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REVIEW

Drug targets beyond HMG-CoA reductase: Why venture beyond the statins?

Ingrid C. GELISSEN[1], Andrew J. BROWN (✉)[2]

[1] *Faculty of Pharmacy, The University of Sydney, Sydney NSW 2006, Australia*
[2] *BABS, School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney NSW 2052, Australia*

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Abstract **In this review, we aim to convey a brief, select history of the development of cholesterol-lowering therapies. We focus particularly on the highly successful statins as well as setbacks that should serve as cautionary tales. We go on to preview recent developments that may complement, if not one day replace, the statins. Our focus is on pharmacological interventions, particularly those targeting the cholesterol biosynthetic pathway. Also, we examine therapies under current investigation that target the assembly of atherogenic lipoproteins (via apolipoprotein B or microsomal triglyceride transfer protein), the stability of the low-density lipoprotein-receptor (via PCSK9, proprotein convertase subtilisin kexin 9), or are designed to increase high-density lipoprotein-cholesterol (via inhibition of cholesteryl ester transfer protein).**

Keywords statins, cholesterol-lowering drugs, side-effects, adverse reactions, cholesterol synthesis

Statins – A remarkable success story

First isolated from a mold (thanks to the heroic efforts of Akira Endo), statins have come a long way to become one of the most prescribed medicines in the world. Statins were discovered and developed against the background of a worrying coronary epidemic in industrialized countries, and mounting evidence that cholesterol was the culprit, or at least a nefarious accomplice.

In 1913, Nikolai Anitschkov was reportedly the first to make a connection between cholesterol and heart disease, inducing atherosclerosis in rabbits by feeding them cholesterol [reviewed in (Li, 2009)]. Several decades later, in the 1950s, key epidemiological studies (notably the Seven Country Study and the Framingham Study) firmly established raised blood cholesterol levels as a major risk factor for heart disease (Frantz and Moore, 1969). Thus, there became a pressing need to develop drug therapies to lower blood cholesterol levels, and hence combat the rising tide of heart disease. Existing compounds such as niacin, bile acid resins, and fibrates (all still in clinical use today) were having some success, but their ability to lower blood cholesterol levels was relatively modest, and not without side-effects. The first drug to directly target the cholesterol biosynthetic pathway, triparanol (trade name: MER/29), was approved in 1960. Triparanol inhibits one of the penultimate steps in cholesterol synthesis, resulting in the accumulation of desmosterol (Fig. 1). However, it was withdrawn two years later, due to many cases of cataract formation, hair loss, and skin reactions [reviewed in (Li, 2009)]. These adverse reactions were attributed to the accumulation of hydrophobic cholesterol precursors, particularly desmosterol. This cautionary tale of triparanol steered researchers' efforts away from the distal end of the cholesterol biosynthesis pathway, to an earlier step where the precursor was water-soluble and so should not accumulate. That step, catalyzed by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC1.1.1.34), was known to be a key rate-controlling step in cholesterol synthesis (Bucher et al., 1960).

In 1973, after a year of painstaking work assaying more than 3800 fungal strains, Akira Endo's team finally discovered a potent HMG-CoA reductase inhibitor, mevastatin (also called compactin) (Endo, 2010). A team at Merck pharmaceutical company developed a chemically-related compound, lovastatin, and in 1987 under the trademark Mevacor, it became the first statin to reach patients [reviewed in (Li, 2009)]. Over subsequent years, a number of statins

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Correspondence: Andrew J. BROWN
E-mail: aj.brown@unsw.edu.au

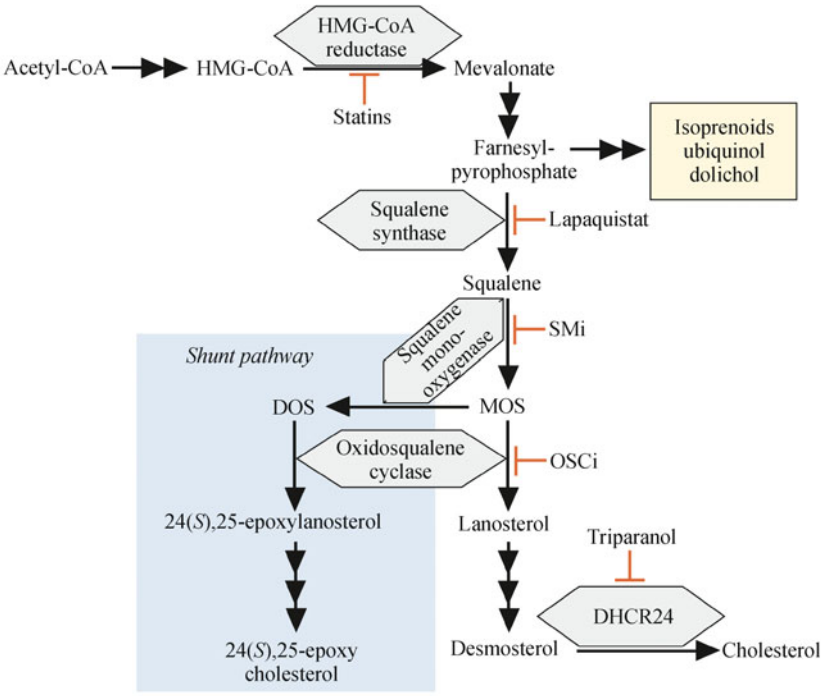

Figure 1 The cholesterol biosynthesis pathway. Some of the major points that are or have been considered drug targets for cholesterol-lowering are indicated. SMi, Squalene monooxygenase inhibitor; OSCi, Oxidosqualene cyclase inhibitor; DHCR24, 24-dehydrocholesterol reductase (also called Seladin-1). See the text for further details.

have become available, usually with superhero names like Zocor (simvastatin), Lipitor (atorvastatin), and Crestor (rosuvastatin); each tending to be more potent than their predecessors.

From their humble fungal beginnings, statins are now considered the cornerstone in the treatment of cardiovascular disease, providing benefit for the great majority of patients. They are probably the most intensively and extensively tested class of drugs as illustrated by a recent meta-analysis of data from 170,000 participants in 26 randomized statin trials (Baigent et al., 2010). Not only are they among the most commonly prescribed class of drugs around the world, but their use is still increasing. For example, in Australia, which has comprehensive and readily accessible prescription data, the proportion of prescriptions for statins has increased threefold in the past 12 years (Brown, 2010). There is a strong likelihood that statin usage will continue to climb, given the opening up of large new markets like China and India, and considering that in traditional markets a substantial proportion of patients who are at high risk of cardiovascular disease are currently not prescribed statins (Webster et al., 2009). Furthermore, results from the JUPITER trial indicate that statins may even benefit people with normal cholesterol levels (Ridker et al., 2009). Moreover, statin therapy is expanding beyond the traditional cardiovascular area, with statins currently being tested for a remarkable diversity of diseases including Alzheimer's disease, asthma, lupus, sepsis, renal diseases, and various cancers (Anon, 2010).

How do statins work?

Statins are reversible, competitive inhibitors of a key rate-limiting step in cholesterol synthesis, catalyzed by HMG-CoA reductase. They preferentially affect the liver cell and cleverly exploit cholesterol homeostasis. In response to inhibited cholesterol synthesis, the liver cell attempts to maintain cholesterol status by upregulating the receptor for the major cholesterol-carrying particle in the circulation, low-density lipoprotein (LDL). This upregulation occurs via a transcriptional pathway mediated by sterol regulatory element binding proteins (SREBPs) (Fig. 2). The elegant cell biology of the SREBP pathway has been elucidated over the past couple of decades by the laboratory of Goldstein and Brown [reviewed in (Goldstein et al., 2006)], who incidently also discovered the LDL-receptor. Since the LDL-receptor is responsible for the clearance of LDL from the bloodstream, its upregulation results in decreased blood cholesterol levels. Besides inhibiting cholesterol synthesis, statins also inhibit the formation of important non-sterol products downstream of HMG-CoA reductase. These are involved in a wide variety of cell processes, including cell growth and differentiation (prenylated proteins including the small GTPases), glycosylation (dolichol), and electron transport (ubiquinol). Inhibition of these products probably accounts for the non-cholesterol-related benefits often ascribed to statins. These so-called "pleiotropic effects" include reduction of the

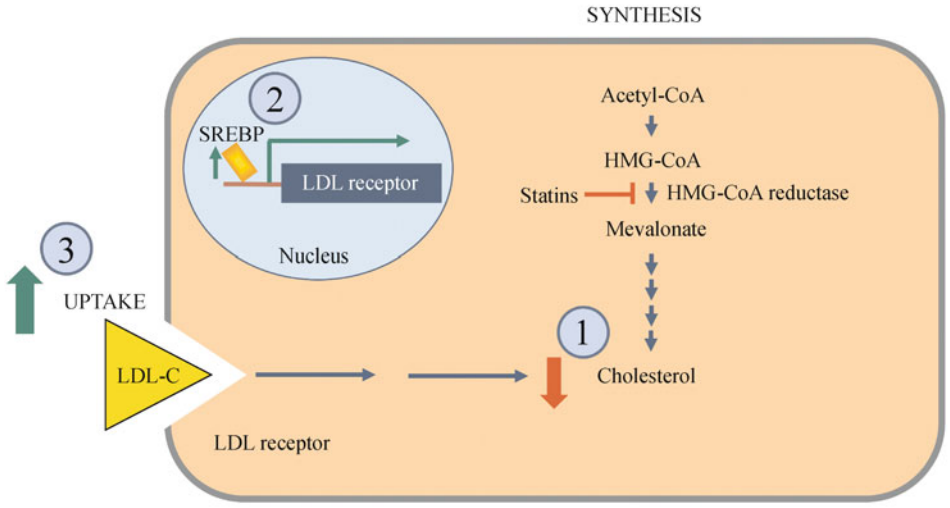

Figure 2 Statins decrease LDL-cholesterol levels by exploiting cholesterol homeostasis in the liver. 1. Decreased cell cholesterol levels lead to 2. Induction of LDL-receptor expression that 3. Increases uptake of LDL-cholesterol (LDL-C), thereby balancing cholesterol levels in the cell, and decreasing blood cholesterol levels.

inflammatory risk marker, C-reactive protein (Ridker et al., 2009), although the idea that statins reduce cardiovascular risk beyond that expected from the extent of LDL-cholesterol lowering remains somewhat contentious (Robinson et al., 2005). Inhibition of these non-cholesterol molecules are also thought to contribute to some of the adverse effects reported for statins.

Why look beyond the statins?

Although statins are considered comparatively safe, like any other drug they are not free from adverse effects. Often these manifest in combination with other drugs. Most documented are raised liver enzymes and muscle-related symptoms– usually muscle pain (myalgia) and inflammation (myositis). A rare side-effect is rhabdomyolysis – the breakdown of skeletal muscle resulting in the release of muscle protein (myoglobin) into the bloodstream, which can cause kidney damage or in extreme cases, kidney failure. A sombre reminder of this rare side-effect of statins came in 2001, when cerivastatin (Baycol) was voluntarily withdrawn due to reports of fatal rhabdomyolysis, usually seen in combination with fibrate therapy (Staffa et al., 2002). Other reported side-effects are not always well-recognized by health professionals, and include cognitive and memory loss, mood disorders, sexual dysfunction, and perturbed glucose metabolism (Golomb and Evans, 2008). A meta-analysis found a slight but significantly increased risk of diabetes with statin treatment (Sattar et al., 2010).

While hard data on the frequency of statin side-effects is elusive, estimates suggest that the rate of myalgia in statin-treated patients may be as high as 10% or even 20% (Bruckert et al., 200; Wenner Moyer, 2010). In a recent survey, statins received the second most complaints of side-effects (Britt et al., 2008). Even rare side-effects become a potential public health issue if the drug is as commonly-prescribed as the statins. Moreover, side-effects increase with the potency and dose of statin used (Silva et al., 2007). Importantly, both have risen markedly over the last decade or so (Brown, 2010). With statin use, dose and potency all increasing, the number of adverse reactions can also be expected to escalate. This is recognized as an important area of investigation, with numerous trials underway to ameliorate the side-effects of statins, particularly those involving ubiquinol (Coenzyme Q) and muscle-related effects (Anon, 2010).

Another major challenge is non-adherence to statin therapy. Statin discontinuation rates are high in both primary and secondary prevention (Bates et al., 2009). In a recent survey, the most common reason patients stopped statin therapy was side-effects (although these were not validated) (McGinnis et al., 2007). Non-adherence is associated with adverse health outcomes and increased costs of healthcare (Bates et al., 2009). There is increasing evidence that taking statins intermittently may cause more harm than not taking statins at all. This has been termed the "Statin Rebound Phenomenon" and has been especially observed after an acute vascular event, when statin discontinuation results in a worse outcome than in those patients who never received statins (Daskalopoulou, 2009). Evidence has also been presented for the existence of a rebound inflammatory effect after statin cessation (measured as C-reactive protein) (Sposito et al., 2009). The statin rebound effect is caused by the exquisite nature of feedback control, and can be reproduced in cultured cells (Wong et al., 2007). The inhibitory effect of a statin on cholesterol synthesis leads to a compensatory increase in expression of several key cholesterol biosynthetic enzymes, including HMG-CoA reductase, which are regulated by

SREBP. Hence, removal of the statin results in a concentration-dependent surge in cholesterol synthesis (Wong et al., 2007). Accordingly in normal subjects, statin cessation caused a rebound of serum cholesterol levels, monocyte HMG-CoA reductase activity and sterol synthesis (Stone et al., 1989).

The induction of HMG-CoA reductase [via a combination of increased SREBP-mediated gene transcription and protein stabilization (DeBose-Boyd, 2008)] has been held responsible for the failure of statins to decrease LDL cholesterol on a continuous, long-term basis in some patients – although this so-called "statin escape" phenomenon is controversial and may relate to poor compliance in many cases (Yeshurun et al., 2005). Given the widespread problems of intermittent statin use due to poor compliance, the statin rebound phenomenon may affect many patients. Improving compliance is desirable of course, but has proved difficult (Schedlbauer et al., 2004). Therefore, there is impetus to develop drugs superior to the statins, avoiding their side-effects. This is particularly important in patients at greater risk of adverse reactions (e.g., the elderly taking a number of medications), or for whom near-complete inhibition of cholesterol synthesis would be inadvisable (e.g., children or adolescents). Furthermore, should we be satisfied with the 20%–30% reduction in coronary events often ascribed to statins? In the following section, we discuss some of the ways of complementing or even replacing statins, with particular emphasis on other enzymes in the cholesterol biosynthesis pathway, as well as some new therapies.

Targeting cholesterol synthesis beyond HMG-CoA reductase

We have focused on three enzymes in cholesterol synthesis (Fig. 1). For other enzymes in this pathway that could also be drug targets, the reader is referred to a recent review (Rozman and Monostory, 2010).

Squalene synthase

After HMG-CoA reductase, squalene synthase (EC2.5.1.21) is probably the most intensively investigated drug target in the cholesterol biosynthesis pathway (Seiki and Frishman, 2009). This enzyme catalyzes the committed step to cholesterol synthesis, converting farnesyl pyrophosphate to squalene (Fig. 1). Because squalene synthase sits at a branch point, inhibitors of this enzyme maintain or even increase flux into non-sterol products (e.g., ubiquinol, dolichol, and other isoprenoid lipid anchors). As a result, squalene synthase inhibitors are considered to have an advantage over statins in terms of lacking myotoxic side-effects (attributed to inhibition of non-sterol products by statins, as discussed earlier). On the other hand, this positive feature has been overshadowed by concerns of its safety profile. In fact, at least in animal models, squalene synthase inhibitors can increase farnesyl and non-sterol products, resulting in acidosis, due to overexcretion of farnesol-derived dicarboxylic acids. Out of the several squalene synthase inhibitors investigated, Takeda's drug, lapaquistat (TAK-475), was at the most advanced stage of development. In 2008, Takeda discontinued development of lapaquistat after Phase III trials, judged on the profile of the compound not being "superior to existing marketed drugs from both efficacy and safety viewpoints" (Anon, 2008).

Squalene monooxygenase (SM)

Squalene epoxidase or monooxygenase (EC1.14.99.7) is a non-metallic, flavoprotein monooxygenase that catalyzes the first oxygenation in cholesterol synthesis; that is, the conversion of squalene to monooxidosqualene (MOS) (Chugh et al., 2003). It also converts MOS to dioxidosqualene (DOS) as the first step in the shunt pathway (Fig. 1), ultimately leading to synthesis of a potent oxysterol regulator, 24(*S*),25-epoxycholesterol (24,25EC) (Abe et al., 2007). SM is the primary drug target to inhibit the growth of pathogenic fungi (Ruckenstuhl et al., 2007). It is also the target by which certain foodstuffs (e.g. garlic, wine, green tea) may reduce blood cholesterol levels (Chugh et al., 2003). SM inhibitors have been developed which lower cholesterol levels in animal models. Moreover, inhibiting cholesterol synthesis at SM (but not at HMG-CoA reductase) decreased gallstone formation in a mouse model (Clarke et al., 2004). SM inhibitors have yet to be tested in humans, and perhaps deserve more attention as a possible cholesterol-lowering therapy (Chugh et al., 2003).

Oxidosqualene cyclase (OSC)

Lanosterol synthase or oxidosqualene cyclase (EC5.4.99.7) is a particularly interesting target. While complete inhibition ablates cholesterol synthesis, partial inhibition decreases it but importantly, stimulates production of 24,25EC (Brown, 2009). This is because the partial inhibition of OSC causes the intermediate MOS to accumulate, giving SM a greater chance to act again to form DOS (Fig. 1). Since OSC favors DOS over MOS, this ensures a greater flux through the shunt pathway, producing more 24,25EC. This oxysterol reduces cell cholesterol levels at three points (Brown, 2009): inhibiting synthesis and uptake (via suppressing SREBP activation), while stimulating export (by serving as a potent ligand for the liver X receptor, LXR), making it a very attractive means to limit cell cholesterol levels. Of note, statins and inhibitors of other early steps of the cholesterol biosynthesis pathway (up to and including SM), inhibit production of this potent oxysterol regulator (Wong et al., 2004).

OSC inhibitors decrease cholesterol levels in various animal models by ~20%–60% (hamsters, squirrel monkeys, and minipigs), although these studies did not set out to study the effect of partial OSC inhibition per se (Eisele et al., 1997;

Morand et al., 1997; Telford et al., 2005). Importantly, one of these studies (Morand et al., 1997) showed that an OSC inhibitor had advantages over statin alone in that it did not reduce coenzyme Q10 levels in liver and heart of hamsters, and significantly did not trigger an overexpression of hepatic HMG-CoA reductase. In an elegant proof-of-concept study, partial siRNA-mediated knockdown of OSC expression in mice enhanced LXR activity and selectively upregulated LXR-target genes involved in reverse cholesterol transport (Dang et al., 2009).

A concern with high doses of OSC inhibitors is increased risk of cataracts (Funk and Landes, 2005). However, low doses of OSC inhibitors, required for partial inhibition, should theoretically avoid side-effects in humans. Instead of blocking the entire mevalonate pathway with statins, the idea of manipulating a self-governing pathway for the production of a physiological regulator, that can augment cholesterol removal and decrease uptake and synthesis, is attractive and warrants further evaluation (Wong et al., 2007).

Other drug targets beyond cholesterol synthesis

Over the years, there have been numerous lipid-related targets that have been proposed for treating cardiovascular disease. Our focus below is on those recent targets for which drug development have progressed furthest, particularly as it relates to lowering LDL-cholesterol or raising HDL-cholesterol. We have briefly summarized the mechanism behind each approach, and presented some key findings from experiments in animals and humans.

Apolipoprotein B (apoB)

ApoB is the structural protein component of very low-density lipoprotein (VLDL) and LDL, serving as the ligand for clearance from the circulation by the LDL-receptor. Inhibiting apoB secretion and hence reducing the formation of atherogenic lipoproteins has been investigated. One of the more advanced products is a second generation antisense oligonucleotide, marketed under the trade name Mipomersen, which is currently being tested in Phase III trials (Visser et al., 2010). Mipomersen is administered subcutaneously and has been shown to achieve a ~25% reduction in LDL-cholesterol in patients with familial hypercholesterolemia, when co-treated with conventional lipid-lowering therapy (Akdim et al., 2010; Raal et al., 2010). Side-effects of Mipomersen include injection site reactions, flu-like symptoms, and increases in the liver injury marker aminotransferase, the latter being reported in a significant proportion of patients (~10%). The cause of this is currently unknown and warrants close observation as it could potentially indicate the development of hepatic steatosis, which might be anticipated if hepatic lipids cannot be secreted and hence accumulate in the liver. Hepatic steatosis is a clinical feature of familial hypobetalipoproteinemia which can be caused by a number of genetic defects, including deficiency of ApoB (or MTTP or dominant-negative PCSK9). However, overall, very low levels of apoB-containing lipoproteins appear to offer cardioprotection in these patients (Sankatsing et al., 2005).

Microsomal triglyceride transfer protein (MTTP)

Another approach to decrease atherogenic lipoproteins is by inhibition of MTTP. Expressed in the liver as well as the intestine, MTTP is involved in the assembly of VLDL and chylomicrons by transferring triglycerides onto ApoB. Pharmacological inhibition of MTTP has been successful with the compound lomitapide (also known as AEGR-733 or BMS-201038), which has been shown to significantly reduce LDL-cholesterol as well as circulating apoB (both dropped by ~50% in a hypercholesterolemic cohort (Cuchel et al., 2007)). However, the drug has significant side-effects, the most serious being elevated liver aminotransferase levels and hepatic steatosis (Cuchel et al., 2007). Inhibition of MTTP may have some potential if future compounds are successful in avoiding liver-related side-effects. Consequently, there are efforts to develop intestine-specific inhibitors (Mera et al., 2010).

Proprotein convertase subtilisin kexin 9 (PCSK9)

Another target that so far does not seem plagued with undesirable side-effects is PCSK9 (EC3.4.21). PCSK9 is secreted mainly by the liver and acts by degrading the LDL-receptor by a mechanism that is thought to include intracellular as well as extracellular pathways. This intriguing protein was first discovered in a cohort of French families that suffer from familial hypercholesterolemia but showed no mutations in either the LDL-receptor or apoB (Abifadel et al., 2003). Two gain-of-function mutations in PCSK9 were associated with elevated LDL-cholesterol. Many other studies have since supported these findings and PCSK9 has become a promising target in managing hypercholesterolemia.

Specific inhibition of PCSK9 activity using traditional pharmaceutical approaches has not been successful to date. However, gene silencing strategies to reduce PCSK9 expression are under intense investigation and are showing promising results [reviewed in (Abifadel et al., 2010)]. Frank-Kamenetsky et al. (2008) used siRNA delivered specifically to the liver by a lipidoid nanoparticle in several animal models, including non-human primates. A single dose of siRNA resulted in a rapid, prolonged (up to 3 weeks after delivery) and significant reduction of PCSK9 as well as LDL-cholesterol, without affecting HDL or triglycerides. Besides gene silencing, monoclonal antibody therapy has also yielded promising results. Chan et al. (2009) reported on the effectiveness of a neutralizing anti-PCSK9 monoclonal antibody in rodent and primate models that inhibits PCSK9 binding to the LDL-receptor. A single injection of this

antibody to cynomolgus monkeys produced an 80% reduction in LDL-cholesterol, which importantly was maintained over 10 days. Statins alone have been shown to increase PCSK9 expression (Dubuc et al., 2004) which indicates that their ability to reduce LDL-cholesterol levels is probably self-limiting. In this context, it is significant that PCSK9 modulation by the monoclonal antibody in a cell culture system together with a statin had at least an additive effect on increasing LDL-receptor levels (Chan et al., 2009). So far, no significant side-effects have been described following PCSK9 reduction using either approach. However, the fate of the antibody-protein complex has not been determined (Steinberg and Witztum, 2009). Therefore, the effects of long-term administration need to be assessed to ensure the enduring safety of these therapies.

Raising HDL

The terms 'Good' (HDL) and 'Bad' (LDL) cholesterol may be in common parlance; although their precise meaning in terms of lipoprotein metabolism tends to be more esoteric. Niacin and fibrates have long been known to increase HDL. But in marketing terms, what would be better than hitting "Bad" cholesterol with a statin while at the same time increasing levels of "Good" cholesterol? This was the intention of combining a statin with an inhibitor of cholesteryl ester transfer protein (CETP). CETP is a glycoprotein secreted predominantly by the liver and bound to HDL in plasma. It facilitates the transfer of cholesteryl esters from HDL to LDL and VLDL, in exchange for triglycerides. Hence, a reduced activity of CETP is associated with higher levels of HDL cholesterol. Indeed, naturally occurring gene variants in CETP which result in reduced CETP function are associated with modest increases in HDL and a slightly decreased risk of cardiovascular events (Anon, 2006) [reviewed in (Thompson et al., 2008)]. Although it should be noted that homozygotes for CETP inactivating mutations leading to increased HDL-cholesterol (common in Japan), do not necessarily have a decreased risk for cardiovascular disease (Hirano et al., 1997). Nevertheless, pharmacological inhibitors of CETP have now been extensively investigated in combination with statins.

The first CETP inhibitor developed, Torcetrapib, failed due to off-target effects and was withdrawn during Phase III clinical trials after increased mortalities in the combined treatment group relative to the statin alone group (2006). It was discovered that the drug increased blood pressure, probably by raising aldosterone production (Barter et al., 2007). Considering these unexpected serious side-effects, results from human trials with two other CETP inhibitors are being heavily scrutinized. Dalcetrapib is undergoing Phase II and III trials and so far is not displaying any of the side-effects that were experienced with Torcetrapib. This compound is different from Torcetrapib in that it induces a conformational change in CETP, leading to a ~50% inhibitory capacity at concentrations < 10 μmol/L (Robinson, 2010). In addition, it increases synthesis of a major apolipoprotein in HDL, apoA-1, in the liver. Phase II trials measuring lipoprotein levels after single therapy with Dalcetrapib or in combination with statins, suggests an increase in HDL-cholesterol levels of up to a third while also increasing apoA-1 plasma concentrations. Large-scale Phase III trials looking at long-term treatment and coronary endpoints are currently underway. Anacetrapib is another CETP inhibitor under investigation and a recent trial (Cannon et al., 2010) in a large cohort of patients with high risk of cardiovascular disease showed a greatly improved lipoprotein profile (increased HDL-cholesterol as well as reduced LDL-cholesterol) with no significant side-effects. The efficacy of this strategy in terms of its protective effect on cardiovascular events remains to be determined. However, it seems from the persistent interest that CETP inhibitors may be around for some time yet.

Conclusions

Although there are new therapies on the horizon that may one day replace the statin class of drugs (Wenner Moyer, 2010), the statins are not going anywhere anytime soon. These highly successful drugs will undoubtedly remain the cornerstone of treating cardiovascular disease for some years to come. However, with the expiration of major patents – most notably for Lipitor in 2011 – this is an opportune time to consider alternatives, either in combination with a statin or as a monotherapy. After all, there is an urgent need for cholesterol-lowering therapies for patients who do not tolerate statins.

Why haven't inhibitors targeting enzymes beyond HMG-CoA reductase been pursued further? It seems that research into inhibitors of the cholesterol biosynthesis pathway has been wound down, possibly because from both a marketing and therapeutic perspective, there is not a sufficient point of difference with the statins. There may also be lingering safety concerns of targeting the distal end of the pathway, with the spectre of triparanol still lurking in the shadows. Here, we have also previewed some of the more promising therapies waiting in the wings. Rather than being directed at the cholesterol biosynthesis pathway, these diverse approaches target the assembly of atherogenic lipoproteins (via apoB or MTTP), the stability of the LDL-receptor (via PCSK9), or are designed to increase HDL-cholesterol (via CETP inhibition). Time will tell if these or other new therapeutics will match or perhaps even surpass the success of the statins.

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