(REVIEW ARTICLE)

Multifunctionality of statins as antimicrobials

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Abstract

The potential of statins as antimicrobials has been recognized for more than a decade; unfortunately, minimal progress in exploring this promise. This mini review highlights the structure of statins and their relevance as antimicrobial co-drug. The current information on the inhibition mechanism of statins on bacterial, fungal, viral, and biofilms is also described.

Keywords: Fungi 1; Sar-CoV-2 2; Antimicrobial resistance 3; Lactone 4; Biofilm 5; Cholesterol 6

1. Introduction

Antibiotic resistance, a top health threat, has a high economic cost due to longer hospitalization with more intensive care that could force individuals into extreme poverty. Antimicrobial resistance (AMR) is 3rd in the CDC's top 9 health threats which estimates two million AMR cases and 23,000 deaths/yr [1]. AMR also has a high economic cost due to longer hospitalization with more intensive care that could force individuals into extreme poverty [1]. Globally, the United Nations projects that by 2050, the AMR-associated mortality rate could reach 10 million/yr outstripping that of cancer and diabetes combined [2]. This threat is taking on an added urgency with evidence that AMR is being fueled in the current SARS-CoV-2 pandemic. Reports indicate a high prevalence of SARS-CoV-2 patients (>90% in some cohorts) are treated with antibiotics to prevent and/or treat secondary pneumonias and sepsis, and large population segments are self-medicating with antibiotics [3-5]. Overall, this AMR threat is triggered by inappropriate antibiotic prescribing, abuse of antibiotic usage, and a lack of viable drug alternatives due to the low rate of return on investment, which disincentivizes pharmaceutical companies to develop new antibiotics. The most expeditious route to a rapid deployment of novel treatments is to repurpose existing FDA approved non-antibiotic drugs [6, 7]. One class of drugs that has high potential for re-purposing as co-drugs in the treatment of AMR are statins. Statins are a class of drugs which may have utility, since they appear to have intrinsic antimicrobial activity [8, 9] [10-14]. However, their application for use as antimicrobials is hampered by: 1) the lack of statin testing against AR threat organisms; 2) lack of testing at physiologically relevant concentrations in host-congruent environmental conditions; 3) lack of testing for biofilm formation, a readout for phenotypic antibiotic resistance; 4) lack of studies correlating chemical structure with utility as co-drug suicide inhibitors of β-lactamase/carbapenemase and/or synergistic activity with other antimicrobials, and 5) correlating the *in vitro* activity with in vivo efficacy. This review is centered on determining statin's relevance as an antimicrobial co-drug.

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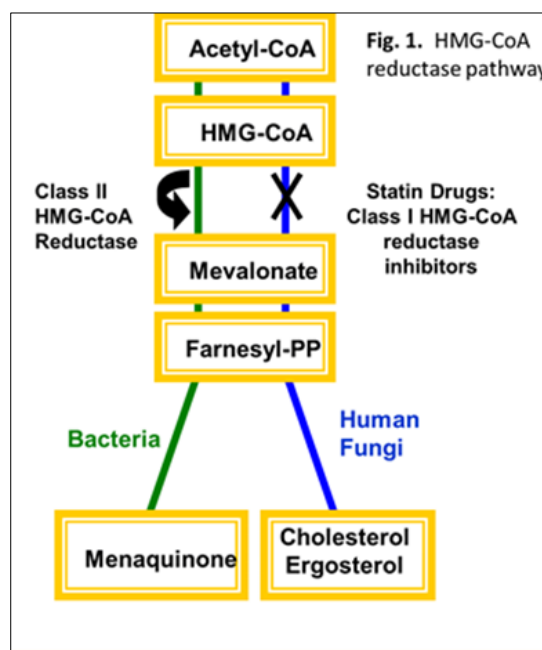
2. Structure and chemistry of statins

Figure 1 HMG-CoA reductase pathway

As one of the most widely prescribed drugs, statins are used as reversible inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase for the treatment of hypercholesterolemia, (HMGCR; Figure 1) and to treat obesity, as serine lipase inhibitors [15, 16]. Generally, statin structures share a pharmacophore, but they vary with regards to whether they are hydrophilic, e.g. rosuvastatin, or lipophilic (e.g. atorvastatin and simvastatin; Figure 2) [17, 18]. In addition, they contain a lactone ring (β or δ). The most reactive of the lactones are the β-lactones, such as tetrahydrolipstatin. β-lactone's four membered rings can function as an acylating agent, covalently modifying enzymes having serine, cysteine, or threonine in their active site, e.g., penicillin-binding proteins (PBPs), and β -lactamases [19-21]. Thus, these compounds with β-lactone rings could have both intrinsic antibiotic activity and/or function as β-lactamase inhibitors [8, 20]. Furthermore, lactones can acylate serine β-lactamases, including class D serine β-lactamase OXA-48, a carbapenemase of significant clinical importance. This indicates that tetrahydrolipstatin may have utility as a suicide-inhibitor co-drug [8, 22]. Analogous structure-activity functionality also appears to hold for δ-lactone statins. Statins, e.g. simvastatin, have both a gamma lactone (6-membered lactone) and a plain ester, so they can be regarded as having 2 groups that can be attacked by a serine or cysteine nucleophile [23]. Thus, these carbonyl-lactone compounds would function as acylating agents of serine/cysteine enzymes in a manner parallel to that of β-lactone statins [24]. In addition, the δ-lactone statins exhibit other variations in chemical structure that may contribute to differences in intrinsic antimicrobial activity and/or potential inhibition of β-lactamase/carbapenemase activity. One set of differences between the different members of the δ-lactone statins is whether the compounds are lipophilic (atorvastatin and simvastatin) or hydrophilic (rosuvastatin) (Figure 2). These properties would affect the ability of the statin to intercalate into the membrane and affect PBPs or other target sites. Another structural difference is whether the lactone ring is open (atorvastatin) or closed (simvastatin). The importance of an open- vs. closed-ring δ-lactone is uncertain since *in vivo* studies indicate adjunctive treatment efficacy for both structures. For example, pravastatin, an open δ-lactone ring statin, was effective in the reduction of recurrent urinary tract infections, while both open and closed ring δ-lactones (atorvastatin and simvastatin, respectively; Figure 2) were highly effective in the treatment of periodontitis [25-27]. Further *in vivo* findings correlate enhanced recovery and reduced risk of adverse sequelae and death from surgery, surgical implants, and sepsis in individuals taking statins [28]. Correspondingly, patients on statin therapy have a lowered incidence of fungal infections [9, 29]. In contrast, other studies of patients with sepsis and inflammatory diseases do not support a role of statins in adjunctive therapy [30-32]. These conflicting findings in regard to statin utility need to be addressed by delineating *in vitro* activity under host relevant conditions. In general, statins can be divided into two classes, Type I and Type II statins. Type I statins possess a decalin ring and are derived from fungal metabolites. Type I statins are powerful but are difficult to synthesize and possess a great deal of side effects. Type II statins were created after Type I and are easier to synthesize [33, 34]. There is a consistent structural motif that is shared between both types of statins, allowing them to fit in the active site of the HMG-CoA reductase enzyme. Through

this function, they halt production of mevalonate, a key intermediate in both the cholesterol and menaquinone synthesis pathways [35]
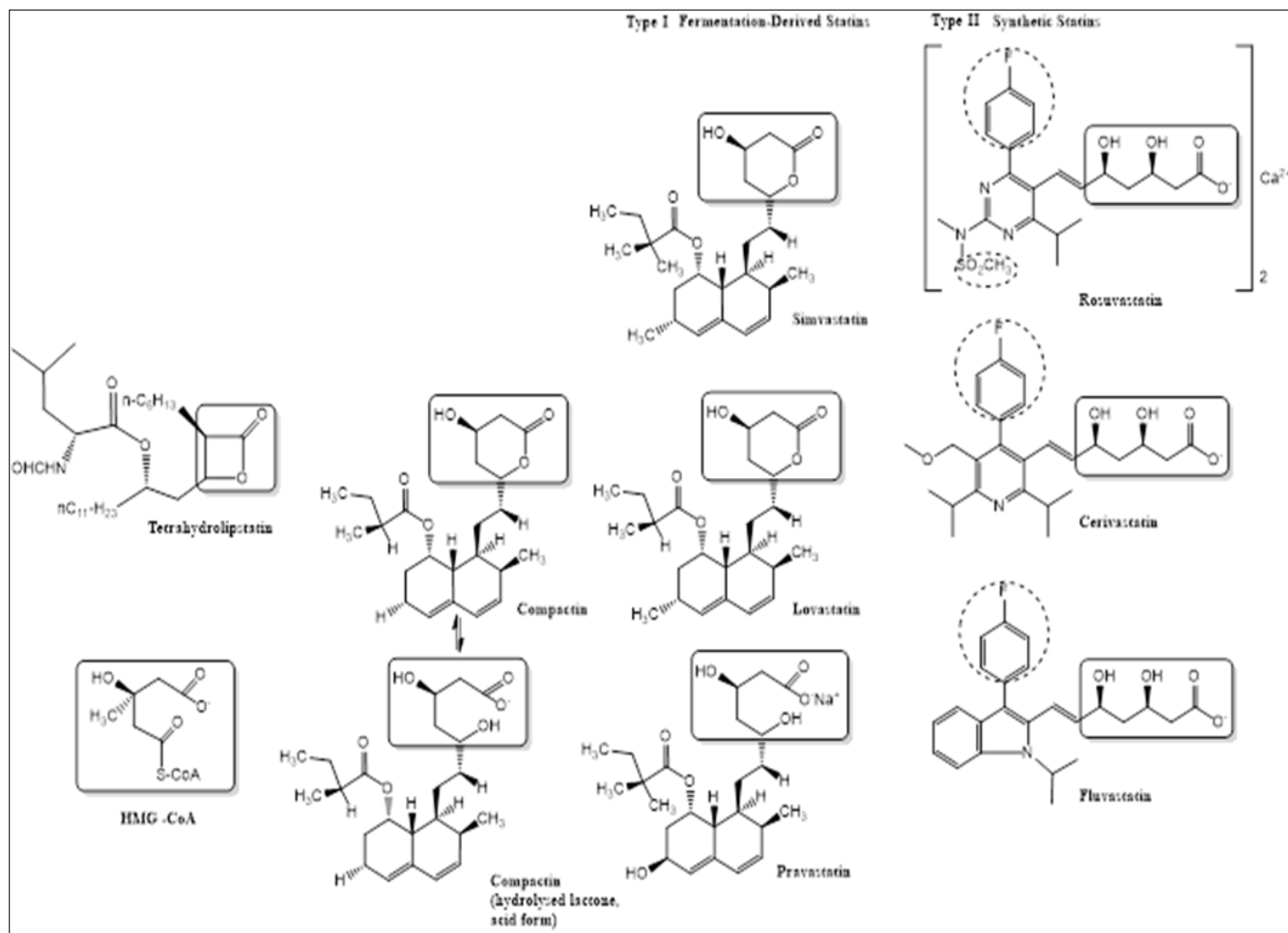

Figure 2 Lovastatin and simvastatin are lactone prodrugs, which rapidly convert to the active free (3R,5R)-3,5-dihydroxypentanoic acids *in vivo*, which in turn inhibit HMG-CoA reductase. All Type II statins have a 4-fluorophenyl group that closely covers the ester side chain from Type I statins, thus significantly contributing to potency. Rosuvastatin has the greatest number of binding interactions with HMG-CoA reductase

3. Statins and biofilms

In addition, biofilm formation is also inhibited at similar statin concentrations [11, 36]. Therefore, statins have the potential to inhibit the growth of drug-resistant bacteria and fungi, as well as interfere with biofilm formation. However, the *in vivo* applicability of these findings is in doubt, since the concentrations used *in vitro* are 100 to 1000 times higher than the maximum plasma concentration (Cmax) [18, 37-40]. The importance of statin concentration was demonstrated by the PI who showed that at or proximate to simvastatin Cmax, biofilm formed by methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) to the extracellular matrix material, fibronectin, was significantly ($p < 0.05$) enhanced, particularly for clinical isolates. The ability to correlate *in vitro* activity to reported *in vivo* efficacy findings is further complicated by the fact that microbes growing in the host do so in highly reduced oxygen conditions (hypoxic to anoxic) [41-45]. These contradictory findings point out the fundamental flaws in antimicrobial testing, i.e., high statin and atmospheric (21%) oxygen concentrations used in *in vitro* studies.

4. Statins microbial growth and resistance

In vitro, statins can inhibit bacterial and fungal growth, as well as modulate biofilm phenotype expression against gram-positive and gram-negative bacteria [10, 36-38, 46]. Simvistatin, atorvastatin, florvistatin, exhibit concentration specific inhibition of bacterial growth. It is hypothesized that this effect is the result of a disruption of the cell membrane through

van der Waals interactions and hydrogen binding from the methyl group of the gem-dimethyl moiety in statins [47]. However, this effect is highly concentration specific, reversing at high statin concentrations. However, as with *in vivo* studies, findings are contradictory due to statin concentrations used, solvent effects and environmental testing conditions. Experimentally, statins are most effective against bacteria with an intact mevalonate pathway, e.g., staphylococci, streptococci, enterococci and fungi, which have class I HMGCR [34, 35, 46, 48, 49]. Importantly, this antibacterial activity extends to AMR bacteria, including vancomycin-resistant enterococci (VRE), MRSA, and both *S. aureus* vancomycin intermediate and resistant strains (VISA and VRSA, respectively). Steroids, including cholesterol, and carotenoids share part of their biosynthetic pathway. Recent findings indicate that statins may have efficacy in treatment of *S. aureus* by blocking staphyloxanthin (carotenoid) associated virulence [46]).

Statins have also been demonstrated to reduce levels of dehydroepiandrosterone (DHEA) [50]. While the exact mechanism that statins can exert this effect is unclear, it is thought that the reduction of cholesterol, in turn, reduces DHEA levels due to cholesterol's role as a substrate in DHEA synthesis [51]. This activity may have utility in the control of staphylococcal infections. Since DHEA levels also impact phenotypic vancomycin resistance and cell wall architecture in *S. aureus,* it is possible that statins may have indirect *in vivo* effects which impact treatment failure [52-54]. Clearly, the co-evolution of microbial communities and their hosts have resulted in the development of complex systems of communication which is just beginning to be explored.

More directly, statins are shown to have utility in the prevention and treatment of infection with *Mycobacterium tuberculosis* (Mtb)*.* Tuberculosis infection is highly dependent upon binding of the Mtb to cholesterol enriched domains of a host macrophage [55]. If the amount of cholesterol in the macrophage decreases, the Mtb infection will be inhibited due to its dependency of binding to cholesterol domains. Therefore, by reducing the amount of cholesterol synthesis, statins can help inhibit tuberculosis infection [56].

In an alternative pathway, certain extremophiles, particularly cyanobacteria and archaebacteria, have been shown to utilize hopanoids, such as bacteriohopanetetrol (BHT). Hopanoids, composed of lipophilic hydrocarbon rings, are structurally similar to sterols and share functions as a membrane lipid as illustrated in Figure 3.

Eukaryotic sterol synthesis may follow one of two routes beginning with the initial cyclization of 2,3-oxidosqualene to lanosterol via oxidosqualene cyclase (OSC) [lanosterol synthase], resulting in cholesterol for vertebrates and ergosterol in fungi. In contrast, plants utilize cycloartenol synthase to convert oxidosqualene into cycloartenol (Figure 3). Recent studies have found that both described pathways are utilized by certain bacterial species (sterol synthesis in diverse bacteria). Interestingly, inhibition of OSC has been proven as a potential treatment for certain bacterial and fungal infections and is serving as a target for novel design strategies for OSC inhibitors.
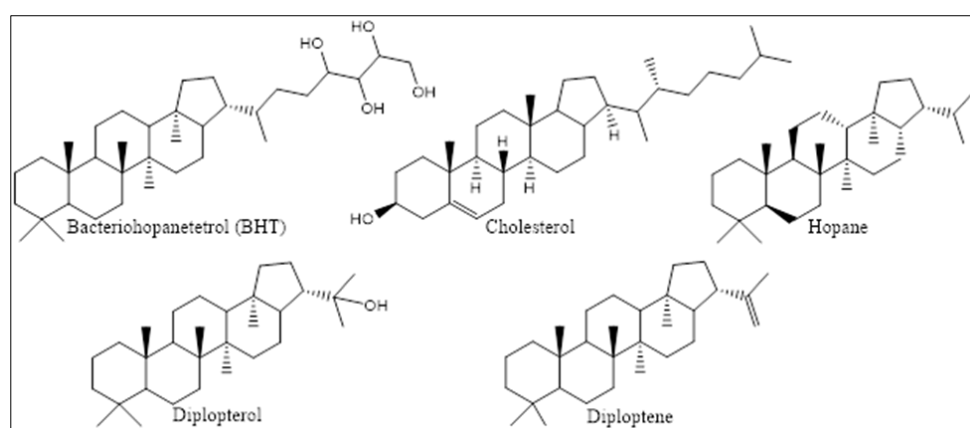

Figure 3 Examples of hopanoids as the functional analogs of cholesterol in bacteria. Hopanoids are some of the most ubiquitous cyclic isoprenoidal lipids in the sedimentary record, and they have been used as molecular proxies for ancient microbial life. Importantly, hopanoid synthesis does not require molecular oxygen, and hopanoids have been reported in sediments predating the enrichment of oxygen in Earth's atmosphere.

4.1. Fungal metabolism

Statins have been demonstrated to have other beneficial effects besides the intended use of inhibiting cholesterol production. Certain statins have been shown to inhibit the production of production ergosterol, a key cell membrane component in fungi and some protozoa (Figure 4). Like humans, these organisms use HMG-CoA reductase for the same

enzymatic step, with the final product being ergosterol, an essential fungal membrane sterol, rather than cholesterol. Statins can exhibit antifungal properties via the reduced production of ergosterol resulting in growth inhibition [10]. Simvastatin reduces ergosterol levels, inhibits growth, and causes loss of mtDNA in *Candida glabrata*. This is an interesting juxtaposition since naturally occurring statins, e.g. lovastatin, are synthesized by a wide variety of fungi [57].
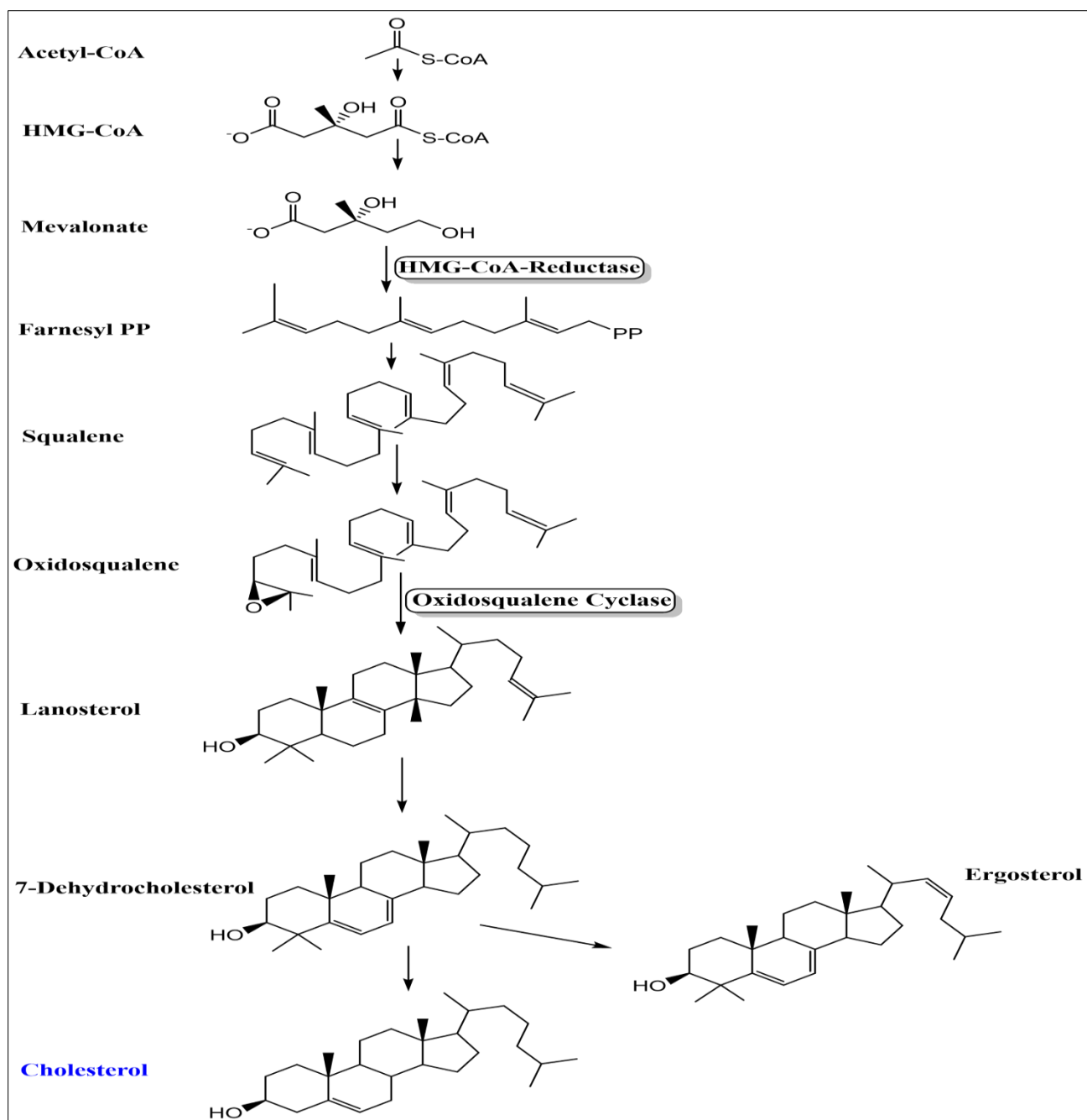

Figure 4 The cholesterol (in vertebrates) and ergosterol (in fungi and leishmania) utilize squalene as starting material, which is obtained from long chain precursors like farnesyl pyrophosphate. Epoxidation of squalene in the presence of squalene epoxidase furnishes squalene epoxide which is successively transformed into lanosterol, and consequently to 7-dehydrocholesterol. The latter is the precursor of cholesterol and ergosterol.

Lovastatin was isolated from the *Aspergillus terreus* cultures in 1979 [58]. Synthesis of the compound relies on the action of polyketide synthases and occurs during secondary metabolism [59]. Select edible non-toxic mushrooms are also a source of statins. It has been shown specifically that varied species of *Pleurotus* contain significant amounts of natural organic compounds, such as lovastatin [60]. The *Pleurotus ostreatus*, also known as the pearl oyster mushroom, is commonly found in European countries year-round and produces lovastatin during secondary metabolism [61].

Similarly, the *Volvariella volvacea*, also known as the straw mushroom, produces lovastatin as a metabolic byproduct [62]. Other notable fungi that produce lovastatin include *M. purpureus, M. pilosus, A. flavipes, A. flavus, A parasiticus, and Accremonium chrysogenum* [63]. Mevastatin, like lovastatin, can be isolated from the *Aspergillus terreus*. It was first isolated from the mold *Penicillium citrinum* in the 1970s, and is created in a polyketide synthase pathway similar to lovastatin [63].

4.1.1. DHEA and fungi

Dehydroepiandrosterone (DHEA) has recently been discovered as the most effective steroid hormone in inhibiting ergosterol synthesis in fungi, along with less effective androstenedione and testosterone. Species studied include ascomycetous fungi *Hortaea werneckii, Saccharomyces cerevisiae* and *Aspergillus oryzae*. This inhibition alters the sterol composition within the fungal plasma membrane, therefore compromising its structure and functions [64]. Researchers have proposed the reason DHEA is the most effective steroid hormone may be due to its constant presence in individuals, regardless of biological sex [65].

4.1.2. Carotenoids and fungi

Carotenoids are important hydrophobic tetraterpenoids that are produced by microbe's bacteria and fungi. Sterols carotenoid derived trisporoids hold functions related to the success of early sexual reproduction and mycelial evolution in Zygomycetes fungi. Acting as pheromones, trisporoids enable the fungi to identify and attract mates [66].

4.2. Bacteria

4.2.1. Cholesterol, Sterol Derivatives-Sterol Glycosides

A wide range of various combinations of sterols and carbohydrates has been well documented in fungi and algae in the form of derivatives sterol glycosides (also known as sterol acyl-glycosides). Although the biosynthesis of sterols is distinctive to eukaryotes, a few bacteria have been shown to possess this ability, or acquire sterols via absorption from the host supply. The discovery of sterol glycol-syltransferase genes has led to identification of steryl glycosides in microorganisms such as bacteria and fungi, and genetic testing has allowed the discovery of function within these species. Studies have shown that muting certain sterol glycosides essential to the pathology of some bacteria and fungi will cause inhibition, proposing a novel method for treating infections such as *Helicobacter pylori*, a gram-negative bacterium which causes chronic gastritis and peptic ulcerations within its human host [67].

H. pylori is a cholesterol auxotroph and is therefore dependent on its host for cholesterol. *H. pylori* obtains cholesterol from the plasma membranes of epithelial and antigen-presenting cells and of cells of its host. The cholesterol is then modified for use in immune evasion [68]. When its cholesteryl alpha-glycoside biosynthesis is inhibited, *H. pylori* is no longer able to modulate the immune system of its host. The cholesteryl α-glycoside has been observed to protect *H. pylori* from the human T cell immune response, thereby avoiding phagocytosis by macrophages [67]. Similarly, *Borrelia burgdorferi*, the agent of Lyme disease, utilizes host cholesterol to produce cholesterol glycosides. Studies investigating whether there is a correlation between elevated blood lipids and Lyme disease severity and chronicity suggest that decreased cholesterol levels may enhance patient prognosis [69]. In addition, steryl glycosides and cholesterol in *Helicobacter* and *B. burgdorferi* as well as certain fungi, e.g., the yeast *Pichia pastoris,* modulates membrane fluidity [67].

4.3. Antifungal activity

The antifungal properties of statins are a burgeoning field of research showing both varied mechanisms of action, and synergism with other more traditional antifungals. One of the primary mechanisms by which statins affect fungal growth relates to their deregulation of the production of ergosterol. Treatment of *C. galabrata* strains with simvastatin and atorvastatin reduced ergosterol production and consequently growth inhibition. Interestingly, reduction in ergosterol production is not solely through inhibition of HMG-CoA reductase. Furthermore, cultures treated with the statin demonstrated an increased incidence of petite mutants that lack the ability to grow under fermentation conditions; showing complete loss of mtDNA when sequenced. This illustrates that in addition to the inhibitory effects of statins on fungal ergosterol production, statins also have a pronounced effect on the activities of many membrane-localized enzymes, and disrupt pathways related to mitochondrial function. This indicates a potential role in the apoptosis cascade as part of their antifungal activity [10]. This is further indicated in study showing lovastatin inducing apoptotic like events in *Mucor racemosus* [70]. Part of this proposed pathway is via the disruption of protein isoprenylation, as seen with atorvastatin effects on *Saccharomyces cerevisiae* which affects downstream cytochrome c function and respiration via heme A production disruption [71-73]. Additionally, lovastatin was shown to affect *Candida albicans* expression of ERG enzymes ERG11 and ERG3 which further disrupts ergosterol production [74]. Interestingly, due to these modifications, there can be some stimulation of virulence factors as such in invasive *Candida* via inhibiting

inhibition of Y-H transition and potentially stimulating biofilm formation *in vitro*. This is an ambiguous affect though in that in this same study potent antifungal biofilm activity was observed both alone, and with azole supplementation [75]. This antibiofilm effect is further seen in other studies with prevention of biofilm growth with simvastatin of both *Candida* spp. and *Cryptococcus* spp. as well nonpathogenic *Candida* spp. [76]. In *in vivo* mouse models survival has also been enhanced by the addition of pravastatin which inhibits farnesol production in *C. albicans* [77]. Statins alone do show pronounced antifungal affects even below minimum inhibitory concentrations; however, these concentrations tend to surpass maximum available concentration ranges within human serum by a significant margin, necessitating their use in conjunction with other antifungal agents for suppression of active infection [78].

5. Synergistic effects of Antifungal Medication

It has been observed that statins exhibit a synergistic effect with azoles. Some statins are ineffective alone reduce the minimum inhibitor concentration of various azoles against *C. glabatra* while statins like fluvastatin which demonstrate synergistic effects when paired with azoles [79]. This synergism is theorized to be due to the downregulation of ergosterol production resulting in increased cell membrane permeability facilitating the uptake of azoles [76]. In *in vivo* studies, mice infected with *C. gatti* show a significant reduction in fungal spores when a combination treatment of fluconazole and atorvastatin is used as compared to mice treated with fluconazole alone. Similarly, *Cryptococcus* infected animals treated with monotherapy of fluconazole were more symptomatic than when treated with combination therapy with atorvastatin [80]. In a retrospective study, type 2 diabetics who had at least one prescription of a statin compound prior to GI surgery showed a clinically significant reduction in positive *Candida* cultures (p=0.03) and length of infection (p=0.01) [81]. However, these effects are not universal across statins. The synthetic statins, i.e., those possessing 4-fluorphenyl group, and/or with high lipophilicity, exhibit the greatest activity. Thus, pravastatin was one of the least potent, and fluvastatin, the most potent in fungal inhibition [75, 82].

Beyond azole antifungals, studies with polyene antifungals in combination with statins have shown variable additive to synergistic effects, depending on target and drug [79]. Further studies have also shown synergistic effects of various statins with allylamines as well as echinocandins, and griseofulvin [83, 84]. However, some studies have shown that specific combinations of antifungals, statins, and pathogen target can occasionally have antagonistic effects, making further studies necessary if combination therapy is to be used in clinical scenarios and furthermore raises the question of cautious antifungal use in individuals currently on statin therapy [85, 86].

6. Viruses and Statins

There is a growing body of research that statins may improve clinical outcomes in certain viral diseases. Retrospective studies have shown reduced complications in patients using statins with influenza [87]. Individuals had a reduced risk of dying if they were already taking a moderate dose of statins before infection, as compared with those who did not take statins, and patients who took statins before or during hospitalization for influenza appear to have a lower risk of dying from the infection. In individuals with hepatitis C virus (HCV) infections the addition of statins to their therapy has improved outcomes [88]. However, this combination therapy must be approached with caution to avoid toxicities, since many statins and antivirals utilize the CYP3A4 system [89]. Fortunately, utilization of the CYP3A4 system is not the sole mechanism for antiviral therapy.

The antiviral effects of statins seem to occur through multiple different processes. Recruitment of lipid rafts for viral entry is frequently a critical step for infection of many viruses [90]. In the case of HIV it was found that statins have demonstrated some activity against HIV-1 by disrupting lipid raft formation via Rho guanosine triphosphatase inhibition [91]. As discussed with antifungals, the inhibition of prenylation also plays a role in the potential antiviral properties, in the case of respiratory syncytial virus (RSV), the requirement of F protein binding to an activated Rho-a needs geranylgeranyl-transferase for the isoprenylation of the molecule to activate. The inhibition of prenylation pathways by the suppression of HMG-CoA reductase was shown in a trial to reduce RSV viral loads [92]. This inhibition of prenylation is also seen to affect viral assembly; statins observed inhibiting HCV via the disruption of geranyl geranylated FL2 protein production [93].

Statins have also been shown to inhibit some viral assembly pathways via the mevalonate pathway [94]. Another potential mechanism of action is statin's influence on the redistribution of cholesterol which occurs via viral infection. An increased level of cholesterol has been observed localized to the endoplasmic reticulum in viral infection as well increased concentrations of mRNA for HMG-CoA reductase. Inhibition of these viral processes with HMG-CoA reductase inhibitors showed disrupted replication of dengue virus (DENV) *in vitro* [95, 96]. Further study has indicated the potential inhibition of viral bud formation via reduced availability of cholesterol, interference with Nef, a regulatory

protein which downregulates MHC-1 presentation on infected cells, and interference in intracellular spread by interference with adhesion molecules, ICAMs in particular [97-99]. Ultimately the body of research into statins as potential antivirals though is currently incomplete. There have been observed deleterious effects like statin's ability to reactivate hepatitis B virus (HBV) infection or increased herpes zoster infection (varicella-zoster virus; VZV) [100, 101]. A greater investigation is needed to elucidate the finer points of their effects.

There is the potential use of statins in the control of downstream events such as clot formation. A growing amount of data shows that intense inflammation, blood clots, and stroke are some of the most severe symptoms of COVID-19. Decades of research have also shown that aside from lowering cholesterol, statins decrease inflammation, reduce blood clots, and prevent damage to endothelial tissue—the thin layer of cells that line blood vessels and other organs. Experimental studies and clinical trials have shown that statins can lower levels of thrombin, an enzyme in blood plasma that assists in blood clotting, and increase levels of thrombomodulin, a protein expressed on endothelial cells that reduces blood coagulation. That tissue is also targeted by COVID-19. One of the other consequences of severe SARS-CoV-2 infection is widespread inflammation in the lungs—also called acute respiratory distress syndrome, or ARDS. Research has shown statins can ameliorate ARDS, although the mechanism has not been fully delineated. Thus, statins may have some utility in the prevention or amelioration of these downstream effects. Direct antiviral effects may be mediated via altered receptor expression. *In vitro* studies indicate that when the cholesterol level in the cell membrane is high, ACE2—the receptor the virus uses to invade cells—sits at a spot on the cell surface primed for endocytosis, and viral entry. However, when cholesterol levels are low, ACE2 is present in a region that isn't primed for endocytosis, thus reducing the efficiency of viral entry.

7. Conclusion

The most efficient approach to fast-track therapeutics for use in the treatment of antibiotic resistant organisms is to repurpose existing FDA-approved drugs. Statins are a class of drugs that may have utility since they appear to have intrinsic antimicrobial activity.

However, their application for use as antimicrobials is hampered by:

- the lack of statin testing against AR threat organisms;
- lack of testing of statins at physiologically relevant concentrations in host-congruent environmental conditions;
- lack of testing for biofilm formation, a readout for phenotypic antibiotic resistance;
- lack of studies correlating statin chemical structure with utility as co-drug suicide inhibitors of β-lactamase/carbapenemase and/or synergistic activity with other antimicrobials.
- correlating the *in vitro* activity with *in vivo* efficacy.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that no conflict of interest exists.

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