New Molecular Targets for Cholesterol-Lowering Therapy

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Accepted for publication February 1, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

The use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) in randomized clinical trials has established that cholesterol-lowering treatment reduces the risk of both cardiovascular and total mortality. This reduction in risk occurs in patients with or without existing cardiovascular disease and in patients with high or average plasma cholesterol concentrations. Aggressive treatment to lower plasma cholesterol has been shown to slow progression of atherosclerosis and in some instances may be as successful as angioplasty in reducing ischemic events. These studies suggest that reduction of plasma cholesterol to levels even below 100 mg/dl might be desirable. New targets for cholesterol-lowering therapy with mechanisms of action different from the statins have been identified. One of these targets is the \( Na^+ \)-dependent bile acid transporter that is expressed in the terminal ileum. This protein is responsible for recycling bile acids from the intestine to the liver. Several compounds that demonstrate the ability to decrease transporter activity and to lower plasma cholesterol have been investigated. Absorption of cholesterol from the small intestine is another potential target. Compounds that inhibit cholesterol absorption may act by interacting stoichiometrically with cholesterol within the intestinal lumen or substoichiometrically, presumably within the enterocyte. Finally, the transcriptional regulation of cholesterol 7\( \alpha \)-hydroxylase by members of the nuclear receptor superfamily provides at least two other molecular targets for cholesterol-lowering drugs.

Reduction in the concentration of serum lipids, especially cholesterol, is a major goal in several primary and secondary prevention initiatives. That cholesterol-lowering drugs decrease mortality due to cardiovascular disease is unequivocal. The use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) in clinical trials over the last 11 years supports this conclusion in a variety of populations, including patients with or without established cardiovascular disease and patients with severe or only moderate hypercholesterolemia. The Scandinavian Simvastatin Survival Study Group (1994) studied patients with known cardiovascular disease who had serum cholesterol levels above 212 mg/dl. The mean serum cholesterol level was 263 mg/dl and the mean low-density lipoprotein (LDL) cholesterol level was 189 mg/dl. Treatment with simvastatin over the 5-year study reduced total and LDL cholesterol by 25 and \( 35\% \), respectively, and this was associated with a reduction in cardiovascular mortality of \( 28\% \). The West of Scotland Coronary Prevention Study (Shepherd et al., 1995) studied men who were hypercholesterolemic (mean total cholesterol was 272 mg/dl and mean LDL cholesterol was 192 mg/dl) and had no history of cardiovascular disease. In these patients, treatment with pravastatin lowered these values by 20 and \( 26\% \), respectively. During the 4.9 years of this study, there was a \( 28\% \) reduction in deaths due to cardiovascular events. Patients in the Air Force/Texas Coronary Atherosclerosis Prevention Study (The AFCAPS/TexCAPS Research Group, 1998) included individuals with average cholesterol levels (mean total cholesterol was 221 mg/dl and LDL cholesterol was 150 mg/dl, the 51st and 60th national percentiles, respectively). Lovastatin reduced these levels by 20 and \( 25\% \), respectively. Treated patients had a \( 37\% \) lower incidence of a first acute major coronary event (myocardial infarction, unstable angina, or sudden cardiac death) over the 5-year study. Two recent meta-analyses have examined 16 (Hebert et al., 1997) and 17 (Ross et al., 1999) clinical trials using statins and more than 29,000 patients. Both analyses found that cholesterol-lowering treatment reduces overall mortality by \( 20 \) to \( 30\% \).

Current treatment guidelines, established by the National...
Cholesterol Education Program (NCEP), Adult Treatment Panel II, recommend pharmacologic intervention in individuals without coronary heart disease who have fewer than two risk factors if LDL cholesterol is ≥190 mg/dl with a treatment goal of <160 mg/dl; if two or more risk factors are present, the treatment threshold is ≥130 mg/dl, with a goal to reduce this to <130 mg/dl. Drug treatment is recommended in patients with known coronary heart disease if LDL cholesterol is >100 mg/dl, with a goal of ≤100 mg/dl.

Epidemiologic estimates place ~50 to 60 million Americans within these treatment parameters. Emerging data indicate that slowing the progression of atherosclerotic plaques requires aggressive therapy and may be significant only when LDL cholesterol levels below 100 mg/dl are obtained. In the Post Coronary Artery Bypass Graft Study, aggressive treatment to an average of 95 mg/dl LDL cholesterol achieved a significant slowing of atherosclerosis where moderate treatment to an average of 134 mg/dl was less effective (The post coronary artery bypass graft trial investigators, 1997). However, in another study (Pitt et al., 1999), aggressive treatment with atorvastatin resulted in a 46% reduction in LDL cholesterol to an average of 77 mg/dl, which was associated with 36% fewer ischemic events than with patients undergoing angioplasty. These data may indicate that the NCEP guidelines are too high in patients with cardiovascular disease. Therefore, there is an extraordinarily large population of individuals who could benefit from, indeed may require, a large reduction in plasma cholesterol. Although currently available drugs are certainly effective, it would be desirable to have additional agents with different mechanisms of action that could be used separately or combined with the statins.

**Current Pharmacological Targets**

Plasma cholesterol levels are determined by inputs from both diet and de novo biosynthesis, utilization of cholesterol, especially in the liver and steroidogenic tissues, and excretion of either cholesterol or bile acids. The most effective currently available drugs that lower plasma cholesterol are the aforementioned statins. This class of drugs is derived from the initial work of Endo et al. (1976), who isolated a molecule from fungi that they named compactin. Compactin was shown to be a competitive antagonist of HMG-CoA reductase with a $K_m$ in the nanomolar range (Endo et al., 1977). Modification of the basic structure of compactin has produced the current group of available statins: lovastatin, pravastatin, simvastatin, fluvastatin, atorvastatin, and cerivastatin. These compounds are less potent than the parent compound but have reduced hepatotoxicity and greater bioavailability (Endo, 1985; Moghadasian, 1999). Statins almost completely inhibit the conversion of HMG-CoA to mevalonate, which blocks subsequent biosynthesis of cholesterol. At maximum doses, plasma LDL cholesterol levels are reduced by as much as 50% (Pitt et al., 1999). The clinical pharmacology of these compounds has been reviewed elsewhere (Moghadasian, 1999), but in general they have good oral bioavailability and relatively long half-lives that permit once or twice a day dosing. Statins block both cholesterol biosynthesis and production of isoprenoids, which are important C5 building blocks necessary for the biosynthesis of ubiquinone and vitamin $K_2$. Isoprenoids are also involved in post-translational modification of a number of proteins; such modification plays a role in protein localization within cells. This makes the relatively low frequency of adverse effects, 1 to 2%, almost surprising; meta-analysis has not found an increase in cancer or other morbidities, but most studies have only been followed for 5 to 7 years (Hebert et al., 1997).

Certainly there are choices other than statins for reducing plasma cholesterol, including niacin and gemfibrozil. These agents interfere with the biosynthesis and release of very low-density lipoprotein (VLDL), the precursor of LDL, and thereby reduce plasma LDL cholesterol. However, the side effect profile and the magnitude of the reduction in cholesterol are not as good as the statins.

Another alternative to statins is the bile acid-binding resins that reduce plasma cholesterol levels by increasing fecal loss of cholesterol metabolites. Bile acids are almost quantitatively recycled from the small intestine back to the liver. Binding resins such as cholestyramine and colestipol are polyanionic exchange resins that nonspecifically bind bile acids within the lumen of the small intestine and reduce the mass of bile acids returning to the liver. This in turn increases the hepatic requirement to synthesize replacement bile acids de novo from cholesterol; this substrate cholesterol is obtained from plasma or tissue stores and thereby reduces circulating cholesterol. The major drawback to these agents is poor compliance caused by the mass and the impalpability of the resin and the nonspecific nature of the ion trapping. In addition, reductions in total plasma cholesterol of only ~10% and LDL cholesterol of 13% are typical with this class of agents (Schulman et al., 1990). The low incidence of systemic adverse effects of these nonabsorbed compounds has encouraged development of more specific biopolymer-based resins that bind more bile acid per gram of resin (Lee et al., 1999).

**New Pharmacologic Targets**

**Bile Acid Transporter.** In the last decade, a number of attractive intracellular molecules that might serve as pharmacologic targets for new cholesterol-lowering therapies have been identified. As evidenced by more than 25 years of experience with cholestyramine, increasing excretion of bile acids provides an effective means of reducing plasma cholesterol. This is because >95% of the bile acids released into the small intestine are recycled back to the liver. This reduces the amount of cholesterol that must be used for de novo bile acid biosynthesis. Some bile acid is reabsorbed passively during the absorption of dietary lipids, but ~60% is reabsorbed by a very efficient and high capacity transporter (Krag and Phillips, 1974), known variously as the apical sodium-dependent bile acid transporter (ASBT) or ileal bile acid transporter reviewed in Love and Dawson (1998). ASBT is expressed at the highest levels in the distal half of the ileum and the kidney (Craddock et al., 1998). The ASBT protein crosses the plasma membrane multiple times and was initially thought to have seven transmembrane domains reminiscent of the G-protein coupled serpentine receptors. More recent evidence suggests that the topology of the protein might have eight or nine transmembrane segments (Hallen et al., 1999). In any case, ASBT is able to transport bile acids and $Na^+$ at a 1:2 stoichiometry and efficiently remove essentially all of the bile acid from the lumen of the small intestine (Weinman et al., 1998). These bile acids are...
ultimately passed into the hepatic portal circulation where they are re-extracted from the plasma via another transport protein called the Na\(^+\)-taurocholate cotransporting polypeptide (Meier et al., 1997). There is a large amount of cholesterol in this metabolized form circulating through this pathway every day. Of the total bile acid pool of approximately 5 g, about 0.5 g is synthesized to replace that lost through excretion. However, the total bile acid pool flows through this pathway 8 to 10 times per day, yielding a total circulation of 40 to 50 g per day (Vlahcevic et al., 1990).

ASBT offers an attractive target for a new class of cholesterol-lowering drugs. An antagonist of the transporter would be expected to increase excretion of bile acids, causing increased conversion of hepatic cholesterol to bile acids that would subsequently reduce hepatic and ultimately plasma cholesterol. Because the binding of bile acids occurs within the lumen of the small intestine, a drug that acted to block the transporter would not need to be absorbed. This reduces the potential for systemic adverse effects and, at least theoretically, increases the specificity of such an antagonist. Fears et al. (1990) suggested that inhibition of specific bile acid reabsorption in the intestine could lead to a new class of cholesterol-lowering drugs. These investigators synthesized a series of unique bile salts and demonstrated that one of these, BRL 39924A (Fig. 1), reduced bile acid transport in vitro by 59%, but the specificity of these compounds was not investigated. At the highest dose tested, BRL 39924A caused a significant reduction in plasma cholesterol in hyperlipidemic guinea pigs and rats. Wess et al. (1994) synthesized several compounds that inhibited Na\(^+\)-taurocholate transport in isolated brush border membrane preparations as well as in vivo perfusion studies. The most potent of these, R14, is illustrated in Fig. 1. Root et al. (1995) synthesized 2164U90 (Fig. 1) and showed that this molecule blocked Na\(^+\)-dependent bile acid transport but had no effect on Na\(^+\)-dependent glucose uptake. Twice daily oral administration of 2164U90 to lipid-fed rats caused a 67% reduction in serum lipids (VLDL + LDL) while causing a small increase in high-density lipoprotein (Lewis et al., 1995). Ichihashi et al. (1998) synthesized a compound, S-8921 (Fig. 1), that inhibited both ileal bile acid transport and cholesterol absorption in the intestine. In rats fed a lipid-enriched diet, this compound decreased elevations in plasma cholesterol by 70% after only 3 days of oral administration. These studies establish that inhibition of ASBT activity can produce a significant reduction in plasma cholesterol, at least in animal models.

How much inhibition of the transporter will be necessary to cause a reduction in plasma cholesterol levels? Some insight into this question might be provided by studies on individuals with mutations in ASBT that produce either no protein or a dysfunctional protein. Studies by Wong et al. (1995) and in our laboratory (Behbod and Loose-Mitchell, 1998) have found at least three alleles that have mutations that render ASBT incapable of transporting bile acids. Heterozygotes that have one normal and one mutated allele might be expected to have 50% of the ASBT-mediated bile acid transport activity of normal subjects. Individuals with this reduced transport may have reduced plasma cholesterol levels. This appears not to be the case; heterozygotes bearing one mutant allele have plasma cholesterol levels that are similar to normal individuals (P.A. Dawson, personal communication). This suggests that the human ileum either has at least twice the necessary transport capacity or that the normal allele is overexpressed by some compensatory mechanism. This observation does suggest that in humans, pharmacologic blockade of >50% of the transport molecules will be required to cause an increase in bile acid excretion.

Potential adverse effects of these compounds remain to be elucidated. By design, such drugs will increase the luminal concentration of bile acids. This differs from the action of the bile acid-binding resins that increase excretion of bile acids by binding and effectively reducing their concentration in the lumen. Bile acids, specifically deoxycholic acid, have been shown to have tumor-promoting capacity in animal models.

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\[\text{Fig. 1. Structure of compounds that have been shown to inhibit the ileal bile acid transporter.} \]
and have been implicated in a variety of intestinal pathologies, including increased rates of colon cancer. Bile acids enhance tumor growth only in the presence of an additional carcinogen, demonstrating that bile acids are not of themselves carcinogenic, but instead promote hyperproliferation and tumor growth (Morvay et al., 1989). Importantly, this tumor-promoting effect can be reversed in vitro and in vivo with a variety of compounds, most notably butyrate, a short-chain fatty acid produced by fermentation of fiber in the gut (Bartram et al., 1995), and ursodeoxycholic acid, a secondary bile acid (Earnest et al., 1994). In addition, the increased luminal bile acids may cause diarrhea due to inhibition of water absorption and increased water excretion (Mekjian et al., 1971) in the colon.

**Cholesterol Absorption.** Blockade of absorption of dietary cholesterol across the small intestine is also an attractive target that might have a significant impact on plasma cholesterol levels. Surprisingly, the precise mechanism by which cholesterol is absorbed from the small intestine is not clear, as recently reviewed by Dawson and Rudel (1999). The original concept was one of simple diffusion with cholesterol crossing the unstirred water layer in conjunction with bile salts. Several lines of evidence argue against this simple model and suggest that a protein or proteins facilitate the absorption of cholesterol. First, Hauser et al. (1998) demonstrated that cholesterol absorption into brush border membrane vesicles was affected by protease treatment. After protease treatment, the kinetics of cholesterol absorption into these vesicles changed from second-order to first-order. The simplest explanation of these results is that there are two processes by which cholesterol is absorbed into these vesicles: the first-order process reflects simple monomeric diffusion of cholesterol, whereas the second-order component reflects a protein-facilitated transport of cholesterol. Second, absorption of cholesterol is far greater than absorption of structurally and chemically related plant sterols such as sitosterol (Salen et al., 1970). Third, the condition of sitosterolemia, an autosomal recessive disorder where plant sterols are readily absorbed by the intestine, also argues for a protein component (one that can be disrupted by a mutation) that mediates cholesterol absorption.

The identity of the putative cholesterol transporter is unknown but is under active investigation by several laboratories. Reconstitution experiments have partially purified a putative transporter (Boffelli et al., 1997). The same laboratory has provided immunological evidence that one of the lipoprotein scavenger receptors, specifically SR-B1 (Hauser et al., 1998), is the cholesterol transporter, but additional studies are needed.

Once cholesterol enters the intestinal enteroocyte, it is esterified by acyl-CoA:cholesterol O-acyltransferase (ACAT), isoform 2 (Oelkers et al., 1998), before its packaging into chylomicrons. It appears that this ACAT-mediated esterification prevents the back-diffusion of cholesterol rather than participating directly in the absorptive process.

Either the putative transporter, interaction between cholesterol and the transporter, or ACAT could be targets for cholesterol-lowering drugs. A number of small molecules, typically plant-derived sterols or analogs thereof, interfere with cholesterol absorption. β-Sitosterol and its 5α-saturated form, sitostanol, are poorly absorbed (~4.5 and <2%, respectively; Heinemann et al., 1993) and have been shown by numerous workers to decrease intestinal cholesterol absorption by ~15%. By administering sitostanol as a lecithin micelle, Ostlund et al. (1999) reported a 37% reduction in cholesterol absorption. Although the mechanism of this reduction is not clear, Ostlund et al. (1999) suggest that sitostanol interferes with the ability of cholesterol to enter a micellar form, which is then taken up by the enteroctye. It is not known whether this micellar uptake is a passive or a facilitated uptake pathway. In an analogous manner, sapo- nins, steroidal glycosides found in a variety of plants, interfere with cholesterol absorption. Two of these saponins, tisqueside and pamaquestudte, were able to inhibit cholesterol absorption in rabbits by ~50% (Morehouse et al., 1999). Interestingly, pamaquestudte was about an order of magnitude more potent than tisqueside with comparable efficacy. The cholesterol/saponin ratio in the excreted lipids ranged from 0.2 to 0.4 for tisqueside-treated animals and 1.5 to 5.2 for pamaquestudte-treated animals. This near-stoichiometric ratio suggests that these saponins interact directly with cholesterol or a few molecules of cholesterol, and they inhibit absorption via that mechanism rather than interfering with a transporter itself.

Nonsterol molecules such as (3R,4S)-1,4-bis-(4-methoxyphenyl)-3-(3-phenylpropyl)-2-azetidinone (SCH 48461) inhibited cholesterol absorption by 68 and 95% in hamsters and monkeys, respectively (Salisbury et al., 1995). In the treated monkeys, this resulted in a 27% reduction in total plasma cholesterol. The 10 mg/kg/day doses used in this study were substoichiometric with respect to the amount of cholesterol excreted, suggesting that SCH 48461 might act directly on the transporter or affect transporter expression. Similarly, recent studies have shown that retinoic acid X receptor (RXR) agonists such as LGN268 are potent inhibitors of cholesterol absorption in the mouse (David Mangelsdorf, personal communication). It is not clear whether the RXR agonists act by inhibiting the transporter itself or decreasing the expression of the transporter.

Several laboratories and pharmaceutical companies have developed ACAT inhibitors and are studying their cholesterol-lowering properties. These compounds are thought to decrease plasma cholesterol levels by interfering with intestinal cholesterol absorption (Uchida et al., 1998; Umeda et al., 1998; Azuma et al., 1999). These agents are effective in reducing plasma cholesterol levels in animal models by 31 to 71%, depending on the particular model used. Success in reducing plasma cholesterol in human trials is lacking.

**Bile Acid Synthesis.** Cholesterol derived from either de novo pools or dietary intake is metabolized to bile acids by a series of more than a dozen enzymes (Schwarz et al., 1998). Quantitatively, this metabolic pathway is the most important route for elimination of cholesterol carbon from the body.

Bile acids are synthesized by two biochemical pathways. The first, called the acidic or classical pathway, is contained within the liver and begins with the hydroxylation of cholesterol by cholesterol 7α-hydroxylase (Cyp7a). 7α-OH cholesterol is then subsequently hydroxylated at carbon 27, leading to the dihydroxy primary bile acid chenodeoxycholic acid. Alternatively, hydroxylation of 7α-OH cholesterol at both carbon 12 and carbon 27, via a branchpoint in the biosynthetic pathway, leads to the trihydroxy bile acid, cholic acid. A different pathway begins with 27-hydroxylation of cholesterol, rather than 7α-hydroxylation. The quantitative impor-
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tance of this pathway has only recently been appreciated, and in most species studied to date accounts for as much as 50% of bile acid biosynthesis (Vlahcevic et al., 1997). The enzyme that mediates this reaction, sterol 27-hydroxylase, is expressed in numerous extrahepatic sites, such as spleen and endothelial cells. The product of this reaction, 27-hydroxy cholesterol, is taken into the liver and further hydroxylated by oxysterol 7α-hydroxylase (Cyp7b). Additional biotransformation produces chenodeoxycholic acid.

From a pharmacologic perspective, most interest has focused on cholesterol 7α-hydroxylase, the rate-limiting enzyme in the classic pathway. This enzyme is regulated by numerous hormones and physiologic conditions but, perhaps most importantly, is regulated by plasma cholesterol levels and bile acids. In the rat, ingestion of a cholesterol-enriched diet increases cholesterol 7α-hydroxylase, which leads to increased bile acid biosynthesis. Bile acids, recycled to the liver from the intestine via ASBT, are potent inhibitors of cholesterol 7α-hydroxylase (Pandak et al., 1991). Increasing expression of cholesterol 7α-hydroxylase via adenoviral vectors (Spady et al., 1995, 1998) causes marked decreases in plasma cholesterol. Rabbis that over-express cholesterol 7α-hydroxylase mRNAs by 7-fold are resistant to increases in plasma cholesterol caused by a cholesterol-enriched diet (Poorman et al., 1993). These experiments suggest that pharmacologically increasing Cyp7a expression might lead to reductions in plasma cholesterol.

The details of the molecular regulation of Cyp7a have only recently been discovered and most of this work has not been corroborated in the human. However, some intriguing molecular targets have been identified (Fig. 2). In rodents, two nuclear receptors play opposing roles in regulating Cyp7a expression. Liver X receptor (LXR), a member of the steroid receptor superfamily, is highly expressed in liver. Two isoforms of LX a, α and β, have been identified; the α isoform is responsible for regulating Cyp7a (Peet et al., 1998). Although the identity of the endogenous ligand for LXR has not been completely established, studies by Janowski et al. (1996) and Lehmann et al. (1997) demonstrated that 24,25-epoxy cholesterol and other oxysterols were potent agonists. LXR agonists form a heterodimer with RXR and then bind to a response element in the Cyp7a promoter; this increases transcription of Cyp7a (Peet et al., 1998) and leads to increased metabolism of cholesterol to bile acids that can then be excreted. Opposing this stimulatory pathway is another member of the nuclear receptor family, the farnesyl X receptor (FXR). FXR also forms a heterodimer with RXR (Zavacki et al., 1997). The ligand for FXR is a bile acid; the most potent found to date is chenodeoxycholic acid (Parks et al., 1999; Makishima et al., 1999; Wang et al., 1999). Transcriptional activity of the Cyp7a promoter is reduced by liganded FXR; this effect may be mediated by interaction with other transcription factors rather than direct binding to the Cyp7a promoter.

Thus either LXR agonists or FXR antagonists would be expected to increase Cyp7a expression and enhance bile acid excretion. A caveat to this hypothesis is that DNA sequences that bind LXR are not present in the proximal promoter of human Cyp7a (Chen et al., 1999); therefore the magnitude of the Cyp7a induction by LXR agonists may be smaller in humans than in rodents.

To be effective as cholesterol-reducing agents, however, any drugs that increase bile acid biosynthesis and their subsequent release into the small intestine must overcome the transport capacity of ASBT in the ileum. Again, the effect of chronically elevated bile acid concentration on the distal portions of the intestine remains to be determined.

**Conclusion**

Development of additional cholesterol-lowering agents with mechanisms of action distinct from statins will probably be necessary to achieve cholesterol target levels in many individuals. Several attractive pharmacologic targets have been identified, and agents that act on those targets, when used alone or in conjunction with a statin, should be very effective cholesterol-lowering therapies.

**References**


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