dl) [3,9,10••] and 21% have hypertriglyceridemia (> 150 mg/dl) [3,10••]. Thus, as important as elevated LDL cholesterol is as a risk factor for CHD, it is important to recognize that the most common spectrum of lipid abnormalities, one which is present in 45 to 50% of men with CHD, is atherogenic dyslipidemia [11,12], which includes borderline high-risk LDL cholesterol (eg, 130 to 159 mg/dl), elevated triglycerides, small dense LDL particles and low HDL cholesterol.

Although the HMG-CoA reductase inhibitors (statins) are effective in lowering LDL cholesterol, and somewhat effective in reducing triglycerides, they have only minimal effects on HDL cholesterol [2•,5•,13••,14••,15]. Indeed, although numerous clinical trials have demonstrated that LDL cholesterol reduction can significantly reduce the risk of CHD, a great number of treated subjects who achieve substantial LDL cholesterol reduction still experience a clinical event [2•,3,13••,14••,15,16•,17•,18]. Therefore, with the goal of developing a therapy for treating patients with dyslipidemias that extends beyond primary hypercholesterolemia, the pharmaceutical industry has targeted inhibition of microsomal triglyceride transfer protein (MTP) as a mechanism for reducing not only plasma total and LDL cholesterol, but also plasma very low density lipoprotein (VLDL) cholesterol and triglycerides.

MTP, which is located within the lumen of the endoplasmic reticulum (ER) in hepatocytes and absorptive enterocytes, is a heterodimeric protein consisting of a 97-kDa subunit, which confers all of the lipid transfer activity of the heterodimer, and a 58-kDa multifunctional protein disulfide isomerase [19••]. MTP plays a pivotal, if not obligatory role in the assembly and secretion of triglyceride-rich, apolioprotein B (apoB)-containing lipoproteins (VLDL and chylomicrons) from the liver and intestine, and also catalyzes the transport of triglycerides, cholesteryl ester and phospholipids between membranes [19••,20,21]. Although

the exact role of MTP in the assembly of apoB-containing lipoproteins is still under investigation [21,22,23•], MTP is proposed to transport lipids from the ER membrane to the growing apoB polypeptide chain in the lumen of the ER, allowing proper translocation and folding of apoB to occur [19••,20-22,23•,24••]. Hence, inhibition of MTP should reduce plasma lipids by preventing triglyceride-rich, apoB-containing lipoprotein assembly in the liver and intestine.

The initial suggestion that MTP inhibition could be a viable lipid-lowering therapy came with the discovery that functional MTP is absent in individuals with abetalipoproteinemia, a genetic disorder characterized by low plasma cholesterol and triglycerides, due to a defect in the assembly and secretion of apoB-containing lipoproteins [25••,26••]. A similar phenotype is observed in MTP knockout mice [23•,27••]. However, abetalipoproteinemia represents an extreme example of MTP inhibition and is not without its clinical sequalae, all of which presumably are related directly or indirectly to fat malabsorption (steatorrhea), vitamin malabsorption, and hepatic and intestinal steatosis [26••,28]. A less severe, and probably more relevant example of the consequences of therapeutic MTP inhibition is a related genetic disease, hypobetalipoproteinemia, caused by mutations in apoB [29]. Heterozygous individuals with this disease possess half of the normal levels of apoB-containing lipoproteins, lack the clinical signs and symptoms of abetalipoproteinemia and have a prolonged lifespan [24••].

High-throughput screening and robotics-assisted parallel synthesis for identification of potent developmental candidates

To identify potent MTP inhibitors, various members of the pharmaceutical industry have devised relatively similar two-stage, empirical screening protocols for compound evaluation. The primary difference is the order of *in vitro* versus cell culture evaluation. At Pfizer, for example, in the first stage of the protocol, compounds were evaluated for their ability to inhibit apoB but not apoA1 secretion from HepG2 cells in a high-throughput, 96-well multiplexed format [30••]. In the second stage of the protocol, confirmed apoB secretion inhibitors were evaluated for their ability to inhibit the MTP-mediated transfer of radiolabeled triolein from synthetic phospholipid donor liposomes to acceptor liposomes [30••]. Using this two-stage screening protocol, Pfizer scientists identified compound **1** (Figure 1) as a potent inhibitor of apoB secretion (IC_{50} = 200 nM) but not of apoA1 secretion [31••]. Inhibition of apoB secretion was determined to be through the action of inhibiting MTP (rat MTP IC_{50} = 250 nM).

With the rather simple structure of compound **1**, Pfizer scientists pursued a robotics-assisted parallel synthesis strategy as a means of exploring alternatives for the 4-toluidine moiety, with the goal of improving potency. Unfortunately, the biphenyl carboxylic acid moiety of compound **1** was not suitable for automated organic synthesis. Attempts to activate the acid group for amide formation resulted in exclusive formation of fluorenone **2** (Figure 1). With the intent of deactivating the aromatic ring and, thus, suppressing fluorenone formation, the 4'-trifluoromethyl-2-biphenyl carboxylic acid **3** (Figure 1) was utilized. In this case, amide formation was uneventful and the resulting compound **4** (Figure 1) remained a potent inhibitor of apoB secretion (IC_{50} = 170 nM) and MTP activity (rat MTP IC_{50} = 90 nM). Using a novel parallel synthesis paradigm, compound **5** (Figure 1) was identified (rat MTP IC_{50} = 30 nM). Removal of the Boc protecting group resulted in a tetrahydro-isoquinoline, which served as an advanced template for further parallel synthesis. Using this paradigm, 500 compounds were prepared in a period of 4 months, leading to the discovery of amide **6** (Figure 1; rat MTP IC_{50} = 7 nM).

Despite improved inhibitory activity in vitro, compound **6** displayed only weak triglyceride lowering activity when administered orally to rats. This lack of efficacy was presumed to be due to poor solubility and rapid clearance. Using in vitro hepatic microsomal clearance as a guide, followed by screening for triglyceride lowering in vivo, CP-346086 (**7**, Pfizer Inc; Figure 1) was ultimately identified as a potent (IC_{50} = 2.0 nM) and orally efficacious MTP inhibitor [31••,32••,33,101].

Bristol-Myers Squibb also reported success in identifying potent and efficacious MTP inhibitors via a similar high-throughput screening (HTS) and parallel synthesis paradigm [24••]. HTS of their chemical library yielded compound **8** (Figure 2), which inhibited MTP activity (IC_{50} = 2.2 μM) and HepG2 cell apoB secretion (IC_{50} = 1.8 μM) with no effect on apoA1 secretion (IC_{50} > 30 μM). The fluorene analog **9** (Figure 2) was also identified by HTS, but its MTP inhibitory activity was much weaker (IC_{50} = 36 μM). Proposing that the fluorenyl moiety overlaps with the diphenylmethyl moiety of compound **8**, 'hybrid' analogs of **8** and **9** were prepared, yielding compounds with significantly improved MTP inhibitory activity, eg, compound **10** (Figure 2; MTP IC_{50} = 36 nM). Modification of the isoindolone moiety of compound **10** revealed that benzamide **11** (Figure 2; MTP IC_{50} = 23 nM) was a suitable replacement. Optimization of compound **11** was carried out by an automated organic synthesis paradigm to yield BMS-201038 (**12**, Figure 2; MTP IC_{50} = 0.5 nM) [24•,102]. Of particular interest is the similarity of this molecule to the Pfizer series in which the 4'-trifluoromethyl-2-biphenyl carboxylic acid moiety was also found to aid optimal MTP inhibitory activity.

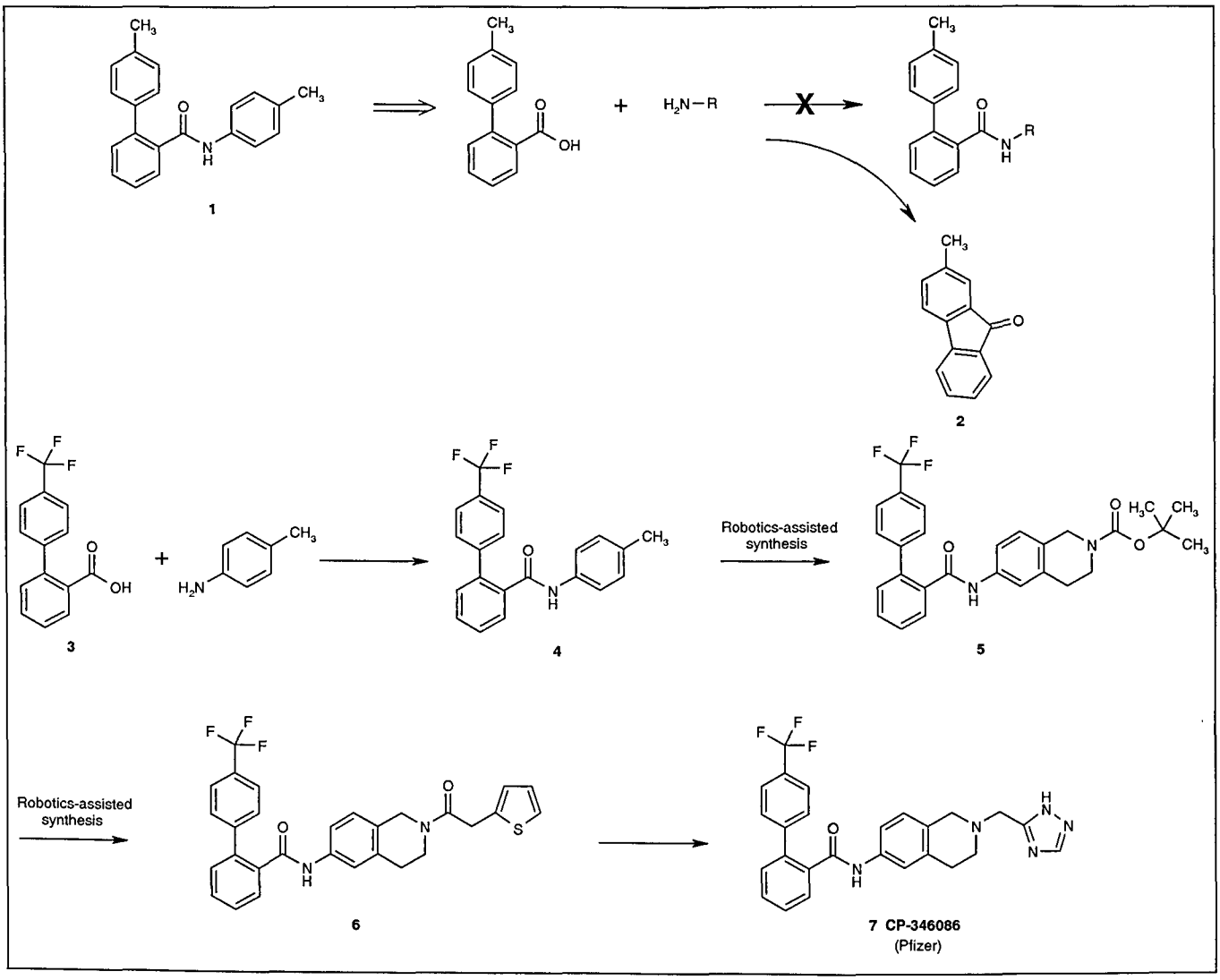
Convergent development of structure-activity relationships across the industry

Inhibition of MTP as a means of treating dyslipidemia is being pursued by many other pharmaceutical companies, including Bayer [34,35••,103], Boehringer Ingelheim [104], Glaxo Group Ltd [105], Janssen Pharmaceutica [106], Japan Tobacco [107], Meiji Seika Kaisha Ltd [108], Norvartis [36••,109] and Wakunaga Pharmaceutical Co Ltd [110]. Representative structures from some of these pharmaceutical companies are shown in Figure 3. It is worth noting that the 4'-trifluoromethyl-2-biphenyl carboxylic acid moiety is a re-occurring group in many of these structures.

In vitro efficacy and mechanism of action studies

Mechanistic studies, using a variety of MTP inhibitors that have entered into development, have provided consistent information regarding the mechanism of action of this class of lipid lowering agents. Developmental prototypes, such as

Figure 1. Strategy followed by Pfizer, leading to the synthesis of CP-346086.



CP-346086, BMS-201038 and BAY-13-9952 (**13**, implitapide, Bayer AG; BMS-201038 and BAY-13-9952 (**13**, implitapide, Bayer AG; Figure 3), all potently and dose-dependently inhibited human and/or rodent MTP-mediated triglyceride transfer between synthetic donor and acceptor liposomes with IC_{50} values of 2 to 10 nM [24••,31••,32••,33,35••]. CP-346086 also inhibited human MTP-mediated transfer of cholesteryl oleate between donor and acceptor liposomes with an IC_{50} value of 1.9 nM, indicating the ability of this compound to equally inhibit transfer of both neutral lipids [32••,33]. However, CP-346086 did not inhibit cholesteryl ester transfer protein (CETP) activity at concentrations of up to 10 µM. Not only does this indicate this compound's specificity for inhibition of MTP-mediated neutral lipid transfer, but it also demonstrates its lack of effect on the physicochemical properties of the donor and acceptor vesicles [32••,33].

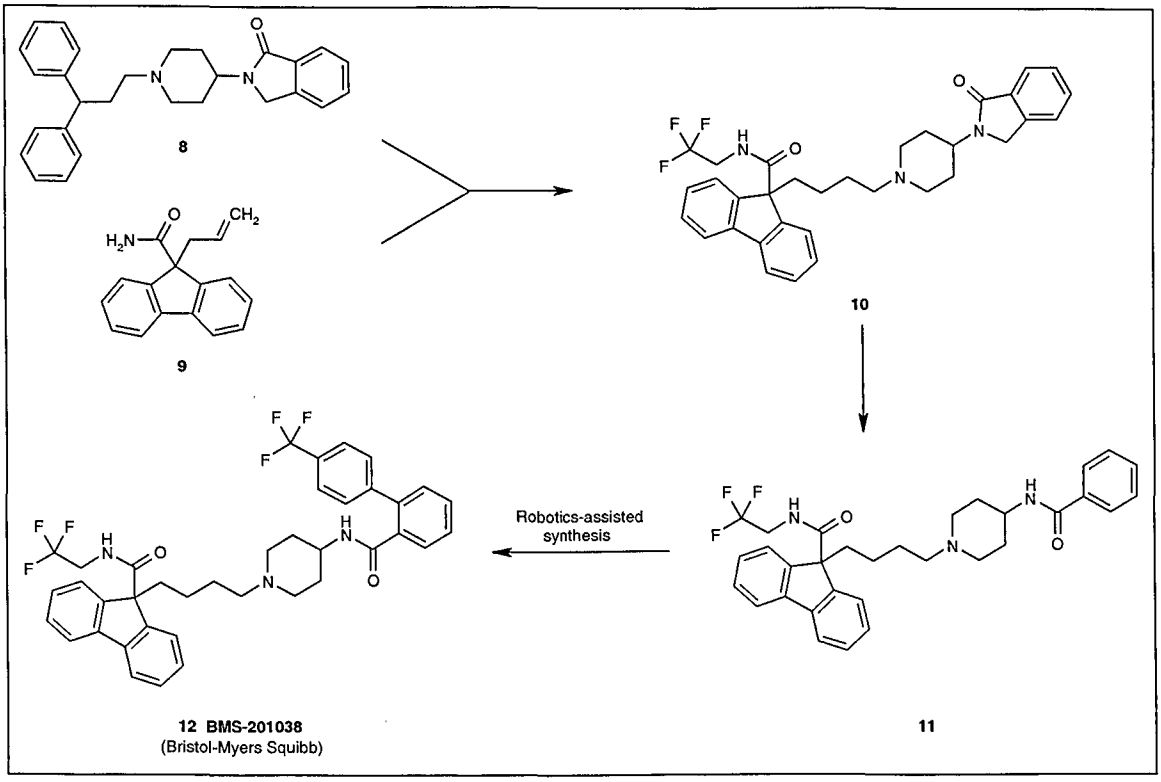
As a consequence of their inhibition of MTP-mediated triglyceride transfer, CP-346086, BMS-201038 and BAY-13-9952 also inhibited HepG2 cell apoB secretion and triglyceride secretion with IC_{50} values of 1 to 3 nM [24••,32••,33,35••,37] without concomitant inhibition of apoA1 secretion [24••,32••,33,35••,37] or cholesterol, fatty acid or triglyceride synthesis [32••,33].

In vivo efficacy and mechanism of action summary

The lipid lowering and anti-atherosclerosis effects of MTP inhibitors have been consistently observed and broadly demonstrated across all series evaluated using a wide variety of representative animal models. The pharmacological effects observed with all MTP inhibitors evaluated are consistent with their mechanism of action.

The effect of MTP inhibition on plasma triglyceride levels is rapid. For example, a single dose of CP-346086 administered orally to rats or mice lowered plasma triglycerides in a dose-dependent manner, exhibiting an ED_{30} of 1.3 mg/kg in both species 2 h after administration [32••,33]. Consistent with its mechanism, CP-346086 did not simultaneously lower plasma cholesterol levels after single dose administration [32••,33].

Figure 2. Synthesis of BMS-201038.



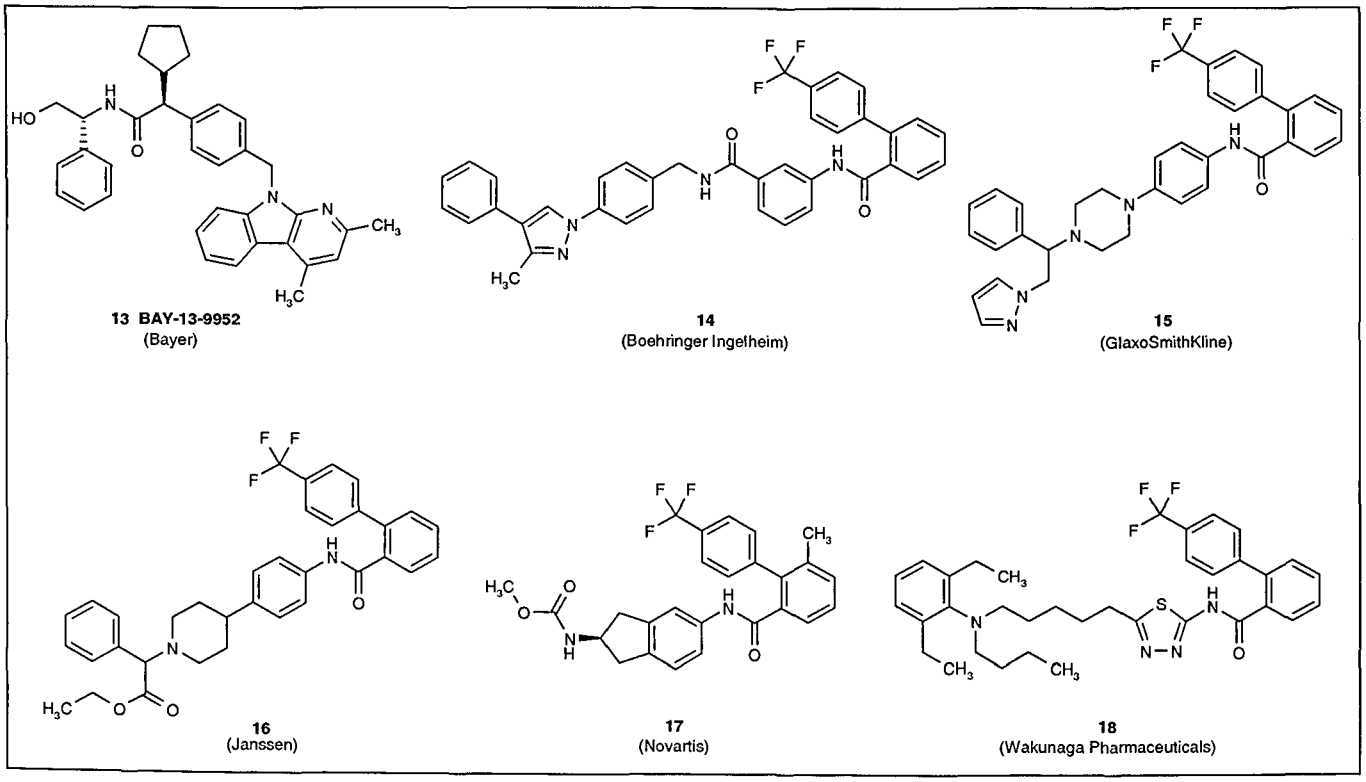
The reduction of plasma triglycerides in animal models by MTP inhibitors is due to inhibition of secretion of apoB-containing lipoproteins. For example, in studies in which mice were treated with Triton WR-1339 to prevent lipoprotein lipase-mediated triglyceride hydrolysis and, hence, VLDL and chylomicron clearance from plasma, single-dose administration of CP-346086 inhibited triglyceride secretion from the liver in fasted animals, indicating that the acute triglyceride lowering described above was an effect of the compound on hepatic triglyceride secretion rather than on clearance of triglyceride-rich particles from the circulation [32••,33]. Similar conclusions were also drawn from studies in rats with BMS-201038 [24••] and in Watanabe heritable hyperlipidemic (WHHL) rabbits with BAY-13-9952 [34,35••]. BMS-201038 and CP-346086 also inhibited lipoprotein secretion in fed rats, indicating their ability to inhibit both hepatic and intestinal lipoprotein secretion [24••,32••,33].

Dose-dependent cholesterol lowering was observed in several animal species after multiple-day administration. For example, after 2-week oral administration to rats, CP-346086 (10 mg/kg/day) lowered plasma triglycerides (62%), total cholesterol (23%), VLDL cholesterol (33%) and LDL cholesterol (75%) [32••,33]. Similarly, oral administration of BAY-13-9952 to obese fa/fa Zucker rats for 4 weeks at a dose of 0.5 mg/kg/day lowered plasma triglycerides (84%) and cholesterol (30%), with 80% reduction in plasma cholesterol noted at a dose of 5 mg/kg/day [35••]. In other studies, 1-week oral administration of BMS-201038 to golden Syrian hamsters dose-dependently decreased plasma triglycerides (35%), total cholesterol (59%), VLDL + LDL cholesterol (51%), and HDL cholesterol (66%) at a dose of 3 mg/kg [24••].

BAY-13-9952 also reduced plasma triglycerides and cholesterol in normolipidemic dogs, with 4-week oral treatment at a dose of 4 mg/kg reducing plasma cholesterol levels by 60% [35••]. Similar efficacy in dogs was also noted with the Novartis MTP inhibitor **17** (Novartis; Figure 3) [36••] and with CP-346086 [32••,33]. Studies conducted with orally administered BMS-201038 (2 mg/kg) in cynomolgus macaque monkeys demonstrated 50% lowering of total cholesterol after 7 days of treatment [37].

The effects of MTP inhibitors were also investigated in homozygous WHHL rabbits, an animal model in which statins have minimal effects. These rabbits have hepatic LDL receptor activity that is < 5% that of normal rabbits, resulting in dramatically elevated levels of LDL cholesterol [24••]. Hence, these rabbits are a model for human homozygous familial hypercholesterolemia (FH), where levels of LDL cholesterol are also very high due to a non-functional or missing LDL receptor [24••]. Studies with BAY-13-9952 administered at 12 mg/kg/day for 4 weeks led to plasma total cholesterol and triglyceride reductions of 70 and 45%, respectively, conditions under which the hepatic VLDL secretion rate was decreased by 80% [34]. BMS-201038 also showed efficacy in the WHHL rabbit, demonstrating an ED_{50} value for total plasma cholesterol and triglyceride lowering of 1.9 mg/kg and a complete normalization of atherogenic apoB-containing lipoprotein particles at a dose of 10 mg/kg [24••].

Figure 3. MTP inhibitors.



Recently, the anti-atherosclerotic and plaque stabilizing potential of MTP inhibitors were studied by Bayer [35••,38,39,40••]. In apoE knockout mice fed a Western-type diet containing BAY-13-9952 for 14 weeks at doses ranging from 1 to 15 mg/kg/day, in addition to reductions in plasma total cholesterol and triglycerides of up to 68 and 61%, respectively, significant reductions in atherosclerotic lesions and lesion lipid content were also observed [35••,38,40••]. The average cross-sectional plaque areas of the aortic root, determined by computer-aided morphometric analysis, were reduced by up to 93%, and lipid content was reduced by up to 99% [35••,38,40••]. This reduction in lesion development and lesion lipid content translated to a dose-related increase in survival time such that, while only one of 25 untreated mice was still alive after 18 months, up to 24 of 25 mice were still alive after 18 months of treatment with BAY-13-9952 [35••,40••]. It is also interesting to note that significant reductions in plaque area (66%) and lipid moieties within the plaque (55%) were observed at the 1 mg/kg-dose, where plasma cholesterol and triglycerides were unaffected [40••]. In addition, orally administered BAY-13-9952 (12 mg/kg/day) reduced fatty streak formation to control levels in New Zealand White rabbits fed a 0.5% cholesterol-enriched diet for 3 months [39].

Finally, because of increasing evidence that delayed clearance of postprandial lipemia is an important contributing factor to the development of atherosclerosis [41], the effect of the MTP inhibitors on postprandial lipemia was studied. In both rats and dogs, compound **17** (1 mg/kg) administered just prior to an oral fat load, effectively prevented the elevation of plasma triglycerides [36••]. This observation provides convincing evidence that MTP inhibition in the intestinal mucosa can result in substantial reduction of apoB-containing lipoprotein particle assembly and/or release, and can effectively attenuate postprandial lipemia.

Clinical efficacy of MTP inhibitors

CP-346086 showed evidence of activity consistent with its mechanism of action. When administered as a single oral dose to healthy human volunteers, CP-346086 reduced plasma triglycerides and VLDL cholesterol in a dose-dependent manner, with ED_{50} values of 10 and 3 mg, respectively, and maximal inhibition (100 mg) of 66 and 87% when measured 4 h after treatment [32••,33]. In a 2-week, multiple-dose, safety and toleration study in healthy volunteers, CP-346086 (30 mg) administered at bedtime, produced an average decrease in plasma total and LDL cholesterol of 47 and 68%, respectively, relative to either individual baseline values or placebo, with little change in HDL cholesterol [32••,33]. Plasma triglycerides were also decreased by up to 75% immediately after dose administration, but the reduction was transient [32••,33].

Similar efficacy was reported for BAY-13-9952, which produced a dose-dependent decrease in total cholesterol (45%), LDL cholesterol (55%) and triglycerides (29%) after 4 weeks of treatment at an oral dose of 160 mg/day [42••]. BMS-201038 also showed similar efficacy in phase I and phase II clinical trials [43].

In addition to direct effects on plasma lipid levels, MTP inhibitors have the potential to attenuate postprandial lipemia and, thus, favorably affect atherogenesis over and above their effects mediated through reduced steady state

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