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MINI-REVIEW

Biotechnological production and applications of statins

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Abstract Statins are a group of extremely successful drugs that lower cholesterol levels in blood; decreasing the risk of heath attack or stroke. In recent years, statins have also been reported to have other biological activities and numerous potential therapeutic uses. Natural statins are lovastatin and compactin, while pravastatin is derived from the latter by biotransformation. Simvastatin, the second leading statin in the market, is a lovastatin semisynthetic derivative. Lovastatin is mainly produced by *Aspergillus terreus* strains, and compactin by *Penicillium citrinum*. Lovastatin and compactin are produced industrially by liquid submerged fermentation, but can also be produced by the emerging technology of solid-state fermentation, that displays some advantages. Advances in the biochemistry and genetics of lovastatin have allowed the development of new methods for the production of simvastatin. This lovastatin derivative can be efficiently synthesized from monacolin J (lovastatin without the side chain) by a process that uses the *Aspergillus terreus* enzyme acyltransferase LovD. In a different approach, *A. terreus* was engineered, using combinational biosynthesis on gene *lovF*, so that the resulting hybrid polyketide synthase is able to in vivo synthesize 2,2-dimethylbutyrate (the side chain of simvastatin). The resulting transformant strains can produce simvastatin (instead of lovastatin) by direct fermentation.

Keywords Statins · Biosynthesis and genetics · Biotechnological production

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Introduction

According to the World Health Organization, cardiovascular diseases are the leading cause of death. In 2005, about 17.5 million people died from these diseases, accounting for approximately 30% of global mortality. This is related to high levels of cholesterol in plasma, since hypercholesterolemia is primary risk factor of atherosclerosis and coronary artery disease (Kannel et al. 1961). Generally, only one-third of the total body cholesterol is diet-derived; while two-thirds are synthesized by the liver and, to a lesser extent by other organs (Furberg 1999; Alberts et al. 1980). For this reason, control of cholesterol by inhibiting its biosynthesis is an important strategy to lower cholesterol levels in blood, as stated by Manzoni and Rollini (2002) in a previous review on statins.

Statins are a group of drugs that selectively inhibit HMG-CA reductase, the regulatory and rate-limiting enzyme in cholesterol biosynthesis. In this way, these compounds lower cholesterol; particularly low density lipoprotein (LDL) or low density cholesterol ("bad cholesterol"); while slightly increasing high-density lipoprotein cholesterol ("good cholesterol"), thus, preventing plaque buildup inside the arteries. Moreover, statins have emerged at the forefront of preventive drugs for cardiovascular disease because of a substantial clinical trial database demonstrating that statins reduce the risk for coronary artery disease morbidity and death across a broad range of at-risk patient cohorts.

This explains the huge success of statins in the medical and the commercial fields. In 2006, two statins led the Forbes magazine's list of America's 20 Best Selling Drugs, with $ 8.4 and $ 4.4 billion dollars annual sales (respectively), and the forecast is an increase in the usage of statins. However, the main statins are coming off patent, so the competition with generic versions will become

tougher in the coming years where the most efficient production processes will prevail.

In the scientific field, the last years have witnessed important advances in biochemical and genetic aspects of natural statins (lovastatin and compactin). These and other studies have allowed the development of novel biotechnological production processes for these statins and their derivatives (simvastatin and pravastatin).

A different group of studies have shown that statin therapy has biological effects beyond the level of LDL-cholesterol. These new studies have discovered numerous new biological (pharmacological) activities of statins; representing potential application in diseases like cancer, Alzheimer's dementia and age-related bone loss.

Historical development of statins

Statins can be divided into natural statins with their semisynthetic derivatives, and statins of synthetic origin. All natural statins are substituted hexahydronaphtalne lactones. The first statin was isolated in Japan by research scientists of Sankyo Co. Ltd., who were screening fungal cultures looking for compounds that could inhibit HMG-CoA reductase (the rate-limiting enzyme in cholesterol biosynthesis) in rat liver extracts. They found a compound, produced by a strain of *Penicillium citrinum* that was originally named ML236B or mevastatin and later, compactin (Endo et al. 1976). As soon as the potential of this new compound was understood, scientists from Merck initiated their own fungal screens. They isolated a strain of *Aspergillus terreus* that produced a yet more efficient statin: lovastatin (Alberts et al. 1980). Independently, Endo et al. (1979) isolated the same compound in broths of *Monascus ruber*, although this species is not used for industrial production of this metabolite. The chemical structure of lovastatin is identical to compactin, except for an additional methyl group (Fig. 1).

After successful clinical trials in which lovastatin proved to dramatically reduced LDL with few side effects, in 1987, Food and Drug Administration (FDA) approved lovastatin under the marketing name Mevacor. Sankyo developed pravastatin, a more efficient compactin derivative. This company teamed up with Bristol-Myers Squibb to distribute and sell pravastatin (marketed as Pravachol).

On their side, Merck developed a second generation semisynthetic derivative of lovastatin which is still now the second leading statin of the market. This derivative was simvastatin, and although it is produced synthetically from lovastatin, it can also be produced now by biotechnological processes (see corresponding section).

Merck sponsored the Scandinavian Simvastatin Survival Study (1994) with 2,221 patients diagnosed with moderate hypercholesterolemia (200–300 mg/deciliter), who took simvastatin. Results not only showed significant decreases in total cholesterol (25%) and LDL (35%) in patients taking simvastatin, but more importantly, showed 42% decrease in death rate in this group.

This and other impressive results drew other pharmaceutical companies into the statin market. Part of the efforts was directed at manufacturing synthetic statins, and fluvastatin (Sandoz AG, Lescol) was the first fully synthetic statin, followed by atorvastatin (Pfizer), better known by its trade name, Lipitor. This product later became the best selling drug (Kidd 2006).

The structures of synthetic statins are dissimilar and quite different from the natural statins. Only the HMG CoA-like moiety, responsible for HMG CoA reductase inhibition, is common to both natural and synthetic statins (Manzoni and Rollini 2002). As mentioned before atorvastatin (Lipitor) is the most important synthetic statin, followed by fluvastatin (Lescol), rosuvastatin (Crestor), with a much smaller share of the market; and pitavastatin that is presently commercialized in some oriental countries.

Biosynthesis of lovastatin

Early research on *Monascus ruber* indicated that monacolin L and J were intermediates in the lovastatin biosynthetic pathway (Endo et al. 1985). It was shown that monacolin L is the first to be synthesized from nine molecules of acetate and is, in turn, converted to monacolin J by hydroxylation. In the hydroxylation reaction, $^{18}O_2$ was incorporated into monacolin J through the action of a monooxygenase system involving cytochrome P-450 present in the cell-free extract of *M. ruber* (Komagata et al. 1989). Subsequent experiments demonstrated the transformation of monacolin J to lovastatin (Kimura et al. 1990).

Research with *Aspergillus terreus*, using labeled precursors (Chan et al. 1983; Greenspan and Yudrovitz 1985; Moore et al. 1985; Shiao and Don 1987) indicated that the lovastatin biosynthetic pathway also starts from acetate units linked to each other in head-to-tail fashion to form two polyketide chains (Fig. 2). The methyl group is present in some statins in the side chain or at C6 derives from methionine, and is inserted in the structure before closure of the rings (Shiao and Don 1987). The main chain is then cylized and in some statins esterified by a side chain at C8. The oxygen atoms present in the main chain are inserted later by aerobic oxidation (Alberts et al. 1980; Greenspan and Yudrovitz 1985; Moore et al. 1985). Studies carried out in *P. citrinum* and *M. ruber* indicated a similar pathway (Endo 1985; Chakravarti and Sahai1 (2004).

Hence, it had been shown that lovastatin was derived from acetate via a polyketide pathway (Moore et al. 1985).

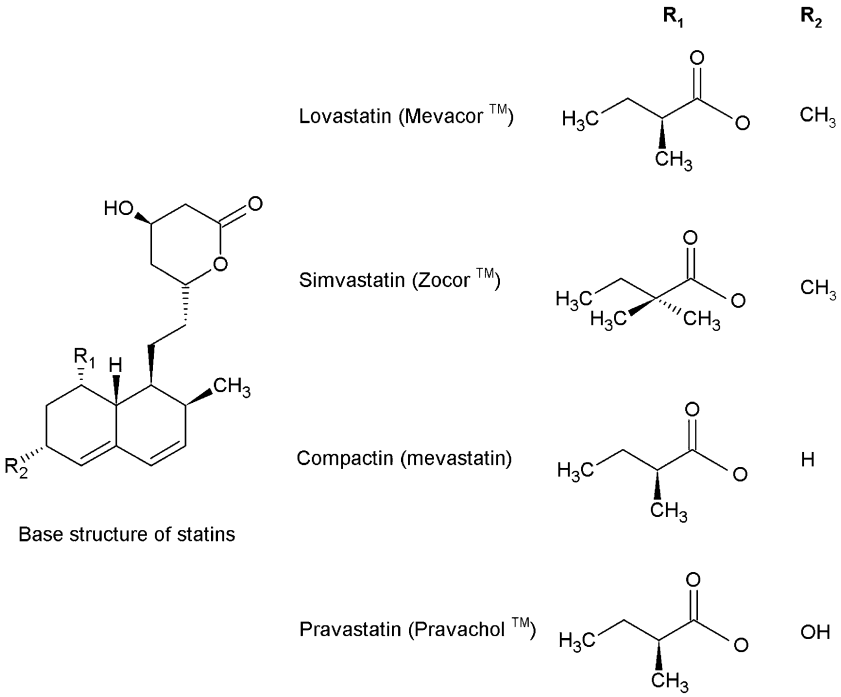

Fig. 1 Chemical structures of natural statins and their derivatives: simvastatin and pravastatin

Pioneering genetic research by Reeves, McAda, and workers at MDS Panlabs Inc., identified a type I polyketide synthase (PKS) gene essential for lovastatin biosynthesis by *A. terreus* (Hendrickson et al. 1999). Its product, now called lovastatin nonaketide synthase (LNKS) has been shown (Ma and Tang 2007) to contain seven active sites (in order: KS = ketosynthase; MAT = malonyl-CoA:ACP acyltransferase; DH = dehydratase; MT = methyltransferase; KR = ketoreductase; ACP = acyl carrier protein; CON = condensation domain). Characterization of the LNKS (*lovB*) gene set the stage for understanding how the carbon skeleton of dihydromonacolin L and lovastatin are assembled. Moreover, since secondary metabolism genes are invariably clustered in microorganisms, this gene also provided a convenient entry into cloning and characterizing the other genes involved in lovastatin synthesis. Using it as a probe, Kennedy et al. (1999) isolated two cosmids, containing the complete lovastatin gene cluster, from a genomic library. They found the presence of 18 genes over 64 kb (Fig. 3). Of these genes, two (*lovB* and *lovF*) encoded PKSs. As previously noted, the *lovB* gene encodes LNKS, while *lovF* encodes a diketide synthase (DKS). The presence of methyltransferase domains in the LNKS and in the DKS indicated that in both cases the methyl groups are likely to be added (S-adenosylmethionine) while the polyketide is being synthesized. Furthermore, the function of the genes could be largely predicted by sequence comparison. Additional understanding of their function was obtained by a loss-of-function mutation strategy, through disruptions of individual genes of the cluster.

It is now clear that the lovastatin biosynthesis cluster contains two type I polyketide synthase genes (*lovB* and *lovF*). In addition, *lovE* encodes a transcription factor regulatory protein with the typical binuclear Zn^{++} finger motif. Its disruption mutants did not produce lovastatin or intermediates, while the overexpression resulted in increased metabolite production. It is assumed that *lovE* regulates lovastatin production at the transcriptional level. However, there is a second gene (*lovH*) with a similar structure (Hutchinson et al. 2000). It is not obvious how lovastatin biosynthesis uses two regulatory genes, since most other secondary metabolites clusters contain one dedicated regulatory gene (Keller and Hohn 1997). On the other hand, *lovA* and ORF 17 encode putative cytochrome 450 monooxygenases.

Synthesis of the main nonaketide-derive skeleton was found to require the LNKS (*lovB*), plus at least one additional protein (enoyl reductase *lovC*) that interacts with LNKS, and is necessary for the correct processing of the growing chain, and production of dihydromonacolin L. In the absence of LovC, LNKS forms the conjugated pyrones 3 and 4 as truncated PKS products (Burr et al. 2007).

LNKS is a multidomain enzyme that contains seven activities, and functions in a way similar to animal fatty acid synthases (FAS) and bacterial type I PKS, i.e., the ketosynthase (KS) performs decarboxylative claisen condensation for chain elongation (Kennedy et al. 1999); the malonyl-CoA:ACP acyltransferase (MAT) selects and transfers the extender unit in the form of malonic esters, while the acyl carrier protein (ACP) serves as the tether (or bind) for the extender unit and the growing chain. In addition, tailoring enzymes such as ketoreductase (KR), dehydratase (DH), methyltransferase (MT), and enoylreductase modify the carbon backbone and introduce struc-

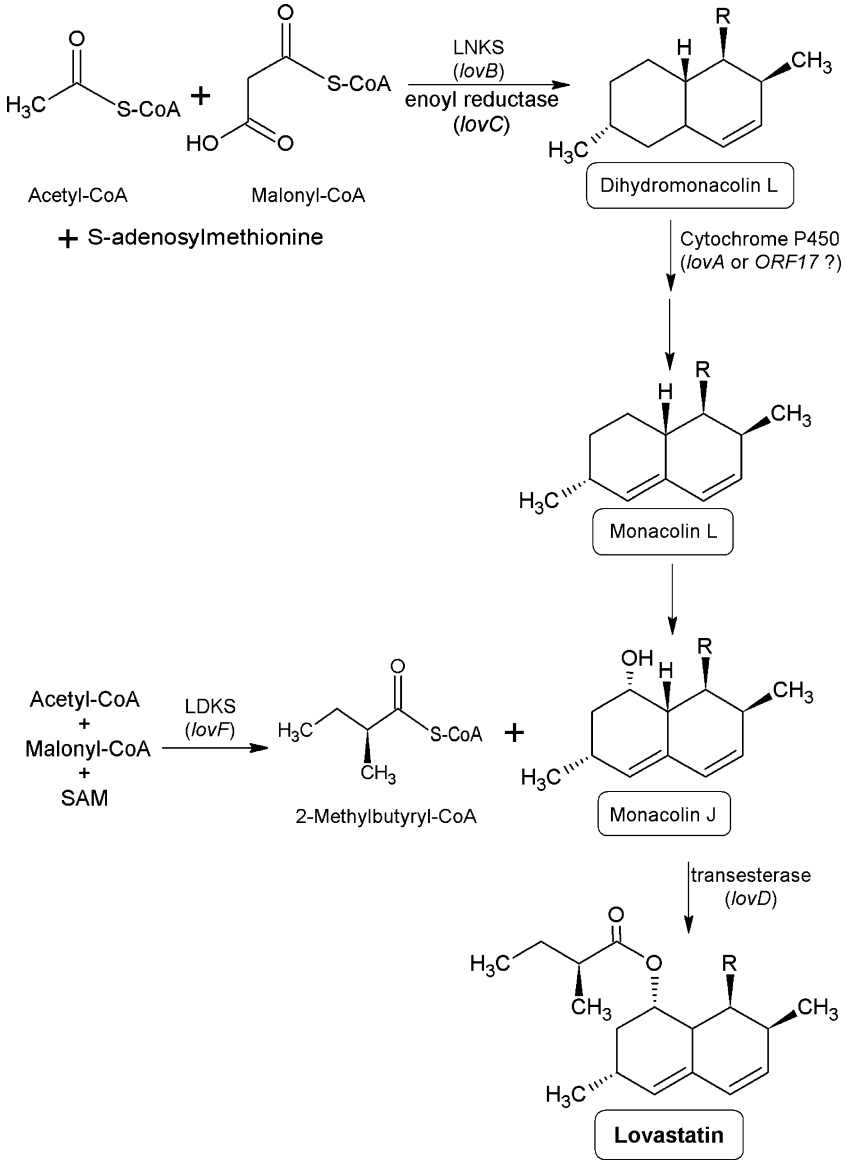

Fig. 2 Lovastatin biosynthetic pathway, showing enzymes involved and their encoding genes

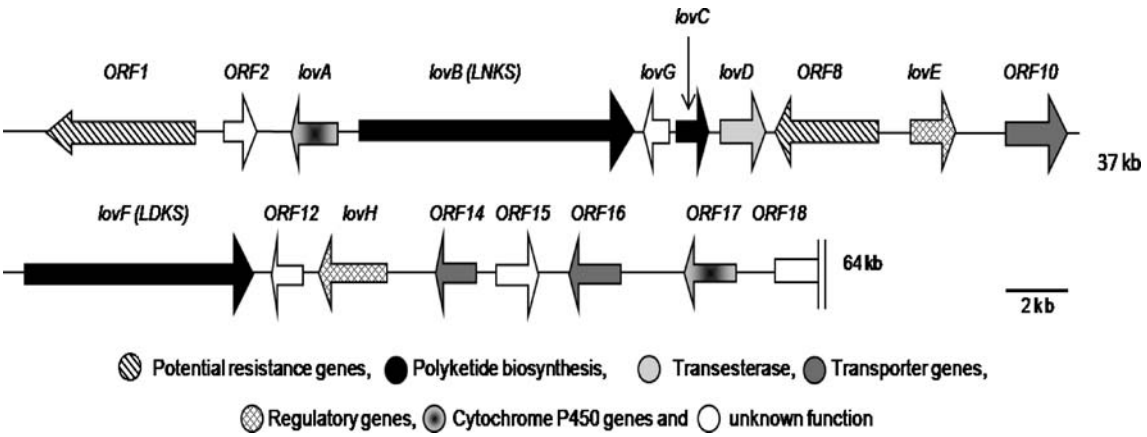

Fig. 3 Lovastatin biosynthetic gene cluster (modified from: Kennedy et al. 1999; Hutchinson et al. 2000)

tural diversity (Staunton and Weissman 2001). However, LovB is a reducing iterative PKS that is mechanistically different from the modular, bacterial type I PKS. The minimal PKS domains in LovB are used repeatedly to synthesize the nonaketide decalin core from nine malonyl-CoA units (Fig. 2). Varying degrees of polyketide tailoring modifications are performed after each condensation step by a different combination of LovB catalytic domains (including the abovementioned dissociated enoylreductase, LovC) to afford the key intermediate dihydromonacolin L. LovB also contains a C-terminus domain with sequence similarity to a nonribosomal-peptide synthase condensation (CON) domain. The function of the CON domain in dihydromonacolin L biosynthesis is not known (Ma and Tang 2007). Recently, reconstitution of the catalytic domains as individual enzymes has been used to analyze the biochemical and structural features of this and other PKS. Ma and Tang (2007) examined the minimal PKS components of the lovastatin nonaketide synthase by obtaining dissociated mono- and didomain proteins. They showed that most of the domains examined can be expressed as standalone proteins, except the KS domain. Also, that the LovB MAT displays strong substrate selectivity towards malonyl-CoA over acetyl-CoA. The LovB MAT displayed significantly different properties when compared to the mammalian FAS MAT domain, especially in acyl-CoA substrate specificity. It is interesting to note that the broad specificity of the KS and MAT domains toward heterologous ACP domains may also provide opportunities for combinatorial biosynthesis of novel polyketide entities.

The transformation to monacolin J requires CYP450 oxygenases, probably encoded by genes *lovA* and ORF 16. The five carbon side unit side chain is synthesized by the other polyketide synthase, the product of *lovF* (also known as LDKS), through a single condensation between an acetyl-CoA and a malonyl-CoA. The LDKS consists of seven linearly arranged domains, in order: KS, MAT, DH, MT, ER (enoylreductase), KR, ACP. The condensed diketide undergoes methylation and reductive tailoring by the individual LovF catalytic domains to yield α-S-methylbutyryl tyioester covalently attached to the phosphopantetheine arm of the acyl carrier protein domain of LovF (Kennedy et al. 1999).Gene *lovD* encodes the 2-mehtylbutyryl/monacolin J transesterase that catalyzes the last step that joins together the two polyketide components of lovastatin, i.e., transacylates, the acyl group from LovF to the C8 hydroxyl group of monacolin J to yield lovastatin (Fig. 2). One particularly unique feature of this type of highly reducing PKSs is the lack of a built-in offloading domain that facilitates the release of completed products. This is in sharp contrast to bacterial type I or fungal nonreducing PKSs, in which a dedicated thioesterase domain is appended at the end of the megasynthase and catalyzes the release of polyketides. As mentioned before, transfer of the diketide side chain from LovF to monacolin J was proposed to be catalyzed by a dissociated acyltransferase LovD (Kennedy et al. 1999). Using the strategy of reconstitution of the catalytic domains as individual enzymes, Xie et al. (2009a) demonstrated that protein-protein interactions between LovF and LovD play a key role in facilitating rapid offloading of the diketide substrate from LovF to LovD and ensure efficient biosynthesis of lovastatin. Also that only the completely tailored, R-*S*methylbutyryl-ACP is accessible by LovD (that is, none of the acyl intermediates are transferred). A possible mechanisms that may account for this phenomenon is that the methyl transfer, ketoreduction, dehydration, and enoyl reduction steps may take place very rapidly following exit of the acetoacetyl-ACP from the KS active site. Another possibility is that the acyl-ACP may be inaccessible by LovD during the tailoring steps.

From an applied stand point, it can be seen that this knowledge is important for strains genetic improvement. For example, promoter sequences from lovastatin associated genes can be used to configure reported based selection in *A. terreus* to rapidly identify improved lovastatin-producing strains. Askenazi et al. (2003) constructed a strain containing the *lovF* promoter fused to the ble gene (phleomycin resistance). This transformant was mutagenized and plated in agar medium with phleomycin. The resulting resistant population displayed a significant increase in lovastatin yield, showing that this is a sophisticated and effective rational selection system. On the other hand, it is interesting to note that *lovB* could be manipulated to generate new compounds. However, manipulation of *lovF* to produce compounds with different side chains is easier to envisage. The deletion of activities in this gene or perhaps the addition of further modules could allow the production of various lovastatin analogs in a predictive manner, which would represent biotechnological processes to substitute chemical strategies to synthesize valuable derivatives like simvastatin (see corresponding section).

Biosynthesis of compactin

Studies on the ^{13}C incorporation in lovastatin and compactin, carried out in *P. citrinum* and *M. ruber*, indicated a similar pathway (Endo et al. 1985; Chakravarti and Sahai1 (2004). Later, Abe et al. (2002) cloned and characterized the compactin gene cluster from *Penicillium citrinum* using similar strategies. Nine genes, *mlcA* to *mlcH* and *mlcR* (regulator) clustering in a 38-kb region, were transcribed when compactin was produced. The predicted amino acid sequences encoded by these nine genes were similar to those encoded by the genes for lovastatin biosynthesis

(Fig. 4). Later, the pathway-specific transcriptional activator MlcR-binding sequence was identified and located in the promoters of *mlcA* and *mlcC*, and other genes of the cluster (Baba et al. 2008). It is important to note that in the case of compactin too; the introduction of extra copies of the regulator gene *mlcR* enhanced the metabolite production (Baba et al. 2009).

Lovastatin and compactin production

Lovastatin (mevinolin or monacolin K) was obtained from a strain isolated from the soil and classified as *A. terreus* at CIBE Laboratories in Madrid (Spain; Alberts et al. 1980); and it was also obtained by *M. ruber* (named monacolin K). A few years later, lovastatin was also obtained from 17 strains of different species of the genus *Monascus* (in particular *M. ruber, Monascus purpureus*, *Monascus pilosus*, *Monascus vitreus*, and *Monascus pubigerus* (Negishi et al. 1986). It is worth noticing that genus *Monascus*, particularly the species *Monascus anka* and *M. purpureus*, is traditionally employed in Asian countries as "red koji" for fermented food (red yeast rice) and beverage production, as well as for red dye (Lin et al. 2008).

However, commercial production of lovastatin is based on *A. terreus* batch fermentation and most of the literature deals with this species (Novak et al. 1997; Manzoni et al. 1998; Kumar et al. 2000; Casas-López et al. 2004; Rodríguez Porcel et al. 2007; Bizukojc and Ledakowicz 2008). *A. terreus* fermentations are typically carried out at≈ 28 °C and pH5.8–6.3, and the dissolved oxygen level is controlled at≥40% of air saturation (Kumar et al. 2000). Batch fermentations generally run for approximately 10 days. Growth of *A. terreus*, and lovastatin production were studied by Casas-López et al. (2003). Results showed that production was influenced by the type of carbon source (lactose, glycerol, and fructose) and the nitrogen source (yeast extract, corn steep liquor, and soybean meal) used and the C:N mass ratio in the medium. Use of a slowly metabolized carbon source (lactose) in combination with either soybean meal or yeast extract under N-limited conditions gave the highest titers and specific productivity. The maximum value of the lovastatin yield coefficient on biomass was 30 mg/g using the lactose/soybean meal and lactose/yeast extract media. The optimal initial C:N mass ratio for attaining high productivity of lovastatin was 40.

Some studies have used response surface methodology to identify the impact of the medium composition on lovastatin production (Lai et al. 2003; Casas-López et al. 2004). Since its discovery, lovastatin production has been studied in shake flask and lab-scale bioreactors, using complex and defined media. Novak et al. (1997) and Kumar et al. (2000) proposed different fed-batch strategies for the improvement of lovastatin formation, while Rodríguez-Porcel et al. (2007) suggested a two-stage feeding strategy. Pellet morphology and its relationship to the hydrodynamic conditions in the reactor and the aeration of the medium in the lovastatin fermentation have also been investigated (Casas-López et al. 2005; Rodríguez-Porcel et al. 2005). Very recently, the effect carbon and nitrogen sources and fermentation parameters on lovastatin production were reviewed by Bizukojc and Ledakowics (2009).

However, *A. terreus* is also capable to biosynthesize other compounds that complicate purification stages in industrial production. One predominant co-metabolite is the benzophenone sulochrin, reported to also arise from a polyketide pathway. This compound was targeted for suppression by classical mutagenesis and screening and this gave rise to increased lovastatin production (Vinci et al. 1999). The same end has been obtained using genetic engineering tools i.e., gene disruption (Couch and Gaucher 2004). Another important cometabolite is geodine, which is also a product of a polyketide pathway. Some researchers have explored the strategy of decreasing geodin formation during the lovastatin fermentation by manipulating fermentation parameters, particularly a decrease in aeration and pH control (Bizukojc and Ledakowicz 2008; Bizukojc and Ledakowics 2009).

The industrial production process for the production of lovastatin was set up in 1980 using an *A. terreus* (ATCC 20542; Mevacor, Merk). The process development involved the analysis of different fermentation parameters such as culture homogeneity, effect of various carbon sources, pH, aeration, and agitator design. Producer strain reisolation together with pH control and slow use of the carbon source, in particular glycerol, yielded a fivefold increase with respect

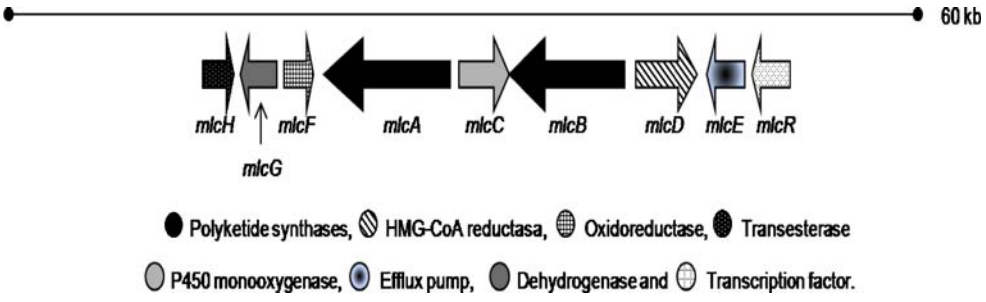

Fig. 4 Compactin biosynthetic gene cluster (modified from: Abe et al. 2002)

to the initial yields. Apparently, the production level reached at that time was 180 mg/l (Buckland et al. 1989). Most of the industrial production processes use stirred tank reactors, fed continuously or discontinuously with medium that contains the nitrogen source (Monaghan et al. 1981).

Similar studies with *P. citrinum* have developed the fermentation technology for industrial production of compactin (Hosobuchi et al. 1993a, 1993b; Chakravarti and Sahai1 2004). Several important factors affecting compactin production, such as fungal morphology, pH, surfactants, medium composition, and feeding strategies have been studied and optimized (Chakravarty and Sahai1 2004). In the case of *P. citrinum* or *P. cyclopium*, pellet-like fermentation broth with low apparent viscosity (<20 cp) yields a higher compactin concentration than filamentous broth (Hosobuchi et al. 1993c).

Important efforts are continuously performed to find higher lovastatin or compactin yielding strains for commercial production. Strains are generally mutagenized, by either ultraviolet or chemical treatment, to obtain higher yielding strains by random or rational selection. The efficiency of this strategy can be seen by the enormous increase in industrial strains production. Apparently, the productivity of *A. terreus* ATCC 20542 (the original lovastatin producer) industrial descendants (mutants) is now around 15 g of lovastatin per liter (Metkinen 2009). Likewise, productivity of compactin industrial strains has increased to around 15 g/l. A mutant strain derived from *Streptomyces carbopilus*, with resistance to 3 mg/l of compactin and 80% conversion yield was obtained by Metkinen (2009). However, recent advances in knowledge of lovastatin and compactin biosynthesis and cloning of the corresponding genes (see corresponding sections) are stepping stones towards enhanced commercial production of lovastatin and compactin by molecular genetics improvement strategies (Barrios-González et al. 2003).

Lovastatin production by solid-state fermentation

Although industrial production of lovastatin (and other secondary metabolites) is conventionally performed by liquid submerged fermentation (SmF), solid-state fermentation is rapidly becoming an alternative industrial production system. Solid-state fermentation (SSF) is a microbial culture system that has been used in several oriental countries since antiquity, to prepare diverse fermented foods from grains like soybeans or rice (Hesseltine 1977a, 1977b). In the last 20 years, different, nontraditional SSF systems have been developed for new uses (Barrios-González and Mejía 1996, 2007). Several comparative studies have shown that SSF often presents advantages over SmF, including higher and faster product yield and improved processing (Balakrishna and Pandey 1996; Robinson et al. 2001; Hölker and Lenz 2005; Barrios-González and Mejía 2007; Pandey et al. 2007). Moreover, some antibiotics are only produced in SSF, even though the corresponding producer fungi can be readily cultivated in SmF (Bigelis et al. 2006; Hölke et al. 2004). The reason for this different physiology in SSF is not fully understood, but it is often called "physiology of solid medium". Some years ago, Biocon India Ltd. started what was going to become a very successful industrial-scale production of lovastatin (and other secondary metabolites) by SSF. Later, the United States Food and Drug Administration (US FDA) approved SSF for generating clinical drugs of fungal origin (Suryanarayan 2003).

Two types of SSF systems are distinguished on the basis of the nature of the solid phase used: (a) SSF on natural solid substrates, which is the most common system, and (b) SSF on impregnated inert supports. Biocon produces lovastatin using the former system, cultivating *A. terreus* on wheat bran SSF. Apparently, the technology is based on the use of the Plafractor, a large-scale SSF bioreactor, although these fermentations can also be performed in other types of SSF reactors (Durand 2003; Giovannozzi-Shermanni and Tiso 2007); or even in tray fermenter, where the solid culture is loaded in trays in thin layers and placed one above other with a gap of a few centimeters into a thermostated room.

The second SSF system involves cultivation of microorganisms on an inert support impregnated with a liquid medium. This method has several potential applications in both scientific studies and may also have potential as a high-production commercial system (Ooijkaas et al. 2000). Although the most popular inert support is sugar cane bagasse, recently, Baños et al. (2009) developed a novel lovastatin production process by SSF on an artificial inert support: polyurethane foam (PUF). Using a wild strain of *A. terreus* (TUB F-514) in this system, physiology of solid medium was clearly manifested, as a 30-fold higher lovastatin production was obtained, compared to that obtained in SmF under analogous conditions (20 mg/gdc (g dry culture) vs 0.62 mg/ml). Moreover, each mg of mycelium from SSF produced 815 µg of lovastatin, whereas each mg of mycelium from SmF only produced 54.7 µg of lovastatin; a specific production more than 14 times higher in SSF.

To gain insight into the molecular events responsible for the difference in the lovastatin production levels between SmF and SSF, the authors performed an expression analysis of two genes (*lovE* and *lovF*) involved in the lovastatin biosynthesis (Barrios-González et al. 2008). Results showed that the higher lovastatin production in SSF correlated with higher transcript accumulation levels for both genes, and for a longer period. *lovE* transcript showed

4.6-fold higher accumulation levels (transcription) than in SmF. Similarly, *lovF* transcript showed a twofold higher accumulation than the highest level detected from mycelium grown in SmF. These results showed, for the first time, that the higher production of lovastatin in SSF is, at least partially, due to a higher transcription of its biosynthetic genes and, most importantly, that this higher transcription levels are the result of higher levels of *lovE* transcription factor (Barrios-González et al. 2008). Moreover, it is very probable that this is an important underlying cause of the higher production reported for other secondary metabolites in SSF.

Later, this group performed basic studies on the environmental stimuli, sensed by the mycelium in SSF, which induce the physiological changes that lead to higher lovastatin production. This allowed the design of genetic improvement methods to generate overproducing strains particularly suited for SSF. Applying these methods they rapidly obtained a mutant (OxB9), from the wild strain of *A. terreus* (TUB F-514), that produced 27.9 mg of lovastatin/gdc in this PUF SSF system (Baños et al. manuscript in preparation).

Production of compactin by SSF has also been reported recently. Shaligram et al. (2008) achieved production of 0.815 mg/gdc by a wild strain of *Penicillium brevicompactum* in a wheat bran-based SSF.

Pravastatin production

Although compactin is not used as a medicine, it is an important source for producing pravastatin, a more efficient derivative (Sankyo Squibb). At first stage, a strain of *Penicillium citrinum* produces compactin (SmF). Pravastatin is normally produced in a second stage by biotransformation (hydroxylation) of compactin by *Streptomyces carbophilus* (Serizawa et al. 1983, 1997). In the course of developing more efficient processes for the industrial production of pravastatin several research groups have performed studied to achieve a higher conversion rate by adding high concentrations of compactin into the culture medium. One important problem is that increasing the concentration of compactin in the culture medium inhibits cell growth and, at still higher concentrations, causes autolysis of the mycelia, resulting in a decrease in the conversion of compactin into pravastatin (Masahiko et al. 1993). The approach of Park et al. (2003) was designing a process where compactin was fed continuously, keeping its concentration constant at at 100 mg l^{-1}. Pravastatin production kinetics was about 1.5-fold higher than that on intermittent feeding of compactin (reaching 1.5 g of pravastatin per liter). On the other hand, Chen et al. (2006) designed a rational screening method to isolate compactin-resistant microbial strains with high hydroxylation activity at the 6β position of compactin. About 2% of target microorganisms were obtained by this screening method. Interestingly, no detectable byproducts were found in the cultures of the best isolates.

It is important to note that Ykema et al. (1999) developed a one-step biosynthesis process to produce pravastatin, by transforming the *P. citrinum* strain with a *S. carbophilus* hydroxylase gene that converts compactin to pravastatin. Some of the transformants obtained in the examples of this patent produced up to 9.5 mg/L, which is rather low. Possibly, a low compactin-producing strain was transformed and used for the examples of this patent.

Simvastatin production by biotechnological methods

With the development in the research of lovastatin and compactin biosynthesis and genetics, more and more attention has been paid to the possibility of obtaining the biosynthesis of the semisynthetic derivatives (mainly simvastatin and pravastatin) by biotechnological processes. For example, simvastatin could be produced by direct fermentation with combinatorial biosynthesis methods, and could also be synthesized from monacolin J with the acyltransferase LovD.

Simvastatin, a semisynthetic derivative of lovastatin, is marketed by Merck as Zocor and is the second-best-selling drug in the USA, with annual sales in 2005 tipping USD$ 12 billion (Kidd 2006). The molecular difference between lovastatin and sinvastatin resides in the side chain on the C-8 carbon position (Fig. 1). In this position, lovastatin carries a 2-methylbutyrate moiety, while sinvastatin has a 2,2-dimethylbutyrate (DMB) moiety. Simvastatin is traditionally prepared chemically by direct alkylation of lovastatin. However, chemical reaction conditions are very rigid, and the final product is difficult to purify, also the pressure of labor protection and environment protection is very high. These multistep processes are laborious, thus contributing to simvastatin being nearly five times more expensive than lovastatin.

One biotechnological approach to its production would be the enzymatic synthesis of simvastatin from monacolin J (MCJ), with the acyltransferase LovD. In 2006, Xie et al. cloned *lovD* gene from *Aspergillus terreus*, and overexpressed this enzyme in *E. coli*. In this way, they were able to characterize this acyltransferase that selectively transfers the α-methylbutyryl group (from the LDKS) to the C8 hydroxyl group of MCJ, to yield lovastatin (Fig. 2). The authors showed that LovD has broad substrate specificity towards the acyl group. Most notably, LovD was able to catalyze the direct acylation of monacolin J by acyl-CoA thioesters or membrane permeable thioesters acyl donors like N-acetylcysteamine (SNAC) thioesters. This work showed the feasibility of using LovD as a biocatalyst for a single-step

synthesis of simvastatin using α-dimethylbutyryl-S-NAC (or α-dimethylbutyryl-S-methylthioglycolate) as a substrate. However, the acylation reaction proceeded with poor turnover, due to substrate inhibition at increasing concentrations of MCJ. This was a result of a weak binding of these acyl donors to lovD, so the second substrate (MCJ) became a competitive inhibitor of DMB-S-NAC.

In a subsequent study (Xie and Tang 2007), the authors identified kinetically superior acyl donors. They found that increasing the thioester chain length by 1 to 3 C resulted in an important increase in affinity (<Km). Kinetic studies showed that this increased affinity and turnover prevented substrate inhibition by MCJ. For economic reasons, the selected acyl donor was DMB-S-methyl mercaptopropionate (DMB-S-MMP). Still, in this in vitro enzymatic process the authors could not get more than 60%, at moderate monacolin J concentrations (>5 mM); due to the competing inverse reaction, which increased the rate of conversion of simvastatin to MCJ, as simvastatin concentrations increased. This made it an inefficient and unviable process.

Surprisingly, when the authors analyzed the in vivo process (i.e., using the whole-cell as biocatalyst) they found that the inverse reaction was 75-fold slower, indicating that this could be a viable process. This convenient phenomenon was due to a detoxifying mechanism, acting like an efflux pump, constantly taking simvastatin out of the cell, together with the permeation barrier that the cell membrane represents to simvastatin. Using this whole-cell biocatalytic process it was possible to obtain 99% conversion rate, to get 10 to 15 mM or 4 to 6 g/L.

In another work (Xie et al. 2007), the process was made faster and cheaper when they deleted gene *bioH* from *E. coli*, generating strain YT2. This gene encodes an esterase that was degrading the side chain precursor DMB-S-MMP. In this way, 15 mM MCJ and 25 mM DMB-S-MMP were transformed in 15 mM simvastatin in 12 h (after additions started). A further improvement of the process consisted in an increase in the concentration of soluble LovD expressed in *E. coli* (Xie et al. 2009b). It was observed that 50% of LovD enzyme, synthesized by the bacteria, remained as insoluble aggregates. The authors applied a strategy of homology modeling and Cys replacement in rational protein engineering. Cys is the only natural amino acid that contains a reactive sulfhydryl group that can form intra- and intermolecular disulfide bonds, leading to insoluble protein aggregates in *E.coli*. In this work, Cys40 and Cys60 of LovD were replaced by alanine and asparagine, respectively, by site directed mutagenesis. The double mutant C40/A and C60/N showed 50% increase in LovD solubility and a corresponding 50% increase in whole-cell LovD activity. Under this conditions, the whole-cell biocatalytic process quantitatively (>99%) transformed MCJ to simvastatin to produce 18 g/L in approximately 18 h (after addition started).

A different approach would be to engineer *A. terreus* so that it can directly produce simvastatin (instead of lovastatin) by fermentation. Van den Berg et al. (2007) used this last approach. The authors propose inactivation of *lovF* gene, to prevent internal accumulation of (lovastatin's side branch) 2 methylbutyrate (2 MB); together with the engineering of the *A. terreus* strain so that it will synthesize DMB (the side chain of simvastatin).

Initially, the authors showed that *A. terreus* (LovD) can also use DMB as a side chain and hence produce simvastatin instead of lovastatin, although DMB is not normally produced by this fungus. Instead, *A. terreus* synthesizes 2 MB by means of LovF (also known as LDKS); through a single condensation between an acetyl-CoA and a malonyl-CoA. However, the authors observed that, in this reaction, *A. terreus* (LovF) can also incorporate methylmalonyl-CoA to produce DMB and then produce simvastatin; although in a small proportion, since LovF preferentially accepts malonyl-CoA. This problem was solved in two stages by modifications in the fungal strain: (a) engineer *lovF* gene with a preference for synthesizing DMB (from methylmalonyl-CoA); and b) provide *A. terreus* with a pathway for in vivo methylmalonyl-CoA production. *A. terreus* was engineered with a pathway for in vivo methylmalonyl-CoA production. This was done by cloning the *Rhizobium* sp malonyl-CoA synthetase gene, whose enzyme product catalyzes the reaction malonate −> malonyl-CoA, and shows an unusually high substrate tolerance, so in vivo produces methylmalonyl-CoA when fed with methylmalonate (external feeding). This gene was PCR amplified and cloned under the control of the *A. nidulans gpdA* promoter (constitutive), and subsequently integrated into the genome of *A. terreus*. Methylmalonyl-CoA was produced by the transformants when the cultures were fed with methylmalonate.

In a second stage, the authors engineered *lovF* to an increased activity to form DMB (from methylmalonyl-CoA). As explained before, polyketide synthase LovF is composed of the following domains in order:

KS − MAT − DH − MT − KR − ER − ACP

(KS = ketosynthase; MAT = malonyl-CoA:ACP acyltransferase; DH = dehydratase; MT = methyltransferase; KR = ketoreductase; ER = enoylreductase; ACP = acyl carrier protein)

The authors engineered this gene to form a protein that showed increased activity to form DMB. It was constructed by substituting the MAT domain from *lovF* with the MAT domain of deoxyerythronolide B synthase from *Saccharopolyspora erythreae*. This construction was cloned to generate the P_{gpdA}-lovF/hybrid PKS- T_{penDE} expression cassette (i.e., under the control of *A.nidulans gpdA* promoter). This construction was then transformed into a

lovF deficient (*lovF*⁻) *A. terreus* strain, already harboring the methylmalonyl-CoA synthesis pathway. The authors also tested an alternative hybrid PKS constructed by exchanging the MT fragment from *lovF*.

Finally, transformants of *A. terreus* with either type of hybrid PKS produced simvastatin in fermentations fed with methylmalonate. Although this is a very interesting work, the authors of this patent use (in their examples) a low producing *A. terreus* strain (ATCC 20542) and do not mention specific yields.

Current and potential uses of statins

The marked lipid-lowering effects of statins have led to a substantial reduction in coronary events, as revealed by clinical, epidemiological and pathological studies (Farnier and Davignon 1998; Galán et al. 2004; Nash 2005; Moride et al. 2008; Seenivasan et al. 2008). In addition, these compounds can prevent stroke and reduce the development of peripheral vascular disease (Maron et al. 2000). Statin therapy has biological effects beyond the level of LDL-cholesterol reduction, including atheromatous plaque stabilization, modification of the atherosclerosis progression, improved endothelial functions, modulates inflammatory responses, and prevent thrombus formation (Wang et al. 2008; Seenivasan et al. 2008).

The multiple effects (i.e., pleiotropic effects) of statins have received increasing recognition and may have clinical applicability across a broad range of cardiovascular and noncardiovascular conditions (Davignon and Leiter 2005). This is related to the fact that statins can also inhibit the synthesis of isoprenoids (farnesylpyrophosphate and geranylgeranylpyrophosphate), which are important lipid attachments for intracellular signaling molecules. Therefore, it is thought that statins might exert "pleiotropic" effects through direct inhibition of these small GTP-binding proteins (Ras, Rho, Rac, and Rap; Fig. 3). Given that the isoprenylated` proteins control diverse cellular functions, it is not surprising that statins might have additional effects beyond lipid lowering (Wang et al. 2008). Recent data indicate that they can directly affect the proliferation/apoptosis balance, down regulating inflammatory chemokines, and the cytogenic messages mediated by these G proteins (Seenivasan et al. 2008).

Due to multiple functions, these wonder drugs have emerged as possible medicines for many other chronic disorders.

The metabolic syndrome (MtS) and diabetes mellitus (DM). Metabolic syndrome is the name given to the collection of risk factors such as dyslipidemia, elevated blood glucose, high LDL cholesterol, and high blood pressure. It is strongly associated with type 2 diabetes and predispose to cardiovascular disease.

MtS and diabetes are associated with underlying inflammatory processes (Nash 2005). The statins may have a modest antihypertensive effect, promote coronary collateral circulation, improve survival in heart failure, and may have favorable effects on glucose metabolism and insulin sensitivity (Massy and Guijarro 2001). Several trials of statin therapy have shown that these agents reduce the risk of cardiovascular events in patients with known DM (Nash 2005).

Cancer Several studies indicated that cancer incidence rates were lower in patients receiving statins. The AFCAPS/TexCAPS trial, found a significantly decreased incidence of new melanomas when evaluating the efficacy of lovastatin in preventing cardiovascular disease. A reduced number of deaths from cancer occurred among patients involved in several large trials of statins for prevention of cardiovascular events (Dulak and Józkowicz 2005). Furthermore, recent observational studies have found large reductions in the risk (20–55%) of site-specific cancers (colorectal, breast, prostate, lung, and pancreatic) with the use of statin therapy (Bonovas et al. 2006; Glynn et al. 2008).

The effect of statins on Ras and Rho protein prenylation might explain this effect on tumor cells, since mutations in Ras have been detected in approximately 30% of human cancers (Fig. 5). In vitro studies have confirmed this effect; since statins inhibited proliferation and triggered apoptosis of tumor cells (Dulak and Józkowicz 2005; Glynn et al. 2008). However, there are still contradictory results (Glynn et al. 2008; Bonovas et al. 2006)

Osteoporosis Statins are also emerging as wonder drugs for bone disorders, such as osteoporosis (Pahan 2006). One of the recent trends is that lovastatin could be used for the treatment of bone fractures. The role of statins on bone formation was shown for the first time by Mundy et al. (1999). Later, epidemiological studies showed that statins have beneficial effects on bone mineral density, reducing the risk of fracture (Edwards and Spector 2002; Pahan 2006). Simvastatin, mevastatin, fluxastatin, and lovastatin have been shown to stimulate bone formation (Edwards and Spector 2002). However, the mechanism of the effect of statins on bone remains to be elucidated (Cummings and Bauer 2000).

The Alzheimer's disease Epidemiological studies suggest that statins reduce the risk of developing Alzheimer's disease (AD; Pedrini et al. 2005; Höglund et al. 2004). Two large retrospective studies suggest that patients taking lovastatin or pravastatin had an approximately 70% lower risk of developing AD (Wolozin 2002). The AD is characterized by accumulation of extracellular and vascular β-amyloid (Aβ) in the brain, which is toxic to many

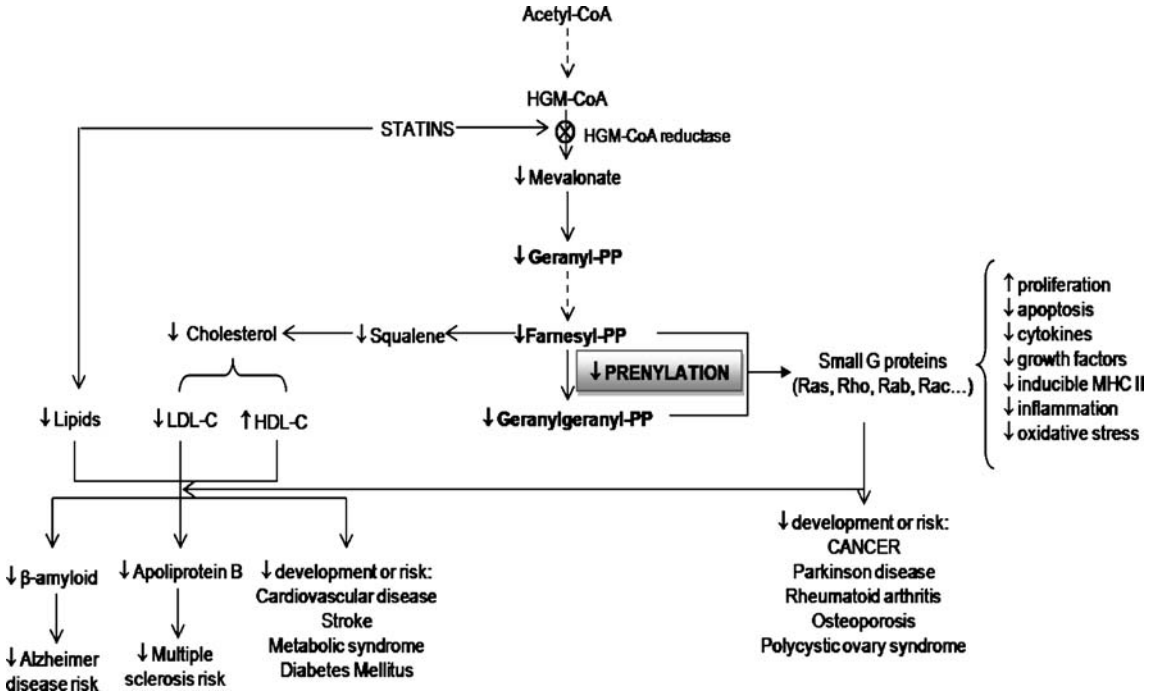

Fig. 5 Model explaining the great variety of biological effects of statins; and hence, current and potential uses. Inhibition of HMG-CoA reductase not only reduces cholesterol levels, but also of isoprenoid intermediates, affecting G proteins (i.e., Ras) prenylation. This can result in the modulation of signal transduction from receptors to gene expression, directly affecting proliferation/apoptosis balance, inflammatory chemokines, and the cytogenic messages mediated by G proteins. Modified from: Cummings and Bauer 2000; Davignon and Leiter 2005; Massy and Guijarro 2001

neurons (Pedrini et al. 2005). In humans, lovastatin has been shown to reduce Aβ levels in blood of patients by up to 40% (Wolozin 2002). Studies in wild-type guinea pigs and in transgenic mice have demonstrated that chronic statin treatment can attenuate cerebral amyloidosis, suggesting that statins may exert their risk-reducing effects in this way (Fassbender et al. 2001; Pedrini et al. 2005). Several studies have suggested an association between Alzheimer's disease and cholesterol metabolism (Höglund et al. 2004; Fig. 5), although the mechanisms underlying this apparent risk reduction by statins are poorly understood (Wolozin 2002).

Parkinson's disease Deregulated lipid metabolism may be of particular importance for central nervous system injuries and disorders, as this organ has the highest lipid concentration next to adipose tissue (Adibhatla and Hatcher 2008).

Although lower LDL levels are linked to Parkinson's disease (PD) risk (Huang et al. 2007), statins may have potentially beneficial effects on this neurodegenerative diseases due to their anti-inflammatory properties (Becker et al. 2008).

Multiple sclerosis Clinical trials have demonstrated that statins can reduce morbidity in patients with multiple sclerosis (MS; Wang et al. 2008). An open-label clinical trial assessing simvastatin in MS revealed a significant decrease in the number and volume of new lesions and a favorable safety profile. Several reports have demonstrated that lovastatin and other statins prevent and reverse chronic and relapsing experimental autoimmune encephalomyelitis, an animal model of MS (Pahan et al. 1997; Neuhaus and Hartung 2007). This was attributed to the immunomodulatory properties of statins and to their induction of a bias toward Th2 cell antiinflammatory cytokine production (Davignon and Leiter 2005).

Rheumatoid arthritis A recent clinical trial has demonstrated immunomodulatory and antiinflammatory effects for statins in patients with rheumatoid arthritis (RA). An extensive in vitro data set defines roles for statins in modifying endothelial function. In vivo data in several inflammatory models suggest that such effects might have immune-modulatory potential (McCarey et al. 2005) and support the hypothesis that statins may be protective against the development of RA (Jick et al. 2009). A good or moderate clinical response was seen in 31% of patients treated with atorvastatin compared to 10% in the placebo group (Turesson et al. 2008). The evidence suggests that statins should be considered in patients with severe RA and an unfavorable cardiovascular-risk profile (Leung et al. 2003; Turesson et al. 2008). Moreover, further

larger studies are required to confirm definitively the safety of statins in patients with RA (McCarey et al. 2005).

Several reports have indicated many potential applications. A couple of studies showed that long-term use of statin leads to a reduced risk of depression in patients with coronary artery disease, possibly by a NO-mediated mechanism (Pahan 2006). Polycystic ovary syndrome frequently coexists with other cardiovascular risk factors. Statins are likely to not only improve the dyslipidemia, associated with this condition, but may also exert other beneficial metabolic and endocrine effects (Kodaman and Duleba 2008). In the treatment of patients with progressive renal disease, the statins can provide protection against kidney diseases characterized by inflammation and/or enhanced proliferation of epithelial cells, like in glomerulonephritis. Moreover, lovastatin therapy has been shown to prevent creatinine clearance decline and to slow renal function loss, particularly in case of proteinuria, and its favorable effect may depend only partially on the attenuation of hyperlipidemia (Seenivasan et al. 2008).

Trends and prospects

A greater understanding of the causes and consequences of heart disease has led to a new emphasis on prevention, with strict new guidelines for disease diagnosis; and doctors taking preventive measures in younger age groups and patients at cardiovascular risk, but without high cholesterol levels. This is increasing the prescription of statins (Mistry 2007; Medco News Alert). It is predictable that in a near future statins will be prescribed for the treatment of other diseases, increasing the demand further.

Although statins have triggered the biggest pharmaceutical boon, the situation is changing fast, since the patents covering the leading statins have began to expire. The patent of lovastatin expired in 2001, while pravastatin and simvastatin came off patent in 2006. The patent of the synthetic Lipitor (atorvastatin) will expire in 2010, Lescol (fluvastatin) in 2011, and Crestor (rosuvastatin) in 2012 (Kidd 2006).

It is foreseen that the competition with generic versions together with the technological advances, like the ones described in this mini review, will reduce the high statins prices. This in turn could increase demand, since it would help to remedy undertreatment of cholesterol problems (Herold 2007); or even by making some new uses economically viable.

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