



Targeting Redox Enzymes of Cholesterol Biosynthesis: A Step in the Right Direction Towards Ending Cardiovascular Disease

Taylor M Borah, Alexandria C Guerra and Kevin Francis*

Department of Chemistry, Texas A&M University Kingsville, USA

*Corresponding author: Kevin Francis, Department of Chemistry, Texas A&M University Kingsville, Kingsville, TX, USA, Tel: 361-593-2923; Email: kevin.francis@tamuk.edu

Review Article

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Abstract

Global occurrence of cardiovascular diseases is a serious health concern resulting in over 2 million deaths annually. A major cause of cardiovascular disease is elevated levels of blood plasma cholesterol which leads to the precipitation of lipoproteins and arterial plaque formation. This review details treatment of hypercholesterolemia that target redox enzymes involved in the biosynthesis of the lipid. It begins with a brief discussion of both high- and low-density lipoproteins that are the vehicles for cholesterol transport in humans. An overview of the five stages of cholesterol anabolism in the liver is then provided focusing on the two rate-limiting redox enzymes involved in the pathway. These enzymes are 3-hydroxy-3-methylglutyl CoA reductase (HMGR) and squalene monooxygenase (SMO). The structure and catalytic mechanisms are presented in great detail to lay the foundation for a presentation of common cholesterol lowering drugs. The first of these are the statins that competitively inhibit HMGR and have shown the greatest success in both the treatment and prevention of cardiovascular diseases. An inhibitor of SMO called terbinafine is then discussed. This drug molecule is a fairly effective anti-fungal but is not currently approved in the U.S. to treat high cholesterol. However, it has been shown to lower low-density lipoproteins and exhibits promise to provide the framework for the development of new cholesterol lowering treatments to target SMO. It is hoped that this review article will raise awareness and spur future research into the treatment of hypercholesterolemia to treat and/or prevent cardiovascular disease.

Keywords: Cholesterol Biosynthesis; High and Low Density Lipoproteins; 3-Hydroxy-3-Methylglutyl Coa Reductase; Squalene Monooxygenase; Coenzyme A; Flavin Adenine Dinucleotide; Nucleotide Adenine Diphosphate; Statins; Terbinafine

Abbreviations: CoA: Coenzyme A; Acetyl-CoA: Acetyl CoA; HMG-CoA: 3-Hydroxy-3-Methylglutyl Coenzyme A; HMGR: 3-Hydroxy-3-Methylglutyl Coenzyme A Reductase; NADPH: Reduced Nicotinamide Adenine Dinucleotide Phosphate; FAD: Flavin Adenine Dinucleotide; SMO: Squalene Monooxygenase.

Introduction

Elevated blood cholesterol is a high-risk factor and one of the leading causes for both the development of

cardiovascular disease and the occurrence of strokes. The World Health Organization (WHO) defines "high" as blood plasma concentrations of >5.0 mM (190 mg/dL) of total cholesterol. According to the WHO, a third of global cases of ischemic heart disease is attributed to high cholesterol and this leads to >2.5 million annual deaths [1]. Arteriosclerotic cardiovascular disease remains a problem in countries of all economic levels despite recommendations and goals set by the WHO [2]. In humans, the liver is primarily responsible for generating cholesterol which circulates through the blood stream. Cholesterol production by the liver is intended

to repair damaged cells, break down ingested fat, and create hormones for a variety of signaling responses. The hydrophobic nature of the lipid leads to aggregation of the molecule and the formation of plaques in patients suffering from hypercholesterolemia [3]. This hinders healthy blood

flow from the vessels to the coronary arteries preventing the supply of oxygen to tissues. This causes cell death, and if plaque formation over time causes a complete blockage of blood flow, cardiac arrest occurs.

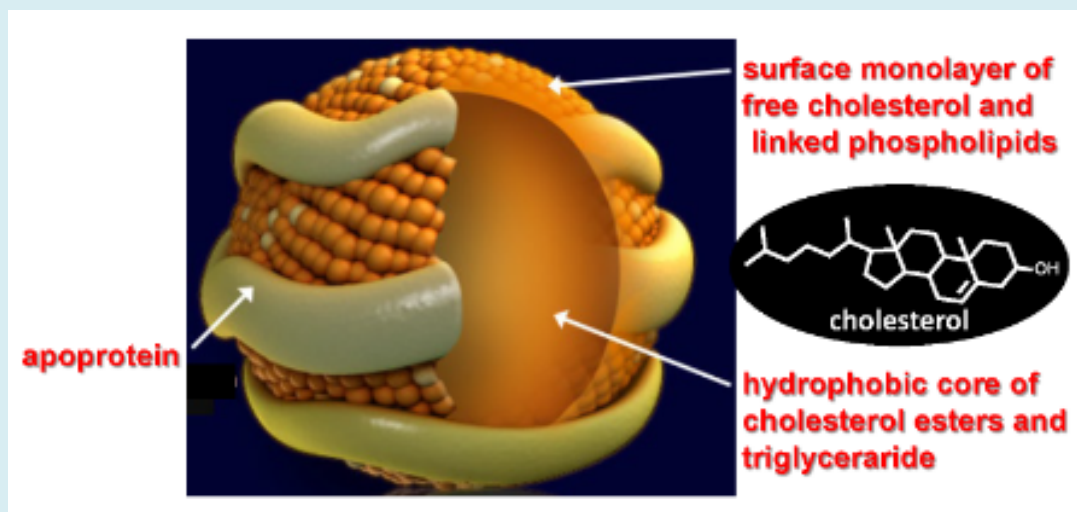


Figure 1: General Lipoprotein Structure. The hydrophilic surface allows the particle to be freely transported throughout the blood stream. Cholesterol attaches to protein through ester linkages that are cleaved by phospholipases when the lipid is needed. This figure was modified from reference [4].

As shown in Figure 1, cholesterol largely contains non-polar covalent bonds between carbon and hydrogen atoms. It therefore has limited solubility in the aqueous environment of blood plasma. Thus, cholesterol does not freely circulate throughout the blood stream, but rather is associated with small spherical proteins called lipoproteins. A detailed account of lipoproteins is beyond the scope of this review, so interested readers are referred to [4] and the references therein. Briefly, cholesterol is transported by the two physically smallest of the seven general classes of lipoproteins. These two classes are the low- and high-density lipoproteins (LDL and HDL, respectively) and have particle sizes of between only 5-30 nm in diameter [4]. HDLs are commonly referred to as “good” cholesterol and LDLs as “bad” though these terms are misnomers because the sterol molecules associated with the proteins are identical in each case.

The general structure of lipoproteins is shown in Figure 1. They resemble micelles and contain a hydrophilic surface to increase blood plasma solubility and a hydrophobic core where the bulk of cholesterol molecules are transported. Cholesterol attaches to lipoproteins mainly as ester linkages to the proteogenic core. LDLs hold the majority of cholesterol transported through humans and most consist of a 512 kDa protein core called Apo B-100 [5]. Accumulation of LDLs in

the blood stream leads to sterol deposits, plaques and the risk of cholesterol molecules breaking off into segments. Water insoluble pieces form clots, which will physically block off access of blood to the arteries. This can result in pulmonary embolism or venial thrombosis [6]. In order to heal and repair damaged cells, LDLs collectively embed within the vessels of the heart. Phospholipase C (E.C. 3.1.4.11) will liberate cholesterol molecules from LDLs when needed by catalyzing ester hydrolysis to cleave the covalent bond linking it to the protein [7].

HDLs are generally larger than LDLs and play an important role in transporting cholesterol away from peripheral tissues and back to the liver for expulsion from the body [4]. HDLs are more tightly packed than LDLs and consist of multiple core apoproteins ranging between 6.6-34 kDa [4]. Tight packing of the apoproteins give particle sizes of between 5-10 nm in diameter [8]. As their name implies, they contain more cholesterol molecules per particle. Since their physiological role is to transport cholesterol out of the body, they are generally beneficial to human health [5,9]. Given the beneficial effects of HDLs, drug treatments specifically designed to increase their level in blood plasma were developed. These include both niacin and fibrate therapies and specific cholesteryl transferase inhibitors such as Anaceptrib® [10]. However, clinical trials were halted

since they were shown to have little if any effect on the incidence of heart attacks or cardiovascular disease [11,12].

Instead, strategies aimed at inhibiting cholesterol biosynthesis have proven to be quite effective in both treating and preventing cardiovascular diseases. This review will focus on two drugs that target redox enzymes involved in cholesterol production in the liver. We start with an overview of the anabolic pathway of cholesterol biosynthesis in humans. The role of statins such as Lipitor® and Crestor® in lowering LDL levels will then be presented through a detailed presentation of the structure and inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). This enzyme is involved in the mevalonate pathway

that contributes to cholesterol production in hepatocytic cells. We then move onto a similar discussion of the drug terbinafine (brand name Lamisil®). This drug was developed to treat fungal infections, but has been demonstrated to also lower cholesterol in humans. The drug targets squalene monooxygenase (SMO) which is another redox enzyme involved in cholesterol biosynthesis. Detailed descriptions of the structure and inhibition of SMO by the drug will be outlined. Before we start our discussion, structures of the coenzymes used by HMGR and SMO are presented in Figure 2 since they may not be familiar to all readers. We hope this review is informative and may stimulate further research in the treatment and prevention of cardiovascular diseases.

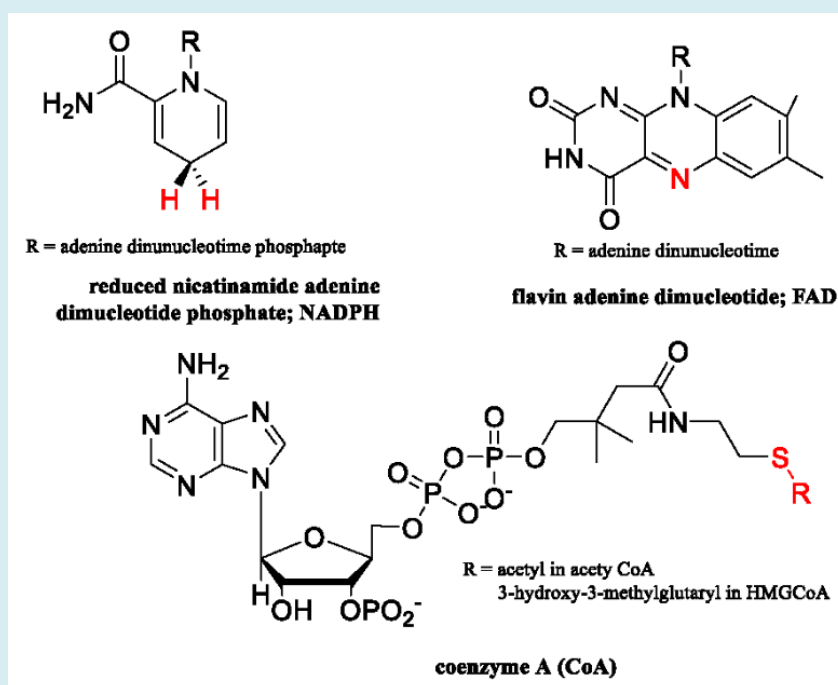
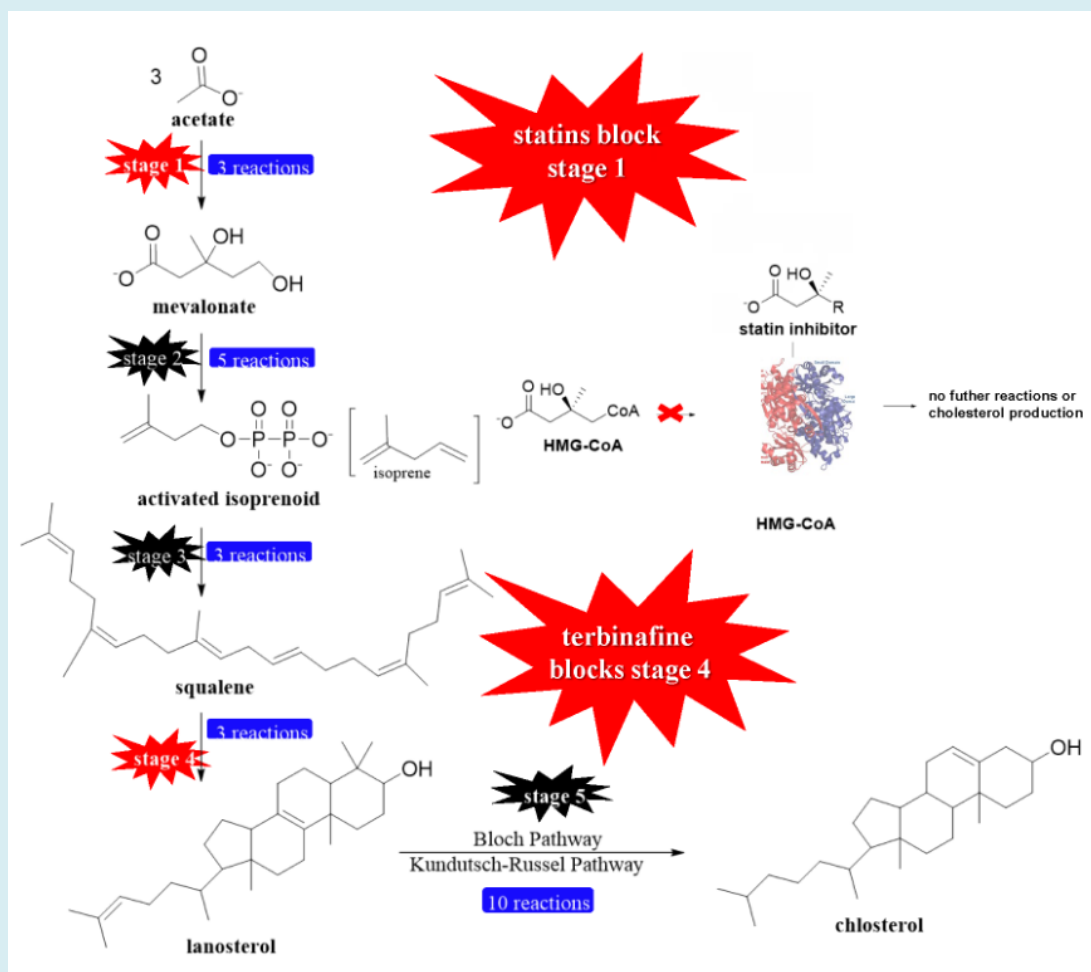


Figure 2: Structures Coenzymes Mentioned in Study Reactive atoms are highlighted in red. Please note that the stereochemistry of hydride transfer in the reductive half reactions of NADPH is enzyme dependent and only one hydride ion is transferred to reduce substrates.

Cholesterol Biosynthesis in Liver and Intestine

The liver is primarily responsible for maintaining homeostatic blood plasma cholesterol levels [13]. Roughly 75-80% of cholesterol in humans is synthesized using acetyl-CoA precursors while the other 20-25% is derived from dietary consumption of animal products. New cholesterol molecules are produced in hepatic cells for transport by LDLs. These lipid molecules are transferred back to the liver by HDLs where they are converted to bile acids and excreted

by the digestive system [14]. Dietary cholesterol is absorbed by the small intestine, incorporated into lipoprotein particles called chylomicrons and transported to the liver for incorporation into LDLs [15]. Given the crucial physiological role of cholesterol, nearly all cell types can synthesize the lipid. However, the predominant pathway of cholesterol biosynthesis takes place in hepatic cells starting from acetyl-CoA units produced during catabolism of fats, proteins and carbohydrates.



Scheme 1: Overview of Cholesterol Biosynthesis Cholesterol is produced from acetate in five stages. Statins target stage one whereas terbinafine targets stage four. Stereochemical configurations are omitted from the structures for simplicity. The inset shows the structure HMGR which statins competitively inhibits to block cholesterol production.

Scheme 1 shows a simplified illustration of this pathway that highlights the stages targeted by cholesterol lowering drugs. Elucidation of the metabolic pathway for cholesterol biosynthesis was pioneered by Bloch [16], Kundutsch and Russel [17] and others. Early experiments were conducted by Konrad Bloch who gained considerable experience in isotopic labeling experiments in the late 1930s and early 1940s as a graduate student at Columbia University. The technology was new at the time and his early projects involved the platinum catalyzed deuteration of alkenes to synthesize 2H-labeled biomolecules as metabolic tracers. These isotopically labeled molecules effectively mapped metabolic processes under different physiological conditions [18]. Bloch later moved to the University of Chicago and set out to determine where all 27 carbon atoms of cholesterol and other sterols are derived. His work on this topic spanned a couple of decades and led to not only a detailed understanding of cholesterol anabolism, but the identity of enzyme targets for treatment

of cardiovascular diseases.

Early experiments started with ^{13}C -labeled acetate fed to pyruvate-deficient mutants of *Neurospora crassa*. This led to the isolation of labeled ergosterol [19] and the hypothesis of a pathway involving acetate being converted into a cyclized intermediate before transforming into cholesterol. In the 1950s, Bloch and fellow researcher, R. G. Langdon, demonstrated that acetate is converted into squalene through a total of 11 reactions [20]. Through additional ^{13}C labeling studies, Bloch was then able to pinpoint the origin of the hydroxyl groups found in sterols and to establish it is critical to convert squalene to cholesterol [19]. In 1960, Kundutsch and Russel also used isotopic labeling studies to discover an alternate pathway that converts squalene to cholesterol. Their pathway differs in only a few small details [17]. The Bloch and Kundutsch-Russel pathways do not contradict each other but rather reflect the evolution of cellular flexibility to adapt to

changing metabolic demands under different physiological conditions [13].

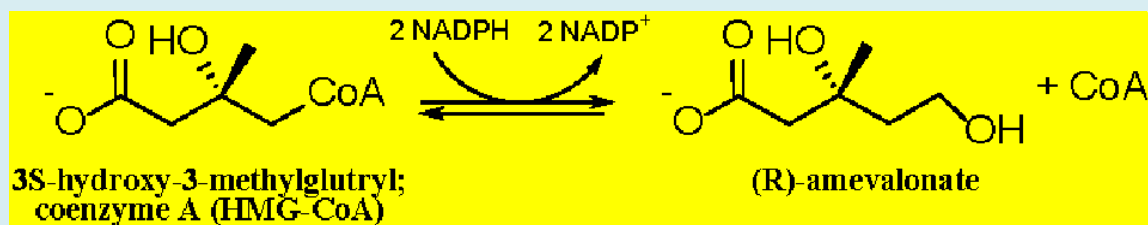
Cholesterol production can be divided into five general stages and involves 24 enzymatic reactions. The first stage is referred to as the mevalonate or isoprenoid pathway and, in the context of cholesterol anabolism, involves three reactions (mevalonate is also a precursor for many vitamins, coenzymes and steroids) [21]. All carbon atoms fed into stage one are derived from acetate and carried into the cell by acetyl-CoA. Importantly, the last reaction of stage one involving the reduction of 3-hydroxy-3-methylglutyl coenzyme A (HMG-CoA) and is effectively targeted by statins to reduce human LDL levels as discussed below. Stage two involves five reactions and converts mevalonate to activate isoprenoids [21]. The next stage links these isoprenoids to generate an unsaturated hydrocarbon of 30 carbon atoms known as squalene [20,22]. In stage four, squalene undergoes three reactions to form a fused-cyclic molecule called lanosterol. The first of these reactions is also targeted by another drug, terbinafine [23,24]. Lanosterol is converted to cholesterol in stage five by either the Bloch or Kundutsch-Russel pathway. Both of these occur in ten enzyme catalyzed reactions [16,17,19].

Targeting enzymes involved in the metabolic pathway illustrated in Scheme 1 coupled with diet and exercise effectively lowers the risk of both cardiovascular diseases and stroke [25]. Blocking the metabolic production of cholesterol significantly lowers blood plasma LDL levels since it limits the number of molecules to load onto proteins. This stimulates the liver to produce both phospholipases and bile acids that degrade LDLs for expulsion from the body through the digestive tract [7,10]. It also leads to increased levels of

LDL receptors on hepatic cells that take up LDLs and reduce their circulation in the blood stratum [26]. The sections below discuss the targeting of two redox enzymes involved in cholesterol anabolism; namely HMG-CoA reductase (HMGR; E.C. 1.1.1.34) and squalene monooxygenase (SMO; E.C. 14.13.132). Before starting this discussion, we shall outline the current knowledge of the structural and mechanistic properties of these enzymes.

Structure and Mechanism of HMGR and SMO

Enzyme Target of Statins: HMGR catalyzes the rate controlling reaction of the mevalonate pathway as shown in Scheme 2. This reaction involves the stereoselective reduction of the thioester bond of HMG-CoA by NAD (P) H to generate (R)-mevalonate.1 Owing to the critical role of (R)-mevalonate in not only cholesterol production but a plethora of other metabolites, HMGR has been extensively studied for almost a century. The enzyme from the eubacteria *Pseudomonas mevalonii* has been most thoroughly characterized since it is cytosolic and therefore, easy to work with. It was found to be overexpressed simply by growing the bacterium in media containing mevalonate as the sole carbon source [27] and is also readily purified to high levels [28]. The first crystal structure of HMGR was solved in 1995 [29]. Many others have been solved since from a variety of sources [21,28,30,31]. Eukaryotic HMGRs are membrane bound so contain both a transmembrane domain and a catalytic domain. They only share 14-20% overall sequence similarity with prokaryotic HMGRs, but have several conserved active site residues. They are also proposed to utilize the same general catalytic mechanism based on numerous kinetic, structural and computational studies [28,31].



Scheme 2: Reaction Catalyzed by 3-Hydroxy-3-methylglutaryl CoA Reductase (HMGR). Structures of NADPH and CoA are shown in Figure 1.

Human HMGR is a tetramer of two obligate dimers that form quite similarly to the dimeric structure of *P. mevalonii* enzyme [28]. Figure 3 shows the general structure of the HMGR dimer as well as the conserved residues in of the active site of the enzyme. A so-called, “flap domain” runs along one side of the active site to position a catalytic lysine residue in proximity of HMG-CoA (Lys 267 in Figure 3) [32]. Closing of the flap positions other catalytic residues as illustrated in

Figure 3. The carboxylate of Asp767 in the human enzyme is 3.6-3.7 Å away from Glu767 leading Istavan et al to propose that it raises the pKa value of the side chain to such an extent that it can serve as a general acid for the reaction [31]. The equivalent residues in the bacterial enzyme are Asp283 and Glu83. The proposed role of Glu was later confirmed by quantum mechanical/molecular mechanical models of both the bacterial and human HMGR reactions [33]. A final

conserved residue that is present in all HMGRs is a histidine (His381 in Figure 3) that acts as a general acid for the

cleavage of the thioester bond linking the glutaryl moiety to CoA [28,29,31].

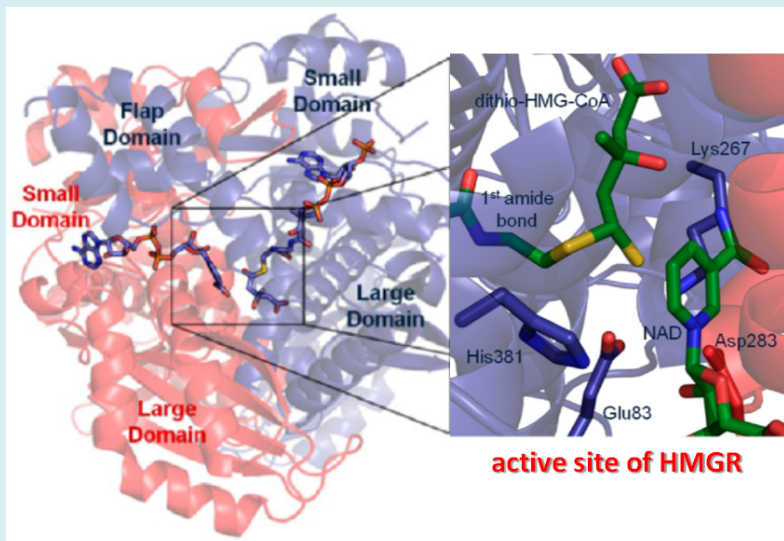
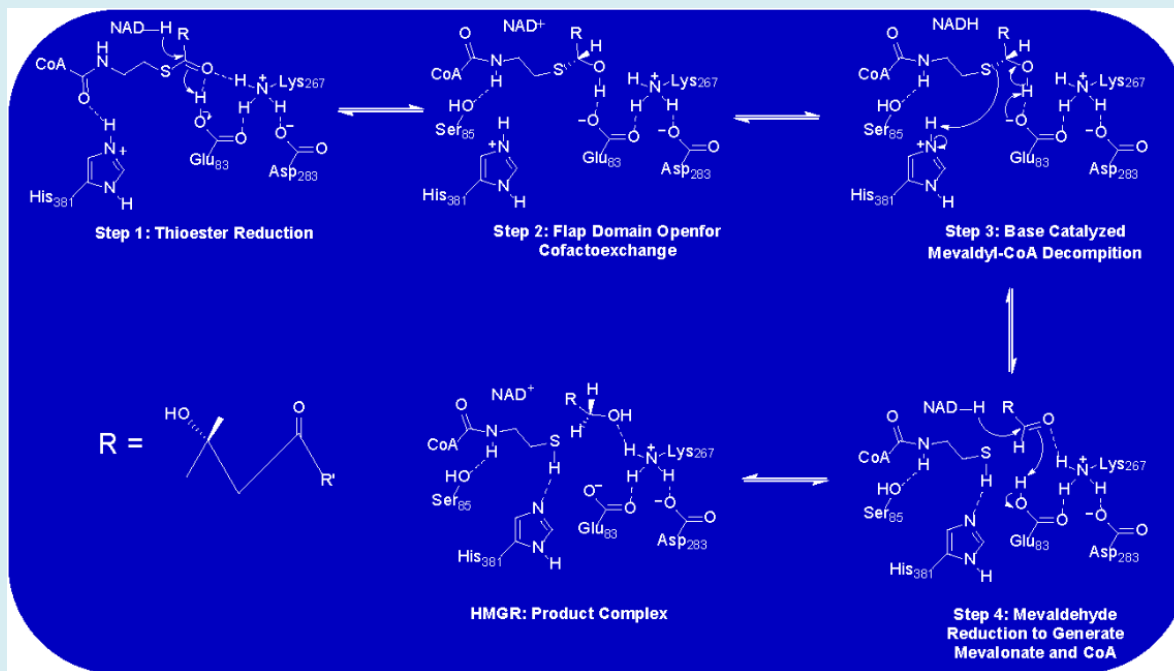


Figure 3: Structure of HMGR dimer. The structure shown is for the enzyme from *P. mevalonii* bound to a tetrahedral dithiohemiacetal intermediate (PDB ID: 1QAX) and shows the active site residues which are conserved in all HMGRs. The figure was modified from [28].



Scheme 3: Catalytic Mechanism of HMGR. The mechanism is proposed based on decades of mechanistic studies [28]. See main text for a description of the chemical details of the reaction.

Elucidation of the complete catalytic mechanism for the HMGR reaction took several decades and was the culmination of many kinetic, structural, computational and molecular biology experiments [28]. These studies spanned over the

course of more than 70 years after Bloch's work revealed that the HMGR reaction controlled the rate of human cholesterol biosynthesis. The generally accepted mechanism proposed for the HMGR is presented in Scheme 3. Residue numbers

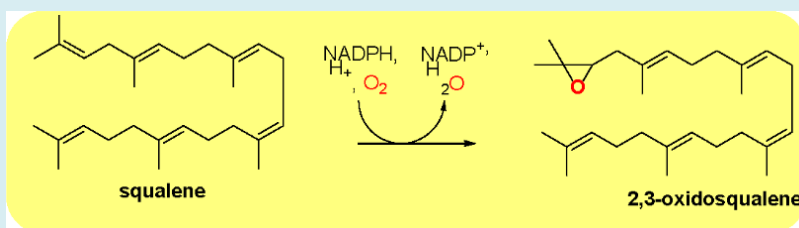
corresponding to the most extensively studied *P. mevalonii* enzyme. All available evidence suggests eukaryotic HMGRs, including that from humans, utilize an identical catalytic mechanism [28,33].

The overall reaction involves a four-electron reduction of HMG-CoA through two separate hydride transfer reactions between the substrate and NAD (P) H. Flap closing in the ternary complex of HMGR initiates catalysis. A hydride ion is first transferred from NAD (P) H to HMG-CoA to reduce the thioester bond to an alcohol (Step 1 in Scheme 3). Reduction occurs with a concomitant proton transfer from Glu83 to the carbonyl oxygen of the thioester of CoA. In the next step of the reaction, the flap domain of HMGR opens to allow for NAD(P)⁺ to leave the active site and a new reduced cofactor to enter for the next hydride transfer step to occur (Scheme 3, Step 2). Evidence for this step came from both kinetic and structural studies of HMGR with dithio-HMG-CoA as substrate. This analogue was predicted to be a competitive inhibitor for the enzyme but was instead found to be a slow substrate [34]. Structures of the enzyme in complex with dithio-HMG-CoA revealed the conformational changes that occur during cofactor exchange. This involves the formation of a hydrogen bond between a conserved serine (Ser85 in the *P. mevalonii* enzyme) and the amido hydrogen of CoA. The active site glutamate (Step 3 in Scheme 3) then acts as a general base to abstract the hydroxyl proton of mevaldyl-CoA. This occurs with a concerted sulfide protonation by an active site histidine residue (His381 in Scheme 3). This

liberates CoA and leads to a second NAD (P) H dependent reduction to produce the carboxylic acid of mevalonate (Step 4 in Scheme 3).

Enzyme Target of Terfenadine: SMO

SMO catalyzes the stereoselective oxidation of squalene to 2,3(S)-oxidosqualene in the first reaction of Stage 4 for cholesterol biosynthesis (as outlined in Scheme 1) [3,20,24]. This reaction is the second slowest step in the metabolic pathway for cholesterol biosynthesis, and like HMGR, has drawn considerable attention by both biochemists and structural biologists alike. As shown in Scheme 4, SMO catalyzes the oxidation of squalene through the incorporation of a single oxygen atom from molecular oxygen to the substrate with the production of water as a byproduct. Since the enzyme produces an epoxide it is also often referred to as squalene epoxidase, but will only be referred to as a monooxygenase in this review article to conform to STREND A nomenclature rules. SMO is a flavin adenine dinucleotide (FAD) dependent enzyme that utilizes NADPH as coenzyme [35]. It is classified as a Group E flavin monooxygenase based on sequence and structural homology to other enzymes in this superfamily [24,36,37]. While it has yet to be rigorously experimentally validated, it is reasonable to assume that SMO utilizes a similar catalytic mechanism as the paradigm Group E flavin monooxygenase, styrene monooxygenase (E.C. 1.14.14.11), as discussed below.



Scheme 4: Reaction Catalyzed by Squalene Monooxygenase (SMO). The oxygen atom incorporated into the product is highlighted in red.

In 2019 Padyana, et al. solved the structures of human SMO in either the absence or presence of the small molecule inhibitors NB-598 and Cmpd-4" [24]. Free enzyme was solved to a resolution of 3.0 Å, ligand bound structures were resolved to 2.3-2.5 Å and comparison of the two provided many insights into inhibition by terbinafine (see TERBINIFINE INHIBITS OF SMO ALSO LOEWERS LDL LEVELS IN HUMAN AND FUNGI below). The overall structure of the enzyme (PDB ID: 6C6P) in complex with NB-190 is shown in Figure 3 and is presented here to focus on its classification as a Group E flavin monooxygenase (interested readers should consult Padyana, et al. [24] for the structure of NB-190 which is omitted here for simplicity). Proper classification

of SMO has important mechanistic implications that have been difficult to experimentally verify due to the laborious techniques required to kinetically characterize the enzyme [24]. Early computational models of SMO were developed using p-hydroxybenzoate hydroxylase (E.C. 1.14.13.2), a Group A FMO, as the starting point [23]. This was based on sequence analysis showing that SMO has conserved residues for a Rosman fold to bind FAD and a similar tertiary structure as p-hydroxybenzoate hydroxylase. Advances in the field of flavin monooxygenase enzymology since 2010 [36,37], along with the long-held demonstration that the enzyme requires an external electron donor for activity [38,39] argues against SMO being Group A enzyme. Instead, mounting evidence

suggests it is a Group E enzyme [36,37]. SMOs contain many conserved aromatic amino acids residues that are essential

to catalysis [40] and several were found in the active site of the enzyme [24].

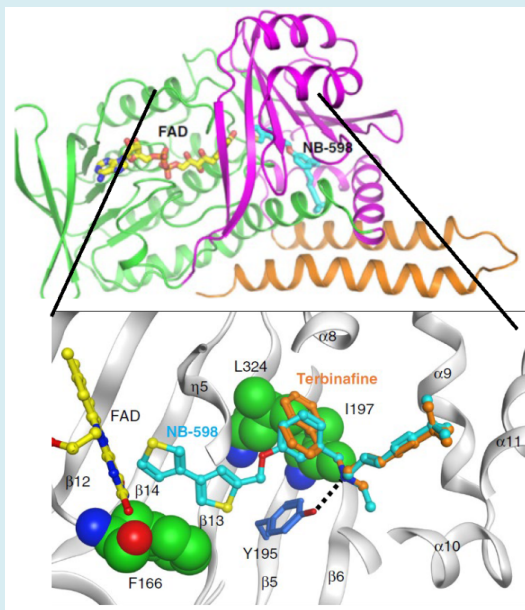


Figure 4: Structure of Human SMO and Computational Model of SMO-Terbinafine Complex. The structure of SMO was solved in complex with the small molecule inhibitor NB-598. This structure was used to create a model of the enzyme with the anti-fungal drug terbinafine. The figure was modified from Padyana, et al. [24].

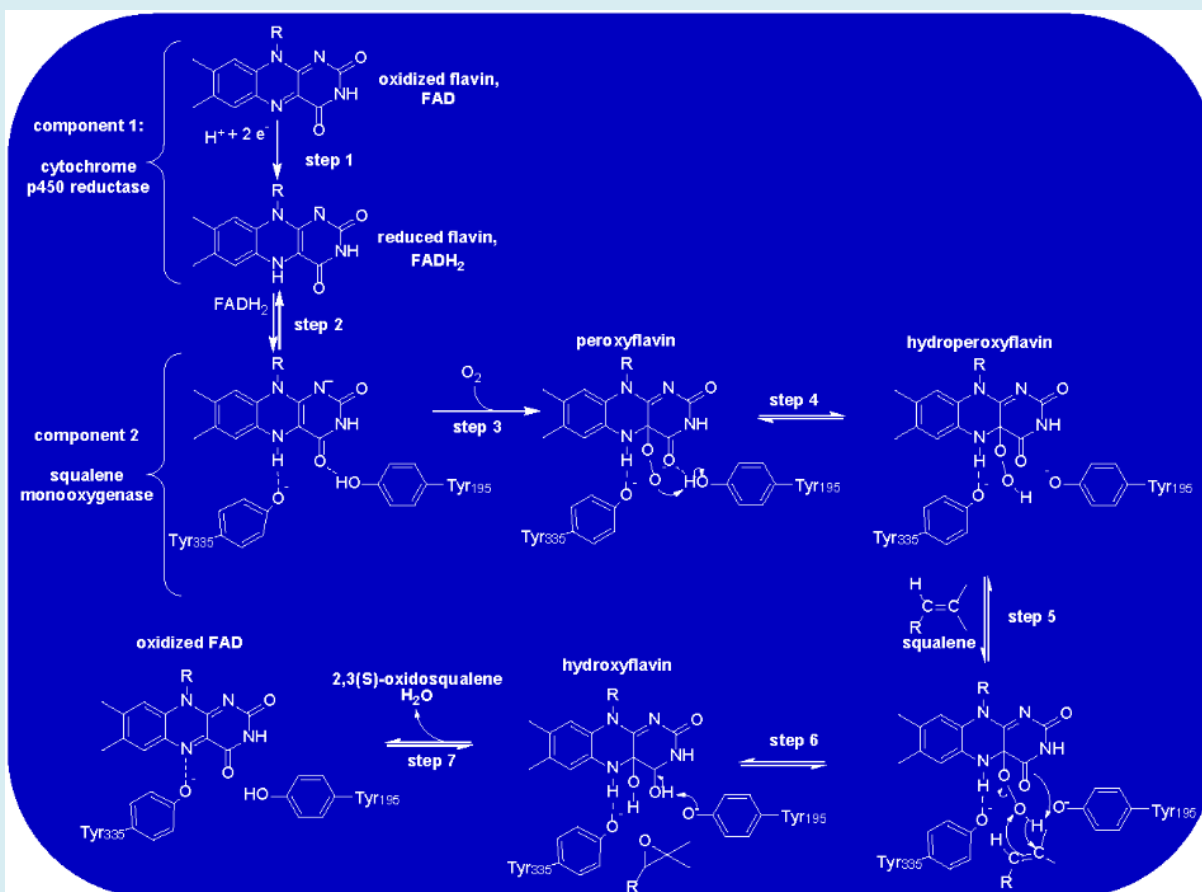
Unlike Group A enzymes, the flavin cofactor of SMO does not remain bound throughout catalytic turnover, but instead is provided by an external reductase [36,37]. Molecular biology experiments have demonstrated that the electrons used to reduce the FAD coenzyme of SMO are provided by a NAD (P) H dependent cytochrome P450 reductase [38,39] and these experiments were confirmed in biochemical studies of the enzyme [24]. Reduced FAD is transferred to SMO for the epoxidation reaction. The requirement of an external reductase coupled with the formation of an epoxide from an alkene strongly supports the classification of SMO as a Group E flavin monooxygenase. Styrene monooxygenase (E.C. 1.14.14.11) [41,42] and zeaxanthin epoxidase (E.C. 1.14.13.90) [43] are the only two other members of Group E. They catalyze the same type of reaction using NAD (P) H as reductant.

Sophisticated liquid choreography/mass spectrometric based assays were developed in [24] for kinetic assays of recombinant SMO overexpressed in baculosomes. This laborious methodology has prevented detailed mechanistic validation of SMO, but given its similarities to other Group E flavin monooxygenases, it is reasonable to assume it utilizes the catalytic mechanism. Scheme 5 proposes such a mechanism for the first time based on extensive rapid kinetic data collected for styrene monooxygenase which rigorously characterized the redox states of FAD during

substrate oxidation [42]. SMO catalysis requires FAD reduction by cytochrome P450 reductase (step 1 in Scheme 5) as shown in [39]. Once reduced FAD is transferred to SMO (Scheme 5; step 2) squalene epoxidation can commence. The crystal structure of human SMO shows that the N(5) position of the isoalloxazine ring of FADH₂ is stabilized through hydrogen bonding with Tyr335. Step 3 would involve atmospheric oxygen reacting with the SMO: FADH₂ binary complex to produce the peroxyflavin form of the coenzyme. Stabilization of a peroxyflavin was clearly observed for styrene monooxygenase through rapid mixing of the reduced enzyme with oxygen. Time resolved flavin spectra showed the characteristic peak of peroxyflavins centered at ~380 nm from 8-100 ms after mixing [42]. Such an intermediate in SMO would be stabilized by the active site residue Tyr195 (Scheme 5; step 3). The same residue would act as a general acid to protonate the peroxyflavin to form the hydroperoxyflavin form of FAD (Scheme 5; step 4). In step 5, squalene would bind to the enzyme for rapid epoxidation (that was too fast to detect with styrene monooxygenase [42]). The nucleophile required to initiate the epoxidation would be provided by the p-electrons of the C4 carbonyl bond of the isoalloxazine ring of the flavin. A concerted reaction following a well-established mechanism [44] between the 2,3 alkene of squalene and the hydroperoxyl group of FAD would then take place as shown in Step 6 of Scheme 5. The isoalloxazine C4 carbonyl would be regenerated through

a Tyr195 catalyzed proton abstraction. This would take place with the concomitant expulsion of water from the hydroperoxyflavin a well-documented process with flavin

monooxygenases [36,37].



Scheme 5: Proposed Catalytic Mechanism of SMO. The mechanism is proposed for the first time in this study based on both extensive mechanistic data collected for styrene monooxygenase [42] and the structural studies of human SMO [24]. Further studies are required to validate this proposal.

Statins Lower LDL levels through Inhibition of HMGCR to Prevent Cholesterol Synthesis

Konrad Bloch and Feodor Lynch were awarded the 1964 Nobel Prize in Physiology or Medicine for their work on cholesterol biosynthesis [18]. Their work ushered in new areas of study for the generation of safe and effective cholesterol lowering drugs to treat cardiovascular diseases [45,46]. It was quickly realized that HMGCR should be a prime target since it catalyzes the committed step of cholesterol biosynthesis and therefore is naturally regulated in human metabolism to maintain cholesterol homeostasis [15]. The earliest statin ever developed to treat hypercholesterolemia was discovered by the Japanese pharmaceutical company

Sankyo (now Dalichi Sankyo Limited) in 1977 [47]. This statin was isolated from the blue-green mold *Penicillium citrinum* and has come to be known as compactin (structure shown in Figure 5). While animal studies in chickens, dogs and monkeys all showed promising cholesterol lowering activity [48], Sankyo discontinued its development in 1980 after the discovery of lymphomas in dogs treated with the drug [49]. Around the same time, Merck, inspired by the compactin studies, isolated another statin from the soil fungus *Aspergillus terreus* [50]. The same statin was also identified from the purple mold *Monascus ruber* and was found to be quite effective in the treatment of hypercholesterolemia without the carcinogenic or other toxic effects observed with compactin [51].

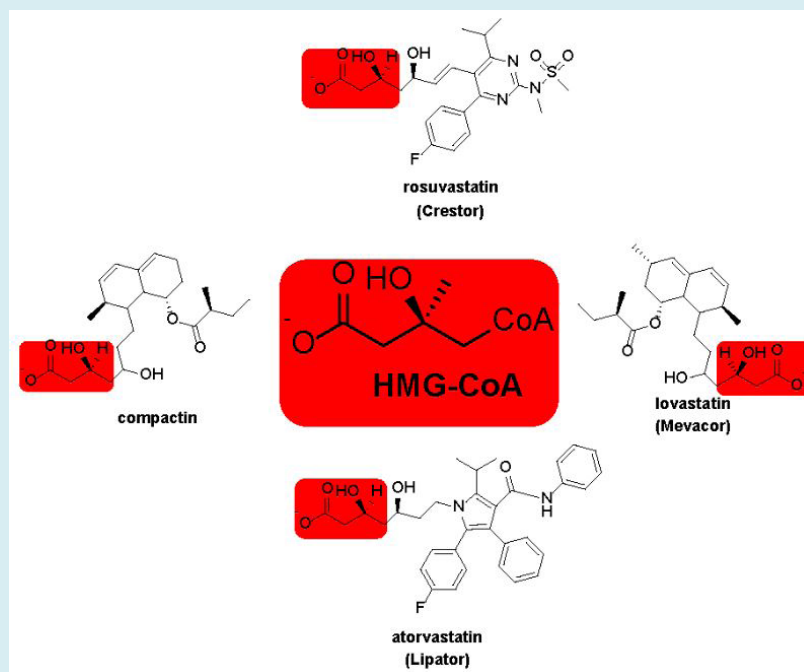


Figure 5: Structure of Some Common Statins. Each is shown in the β -hydroxy form that exists under physiological conditions. Red boxes are to highlight the structural similarities between statins and HMG-CoA, the substrate for HMGR. These similarities give rise to the potent competitive inhibition by statins (K_i in the nM range) to lower cholesterol biosynthesis.

The statin isolated by Merck has come to be known as lovastatin and was carried through clinical trials in the 1980s. Structural similarities between compactin, lovastatin and HMG-CoA (Figure 5) makes it no surprise that statins are potent competitive inhibitors of HMGR [47,50]. As with the HMG-CoA, the carboxylate oxygen of lovastatin (highlighted in red boxes) forms a lactone with the γ -carbon of the drug, but this readily undergoes hydrolysis *in vivo* to give the β -hydroxy acids shown in in Figure 5. Clinical trials were a success and lovastatin got approval from the U.S. Food and Drug Administration in 1987 to be sold under the brand name Mevacor® [49]. Since that time, several statins have been developed and approved for the treatment of high LDL levels. Figure 5 shows a few representative examples and provides both their generic and brand names. Statins have a remarkable potency and competitively inhibit HMGR both *in vitro* and *in vivo* with K_D or IC_{50} values in the low nM range [31]. Crystal structures of HMGR: lovastatin complex show that the drug makes four different active site contacts with the enzyme through hydrophobic and hydrogen bonding interactions with the same amino acids used to bind the HMG-Co A substrate [52]. Inhibiting HMGR activity dramatically decreases mevalonate production (Scheme 1)

and therefore LDL cholesterol levels. This reduction has been demonstrated to reduce the risk of major cardiovascular events by about 25% after each year of statin therapy [53].

Terbinafine Inhibition of SMO also Lowers LDL levels in Humans and Fungi

As with any drug, statin therapy is sometimes met with intolerable side effects which can lead to a discontinuation of treatment. The most common side effect of statins is muscle pain or weakness, and this often leads to patients not remaining compliant with therapy [54]. In such cases, alternate therapies are desirable and the targeting of SMO by terbinafine could provide such a strategy. Currently, terbinafine is only approved to treat fungal infections typically by a topical ointment, though oral therapies are also available (i.e. Terbinex®). A correlation between terbinafine treatment and lower cholesterol levels have been made in some studies [55-57]. We end with a look into the inhibition of SMO by terbinafine since it could lead to more effective ways of treating high levels of LDL in patients suffering from hypercholesterolemia for which statin therapy was unsuccessful.

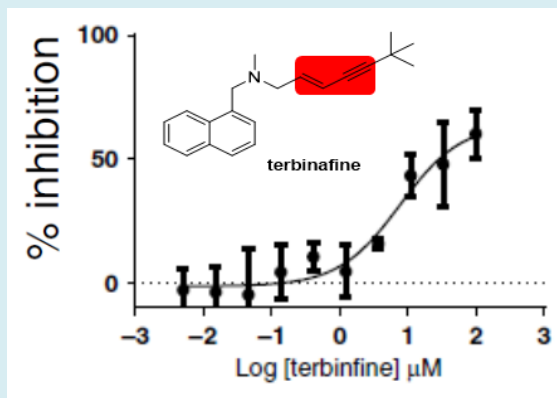


Figure 6: Inhibition of SMO by Terbinafine. The data was taken from Padyana AK, et al. [24] which showed that terbinafine inhibits human SMO with a IC_{50} value of ~ 7 mM. The structure of terbinafine is shown with a box of the enzyme moiety that was shown to be critical for binding for this and other SMO inhibitors.

The inhibition of both human and fungal SMO has been well characterized kinetically and structurally [23,24,38,56-58]. Owing to the inherent difficulties in measuring the kinetics of SMO, the inhibition of the enzyme by terbinafine was not straightforward to characterize. Nevertheless, inhibition is now generally well understood. Fungal SMO is clearly more susceptible to terbinafine inhibition showing KD values in the nM range as compared to the sub-M values for the human enzyme (Figure 6). Comparison of the chemical structures of terbinafine and squalene leads to a reasonable hypothesis that it should competitively inhibit the enzyme. However, kinetic patterns of terbinafine inhibition clearly show that it is a tight binding non-competitive inhibitor [24,38]. Both computational modeling of terbinafine inhibition of SMO [23] and high-resolution crystal structure of the human enzyme in complex with the structural analogs NB-190 and Cmp-4" show many interactions between the drug and active site residues as expected. Non-competitive inhibition patterns could simply be the result of the limited solubility of terbinafine in aqueous solution that prevented substrate displacement.

The inhibition of SMO by terbinafine has similar effects on cholesterol production as does statin inhibition of HMGR. By blocking a rate-controlling step of cholesterol production through enzyme inhibition, cells have nothing to load onto LDLs. This triggers the destruction of LDLs in the liver and has positive clinical outcomes. Inhibition of SMO in fungi prevents cell wall formation and thus survival of the microbe. Weaker terbinafine inhibition of human SMO currently limits its use as a cardioprotective agent. However, the biochemical and structures studies of Padyana, et al. [24] provide a framework on how human SMO inhibitors can be developed to provide new drugs to treat cardiovascular diseases.

Conclusion

Before the work of Konrad Bloch and many other Biochemists in the first half of the 20th century, it was a common belief that cardiovascular events such as heart attacks or strokes were just a normal part of the aging process. It was generally known that high levels of cholesterol in the blood led to plaque formation, but it seemed this could not be avoided. Once the details of cholesterol metabolism were understood, it became apparent that the medical community was not powerless to the aging of the cardiovascular system. Instead, there were twenty enzyme targets that could be hit to lower cholesterol production in the liver. The two most obvious enzymes to target were HMGR and SMO since they catalyze the two slowest steps in cholesterol anabolism. Through close to a century of experiments, effective inhibitors have been developed to lower LDL cholesterol and the incidence of both heart attacks and strokes.

While many successes have been made, there is still much work left to achieve the ambitious goal set by the WHO to reduce global cases of atherosclerotic cardiovascular diseases by 30% by 2030 [1]. This review detailed not only the overall pathway for cholesterol production but the structure and function of HMGR and SMO. We hope it led to an understanding of statin and terbinafine treatments. Great progress has been made in statin therapies, but additional work to improve SMO inhibition could provide alternative and perhaps more effective treatments of hypercholesterolemia. Such treatments would greatly improve the quality of life for millions of patients around the world.

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Footnotes

¹Prokaryotic HMGRs utilize NADH as reducing substrate while eukaryotes use NADPH. As is the case with many nicotinamide dependent enzyme cofactor specify does not alter the catalytic mechanism of the two classes of enzyme. The cofactor will be written as NAD (P) H as is customary in the field.

References

1. Ray KK, Ference BA, Tania S, Blom D, Nichollis S, et al. (2022) World Health Organization Global Action Plan for Primary Prevention of Cardiovascular Disease 2025. *Glob Heart* 17(1): 75.
2. Watkins DA, Msemburi WT, Pickersgill SJ, Kawakatsu Y, Gheorge A, et al. (2022) NCD Countdown 2030: efficient pathways and strategic investments to accelerate progress towards the Sustainable Development Goal target 3.4 in low-income and middle-income countries. *Lancet* 399(10331): 1266-1278.
3. Cory EJ, Russey WE, Montellano PRO (1966) 2,3-oxidosqualene, an intermediate in the biological synthesis of sterols from squalene. *J Am Chem Soc* 88(20): 4750-1751.
4. Feingold KR (2000) Introduction to Lipids and Lipoproteins. In: Feingold KR, et al. (Eds.), *Endotext*, South Dartmouth (MA), USA.
5. Smith LC, Pownall HJ, Gotto AM (1978) The plasma lipoproteins: structure and metabolism. *Annu Rev Biochem* 47: 751-757.
6. Hevonoja T, Pentikainen MO, Hyvonen MT, Kovanen PT, Korpela MA (2000) Structure of low density lipoprotein (LDL) particles: Basis for understanding molecular changes in modified LDL. *Biochim Biophys Acta* 1488(3): 189-210.
7. Bill CA, Vines CM (2020) Phospholipase C. *Adv Exp Med Biol* 1131: 215-242.
8. Segrest JP, Garber DW, Brouillette CG, Harvey SC, Anantharamaiah GM (1994) The amphipathic alpha helix: a multifunctional structural motif in plasma apolipoproteins. *Adv Protein Chem* 45: 303-369.
9. Kuai R, Li D, Chen YE, Moon JJ, Schwendeman A (2016) High Density Lipoproteins: Nature's Multifunctional Nanoparticles. *ACS Nano* 10(3): 3015-3041.
10. Kaur N, Pandey A, Negi H, Shafiq N, Reddy S, et al. (2014) Effect of HDL-raising drugs on cardiovascular outcomes: a systematic review and meta-regression. *PLoS One* 9(4): e94585.
11. Keene D, Price C, Shin MJS, Francis DP (2014) Effect on cardiovascular risk of high density lipoprotein targeted drug treatments niacin, fibrates, and CETP inhibitors: meta-analysis of randomised controlled trials including 117 411 patients. *BMJ* 349: g4379.
12. Bowman L, Hopewell J, Wiviott S, Sammons F, Chen F, et al. (2017) Effects of Anacetrapib in Patients with Atherosclerotic Vascular Disease. *N Engl J Med* 377(13): 1217-1227.
13. Singh P, Saxena R, Srinivas G, Pande G, Chattopadhyay A (2013) Cholesterol biosynthesis and homeostasis in regulation of the cell cycle. *PLoS One* 8(3): e58833.
14. Spady D, Woollett L, Dietschy J (1993) Regulation of plasma LDL-cholesterol levels by dietary cholesterol and fatty acids. *Annual Rev Nutr* 13(1): 355-381.
15. Grundy SM (1978) Cholesterol metabolism in man. *West J Med* 128(1): 13-25.
16. Bloch KE (1983) Sterol structure and membrane function. *CRC Crit Rev Biochem* 14(1): 47-92.
17. Kandutsch AA, Russell AE (1960) Preputial gland tumor sterols. 3. A metabolic pathway from lanosterol to cholesterol. *J Biol Chem* 235: 2256-2261.
18. Pendse A, Lockyer P, Schisler J, Willis M (2011) Konrad Bloch, PhD: Understanding of Cholesterol Metabolism. *Laboratory Medicine* 42(6): 370-373.
19. Bloch K (1987) Summing up. *Annu Rev Biochem* 56: 1-19.
20. Langdon RG, Bloch K (1952) The Biosynthesis of Squalene and Cholesterol. *J Am Chem Soc* 74(7): 1869-1870.
21. Miziorko HM (2011) Enzymes of the mevalonate pathway of isoprenoid biosynthesis. *Arch Biochem Biophys* 505(2): 131-43.
22. Kritchevsky D, Moyer AW, Tesar WC, Logan JB, Brown RA, et al. (1954) Squalene Feeding in Experimental Atherosclerosis. *Circulation Research* 2(4): 340-343.
23. Nowosielski M, Hoffmann M, Wyrwicz LS, Stepniak P, Plewczynski DM, et al. (2011) Detailed Mechanism of Squalene Epoxidase Inhibition by Terbinafine. *J Chem Info Model* 51(2): 455-462.
24. Padyana AK, Gross S, Jin L, Cianchetta G, Narayanaswamy R, et al. (2019) Structure and inhibition mechanism of

- the catalytic domain of human squalene epoxidase. *Nature Communications* 10(1): 97.
25. Ornish D, Brown SE, Billings JH, Scherwitz LW, Armstrong WT, et al. (1990) Can lifestyle changes reverse coronary heart disease?: The Lifestyle Heart Trial. *Lancet* 336(8708): 129-133.
 26. Brown MS, Goldstein JL (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science* 232(4746): 34-47.
 27. Gill JF, Beach MJ, Rodwell VW (1984) Transport of mevalonate by *Pseudomonas* sp. strain M. *J Bacteriol* 160(1): 294-298.
 28. Haines BE, Wiest O, Stauffacher CV (2013) The increasingly complex mechanism of HMG-CoA reductase. *Acc Chem Res* 46(11): 2416-2426.
 29. Lawrence CM, Rodwell VW, Stauffacher CV (1995) Crystal structure of *Pseudomonas mevalonii* HMG-CoA reductase at 3.0 angstrom resolution. *Science* 268(5218): 1758-1762.
 30. Vogeli B, Shima S, Erb TJ, Wagner T (2019) Crystal structure of archaeal HMG-CoA reductase: insights into structural changes of the C-terminal helix of the class-I enzyme. *FEBS Lett* 593(5): 543-553.
 31. Istvan ES, Palnitkar M, Buchanan SK, Deisenhofer J (2000) Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. *EMBO J* 19(5): 819-830.
 32. Tabernero L, Bochar DA, Rodwell VW, Stauffacher CV (1999) Substrate-induced closure of the flap domain in the ternary complex structures provides insights into the mechanism of catalysis by 3-hydroxy-3-methylglutaryl-CoA reductase. *Proc Natl Acad Sci U S A* 96(13): 7167-7171.
 33. Haines BE, Steussy CN, Stauffacher CV, Wiest O (2012) Molecular modeling of the reaction pathway and hydride transfer reactions of HMG-CoA reductase. *Biochemistry* 51(40): 7983-7995.
 34. Steussy CN, Critchelow CJ, Schmidt T, Min JK, Wrensford LV, et al. (2013) A novel role for coenzyme A during hydride transfer in 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Biochemistry* 52(31): 5195-5205.
 35. Ono T, Bloch K (1975) Solubilization and partial characterization of rat liver squalene epoxidase. *J Biol Chem* 250(4): 1571-1579.
 36. Berkel WJH, Kamerbeek NM, Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. *J Biotechnol* 124(4): 670-689.
 37. Huijbers MME, Montersino S, Westphal AH, Tischler D, Berkel WJH (2014) Flavin dependent monooxygenases. *Arch Biochem Biophys* 544: 2-17.
 38. Favre B, Ryder NS (1996) Characterization of squalene epoxidase activity from the dermatophyte *Trichophyton rubrum* and its inhibition by terbinafine and other antimycotic agents. *Antimicrob Agents Chemother* 40(2): 443-447.
 39. Sakakibara J, Watanabe R, Kanai Y, Ono T (1995) Molecular Cloning and Expression of Rat Squalene Epoxidase. *Journal of Biological Chemistry* 270(1): 17-20.
 40. Abe I, Abe T, Lou W, Masuoka T, Noguchi H (2007) Site-directed mutagenesis of conserved aromatic residues in rat squalene epoxidase. *Biochem Biophys Res Commun* 352(1): 259-263.
 41. Kantz A, Chin F, Nallamotheu N, Nguyen T, Gassner GT (2005) Mechanism of flavin transfer and oxygen activation by the two-component flavoenzyme styrene monooxygenase. *Arch Biochem Biophys* 442(1): 102-116.
 42. Kantz A, Gassner GT (2011) Nature of the Reaction Intermediates in the Flavin Adenine Dinucleotide-Dependent Epoxidation Mechanism of Styrene Monooxygenase. *Biochemistry* 50(4): 523-532.
 43. Buch K, Stransky H, Hager A (1995) FAD is a further essential cofactor of the NAD(P)H and O₂-dependent zeaxanthin-epoxidase. *FEBS Lett* 376(1): 45-48.
 44. Filippova TV, Blyumberg EA (1982) Mechanism of the Epoxidation of Alkenes by Molecular Oxygen. *Russian Chemical Reviews* 51(6): 582.
 45. Brown MS, Danav SE, Siperstein MD (1974) Properties of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Solubilized from Rat Liver and Hepatoma. *J Biol Chem* 249(20): 6585-6589.
 46. Dietschy JM, Wilson JD (1970) Regulation of cholesterol metabolism. *N Engl J Med* 282(20): 1128-1138.
 47. Endo A, Tsujita Y, Kursoda M, Tanzawa K (1977) Inhibition of Cholesterol Synthesis in vitro and in vivo by ML-236A and ML-236B, Competitive Inhibitors of 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase. *Eur J Biochem* 77(1): 31-36.

48. Yamamoto A, Sudo H, Endo A (1980) Therapeutic effects of ML-236B in primary hypercholesterolemia. *Atherosclerosis* 35(3): 259-266.
49. Endo A (2010) A historical perspective on the discovery of statins. *Proc Jpn Acad Ser B Phys Biol Sci* 86(5): 484-93.
50. Alberts AW, Chen J, Kuron G, Hunt V, Huff J, et al. (1980) Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc Natl Acad Sci U S A* 77(7): 3957-3961.
51. Endo A, Monacolin K (1979) A new hypocholesterolemic agent produced by a *Monascus* species. *J Antibiot (Tokyo)* 32(8): 852-854.
52. Taberero L, Rodwell VW, Stauffacher CV (2003) Crystal structure of a statin bound to a class II hydroxymethylglutaryl-CoA reductase. *J Biol Chem* 278(22): 19933-19938.
53. Collins R, Reith C, Emberson J, Armitage J, Baigent C, et al. (2016) Interpretation of the evidence for the efficacy and safety of statin therapy. *Lancet* 388(10059): 2532-2561.
54. Ruscica M, Ferri N, Banach M, Sirtori CR, Corsini A (2022) Side effects of statins: from pathophysiology and epidemiology to diagnostic and therapeutic implications. *Cardiovasc Res* 118(17): 3288-3304.
55. Alsuwaidan S, Alajlan A, Alkreathy H (2023) The effects of terbinafine on the lipid profile in humans and rabbits. *Medical Science* 27: 1-11.
56. Belter A, Skupinska M, Pietraszuk MG, Grabarkiewicz T, Rychlewski L, et al. (2011) Squalene monooxygenase - a target for hypercholesterolemic therapy. *Biol Chem* 392(12): 1053-1075.
57. Madan B, Virshup DM, Nes WD, Leaver DJ (2022) Unearthing the Janus-face cholesterologenesis pathways in cancer. *Biochem Pharmacol* 196: 114611.
58. Sagatova AA (2021) Strategies to Better Target Fungal Squalene Monooxygenase. *J Fungi (Basel)* 7(1): 49.

