Pharm Res (2012) 29:3235–3250
DOI 10.1007/s11095-012-0858-6

EXPERT REVIEW

Inhibition of Cholesterol Absorption: Targeting the Intestine

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Received: 10 May 2012 / Accepted: 6 August 2012 / Published online: 25 August 2012

ABSTRACT Atherosclerosis, the gradual formation of a lipid-rich plaque in the arterial wall is the primary cause of Coronary Artery Disease (CAD), the leading cause of mortality world-wide. Hypercholesterolemia, elevated circulating cholesterol, was identified as a key risk factor for CAD in epidemiological studies. Since the approval of Mevacor in 1987, the primary therapeutic intervention for hypercholesterolemia has been statins, drugs that inhibit the biosynthesis of cholesterol. With improved understanding of the risks associated with elevated cholesterol levels, health agencies are recommending reductions in cholesterol that are not achievable in every patient with statins alone, underlying the need for improved combination therapies. The whole body cholesterol pool is derived from two sources, biosynthesis and diet. Although statins are effective at reducing the biosynthesis of cholesterol, they do not inhibit the absorption of cholesterol, making this an attractive target for adjunct therapies. This report summarizes the efforts to target the gastrointestinal absorption of cholesterol, with emphasis on specifically targeting the gastrointestinal tract to avoid the off-target effects sometimes associated with systemic exposure.

KEY WORDS absorption · atherosclerosis · cholesterol · heart disease · targeted therapy

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ABBREVIATIONS

ABC	ATP-binding cassette
ACAT	Acyl CoA cholesterol Acyl transferase
Apo	apolipoprotein
ASO	antisense oligonucleotides
BAS	bile acid sequestrants
CAD	coronary artery disease
LDL	low-density lipoproteins
LXR	liver X receptor
MTP	microsomal triglyceride transfer protein
NPC1L1	Niemann Pick C 1 Like 1
NSAS	nanostructured aluminosilicate
RNAi	RNA interference
SREBP	sterol regulatory element binding protein

INTRODUCTION

Coronary artery disease (CAD) is the leading cause of morbidity and mortality in developed countries and its prevalence is increasing worldwide (1,2). Although CAD is a complex disease with a range of etiologies, the dominant underlying cause is atherosclerosis, the gradual formation of a lipid-rich plaque and thickening of the arterial wall, reviewed in (3). Large world-wide epidemiological studies demonstrate that elevated circulating cholesterol is a key risk factor for the development of CAD (4–9). The primary therapy for hypercholesterolemia is a regimen of HMG CoA Reductase inhibitors (statins), pharmacological agents that inhibit the biosynthesis of cholesterol. The risk of serious coronary events decreases by 22% for every 40 mg/dL reduction in circulating cholesterol carried by low-density lipoproteins (LDL), demonstrated by meta-analyses of large post-market statin trials (10). As the evidence linking LDL-cholesterol levels with CAD risk becomes clearer, there is a clinical imperative for further reductions in LDL-cholesterol (11). Current clinical guidelines

suggest target plasma LDL cholesterol levels of less than 100 mg/dL (2.5 mmol/L) for patients with moderately high risk or high risk for CAD (12–14). Unfortunately, these levels are not achievable with statin monotherapy in every patient population and an estimated 25% of high-risk patients fail to achieve treatment goals (3). Treatment with statins alone may be insufficient in patients genetically predisposed to high LDL-cholesterol levels (15,16), patients taking other medications that interact with statins (17,18), or patients who cannot tolerate the higher doses of statins that may be required for treatment success (19).

The circulating cholesterol pool is derived from two sources: *de novo* synthesis and diet. Although statins are effective at reducing the biosynthesis of cholesterol, they do not inhibit the absorption of cholesterol (biliary and dietary derived) in the intestine. In fact, treatment with statins may increase cholesterol absorption (20–22). Likewise, inhibition of cholesterol absorption is accompanied by increased cholesterol synthesis (23,24). These clinical observations suggest that further reductions in LDL-cholesterol can be achieved by combining statin therapy with agents that target cholesterol absorption (25). Cholesterol absorption is a complex process that occurs in three distinct phases: solubilization in the gastrointestinal lumen, uptake into enterocytes in the proximal jejunum, and intracellular transport and packaging of the cholesterol into chylomicrons for secretion to the lymphatic system. The extent of cholesterol absorption from the intestine varies broadly in humans with values ranging from 15% to 80%, with an average of about 50% (23,26–29). The high variability suggests a genetic component in the regulation of intestinal absorption of cholesterol, reviewed in (30). The intestine processes 1200–1700 mg of cholesterol per day originating from three sources, diet (300–500 mg/day), bile (700–1300 mg/day), and sloughing of the intestinal epithelium (200–300 mg/day) (31–34). Each stage in the cholesterol absorption process can be targeted for pharmacological intervention. Recently, scientific focus has been on developing agents that act in the small intestine without being taken up into the circulatory system. The goal of this approach is to minimize the potential for systemic adverse events and off-target effects. Throughout this paper, we will focus on this concept as we review developments in our understanding of intraluminal and intracellular events in cholesterol absorption and the new potential pharmacological interventions for these targets.

DISRUPTING THE INTRALUMINAL PROCESSING OF CHOLESTEROL

Phytosterols

Plants produce a range of chemicals with structural and chemical similarity to cholesterol, collectively referred to as phytosterols (35). As shown in Fig. 1, plant sterols have the same sterol ring structure but differ from cholesterol in the side chain. Plant stanols are less common in nature, these molecules differ from cholesterol in that they contain a saturated sterol ring as well as side chain modifications (36). Of the more than 250 unique plant sterols discovered, the most common are campesterol, stigmasterol and β-sitosterol (Fig. 1) (35,36). Although the amount of phytosterols in Western diet is similar to the amount of cholesterol (~300 mg/day), there is very little systemic exposure of plant sterols (bioavailability ranges from 0.4%–3.5%) (37–41). Genetic analyses of the rare human disease Sitosterolemia, in which patients hyper absorb both cholesterol and plant sterols, identified two genes in the uncontrolled absorption of sterols: *ABCG5* and *ABCG8* (42,43), these genes encode half-transporters that function together as a barrier to sterol absorption (43,44).

The first clinical studies using crude extracts of plant sterols to decrease plasma cholesterol were published in the 1950s (45,46). Further studies in humans and animal models confirmed that plant sterols reduce the fractional absorption of cholesterol leading to reductions in LDL-cholesterol (24,39,40,47,48). In animal models, plant stanols are more potent inhibitors of cholesterol absorption than plant sterols (47,49–52), and show better effects on improved atherosclerotic lesion progression (53). Because of the demonstrated hypocholesterolemic activity of phytosterols and phytostanols, these agents are incorporated into nutritional products and labeled as functional foods. In many cases, the plant sterols are esterified with fatty acids to increase the incorporation capacity (47). A recent meta-analysis of 84 randomized clinical trials of plant sterols demonstrates that there is a non-linear dose response between LDL-cholesterol reductions and intake of plant sterols (54). The mean 8.8% reduction in LDL-cholesterol is achieved with a daily dose of 2.15 g of phytosterols; there were minimal improvements to LDL-cholesterol lowering at further doses (up to 10 g/day). In humans, LDL-cholesterol lowering is the same when plant sterols or plant stanols are used as the active ingredient.

Many attempts have been made to modify phytosterols to improve their activity (55). Perhaps the most studied modified phytosterol is disodium ascorbyl phytostanol phosphate (also known as FM-VP4), a water-soluble derivative of sitostanol and campestanol prepared by esterification with an ascorbyl-phosphate group (Fig. 2). FM-VP4 effectively reduces cholesterol absorption and circulating cholesterol levels in rats, gerbils and mice (56–59). The cholesterol lowering effect also reduces atherosclerotic lesion formation in a murine model of atherosclerosis, the Apolipoprotein (Apo) E knockout mouse (60). Interestingly, FM-VP4 is also an effective anti-obesity agent, preventing weight gain on a high fat diet (61). Intervention with FM-VP4 in pre-obese

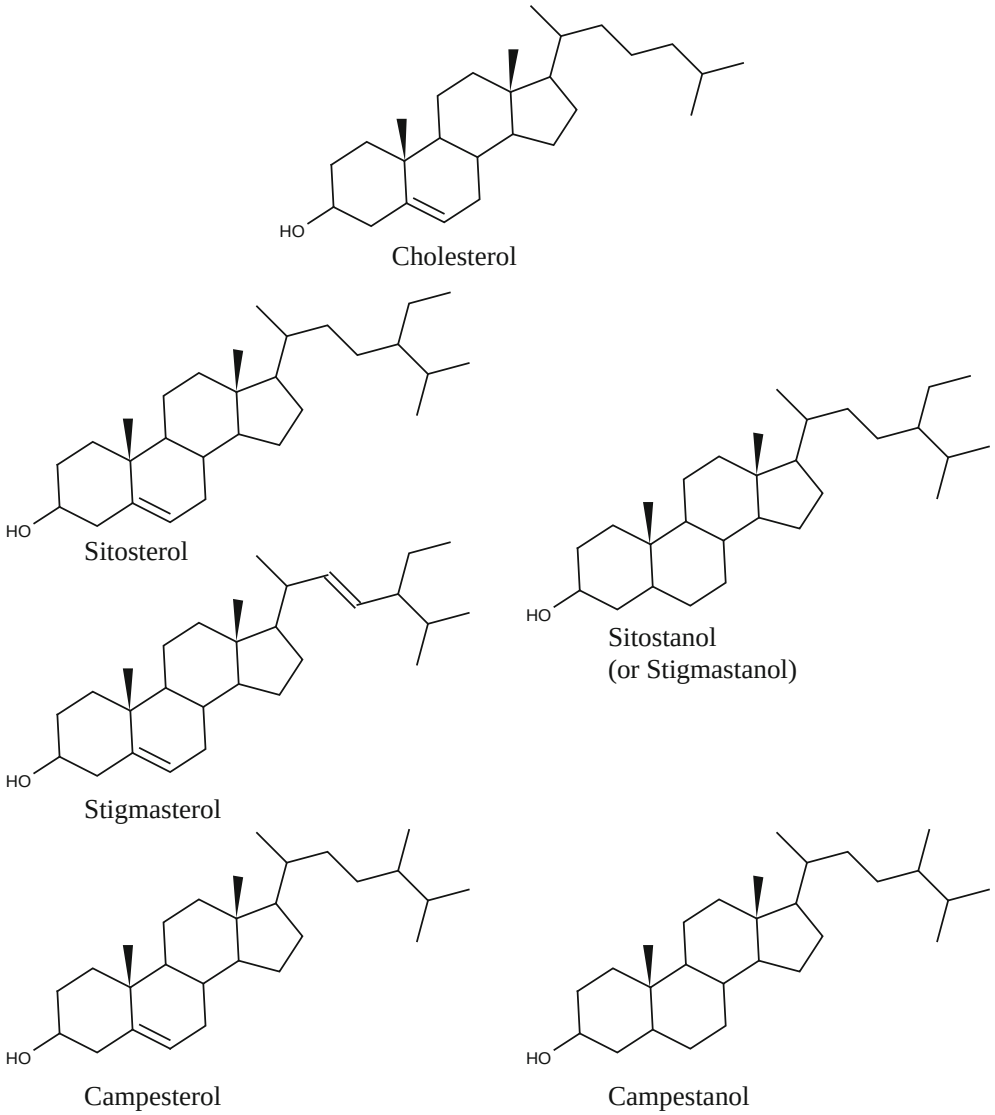

Fig. 1 Chemical structure of cholesterol, three common plant sterols (sitosterol, campesterol, and stigmasterol), and the saturated derivatives, plant stanols (sitostanol and campestanol). Note that stigmastanol is identical to sitostanol.

mice on a high fat diet restores the mice to a lean phenotype as shown by reduced body mass and fat content, perhaps explained by an improved metabolic scope (62). FM-VP4 is also more effective in reducing plasma cholesterol levels than free phytostanols in hamsters (63). Clinically, FM-VP4 is well tolerated, and reduces LDL cholesterol by 7% compared to baseline level, or by 10% compared to placebo (64). The LDL-cholesterol lowering observed in the Phase II trial was not sufficient to warrant further development of FM-VP4.

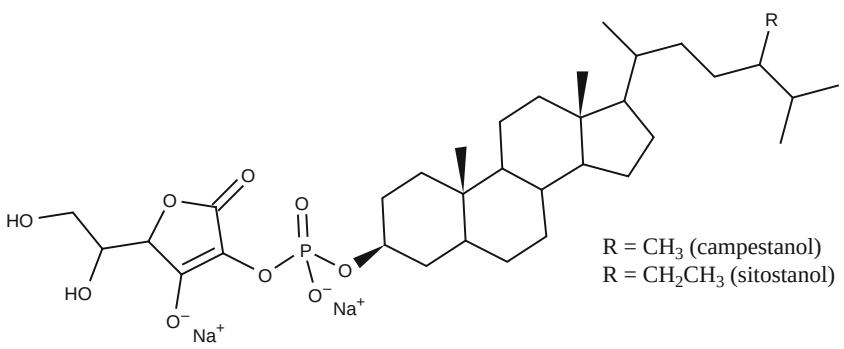

Fig. 2 Chemical structure of the modified plant stanol, FM-VP4. The compound is a mixture of ascorbic acid linked by a phosphodiester bond to campestanol ($R=CH_3$) or sitostanol ($R=C_2H_5$).

Despite extensive clinical evidence that phytosterols are effective cholesterol inhibitors, there is considerable debate about their mechanism of action. The long-held belief is that the poorly water soluble phytosterols compete with cholesterol for incorporation into mixed micelles, comprised of dietary fat, bile acids and sterols (39,65–68). Without partitioning into the micelle phase, cholesterol is unable to cross the unstirred water layer surrounding the intestinal wall and cannot be taken up by enterocytes for subsequent packaging into chylomicrons. This mechanism does not impact fat absorption, as demonstrated in a recent clinical experiment using intubated volunteers. The administration of phytosterols with a meal significantly decreased the transfer of cholesterol to the aqueous (micelle) phase without disrupting triacylglycerol hydrolysis or absorption (69). Recent studies suggest that phytosterols may have other effects that contribute to their LDL-cholesterol lowering properties, though many of the studies have contradictory findings depending on the study design (39). Phytosterols may be agonists for the liver X receptor (LXR) (70,71), a nuclear receptor responsible for up regulating cholesterol efflux pathways throughout the body (72). This is discussed in more detail as an intracellular intervention, below. Phytosterols also suppress *de novo* synthesis of cholesterol in rats, similar to statin therapy (73). In cell culture, phytosterols can interfere with intracellular cholesterol trafficking in some intestinal cell lines (74), but not in hamsters or other cell lines (75,76). The consumption of phytosterols has been associated with reduced production of triacylglycerol-rich ApoB-containing lipoproteins, the precursor to LDL (77). Despite the controversies surrounding the mechanism of action, clinical evidence suggests that these cholesterol analogs inhibit cholesterol absorption with downstream effects on cholesterol metabolism throughout the body (35,39,54).

Bile Acid Sequestrants

The primary mechanism by which humans remove excess cholesterol is catabolism into bile acids. Bile acids are charged cholesterol-derived molecules that are essential for the proper digestion of fat, fat-soluble vitamins, and cholesterol. The human liver catabolizes 500 mg of cholesterol per day into bile acids, while >95% of the bile acids secreted into the intestinal lumen are reabsorbed in the distal digestive tract (78,79). Traditional bile acid sequestrants (BAS) such as cholestyramine and colestipol are large, positively charged resins that non-specifically bind to negatively charged bile acids in the intestine (80,81). Colesevelam, a second generation BAS approved in 2000 (82–84), differs from traditional BAS in that it is a polymer designed to specifically adsorb bile acids. The precipitated bile acids cannot be reabsorbed in the distal ileum and are excreted in feces. By disrupting the enterohepatic circulation of bile acids, the bile acid pool is depleted and the liver increases synthesis of bile acids from cholesterol stores to compensate (85–88). The LDL receptor is up-regulated in response to the reduction in hepatic cholesterol, increasing LDL clearance (89,90). BAS monotherapy can reduce LDL-cholesterol by 9–18% (See Table I) and can improve the LDL-cholesterol lowering capacity of statins by an additional 4–17% (91).

Studies in genetically modified mice show that decreasing the bile acid pool causes cholesterol absorption to drop to <5% (92,93). Based on these findings treatment with BAS is hypothesized to reduce cholesterol absorption, confirmed in animal (94,95) and human studies (96). These findings are contradicted by studies demonstrating that BAS have no effect on neutral sterol excretion (97,98). A time course study in humans shows that BAS reduce cholesterol absorption by 38% upon administration but there is a long-term increase in cholesterol absorption (99). The temporal nature of BAS-mediated inhibition of cholesterol absorption may explain some of the variability in the literature surrounding this field.

Although BAS have been in clinical use for over 40 years and have an excellent safety record, particular patient groups do not tolerate them. Treatment with BAS increases circulating triacylglycerol levels and they are contraindicated for patients with hypertriglyceridemia (100). While the precise mechanism by which BAS induce hypertriglyceridemia has yet to be determined, it is likely through the activation of the SREBP-1c transcription factor, which induces the expression of lipogenic genes, caused by reductions in the hepatic bile acid pool (101). BAS are not absorbed into the circulatory system and consequently are not associated with systemic side effects. The most common undesired effect of BAS therapy is gastrointestinal distress, leading to poor compliance. New BAS, like colesevelam, are more potent and can be administered at lower doses, with fewer side effects (81,102). Future therapies targeting the bile acid pathway will have further improvements to potency, perhaps for particular classes of bile acids. Bile acid feeding studies in mice demonstrate that altering the composition of the bile acid pool can impact cholesterol absorption (103,104). Improved potency will reduce the non-specific binding of BAS to co-administered drugs, reducing the drug-drug interactions that may limit the use of BAS for some patient groups (80,102,105). Other groups are addressing this issue by creating new inhibitors that target the bile acid uptake pathway rather than bile acids themselves (106,107). It remains to be seen whether these new approaches will improve clinical options for hypercholesterolemia.

Sequestration of Cholesterol

In addition to interactions with bile acids, cholestyramine and other molecules bind and sequester cholesterol directly

Table I Summary of Physiological Targets, Drugs, Stage of Development (Current as of June 2012), and Clinical Effect on Circulating LDL and HDL Cholesterol Levels (9,54,64,83,84,118,127,128,148,169,171,174,187,204,215–223)

Drug	Clinical change in LDL-C	Clinical change in HDL-C	Trade name	Company	Dosage form	Delivery	Approval date or phase of development	FDA status as of June 2012	References
Bile acid sequestrants									
Cholestyramine	↓ 20–23%	↑ 8%	Questran	Pharmacia & Upjohn apothecon	powder, oral tablet	GI-specific	1973 1994	discontinued discontinued	(9,213,214)
			Prevalite	Uphser Smith	powder, oral		1996	prescription	
			cholestyramine	TEVA	powder, oral		1998	discontinued	
			Cholybar	Parke Davis	chewable bar		1988	discontinued	
			Locholest	Sandoz	powder, oral		1996	prescription	
Colestipol	↓23%	0%	Colestid Flavored Colestid	Pharmacia & Upjohn Pharmacia & Upjohn	granule or tablet granule	GI-specific	1977 1977	prescription prescription	(214,215)
			Colestipol HCl	Impax Labs	granule or tablet		2006	prescription	
Colesevelam	↓ 9–18% ↓ 13%	↑ 0–4%	Welchol	Daiichi Sankyo	tablet Oral suspension	GI-specific	2000 2009	prescription prescription	(83,84,214,216)
Cholesterol solubility disruptors									
plant sterols	↓9%	0%		Various	Food additive	GI-specific	GRAS	Food additive	(54)
FM-VP4	↓10%	0%		Forbes Medi-Tech	Unknown	GI-specific	Phase II	Terminated	(64,214)
Cholesterol sequestration									
soluble dietary fiber	↓10%	0%	n/a		dietary supplement	GI-specific	GRAS	Food additive	(116)
Surfomer	↓13%	0%		n/a	unknown	GI-specific	Pre-phase I	unknown	(125)
Olestra	↓5%	0%	Olean		spreadable oil	GI-specific	1996	Food additive	(128)
NSAS				Amcol	suspension				
Niemann pick C 1 like 1 inhibitors									
Ezetimibe	↓18%	↑10%	Zetia	Merck	tablet	systemic	2002	prescription	(144,214)
Microsomal triacylglycerol transfer protein inhibitors									
Lomitapide	↓44%			Aegerion		systemic	Phase III	Phase III trial	(185,217)
Implitapide	↓67%			Bayer & Aegerion		systemic	Phase II	Terminated	(217)
JTT 130				Japan Tobacco		systemic	Phase I	Terminated	(217)
CP-346,086	↓72%	0%		Pfizer		systemic		Unknown	(218)
SLx4090				Surface Logix		GI-specific	Phase II	Unknown	(217)
Acyl CoA cholesterol acyltransferase (ACAT) inhibitors									
Avasimibe	↑8–11%	0%		Pfizer		systemic	Phase III	Terminated	(165,217)
Pactimibe	0%	0%		Daiichi Sankyo		systemic	Phase III	Terminated	(167,217)
isotype specific							screening	Unknown	(170)
Apolipoprotein B mRNA antagonists									
Mipomersen sodium	↓36%	0%	KYNAMRO	Genzyme / ISIS	SQ injection	systemic	Phase III	NDA review	(202,219–221)
TKM-APOB				Tekmira	lipid nanoparticle (injected)	systemic	Phase I	Reformulation	(217)

(108,109). One class of compounds with intraluminal cholesterol binding properties is dietary fiber. Dietary fibers are plant-derived complex carbohydrates that are neither digested nor absorbed by the body (110), and yet, epidemiological studies link their consumption with reduced risk of coronary artery disease (111–116). Because of these studies, cardiovascular health guidelines in North America and Europe suggest increasing dietary fiber consumption along with other lifestyle modifications as the primary action for people with hypercholesterolemia (12–14,117). Dietary fiber is generally categorized as being either water-soluble/gel-forming (guar gum, β-glucan, pectin, psyllium) or water insoluble (cellulose, lignins). A meta-analysis revealed that while all dietary fibers reduce the risk of heart disease, it is only water-soluble dietary fibers that contribute to LDL-cholesterol lowering (118). The reductions in LDL-cholesterol are likely through increased clearance of circulating LDL (119), linked with the inhibition of cholesterol absorption (120,121) due to the gel-like properties of the soluble fibers (122,123).

The LDL-cholesterol lowering effects of dietary fiber are modest, compared with pharmacological options. Consequently, a number of synthetic fibers have been tested for their ability to sequester cholesterol. The most studied of these is a copolymer of an 18-Carbon α-olefin and maleic acid (surfomer). Surfomer inhibits cholesterol absorption by 30%–50% in a variety of animal models (124–126). Humans treated with surfomer have a 25% reduction in cholesterol absorption accompanied by a 12% reduction in LDL-cholesterol (127). Despite promising results in these early studies, no further development of surfomer has been reported. Olestra, a non-absorbed sucrose polyester analogue of triacylglycerol used as an anti-obesity agent, also reduces cholesterol absorption by 50% (128,129). Development of Olestra as a hypocholesterolemic agent was discontinued due to gastrointestinal effects and a failure to reduce LDL-cholesterol by 15% in a double blind clinical trial (130).

Recent work in our laboratory has probed the hypocholesterolemic properties of surface modified nanostructured aluminosilicate (NSAS). These compounds belong to the montmorillonite minerals family, commonly referred to as bentonite clays. NSAS have a unique aluminosilicate platelet structure with a high surface area (200–800 m^2 per gram). In contrast to the positively charged bile acid sequestrants, NSAS platelets are negatively charged. Surface protons are incorporated to counterbalance the negative charge in the platelets. NSAS are fine particles that can adsorb water and organic materials both within their interlaminar space and on external surfaces (131), see Fig. 3 for a diagram. Pepto-Bismol contains a different purified montmorillonite clay called Veegum that adsorbs bile acids (132). Studies in rats revealed that the purified protonated form of NSAS reduces cholesterol absorption by 39%, similar to an identical dose of stigmasterol (133). Chronic administration of NSAS reduces circulating cholesterol levels in ApoE knockout mice over 12 weeks leading to reductions in atherosclerotic lesion formation at the aortic root (134). In an *in vitro* lipolysis assay, protonated NSAS specifically adsorbs cholesterol, sequestering it from the aqueous phase of the digestive milieu (109). This differs from cholestyramine,

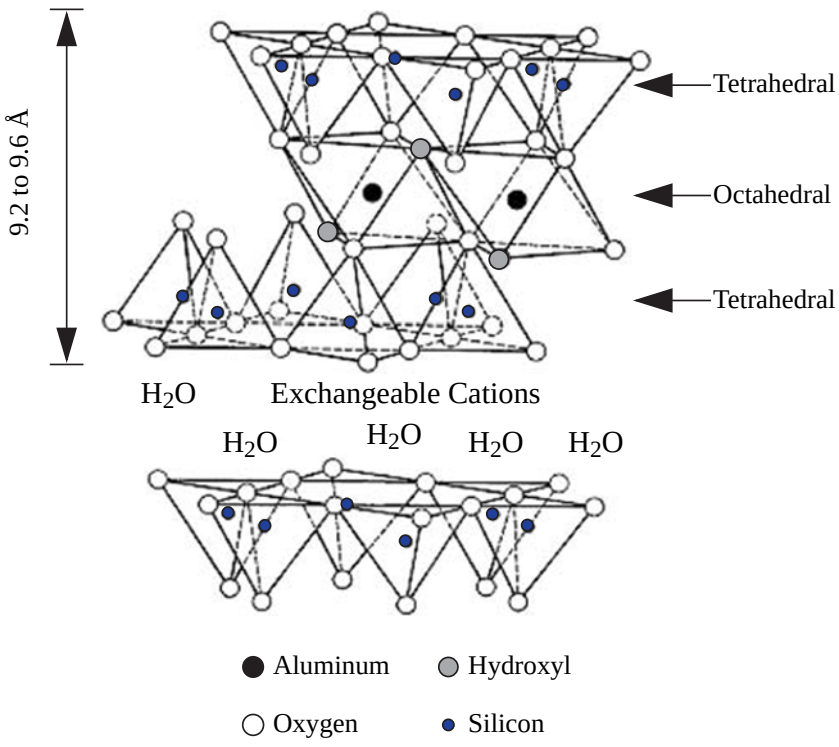

Fig. 3 A diagram of the general montmorillonite crystal structure. Montmorillonite usually has a layer-lattice structure consisting of two sheets of tetrahedral silicon crystals enclosing a sheet of octahedral aluminium crystals. Water and surface cations enter between adjacent silicon sheets causing the material to expand. Substances adsorb to external surfaces or within the interlaminar space. Adapted with permission from Hendricks SB. J Phys Chem. 45(1):65–81. Copyright 1941 American Chemical Society.

which non-specifically binds bile acids, cholesterol, and triacylglycerol (109). Additional studies assessing the toxicity of protonated NSAS and vitamin absorption are currently in progress. The specificity of cholesterol sequestration, the lack of systemic exposure, and the ability to reduce atherosclerotic lesion formation in animal models suggest that protonated NSAS may be a viable adjunct therapy with statins for hypercholesterolemic patients.

INTRACELLULAR PHARMACOLOGICAL TARGETS

Understanding the processes that govern the transcellular movement of cholesterol across the absorptive cells of the intestinal tract is of particular interest for the development of new drugs for the treatment of CAD. In order for cholesterol to be transported into the body, it is packaged in a unique lipoprotein produced by the intestine called a chylomicron. Three complementary pathways must converge for the synthesis of these lipoproteins. (A) Cholesterol must be taken up by the cell and packaged in nascent chylomicrons for secretion into the lymphatic system. (B) Dietary fats must be taken up by the enterocyte, assembled into triacylglycerol, and packaged onto the nascent chylomicron. (C) ApoB-48, the protein scaffold for chylomicrons, must be synthesized in the endoplasmic reticulum and trafficked to the Golgi apparatus for assembly of the mature chylomicron prior to secretion into the lymph. Pharmacological interruption of any of these pathways will affect the efficiency and magnitude of cholesterol absorption.

Ezetimibe

Ezetimibe is the first new pharmacological treatment for hypercholesterolemia since the discovery of statins. Unlike a majority of new drugs, ezetimibe was developed without a clear molecular target (135). Ezetimibe (and its analogues) were discovered while screening for cholesterol esterification inhibitors (discussed in section "Acyl CoA: Cholesterol Acyl Transferase (ACAT) Inhibitors"). Ezetimibe does not directly inhibit the esterification of cholesterol, yet blocks absorption. The development of the chemistry was guided by the cholesterol absorption activity in the cholesterol-fed hamster (135). Using radiolabeled compounds, Davis *et al.* demonstrated that ezetimibe and its active phenolic glucuronide act at the level of the brush border membrane in the small intestine (136). Cholesterol is taken up into the brush border membrane of columnar absorptive cells, called enterocytes, from mixed micelles in the gastrointestinal lumen. Upon uptake into the plasma membrane, cholesterol is subjected to competing molecular pathways (Fig. 4). Two hemi-ATP-binding cassette (ABC) transporters, ABCG5 and ABCG8, dimerize to form a complete transporter that actively effluxes both cholesterol and phytosterols back into the intestinal lumen (137,138). Loss of function mutations to ABCG5/G8 cause sitosterolemia, characterized by unregulated sterol absorption leading to premature CAD and the formation of subcutaneous cholesterol deposits called xanthomas (30,42,43,139,140). In opposition to the actions of ABCG5/G8 is the absorptive pathway that is targeted by ezetimibe.

In 2004, Altmann *et al.* proposed that the target of ezetimibe was a previously uncharacterized protein called Niemann Pick C1 Like 1 (NPC1L1), supported by studies in knockout mice (141). Although this assertion was initially controversial, and several studies refuted the finding, it is now generally acknowledged that ezetimibe inhibits cholesterol absorption by binding to an extracellular loop of NPC1L1 (142). Ezetimibe binding precludes the internalization of NPC1L1, thereby preventing it from chaperoning the transport of cholesterol from the plasma membrane to the endoplasmic reticulum (143–146). Clinical development of ezetimibe demonstrated that 10 mg/day was sufficient to reduce cholesterol absorption by >50%, which reduces LDL-cholesterol by 20% (23). Ezetimibe-simvastatin cotherapy can reduce LDL cholesterol by 25% beyond the LDL-cholesterol lowering of simvastatin alone (147). Ezetimibe monotherapy is accompanied by an increase in endogenous cholesterol production, making it a logical choice for co-therapy with a statin (23,24). Several large post-approval studies have confirmed that ezetimibe and simvastatin cotherapy reduces LDL cholesterol by 16.5% further than simvastatin alone in patients with familial hypercholesterolemia (148). This finding was recently confirmed in a less severe cohort (149). Although ezetimibe has proven to be an effective adjunct therapy for the reduction in circulating LDL cholesterol, neither of these large trials detected a reduction in atherosclerotic burden, leading to questions about how best to assess improvements in cardiovascular health (150,151). Despite continued debate about how best to evaluate progression or regression of atherosclerosis in the clinic, LDL-cholesterol is a long established risk factor for coronary artery disease that is significantly reduced by treatment with ezetimibe, particularly when coupled to treatment with statins.

LXR Agonists

The efflux of cholesterol from enterocytes is driven by the actions of ABC transporters. The heterodimer ABCG5/G8 moves cholesterol back to the intestinal lumen while ABCA1 shuttles cholesterol onto lipid-poor ApoA1 in the portal vein (see Fig. 4). The Liver X Receptor (LXR) exerts transcriptional control over both of these transporters (152). Transgenic mouse models show that induction of *Abcg5* and *Abcg8* genes is sufficient to reduce cholesterol absorption and

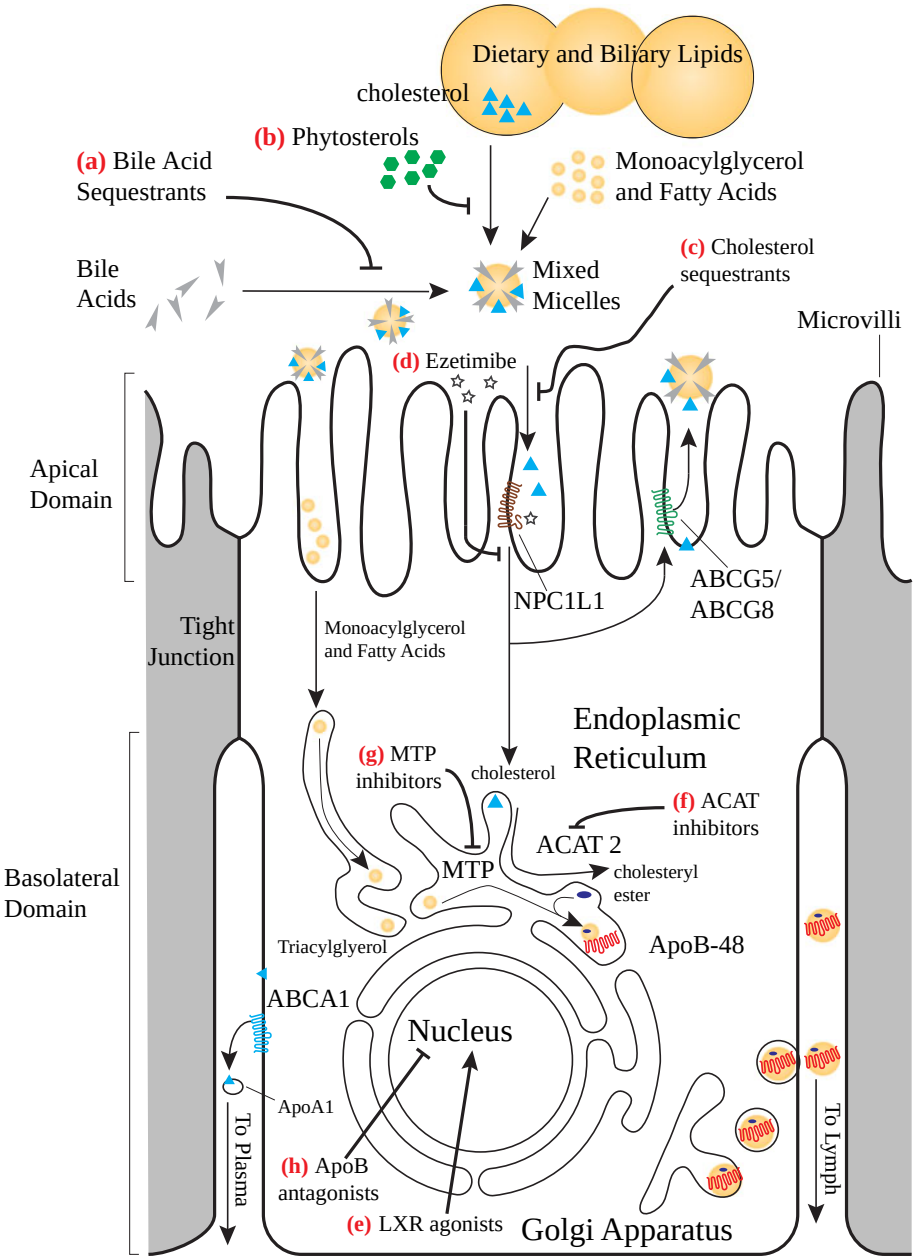

Fig. 4 Pharmacological targets in the gastrointestinal absorption of cholesterol. Cholesterol from the lipid phase is incorporated into mixed micelles comprised of fatty acids, monoacylglycerol, and phospholipids, stabilized by bile acids. Monomers of cholesterol cross the unstirred water layer surrounding the enterocyte and are taken up into the brush border membrane. (**a**) Bile acid sequestrants adsorb bile acids, preventing them from incorporating into mixed micelles. (**b**) Plant sterols compete with cholesterol for incorporation into the mixed micelles and/or stimulate efflux from the cell membrane. (**c**) Cholesterol sequestrants, including nanostructured aluminosilicates (NSAS) and soluble dietary fibers prevent cholesterol from crossing the unstirred water layer. (**d**) Ezetimibe binds to Niemann Pick C1 Like 1 (NPC1L1) and prevents the internalization of the protein, retaining cholesterol in the plasma membrane, where it is effluxed by the dimer of ATP-binding cassette transporters ABCG5/ABCG8. (**e**) Liver X Receptor (LXR) agonists increase the expression of cholesterol efflux genes, including *ABCG5/G8*. (**f**) Acyl CoA Cholesterol Acyltransferase 2 (ACAT2) inhibitors prevent cholesterol esterification in the endoplasmic reticulum, a step that is required for incorporation of cholesterol into nascent chylomicrons. (**g**) Microsomal Triacylglycerol Transfer Protein (MTP) inhibitors block the transfer of newly reassembled triacylglycerol to the ApoB-48 polypeptide as it is being translated in the rough endoplasmic reticulum, degrading the polypeptide and reducing chylomicron formation. (**h**) Antisense oligonucleotides and/or siRNA target mRNA that encodes ApoB-48 to decrease secretion of chylomicrons into the lymphatic system. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (reference 141), copyright 2008. http://www.nature.com/nrm/index.html.

attenuate atherosclerosis (138,153). Mice treated with the LXR agonist T0901397 have reduced cholesterol absorption, suggesting that pharmacological stimulation of LXR is an option for reducing cholesterol absorption (154). First generation LXR agonists are effective at stimulating cholesterol efflux from atherosclerotic lesions (72,155); however,

they also induce the expression of lipogenic genes (156,157) leading to hepatic steatosis. The undesired effects of fatty liver may be addressed through the synthesis of a new generation of LXR agonists, or by targeted drug delivery to the intestine. Despite a number of hurdles to overcome, development of LXR agonists remains an area of active research in cardiovascular disease.

Acyl CoA: Cholesterol Acyl Transferase (ACAT) Inhibitors

Cholesterol that is moved from the brush border membrane to intracellular membranes must be esterified prior to assembly in chylomicrons. The esterification of cholesterol with free fatty acids occurs in the endoplasmic reticulum and is catalyzed by the enzyme Acyl CoA Cholesterol Acyl Transferase (ACAT) (158,159). The inhibition of ACAT is proposed to reduce the absorption of cholesterol by preventing the movement of cholesterol into nascent chylomicrons (160). Three groups simultaneously discovered that there are two isoforms of ACAT in the body: ACAT 1 and ACAT 2 (161–163). ACAT 1 is expressed ubiquitously whereas ACAT 2 is only found in the small intestine and the liver (161,164–166). Clinical development of ACAT inhibitors began before the discovery of multiple isoforms and consequently they target both ACAT1 and ACAT2. The subsequent analysis of knockout mice explains why the first generation of ACAT inhibitors has not been clinically successful (Table I).

Mice lacking ACAT1 or ACAT2 have dramatically different metabolic phenotypes. Mice lacking ACAT 1 cross-bred with murine models of atherosclerosis (LDL receptor knockout or ApoE knockout mice) develop xanthomas and increased atherosclerosis, despite reductions in circulating cholesterol (167,168). In comparison, mice lacking ACAT 2 have a favorable phenotype including reduced cholesterol absorption and resistance to dietary induced hypercholesterolemia and gallstones (158,159). The first ACAT inhibitor to reach clinical development was avasimibe. Despite promising preclinical data, treatment with avasimibe did not improve atherosclerosis (169), and induced the expression of drug metabolizing enzymes (170). The second ACAT inhibitor tested in clinical trials, pactimibe, did not improve atherosclerosis, in fact worsened progression of the disease in two specific analyses (171). It has been widely speculated that these two clinical trials failed to demonstrate the value of ACAT inhibition because they non-specifically inhibited both isoforms of ACAT (172). Although ACAT2 inhibition is hypothesized to be beneficial, based on genetically modified mouse studies (158,160,172,173), none of the isotype-specific inhibitors currently in development (174) have been tested clinically at the time of this review.

Microsomal Triacylglycerol Transfer Protein (MTP) Inhibitors

Although chylomicrons transport cholesterol, they are primarily composed of triacylglycerol (175). Inadequate lipidation of the ApoB polypeptide as it is being translated in the ribosome causes misfolding and subsequent degradation of ApoB-48, blocking the secretion of chylomicrons into the lymphatic system (Fig. 4). Dietary fats are broken down into fatty acids and monoacylglycerol by lipases in the intestinal lumen prior to absorption by enterocytes. Once in the intestinal cell, they are repackaged as triacylglycerol molecules and bind to a transfer protein called Microsomal Triacylglycerol Transfer Protein (MTP) (176). By inhibiting MTP, the lipid transfer actions are disrupted and there is a reduction in the production of triacylglycerol-rich lipoproteins (177). This is an attractive target for new therapeutics as inhibition of intestinal MTP addresses two health issues simultaneously: the absorption of excess dietary fat and the absorption of cholesterol by reducing chylomicron production.

At the time of this review, five inhibitors have entered the drug development process: Lomitapide (also called AEGR-733, BMS-201038), Implitamibe (also called BAY-13-9953), JTT 130, CP 346086, and SLx 4090. Each of these inhibitors reduces circulating cholesterol, ApoB-containing lipoproteins, and triacylglycerol in preclinical animal studies (177–185). In clinical trials, both CP-346086 and Lomitapide reduce circulating cholesterol and triacylglycerol in hyperlipidemic patients (180,186–188). In both studies, a subset of the patients treated with MTP inhibitor had increased gastrointestinal disturbances and mild increases to liver transaminase levels, causing concerns for the continued development of these inhibitors. In animal studies, MTP inhibition can increase triacylglycerol storage in the liver and in the intestine perhaps accounting for both the increases in hepatic liver enzymes and gastrointestinal side effects of MTP inhibition (177,189,190). In order to avoid these complications, new, intestine-specific, MTP inhibitors are being developed that lack systemic absorption, such as SLx4090 (184,191). The viability of inhibiting intestinal MTP as a means of reducing cholesterol absorption will depend on the outcome of studies using these inhibitors.

ApoB Transcription Inhibitors

While MTP inhibition indirectly degrades ApoB, several groups have attempted to target the production of ApoB directly. Circulating levels of ApoB-48 increase after feeding but ingestion of fat does not appear to regulate *APOB* transcription. Small dense lipoproteins containing ApoB-48 are secreted by the intestine, even in the fasted state

(192). Rather, the enterocyte retains a store of small dense chylomicron precursors for rapid response to feeding (193–195). Transcription of *APOB* appears to be constitutively active, and production of ApoB lipoproteins is regulated through degradation (196,197). Because there is no known activator of gene expression to inhibit pharmacologically, *APOB* mRNA has been targeted directly. Both antisense oligonucleotides (ASO) and RNA interference (RNAi) strategies successfully reduce ApoB. These techniques utilize short strands of nucleic acids with complementary sequences to the mRNA of the gene being targeted (198). Although both technologies degrade mRNA after binding to complimentary sequences, ASO and RNAi accomplish this through different mechanisms (199,200). At present, ApoB mRNA targeting has only been utilized to prevent the formation of hepatic lipoproteins (201–204); however, targeting intestinal ApoB production with next-generation silencing therapies is an attractive means of reducing cholesterol absorption.

SUMMARY

Novel combination therapies for coronary artery disease seek to modify risk factors associated with the initiation and progression of atherosclerosis. Statin therapy is effective at lowering LDL-cholesterol and reducing CAD-related morbidity and mortality (10,205–210). Unfortunately, statin use can only reduce cardiovascular events by 33% in the most responsive patient groups, leaving a great deal of cardiovascular risk to be treated (211). Recently, there have been a number of excellent reviews of the clinical promise of raising HDL and reducing inflammation as alternate routes to lowering CAD (72,212–214). Clinical guidelines, driven by empirical evidence of reduced mortality, continue to call for lower levels of LDL-cholesterol in high-risk CAD patients. There is a need to identify new pharmacological targets that can be treated in tandem with statins to reduce LDL-cholesterol. In this paper, we summarize the opportunities and challenges for drug development of cholesterol absorption inhibitors, with emphasis on the advantages of intestine-specific therapies.

ACKNOWLEDGMENTS AND DISCLOSURES

Kishor Wasan is supported by operating grants from the Canadian Institutes of Health Research. Stephen Lee is supported by scholarships from Canadian Institutes of Health Research and from the Michael Smith Foundation for Health Research. Pavel Gershkovich was supported by a scholarship from MITACS. Gerald Darlington is an employee of Amcol International Corporation.

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