ACAT inhibitors: evolution from cholesterol-absorption inhibitors to antiatherosclerotic agents

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Considerable research efforts have focused on the discovery of safe and efficacious acyl-CoA cholesterol acyltransferase (ACAT) inhibitors as cholesterol-lowering and/or antiatherosclerotic agents. Although clinical success remains elusive, recent developments in the molecular biology of ACAT and success in identifying potent and bioavailable inhibitors that are not adrenotoxic has led to renewed hope that ACAT inhibition may ultimately yield agents that will have value in treating human atherosclerotic disease.

Coronary artery disease (CAD) continues to be the leading cause of death in industrialized nations[1]; thus strategies for controlling the risk factors involved in CAD have been the focus of much research in industry and academia[2]. Because an elevated level of circulating plasma low-density lipoprotein cholesterol (LDL-C) has been identified as an independent risk factor in the development of CAD[3], many of these strategies have been directed at lowering levels of cholesterol carried in this atherogenic lipoprotein[4]. Since cholesterol is available to the body through two basic mechanisms – endogenous biosynthesis and absorption from the gastrointestinal tract – much of the research has focused on these two fundamental processes. Efforts directed towards finding safe and effective cholesterol biosynthesis inhibitors have resulted in the development of a large number of potent, specific inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis[5]. Efforts to find a similar enzyme responsible for the esterification of cholesterol in intestinal mucosal cells that was rate-limiting for cholesterol absorption led to the postulation of both cholesterol acyltransferase (ACAT; EC 2.3.1.26)[6] and pancreatic cholesteryl ester hydrolase[7] as candidate enzymes. The seminal work of Norum[8], Heider[9], Field[10] and others[11] ultimately led to the conclusion that ACAT was the primary enzyme responsible for the esterification of cholesterol in intestinal mucosal cells. Subsequent work has suggested that ACAT is the enzyme responsible for synthesis of the cholesteryl esters packaged into very-low-density lipoproteins (VLDL) secreted by liver[12] and in atherosclerotic lesions in animal models[13]. This has led to the hypothesis that inhibitors of this enzyme could lower plasma cholesterol by inhibiting cholesterol absorption and/or reducing VLDL production in liver[14], and that inhibitors that reached the peripheral circulation could directly block atherosclerotic lesion formation[15]. The latter possibility has stimulated interest in the design of a second generation of orally bioavailable inhibitors[16].

ACAT enzymology

ACAT is an integral membrane protein, located in the endoplasmic reticulum (ER) of cells, which catalyzes the esterification of cholesterol by long-chain fatty acyl-CoA derivatives (Figure 1). ACAT activity is regulated primarily by cholesterol concentration in the ER (Ref. 17). ACAT mRNA levels also increase in a tissue-specific manner in response to

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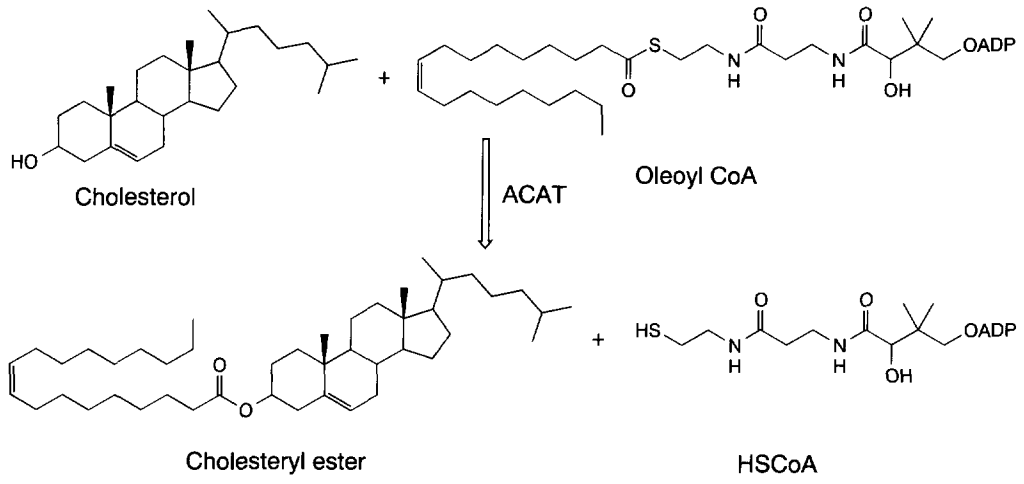

Figure 1. *The reaction catalyzed by acyl-CoA cholesterol acyltransferase (ACAT). CoA, coenzyme A.*

dietary fat and cholesterol[18]. In order to better understand the structure and function of ACAT, considerable effort has been devoted to the cloning and isolation of the pure enzyme[19]. Thus, Chang *et al.*[20] isolated a 4-kb cDNA clone (called K1) from a human macrophage library which was predicted to encode for a 550 amino acid protein. Transfection of this clone into ACAT-deficient Chinese hamster ovary cells restored their ability to esterify cholesterol, suggesting that this protein was human ACAT. Hydrophobicity analysis of the predicted ACAT protein indicated that this protein may contain at least two transmembrane helices and shares peptide sequences homologous with other enzymes involved in the catalysis of acyl transfer. Following this discovery, a second group of investigators suggested that ACAT and liver carboxylesterase were in fact the same enzyme[21]; however, this supposition has been disputed[22].

Studies examining the tissue expression and regulation of ACAT activity have suggested that two isoforms of the enzyme may exist. Using sensitivity to diethyl pyrocarbonate (DEP) as a probe, Kinnunen *et al.*[23] suggested that two subtypes of ACAT existed in rabbit and that these subtypes were differentially regulated by cholesterol feeding. Thus, in normal, chow-fed rabbits, ACAT in liver, pancreas and intestine was not inhibited by DEP, whereas ACAT activity in all other organs tested (adrenal, aorta, kidney, lung, heart, testis, brain) was blocked by 100 μM DEP. Interestingly, after cholesterol feeding, the ACAT activity in the intestine became DEP sensitive, whereas, in liver and aorta, DEP sensitivity was unchanged. Aortic ACAT was the most sensitive to DEP treatment under both conditions, whereas liver required millimolar amounts, suggesting marked differences in the accessibility of histidine residues between liver and aortic ACAT proteins.

The supposition that different ACAT subtypes exist has now been further supported by studies in transgenic mice in which the gene encoding for the ACAT protein discovered by Chang *et al.*[20] was disrupted[24]. Animals homozygous for this mutation were healthy and grew normally; however, peritoneal macrophages from these animals, unlike those from wild-type mice, did not take up acetylated LDL to form foam cells. Importantly, adrenals from these animals were also very depleted in cholesteryl esters, but functioned normally. In contrast, hepatic cholesteryl esters were only 30% lower than those of wild-type mice. In addition, cholesterol absorption was not significantly different in ACAT-deficient mice, whether fed a chow or high-fat, high-cholesterol diet. Although there is not complete concordance between the data from the chemical modification and biological knockout experiments, taken collectively these data support the supposition that at least two ACAT protein subtypes exist and that liver ACAT is a different subtype from adrenal and aortic ACAT. This conclusion is consistent with results found in yeast[25] and some early studies with the Lederle compound CL277082 (**2**; Figure 2)[26]. The existence of ACAT subtypes, if confirmed, may ultimately lead to subtype selective inhibitors which may have pharmacologic and/or toxicologic properties distinct from current inhibitors.

Early efforts directed at lipid lowering

Although the potential of ACAT inhibitors to block atherosclerosis was appreciated as early as 1986 (Ref. 13), early work on ACAT inhibitors was directed at finding agents that would lower plasma total and/or LDL-C by blocking cholesterol absorption[9,26]. Thus, several fatty acid amides [e.g. 57118 (**3**) and 58035 (**4**); Figure 2], expected to mimic the substrate of ACAT, were developed during the early

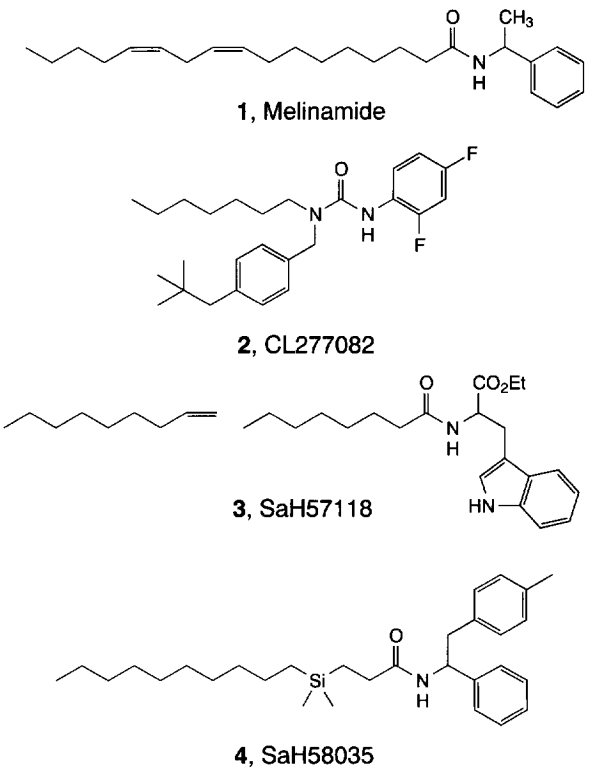

Figure 2. *Early acyl-CoA cholesterol acyltransferase inhibitors.*

work on ACAT inhibitors conducted at Sandoz and found to potently and selectively inhibit ACAT *in vitro* and to block cholesterol absorption and lower plasma total cholesterol in cholesterol-fed animal models *in vivo*[9,27]. In parallel with this work, a series of tri-substituted ureas was developed by DeVries *et al.*[28,29] and found to be potent and selective ACAT inhibitors *in vitro* and very effective at lowering plasma total cholesterol in cholesterol-fed animal models *in vivo*. These two series provided the foundation for much of the early research to determine the utility of ACAT inhibitors as well as the first compounds to be studied clinically. This work has recently been reviewed[30].

Early clinical studies

SaH57118 (**3**) and SaH58035 (**4**) were both studied in Phase I clinical trials and found to be safe at doses as high as 5 g day^{-1} and to reduce cholesterol absorption in hyperlipidemic patients by as much as 70%. However, development was discontinued prior to evaluation of their clinical efficacy[30]. In fact, the only clinical efficacy data available for a fatty acid amide are for melinamide (**1**), which was developed originally as an intestinal cholesterol-absorption inhibitor whose effect on ACAT was not known until after it was marketed[31]. This compound displayed modest cholesterol-lowering ability in clinical trials, reducing plasma total cholesterol 20% in hypercholesterolemic patients after 12 months' administration at a dose of 2.25 g day^{-1} (Ref. 32).

The next ACAT inhibitor evaluated for efficacy in humans was the tri-substituted urea CL277082 (**2**)[33]. When administered to eight healthy male volunteers at a dose of 750 mg day^{-1} for 20 days, no effects were observed on cholesterol absorption, sterol excretion rates or any plasma lipoprotein, despite attaining significant plasma drug levels. Several potential explanations were offered, including the possibility that ACAT was not rate-limiting for cholesterol absorption in humans. This clinical result illustrated a fundamental difference between cholesterol-fed animals and normolipidemic

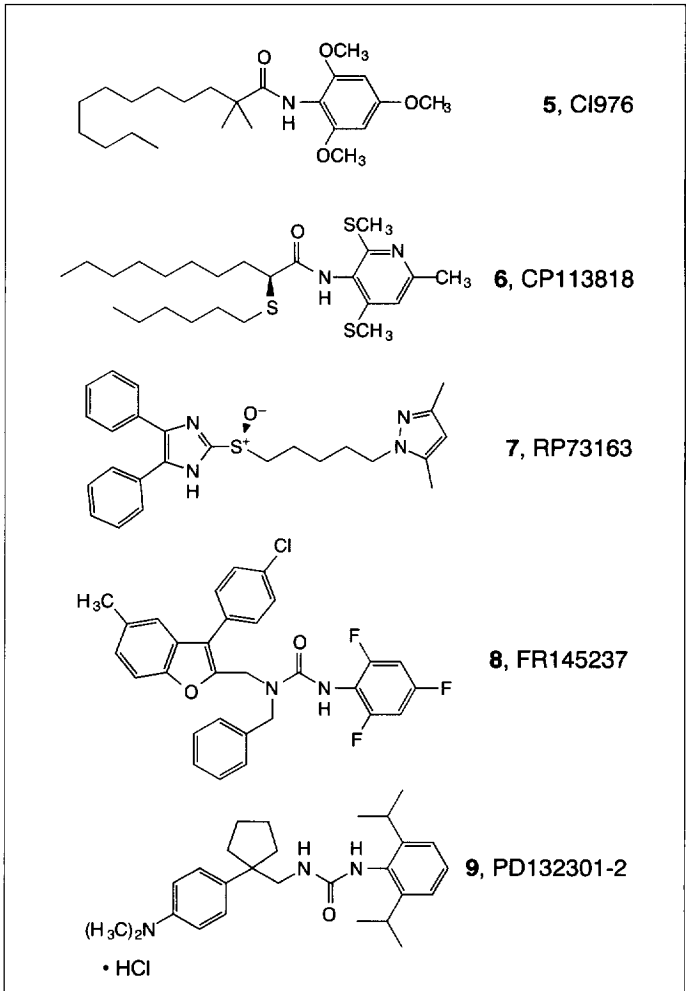

Figure 3. *Bioavailable acyl-CoA cholesterol acyltransferase inhibitors.*

Figure 4. *General histologic appearance and relative macrophage involvement in the iliac femoral artery of control and CI976-treated rabbits. (a) General histologic appearance of atherosclerotic lesions in control animals. Note the significant fibrofoamy intimal lesion. Hematoxylin and eosin stain. (b) Monocyte-macrophage involvement in atherosclerotic lesions of control animals. Monocyte-macrophages are depicted as areas of black immunoprecipitant. RAM11 immunohistochemistry with hematoxylin nuclear counterstain. (c) General histologic appearance of atherosclerotic lesions in CI976-treated animals. Note the qualitative reduction in lesion area. Hematoxylin and eosin stain. (d) Monocyte-macrophage involvement in atherosclerotic lesions of CI976-treated animals. RAM11 immunohistochemistry with hematoxylin nuclear counterstain. In all micrographs the arrows delineate the location of the internal elastic lamina. L, lumen. Bar: 100 μm. (Micrograph kindly provided by Dr Thomas Bocan.)*

volunteers in the response to CL277082 and therefore caused concern that ACAT inhibition may not be a useful means for lowering plasma total cholesterol or LDL-C in humans, or that the benefit(s) of ACAT inhibition may not be evident in nonhypercholesterolemic individuals.

CI976: a key turning point

Despite the discouraging results observed in these early clinical trials, the search for more effective ACAT inhibitors continued. A key turning point in understanding the utility of ACAT inhibitors occurred with the publication of studies in a unique cholesterol-fed rabbit model of atherosclerosis with the potent, specific ACAT inhibitor CI976 (**5**; Figure 3)[15]. In this model, in addition to the naturally occurring hypercholesterolemia-induced lesions of the thoracic aorta, atherosclerotic lesions comparable to human fatty streak lesions were induced in rabbits by chronic denudation of the endothelium in the iliac-femoral artery. Administration of CI976 to these animals not only blocked the progression of the naturally occurring diet-induced lesions in the noninjured thoracic aorta, but also induced the regression of the preestablished lesions in the injured iliac femoral artery (Figure 4). Most notably, this effect was observed even with doses at which CI976 did not affect plasma total cholesterol. Conventional lipid-lowering therapies such as cholestyramine or coadministration of cholestyramine and niacin required substantial reductions in plasma total cholesterol to achieve the same effect as that observed with CI976. These facts taken together led to the conclusion that the effects observed with CI976 were the result of direct inhibition of arterial ACAT.

This result reinvigorated efforts in the ACAT field, spawning a plethora of structurally diverse inhibitors, but with a new focus: finding systemically bioavailable inhibitors that would have the potential of directly affecting atherosclerotic disease at the artery wall[16,34]. Structures for several of the more extensively studied compounds that have been demonstrated to have systemic bioavailability and that displayed efficacy in models of atherosclerosis are shown in Figure 3 [CP113818 (**6**)[35]; RP73163 (**7**)[36]; FR145237 (**8**)[37]; PD132301-2 (**9**)[38]].

A new hurdle: adrenal toxicity

Success in finding systemically bioavailable pharmaceuticals always comes at the risk of discovering target organ toxicity. The first report of toxicity with an ACAT inhibitor stemmed from studies performed with the potent, bioavailable urea PD132301-2 (**9**)[38,39]. Administration of PD132301-2 at a wide range of doses to beagle dogs for 2 weeks resulted in depletion of adrenal cholesteryl esters at all but the lowest dose (6 mg kg^{-1}) as an apparent pharmacodynamic consequence of ACAT inhibition. However, unexpectedly, adrenocortical degeneration, including necrosis in zona fasciculata and zona reticularis, was observed at all doses. This toxicity was associated with decreases in ACTH response which were apparent already at day 6 of dosing. Histopathologic examination of the adrenals from drug-treated dogs revealed effects ranging from multifocal increases in cytoplasmic coarse vacuoles and decreases in cortical cell size at the lowest dose to total ablation of the adrenal cortex in several high-dose (50–800 mg kg^{-1}) treated animals. Further mechanistic studies in guinea pig adrenocortical cells in culture, however, suggested that the observed cytotoxicity was not a result of ACAT inhibition, but of ATP depletion resulting from direct inhibition of mitochondrial respiration[40].

Shortly after this report was published, reports of adrenal toxicity with other potent bioavailable ACAT inhibitors appeared in the literature. Thus, RP73163 (**7**), which had previously been demonstrated to potently inhibit microsomal ACAT *in vitro* (IC_{50} = 86–370 nM) and in whole cell cultures (IC_{50} = 158–314 nM) and to produce high blood levels of drug *in vivo* (4–82 μM at doses of 3–100 mg kg^{-1})[36], when dosed to guinea pigs at 100, 300 and 1,000 mg kg^{-1} for 3 weeks produced dose-related histopathologic changes in the adrenal cortex consisting of diffuse coarse vacuolation of zona fasciculata cells similar to that observed in dogs with PD1323012. Similar to the conclusions with PD1323012, these authors also concluded that the adrenal toxicity was not related to ACAT inhibition, because the pharmacologically inactive enantiomer of RP73163 caused very similar effects. Further mechanistic studies suggested that both RP73163 and its enantiomer were metabolized to a common product which was an inhibitor of cholesterol 17α-reductase. The authors speculated that this may be the source of the coarse vacuolation produced by RP73163, although ACAT inhibition could not be ruled out[41]. Subsequent to this report, it was disclosed that FR145237 (**8**), a potent ACAT inhibitor previously demonstrated to have direct antiatherosclerotic activity at the artery wall[37], also produced adrenal toxicity in normal rabbits after a single 3.2 mg kg^{-1} IV dose[42]. Surprisingly, FR145237 did not induce adrenal toxicity in LDL-receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits, despite producing adrenal drug concentrations equivalent to those found in normal Japanese white rabbits. These authors speculated that, because of the structural similarity between FR145237 and PD132301-2, the toxicity induced by FR145237 may not be due to ACAT inhibition but to inhibition of mitochondrial respiration. No explanation was offered for the lack of sensitivity of WHHL rabbits to FR145237 and further work will be required to understand this finding.

In an effort to further understand the specific intracellular mechanisms involved in ACAT inhibitor mediated adrenal toxicity, Warner *et al.* performed long-term incubations of SaH58035 (**4**) and CP113818 (**6**) with cholesterol-enriched mouse peritoneal macrophages[43]. These authors concluded that ACAT inhibition in these cells induced cell toxicity as a result of the build-up of intracellular free cholesterol concentrations. This was supported by the observations that cell toxicity paralleled the increase in intracellular free cholesterol concentrations and that removal of free cholesterol by the addition of extracellular cholesterol acceptors or by blocking intracellular sterol transport relieved the toxicity induced by ACAT inhibition. Thus, it was proposed that ACAT inhibition *in vivo* could induce cell death by destabilization of the plasma membrane upon cholesterol enrichment, unless sufficient cholesterol acceptors were present. Interestingly, these cytotoxic effects were not observed in an earlier study of SaH58035 in bovine adrenal cortical cells, presumably because of the much shorter incubation time (30 min)[44]. Despite these findings of adrenal toxicity with systemically available ACAT inhibitors, the recent results in ACAT-deficient transgenic mice would tend to support the position that the observed toxicity is not related to ACAT inhibition, because these animals developed normally and had no evidence of adrenal necrosis, although their adrenal cholesteryl esters were markedly reduced[24].

Strategies to avoid adrenal toxicity

Although there was no definitive evidence that the adrenal toxicity observed with these potent, bioavailable ACAT inhibitors was due to ACAT inhibition, in an attempt to better understand the structural factors leading to avoidance of adrenal toxicity, we at Parke-Davis instituted a screening strategy that included routine testing for adrenal toxicity in guinea pigs prior to testing inhibitors in long-term models of atherosclerosis[45]. One of our early strategies to avoid adrenal toxicity was to develop inhibitors that were more hydrophilic, in the hope that this might lead to less drug accumulation in adrenals. PD138142-15 (**10**; Figure 5) is representative of this strategy. PD138142-15 is a relatively weak ACAT inhibitor in rat liver microsomes *in vitro* (IC_{50} = 5.3 μM), but it is highly water-soluble and produces high blood concentrations of drug, unlike most ACAT inhibitors[46]. Despite the low lipophilicity and modest ACAT inhibitory potency displayed by this compound, PD138142-15 induced adrenocortical zonal atrophy at the high doses of 30 and 100 mg kg^{-1} when administered orally to beagle dogs for 13 weeks[47]. Continued application of this screening strategy filtered out several additional compounds [**11–14** (Ref. 48); **15** (Ref. 49)], which possessed promising pharmacokinetic properties but were adrenotoxic in guinea pigs *in vivo*. However, during the course of these studies a structural feature that seemed to prevent adrenal toxicity was discovered. Thus, compounds **16–19** (Figure 6) were identified, which produced robust plasma drug levels (as measured by an enzyme bioassay) yet were not adrenotoxic to guinea pigs when dosed orally at 100 mg kg^{-1} for 2 weeks[48,50]. Interestingly, all of the nontoxic compounds have a phenyl group on the carbon atom adjacent to the amide carbonyl, whereas the toxic analogs are all unsubstituted at this position. The source of the protective effect of this substituent is not known; however, all of the compounds are very potent ACAT inhibitors in both microsomal and cell based assays (IC_{50} as low as 10 nM) and produce high blood levels of drug at the doses administered. The source of the protective effect is still under investigation; however, the available evidence would suggest that it is independent of ACAT inhibition.

The discovery of potent, bioavailable ACAT inhibitors that are antiatherosclerotic but not adrenotoxic has stimulated further efforts to find ACAT inhibitors suitable for clinical study. Recently, a new ACAT inhibitor, CI1011 (**20**), derived from efforts to improve the chemical stability and safety profile of PD138142-15, has been reported to have outstanding activity in animal models of hypercholesterolemia. Although this compound appears to be a weak ACAT inhibitor in the normal microsomal assay, it is highly protein-bound, and removal of bovine serum albumin from the assay system increases its potency 1,000-fold. More significantly, although the compound appears to be a specific ACAT inhibitor, it has demonstrated excellent cholesterol lowering in noncholesterol-fed animal models, whereas other ACAT inhibitors have proved ineffective. For this reason, CI1011 has been selected for investigation in clinical trials[51].

Conclusions

Although over 10 years of research have been devoted to discovering safe and efficacious ACAT inhibitors that might have utility as cholesterol-lowering and/or

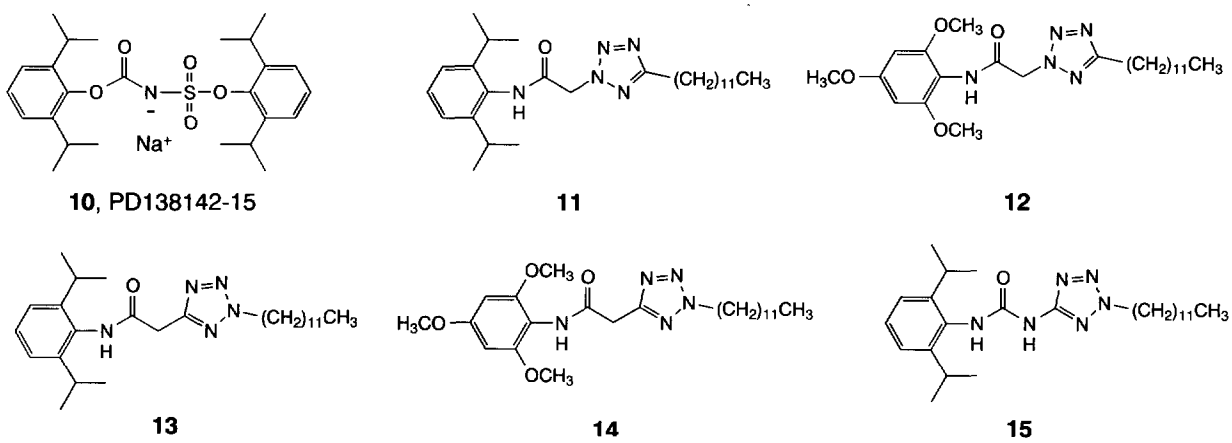

Figure 5. *Adrenotoxic acyl-CoA cholesterol acyltransferase inhibitors.*

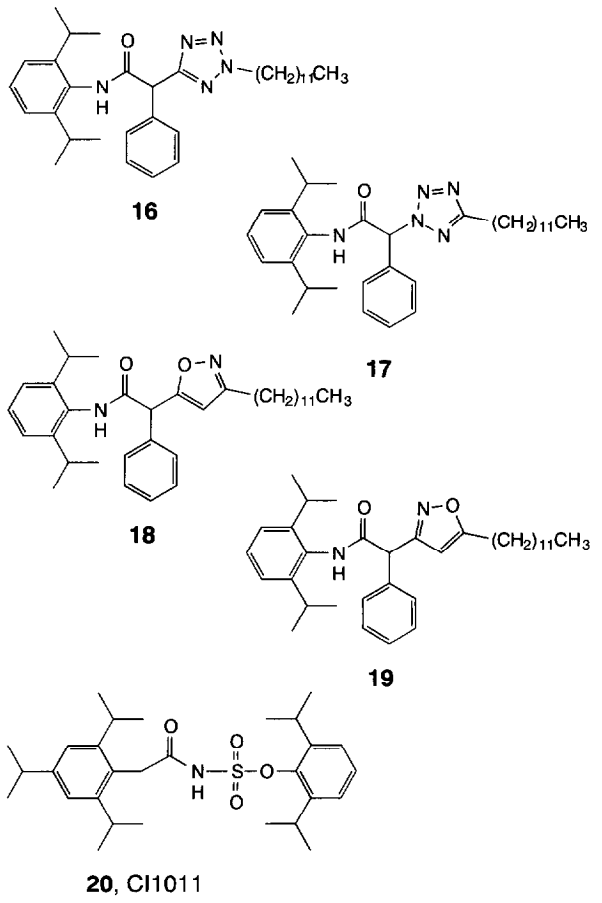

Figure 6. *Nonadrenotoxic acyl-CoA cholesterol acyltransferase inhibitors.*

antiatherosclerotic agents, clinical success has been elusive. However, recent developments in the molecular biology of ACAT and success in identifying potent and bioavailable inhibitors that are not adrenotoxic has led to renewed hope that ACAT inhibition may ultimately yield agents that will have value in treating human atherosclerotic disease, possibly independent of lowering LDL-C. This is important since many patients with coronary heart disease have normal levels of LDL-C and treatment options for these patients are limited. Results of clinical trials with newer agents, such as CI1011, should take us one step further in understanding the role of ACAT in lipoprotein and arterial wall metabolism.

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