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Review Article

Microbial Metabolites and Enzymes Inhibition in Drug Discovery and Development: A Case Study of the Statins, a Class of HMG-CoA Reductase Inhibitors

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ABSTRACT

The discovery and development of the statins provides an insight into the many facets of the drug discovery process and also shows the time and resources that must be devoted to developing a clinically useful drug from an initial concept. It began from the conceptual search for metabolites that could inhibit an important enzyme in cholesterol biosynthesis, HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase, to the isolation of the first active metabolite, citrinin, from a fungus, leading to further search and development. This discovery is a milestone in medicine and a historical portrait, which illustrates the importance of multidisciplinary approach in drug development. The statins are the results of combined efforts of inter alia, pharmaceutical chemists, microbiologists, physiologists, and pharmacologists. This paper gives an account of the discovery of the statins, highlighting the clinical importance of cholesterol, the discovery of HMG-CoA reductase, and subsequent search for its inhibitors. It showcases the clinical importance and limitations of the statins and seeks to serve as a guide and inspiration for future discovery and development of novel, potent and safer cholesterol-lowering agents.

Keywords: Drug discovery, Statins, Microbial metabolites, HMG-CoA reductase inhibitors, Cholesterol-lowering agents, Akira Endo.

Introduction

The statins are a group of revolutionary drugs used for the treatment of hypercholesterolemia.[1] They are indicated for the prevention or treatment of early detected coronary heart diseases such as stroke and heart attack. They act by inhibiting HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase, which is the rate-controlling enzyme in the biosynthesis of cholesterol. Hypercholesterolemia, high blood cholesterol, is one of the major risk factors of atherosclerosis. Atherosclerosis is implicated in the pathogenesis of cardiovascular diseases (CVDs), which according to the World Health Organization (WHO) are the number one cause of death globally.[2] The advent of the statins is a breakthrough in drug discovery and medicine. Since their introduction; the statins have not only revolutionized the management of coronary heart diseases but also decreased its risks and associated mortality.[1]

Methods

We conducted a review of the available literature on the discovery and development of the statins. We used the search terms statins, drug discovery, microbial metabolites, enzymes inhibition, HMG-CoA reductase inhibitors, cholesterol, cardiovascular diseases, cholesterol-lowering agents, on Google Scholar and PubMed search engines. From the generated results, articles that have a definite relationship with the subject matter were included and otherwise were excluded.

To facilitate a focus review, first, we outline the veritable importance of microbial metabolites and enzymes as targets in drug discovery and development. Next, we discuss cholesterol, with a focus on key research that facilitated our understanding of its role in the pathogenesis of some cardiovascular diseases and its biosynthetic pathway. We transition to give accounts of the search for cholesterol-lowering drugs and the various challenges and scientific exploits that were associated with the discovery and development of the statins. Finally, we outline the chemical properties, clinical importance and limitations of the statins, and we leverage this understanding to suggest the potential for development of novel cholesterol-lowering drugs.

Microbial metabolites and enzymes inhibition in drug discovery and development – a perspective

The discovery and development of the statins is an elegant testimony to the importance of microbial metabolites and enzymes inhibition in drug discovery and development. Microbial secondary metabolites, which include terpenoids, alkaloids, phenylpropanoids, aliphatic compounds, polyketides, and peptides, do not play essential roles in growth and reproduction but usually enhance the survivability of the microorganism by serving as "chemical warfare" agents against prey, predators, and competing organisms.[3,4] Owing to their biochemical properties, many secondary metabolites have been isolated and used as drugs (e.g. penicillin G and V, chloramphenicol, streptomycin, and daunorubicin) and/or served as leads for the development of more therapeutically effective drugs (e.g. rifampicin, amoxicillin, and cefuroxime).[5,6] Enzymes are ubiquitous in nature and are indispensable for the normal functioning of life. Researchers have shown that altering an enzyme activity has defined biochemical effects which could be useful in the management of certain diseases.[7] For this reason, enzymes have been important targets in drug design; 47% of

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all current drugs inhibit enzyme targets.[8] Enzyme-targeted drugs mainly exert their action by inhibition (e.g. methotrexate, celecoxib, lamivudine, saquinavir, and neostigmine), activation (e.g. pralidoxime) or allosteric modification (e.g. delavirdine, and nevirapine) of enzymes.

Methotrexate, an antineoplastic agent, is an antimetabolite; an analogue of folic acid, needed for the de novo synthesis of the nucleoside thymidine, required for DNA synthesis.[9] Methotrexate competitively inhibits dihydrofolate reductase (DHFR), an enzyme that reduces dihydrofolic acid to tetrahydrofolic acid; necessary for the synthesis of thymidine, and thus inhibits DNA synthesis and cellular replication.[9,10] Lamivudine, an antiretroviral drug, is a nucleoside analogue; an analogue of cytidine, required for RNA synthesis. Lamivudine, a prodrug, is activated in vivo by phosphorylation to its nucleotide form and acts as a false substrate of viral reverse transcriptase (which transcribes viral RNA into DNA) leading to termination of DNA chain or production of fraudulent DNA. Delavirdine and nevirapine are non-nucleoside reverse transcriptase inhibitors (NNRTIs) used for the management of HIV. Unlike lamivudine which binds to the orthosteric (active) site of the reverse transcriptase, delavirdine and nevirapine bind to an allosteric site, a site distinct from the active site.[11] They bind to a hydrophobic pocket (known as the NNRTI pocket) which is about 10Å away from the active site, causing conformational changes in the three-dimensional structure of the enzyme, which destabilizes the enzyme and reduces its ability to carry out its statutory functions.[11,12] Pralidoxime, an antidote to organophosphate pesticides and chemicals, reactivates phosphorylated acetylcholinesterase. Organophosphates bind to the esteric site of the active site of acetylcholinesterase enzyme, thereby blocking its activity. Pralidoxime binds to the unblocked site of the active site and then displaces the phosphoryl moiety from the enzyme, thus regenerating the fully functional enzyme.[13]

The concepts of enzyme inhibition, activation, and allosteric modification have been greatly exploited for drug discovery, design, and development. Recent progress in this field is associated with the development of the three-dimensional crystal structure of many enzymes, which facilitated structure-based drug design and computer-aided drug design (CADD).[14] Computer-based drug design approaches allow for evaluating molecules at simulated biological targets and modifying such molecules to improve, albeit theoretically, their pharmacodynamics and pharmacokinetics properties. These approaches have become major tools in rational drug design, and have proved successful in developing novel lead compounds.[7] However, such compounds still have to be synthesised and tested experimentally, to confirm the predicted effects against the enzyme targets, before progressing to complex in vivo models and further development. Drug discovery, design, and development involve various steps and procedures (some of which are known to be very laborious and expensive) and entail a cross-fertilization of inter alia, medicinal chemistry, pharmacology, biochemistry, and physiology. The discovery and development of statins is a fine example of classical drug discovery and development; this account of the statins provides an insight into the many facets of the drug discovery process and also shows the time and resources that must be devoted to developing a clinically useful drug from an initial concept.

Cholesterol and cardiovascular diseases

Cardiovascular diseases (CVDs) are the leading cause of death globally.[2] They are a group of disorders of the heart and blood vessels and include coronary artery diseases (CAD), stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, heart arrhythmia, congenital heart disease, peripheral artery disease, thromboembolic disease and venous thrombosis.[15] The pathogenesis of these diseases varies. Coronary artery diseases (CAD), stroke, and peripheral artery disease involve atherosclerosis—a disease in which the inside of an artery narrows due to the buildup of plaque. One of the causes of atherosclerosis is high blood cholesterol.

Cholesterol was first discovered and isolated by the French physician-chemist François Poulletier from bile and gallstones in about 1769.[16] Poulletier's work was never published and it was in 1815 that another French chemist, Michel Chevreul, rediscovered same compound and named it "cholesterine" (solid bile in Greek: chole for bile and stereos for solid).[17,18] Since this time, cholesterol has been the subject of great studies and seminal research in science. Thirteen Nobel Prizes have been awarded to scientists who devoted major parts of their careers to cholesterol research.[19] Cholesterol composes about 30% of all animal cell membranes and is required to maintain membrane integrity. It facilitates intracellular transport and is the precursor for the synthesis of vitamin D and all steroid hormones, including the adrenal gland hormones cortisol and aldosterone, as well as the sex hormones progesterone, estrogens, and testosterone.[20,21] Unfortunately, cholesterol is a Janus-faced molecule. The chemical property that makes it useful in cell membranes, namely its absolute insolubility in water, also makes it harmful; when cholesterol accumulates in the wrong place, such as within the wall of an artery, it cannot be readily mobilized, and its presence eventually leads to the development of an atherosclerotic plaque.[19] The first hint that cholesterol was related to atherosclerosis goes back to the early decades of the 19th century, when Adolf Windaus (awarded Nobel Prize in Chemistry in 1928) in a research on aetiology of atherosclerosis found that atherosclerotic plaques from aortas of human subjects contained over 20-fold higher concentrations of cholesterol than did normal aortas.[22] In the following decades, understanding of the pathophysiology of atherosclerosis improved. Nikolai Anitschkow, a Russian pathologist, reported that cholesterol produced marked hypercholesterolemia and severe atherosclerosis of the aorta in rabbits, following oral administration.[23]

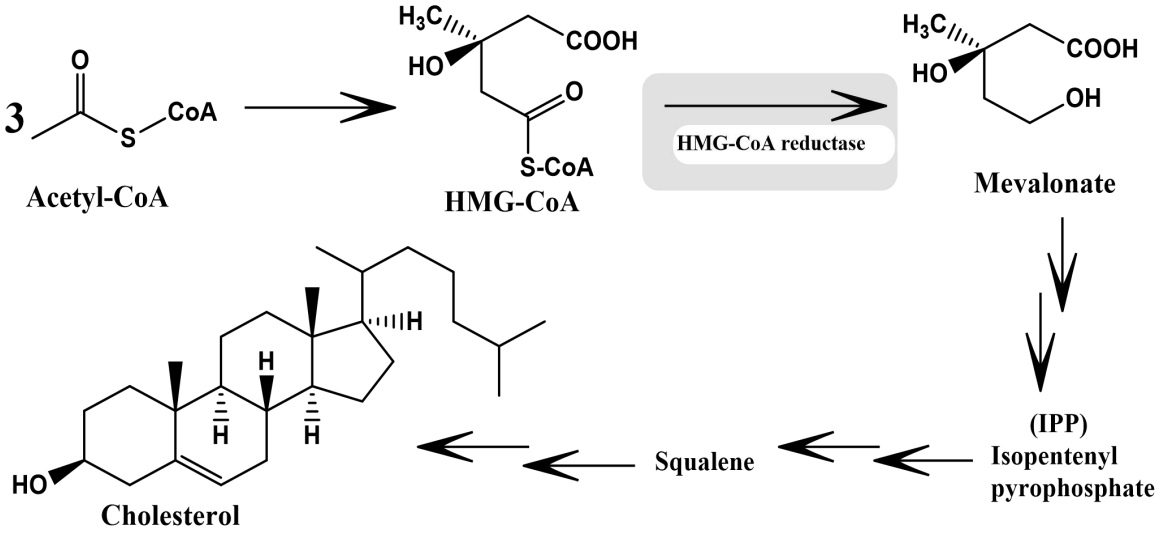

Figure 1: Key stages in the biosynthesis of cholesterol

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These findings were further corroborated by an American scientist, John Gofman "the Father of Clinical Lipidology", who reported that heart attacks were associated with high levels of blood cholesterol, particularly cholesterol contained in low density lipoproteins (LDL).[24] By the 1950s, the correlation between high blood cholesterol levels and coronary heart diseases was established by Ancel Keys' Seven Countries Study at the University of Minnesota and the Framingham Heart Study in Boston University.[25-27]

Cholesterol biosynthetic pathway

The Framingham Heart Study served as a great impetus for researchers to explore ways of lowering blood cholesterol level. Intense efforts were dedicated towards determining the pathway by which cholesterol was synthesized in the body. After a decade of studies, the major outlines of cholesterol biosynthetic pathway were elucidated in 1960 by Konrad Bloch and Feodor Lynen (awarded the Nobel Prize in Physiology or Medicine in 1964).[28,29] Cholesterol biosynthesis involves 30 steps; the five key stages are (1) condensation of three acetate units to form a six carbon intermediate, 3-hydroxy-3-methylglutaryl CoA (HMG-CoA); (2) reduction of HMG-CoA to mevalonate by the enzyme HMG-CoA reductase; (3) conversion of mevalonate to activated isoprene units; (4) polymerization of six 5-carbon isoprene units to form the 30- carbon linear squalene; (5) cyclization of squalene to form the steroid nucleus, with a further series of changes to produce cholesterol (Figure 1).

The reduction of HMG-CoA to mevalonate by the enzyme HMG-CoA reductase was identified as the committed and rate-limiting step in cholesterol biosynthesis.[30,31] Consequently, HMG-CoA reductase became the prime target for the development of cholesterol-lowering drugs.

The search for HMG-CoA reductase inhibitors and discovery of the first statin

Following the determination of cholesterol biosynthetic pathway, scientists in both academia and industry began searching for molecules that would block one of the 30 steps in the synthesis.[32] Many molecules (some homologous to intermediates along the pathway) such as triparanol (MER/29), nicotinic acid, clofibrate and cholestyramine were discovered and evaluated but none of these agents was considered ideal in terms of efficacy and safety.[32-35]

Around this time, in the mid-1960s, Akira Endo, a researcher at Sankyo Research Laboratories, Tokyo became interested in cholesterol research; he wrote to Konrad Bloch in 1965 to enquire about a postdoctoral position in his research team, but the position was not available at the time.[32] Endo, instead spent two years as research associate at the Albert Einstein College of Medicine in New York. During his stay in the USA then, he was surprised at the large number of people living with coronary heart disease and hypercholesterolaemia.[32] In 1968, after returning to Sankyo, Endo began the quest for cholesterol-lowering agents; he hypothesised that, fungi such as moulds and mushrooms would produce secondary metabolites that could inhibit HMG-CoA reductase.[32] He was inspired by Alexander Fleming (penicillin, 1928) and Selman Waksman (streptomycin, 1940), deeply impressed by the versatility of antibiotics, and hoped to find a microorganism that produced an HMG-CoA reductase inhibitor as a defence mechanism against attack by other microbes which relied on sterols as part of their biochemical make up.[5,6,36]

Isolation of citrinin: In April 1971, Endo and co-researchers began to test culture broths of thousands of fungi in search of microbial metabolite HMG-CoA reductase inhibitors.[32] To test his hypothesis, they set up two *in vitro* test systems: (1) inhibition of the incorporation of [^{14}C] acetate into sterols; and (2) inhibition of sterol synthesis from [^{3}H] mevalonate. Fungal broths that were active in the first test but not active in the second were suspected to contain metabolite(s) that would inhibit the conversion of HMG-CoA to mevalonate by HMG-CoA reductase.[37]

A year into this study, after testing 3800 strains of fungi; they found a mould, *Pythium ultimum*, that showed potent inhibitory activity. The isolated active principle, citrinin (first isolated from *Penicillium citrinum*) was a known compound. Citrinin (Figure 2) showed potent HMG-CoA reductase inhibitory and cholesterol-lowering effect in rats.[38,39] However, further research into its therapeutic use was suspended because of its unacceptable toxic profile, which includes hepatotoxicity and nephrotoxicity.[32]

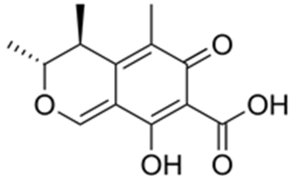

Figure 2: Citrinin

***Discovery of compactin (the first statin)*:** Despite the therapeutic shortcomings, the findings from citrinin research supported Endo's hypothesis and the experience gave him and his team hope and courage for further research. In 1972, they found another active mould, *Penicillium citrinum*, and in July 1973, by incorporating principles of solvent extraction, silica gel chromatography, and crystallization, they isolated three active metabolites from the culture broth of *Penicillium citrinum*.[32] Further studies showed that these metabolites inhibited cholesterol synthesis both *in vitro* and *in vivo*.[40] The most active of the three, ML-236B (called Compactin or Mevastatin)—the first statin, was subjected to further developmental studies.

Compactin, a structural analogue of HMG-CoA and a prodrug which is metabolized to the active carboxylate form (Figure 3), was a highly potent competitive inhibitor of HMG-CoA reductase.[41] Although it did not work in rats (due to concurrent enzyme induction); it showed profound activity in dogs and monkeys and was marked as a drug candidate.[42,43]

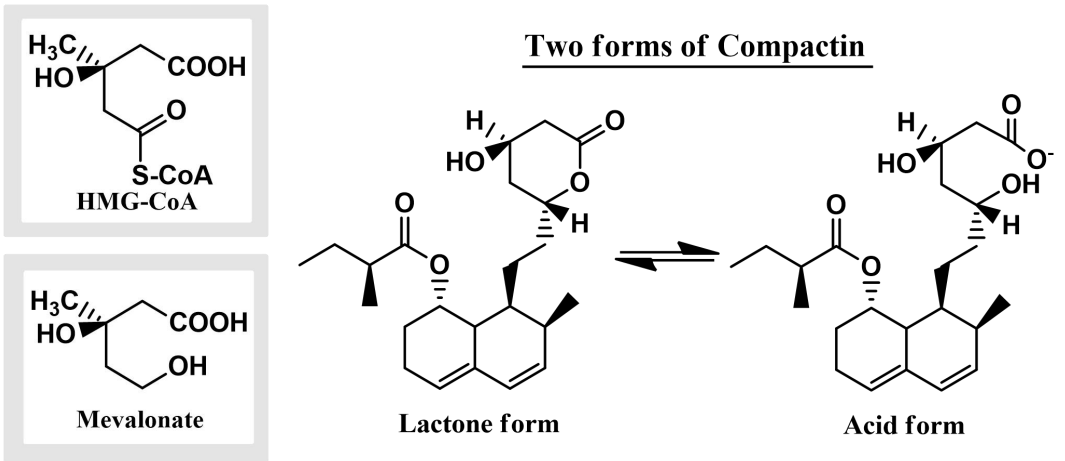

Figure 3: Structural similarity between HMG-CoA, mevalonate and compactin

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In August 1976, Sankyo launched Compactin Development Project; headed by Endo and including pharmacologists, pathologists, toxicologists, organic chemists and microbiologists.[32] However, in August 1980 Sankyo discontinued the clinical development of compactin as long-term toxicity study in dogs indicated that it caused lymphoma at higher doses and as a result was believed to be too toxic for human use.[32] During this period, researchers at Beecham Pharmaceuticals (now GlaxoSmithKline) in England had also isolated compactin from the mould, *Penicillium brevicompactum*; they reported its antifungal and cholesterol-lowering properties but did not develop it further because of its failure to work in rats.[44,45]

***The discovery of lovastatin (the first commercial statin)*:** The discovery of compactin at Sankyo inspired other pharmaceutical companies to begin to search for another statin. In July 1976, Merck Research Laboratories reached a confidential agreement with Sankyo, which provided them with samples of compactin and confidential experimental data.[32] In February 1979, researchers at Merck; headed by Alfred Alberts, isolated a statin (named mevinolin); an analogue of compactin, from the fungus *Aspergillus terreus*.[46] In the same month, Endo (now at Tokyo Noko University) isolated another statin (called monacolin K) from the fungus *Monascus ruber*.[47] Later in the year, monacolin K and mevinolin were identified as the same compound and renamed lovastatin.

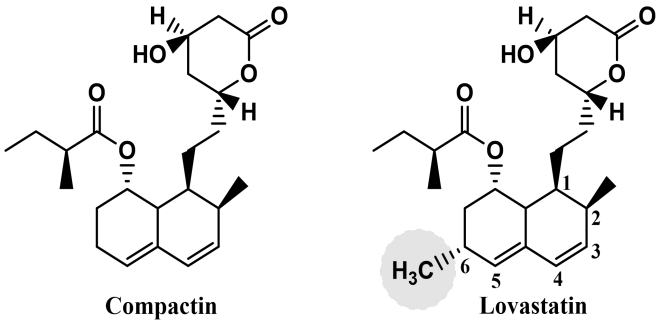

Figure 4: Structural similarity between compactin and lovastatin

In April 1980, Merck began clinical trials of lovastatin, but 5 months later, in September 1980, they discontinued these trials.[48] Lovastatin was thought to possess similar toxicity as compactin—which caused cancers in dogs—due to their structural similarity (differing only by the presence of 6-methyl on the hexahydro naphthalene ring of lovastatin (Figure 4)). In 1981, Michael Brown and Joseph Goldstein (awarded the Nobel Prize in Physiology or Medicine in 1985), who had earlier discovered low-density lipoprotein (LDL) receptors in 1973, reported that lovastatin raised liver LDL receptors, which led to profound fall in plasma LDL levels in dogs and humans.[19,49] These and subsequent studies on lovastatin, which reported very few side effects, led Merck to commence large scale clinical trials of lovastatin in patients at high risk and long-term toxicity studies in dogs in 1984. Following successful clinical trials, lovastatin (marketed as Mevacor) received approval from the US Food and Drug Administration to become the first commercial statin in September 1987, nine years after its discovery.[48]

The development and design of other statins

Subsequent to the approval and clinical introduction of lovastatin, other statins have been developed based on structural modification of lovastatin and mevastatin. Two major approaches have been followed.

I. Chemical modification of fungal metabolites

Development of pravastatin: Following the introduction of lovastatin by Merck, Sankyo revisited compactin (mevastatin). Structural modifications to reduce its toxicity were embarked upon. This involved reducing its lipophilicity (to lower systemic bioavailability) and increasing its hydrophilicity (to improve hepatoselectivity). Introduction of hydroxyl group (OH) at C-6 of the hexahydro naphthalene ring and opening of the lactone ring to its carboxylic acid (COOH) derivative (Figure 5) gave rise to the compound CS-514, with favourable effects, and it was introduced as pravastatin (Pravachol) in 1989.[50]

Development of simvastatin: During the clinical development of lovastatin, Merck was aware of its competitors' similar research into microbial metabolites HMG-CoA reductase inhibitors; therefore it initiated a concurrent search for a more potent synthetic analogue. A chemical alteration of lovastatin gave a more potent compound MK-733 and it was introduced by Merck as simvastatin (Zocor) in 1992.[51] Simvastatin differs from lovastatin only by the presence of an additional methyl (CH_3) group on the butyryl moeity at C-8 of the hexahydro naphthalene ring (Figure 5). It is more lipophilic than and approximately twice as potent as lovastatin and pravastatin. Owing to its higher lipophilicity, it also has higher risk of adverse effects such as myopathy.[52]

The semi-synthetic statins, pravastatin and simvastatin, are sometimes classified together with lovastatin as type 1 statins, due to their having the hexahydro naphthalene ring and butyryl group in common.[53]

II. Development of synthetic statins

The synthetic approach to designing a suitable HMG-CoA reductase inhibitor was predicated on the fact that a well-defined biological concept of competitive enzyme inhibition was in place. The lead compound was HMG-CoA, the natural substrate of the enzyme. Cues were also taken from the structures of the earlier isolated natural statins. Synthetic statins were designed by attaching a ring system (with different substituents) to the pharmacophore of the statins, the 3,5-dihydroxyheptanoic acid unit. This involved replacement of the hexahydro naphthalene ring on the type 1 statins with heterocyclic rings and bioisosteric exchange of the butyryl group with fluorophenyl group. By virtue of this approach, five synthetic statins (sometimes classified as type 2 statins): fluvastatin (Lescol, Novartis 1993); atorvastatin (Lipitor, Warner-Lambert/Pfizer 1996); cerivastatin (Baycol, Bayer 1998); rosuvastatin (Crestor, AstraZeneca 2003) and pitavastatin (Livalo/Livazo, Nissan Chemical Industries/Kowa Pharmaceuticals 2003), have been developed and introduced (Figure 6). Fluvastatin has indole ring system, while atorvastatin, cerivastatin, rosuvastatin and pitavastatin have pyrrole, pyridine, pyrimidine and quinoline based ring system respectively.

The different heterocyclic systems confer unique steric, electronic and physicochemical properties on these compounds which impact their pharmacokinetics and interaction with the enzyme. The fluorophenyl group accords them additional polar interactions that cause tighter binding to the HMG-CoA reductase enzyme.[52] The sulfonamide group of rosuvastatin forms a unique polar interaction with amino acid residues on the enzyme; as a result, rosuvastatin has superior binding affinity to the HMG-CoA reductase enzyme compared to the other statins.[54] Cerivastatin is the most lipophilic and most potent statin. However, due to its relatively severe adverse effects in comparison to other statins, its clinical use has been discontinued since 2001.[55] Artovastatin is the most popular statin and one of the most prescribed drugs of all time. In 2003, it was announced as the best-selling pharmaceutical in history and in 2008, Pfizer garnered US$12.4 billion from artovastatin (Lipitol) sales.[56,57]

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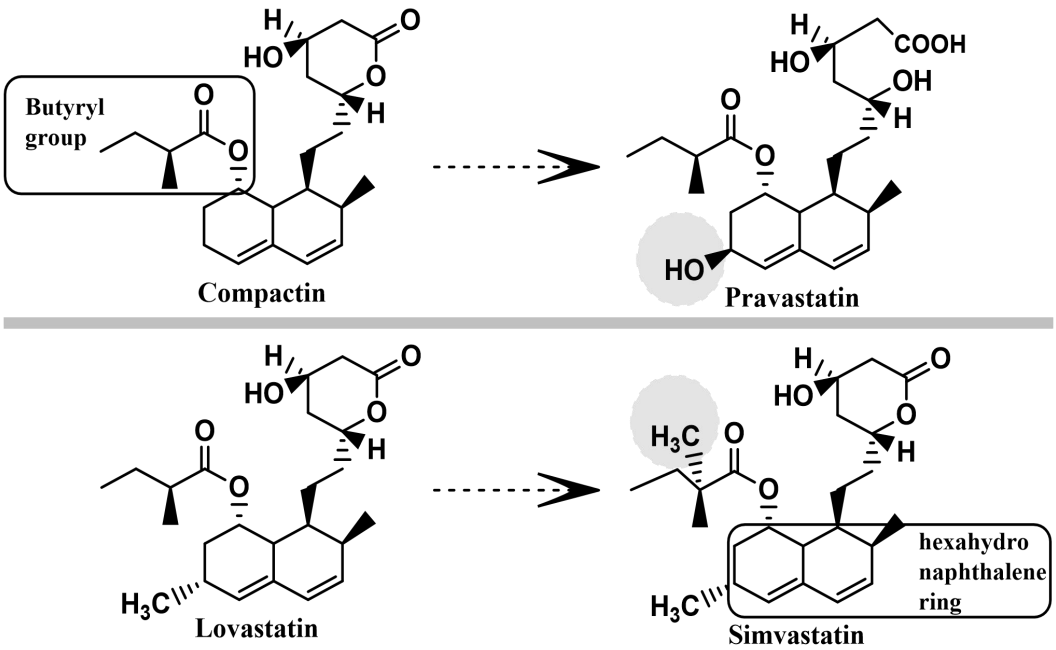

Figure 5: Development of pravastatin and simvastatin

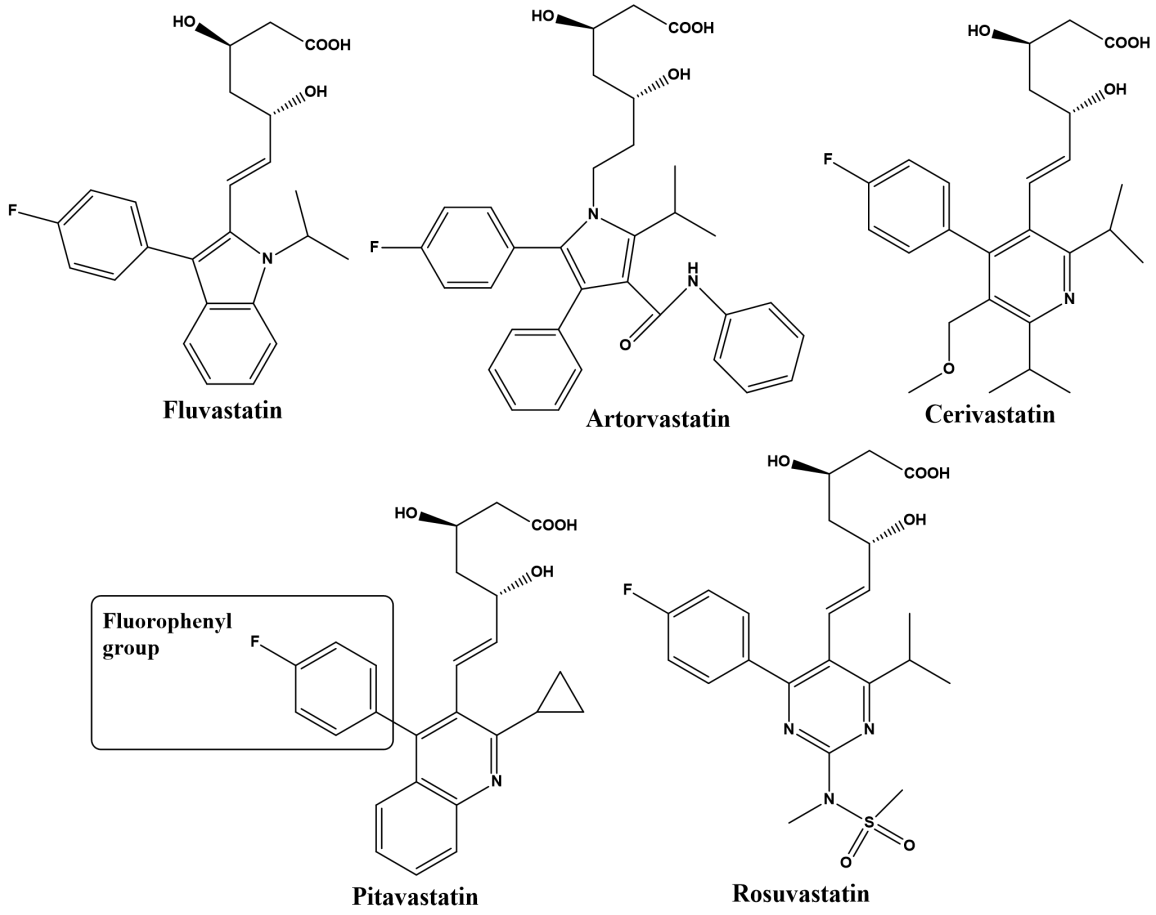

Figure 6: Structures of synthetic statins

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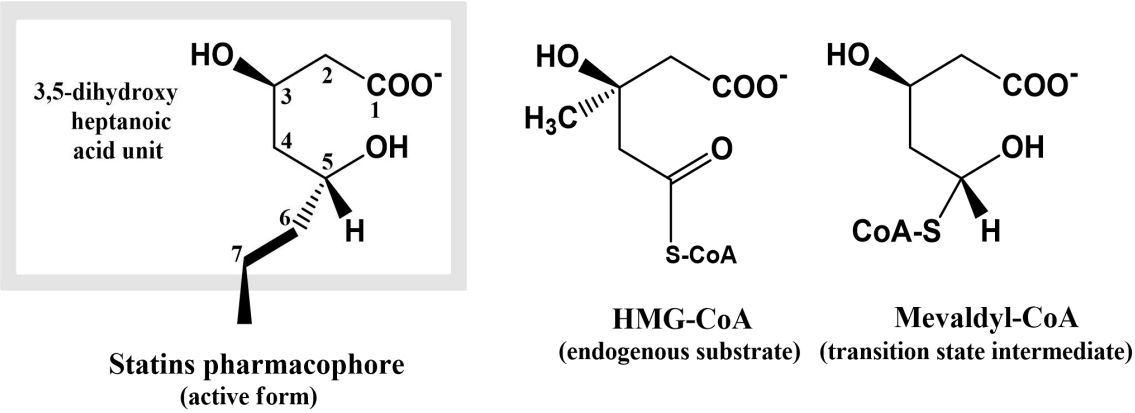

Figure 7: Comparative stereochemistry of the statins pharmacophore, HMG-CoA and Mevaldyl-CoA

The stereochemistry of the statins

The statins act as false substrates of HMG-CoA reductase, thus inhibiting the endogenous substrate, HMG-CoA. This activity is essentially dependent on the statins pharmacophore, the 3,5-dihydroxyheptanoic acid (or heptenoic acid) unit. The functionality of the statins pharmacophore is closely related to its stereochemistry, which reflects that of the endogenous substrate, HMG-CoA, and the transition state intermediate, mevaldyl-CoA, which possess two chiral carbon atoms, C3 and C5 (Figure 7).[58] The statin pharmacophore competes directly with HMG-CoA for the receptor on the active site cavity of HMG-CoA reductase.[52,58] The receptor stereo-selectively binds the 3R,5R conformation; hence all statins must possess this requisite stereochemistry to be effective HMG-CoA reductase inhibitors.[58]

Limitations of statins and further research

Statins are generally well tolerated and incidences of serious adverse effects are rare.[59] The health-compromising adverse effects that may accompany the use of statins include hepatotoxicity, myopathies, rhabdomyolysis, diabetes, insomnia, memory loss, confusion, peripheral neuropathy, impaired myocardial contractility, autoimmune diseases, erectile dysfunction, and mitochondrial dysfunction.[60-64]

Statins are known to primarily inhibit HMG-CoA reductase; blocking cholesterol synthesis and raising LDL receptors in the liver. Further studies have shown that statins also inhibit the biosynthesis of certain prenylated proteins from activated isoprene units. This property is thought to be responsible for the improvement of endothelial function, modulation of immune function and other pleiotropic cardiovascular benefits associated with the statins.[65,66] This has led to recent studies investigating the use of statins in conditions such as inflammation, dementia, lung cancer, sepsis, chronic obstructive pulmonary disease, hypertension and prostate cancer.[67-71]

Conclusion

Since the introduction of lovastatin, the impact of statins on the treatment and prevention of cardiovascular diseases has been staggering. Statins have been investigated in many clinical trials, involving over 90,000 subjects and over 5 years period; the findings from these studies indicated that, treatment with statins lowers plasma LDL levels by 25–35% and reduces the frequency of heart attacks by 25–30%.[32,72] Akira Endo has been widely applauded for his role in the discovery of statins; he received the prestigious Japan Prize in 2006, was awarded the covetous Lasker-DeBakey Clinical Medical Research Award in 2008 and was inducted into the National Inventors Hall of Fame in 2012.[73,74] The story of the discovery and development of statins is a testament to the importance of collaborative research—typical of scientific breakthroughs—in science and highlights the rigorous research and great amounts of time and resources often needed to nurture a lead compound or concept to a clinically approved drug.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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