Antibacterial Effect of Some Eukaryotic Sterol Biosynthesis Inhibitors

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Abstract

Background: Isoprenoids and their derivatives are building blocks for the synthesis of biomolecules with important biological functions such as cholesterol in eukaryotes and lipid carrier undecaprenol, which is involved in cell wall biosynthesis in bacteria. With the global threat of multidrug-resistant bacteria, there is a need for finding new metabolic targets for killing bacteria. In the present study, we examined the impact of eukaryotic sterol biosynthesis inhibitors on the growth of four pathogenic bacteria.

Materials and Methods: Antibacterial effect of HMG CoA reductase inhibitor (simvastatin), farnesyl pyrophosphate synthase inhibitor (alendronate), squalene epoxidase inhibitor (terbinafine), and lanosterol demethylase inhibitor (ketoconazole) were studied against four pathogenic bacteria: two gram-positive bacteria, Staphylococcus aureus and Enterococcus faecalis and two gram-negative bacteria, Escherichia coli and Pseudomonas aeruginosa. Broth microdilution method was used for assessing the antibacterial susceptibility of the components using 96 well plates. MIC and MBC were determined visibly.

Results: MIC of Ketoconazole for Staphylococcus aureus and Enterococcus faecalis were 0.166 and 1 mg/mL, respectively. Terbinafine had a weak inhibitory effect on Staphylococcus aureus (MIC: 8 mg/mL). Ketoconazole and terbinafine had no inhibitory effect on gram-negative bacteria. MBC of Simvastatin for both Staphylococcus aureus and Enterococcus faecalis was 0.5 mg/mL and of Alendronate for Pseudomonas aeruginosa was 6.6 mg/mL.

Conclusion: Our results show that farnesyl pyrophosphate synthase and class II HMG-CoA reductases inhibitors (ketoconazole and simvastatin) have reasonable antibacterial activity against gram-positive bacteria. These two enzymes provide suitable targets for designing new antibiotics based on modifying the chemical structure of currently used drugs to obtain maximum activity.

Keywords: Antibacterial agent, antibiotics, biosynthesis, inhibitors, isoprenoid, sterol

INTRODUCTION

The development of bacterial resistance to antibiotics and the deficit in the findings of new bacterial targets have raised global concern. Isoprenoid biosynthesis is an essential highway that provides a key route for the synthesis of thousands of vital biomolecules such as coenzyme Q in electron transport chains, hopanoids in bacteria and sterols in eubacteria and eukaryotes membranes components, and carotenoids in eukaryotes and prokaryotes. This pathway is vital for bacterial life; therefore, blocking the enzymes involved may kill bacteria. Many of the enzymes that are involved in isoprenoid biosynthesis in eukaryotes are drug targets; for example, simvastatin inhibits the early stage of cholesterol biosynthesis in humans to reduce serum cholesterol, while terbinafine exerts its fungicidal
effect by blocking the end stages of sterol biosynthesis in fungi.\(^5\)

Isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are important intermediate molecules for isoprenoid biosynthesis.\(^6\) The final product of IPP metabolism in eukaryotes is cholesterol, but in bacteria, it is converted to a wide variety of biomolecules including lipid carrier undecaprenol,\(^7,8\) which are, respectively, involved in bacterial cell wall biosynthesis and electron transport.

There are two main pathways for the biosynthesis of IPP in bacteria, glyceraldehyde 3-phosphate (GAP)–pyruvate pathway found mostly in gram-negative bacteria\(^9\) and mevalonate pathway found in gram-positive bacteria.\(^2,8\)

However, analysis of microbial genome sequences has revealed that some gram-negative bacteria encode only enzymes of the mevalonate pathway.\(^10\) In humans and bacteria such as *Staphylococcus aureus*, isoprenoids are formed in the mevalonate pathway. The mevalonate pathway in eukaryotes and eubacteria begins with the production of HMG-CoA from acetoacetoy-CoA and acetyl-CoA by HMG-CoA synthase.\(^11\)\(^-\)\(^13\)

The next step involves HMG-CoA reductase, which converts HMG-CoA to mevalonic acid. Statins target this enzyme in humans to lower blood cholesterol levels.\(^14\) In *Enterococcus faecalis*, HMG-CoA synthesis and subsequent reduction are performed by a dual-action enzyme.\(^14\) Pravastatin has been reported to inhibit purified bacterial HMG-CoA reductase in vitro.\(^15\) Mevalonate is converted to IPP, and then farnesylpyrophosphate synthase condenses IPP and DMAPP to form farnesylpyrophosphate. In humans, bisphosphonates (alendronate) used to treat osteoporosis, strongly inhibit this reaction to induce apoptosis in osteoclasts.\(^16\)\(^-\)\(^17\)

A gram-positive bacteria *Staphylococcus aureus* has been reported to engage FPPS.\(^18\)\(^-\)\(^19\)

Condensing two molecules of FPP yields squalene, which is epoxidized and then cyclized to form lanosterol.\(^20\) Fungal squalene epoxidase is selectively inhibited by the allylamines and terbinafine.\(^21\)

Lanosterol is then converted to zymosterol by sterol demethylase, a reaction that is blocked by azoles class of antifungal drugs such as ketoconazole, miconazole, and clotrimazole.\(^22\)

Some kinds of bacteria such as *Streptomyces* strains contain monoxygenases, which may be homologs to sterol demethylase that is inhibited by azoles.\(^23\)

As shown here, isoprenoid biosynthesis can be a new and promising target for finding new antibiotics. We can find several drugs in drugstores that have been designed for eukaryotic pathogens. Furthermore, there are many similarities in isoprenoid and sterol biosynthesis between eukaryotes and prokaryotes. In the present study, to assess the antibacterial properties of some eukaryotic sterol biosynthesis inhibitors, we selected two gram-positive bacteria having enzymes of the mevalonate pathway and two gram-negative bacteria having alternative pathways as reference.

## Materials and Methods

### Chemicals and microorganisms

Simvastatin, alendronate, terbinafine, and ketoconazole were purchased from pharmaceutical companies (Poorsina or Osve, Iran). The culture medium was purchased from Merck (Germany). The organisms, including *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 25992), *E. coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from Pasteur cell bank.

### Antimicrobial susceptibility assay

At first, a suspension of each bacterium up to turbidity equal to that of a 0.5 McFarland standard was prepared in sterile saline. Different concentrations of each test compound, simvastatin, alendronate, terbinafine, and ketoconazole ranging 8–0.015 mg/mL were prepared by dissolving the statin in absolute methanol.

For measuring minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), we used a serial dilution assay in 96 well plates. At first 25 µl of TSB, then 200 µl bacterial suspension (1.5 × 10\(^5\) bacteria/mL, equal to 1/1000 of 0.5 McFarland standard), and finally 25 µl of drugs were added into wells. Two wells for solvent and growth control were included in each set of the experiment. Plates were incubated at 37°C for 24 h.\(^24\)

All the experiments were performed in triplicate. The MIC was determined as the lowest concentration of the drugs that inhibits bacterial growth so that had no visible turbidity. MBC was determined by subculturing the clear wells (two wells before and after the well that showed MIC) to agar plates that do not contain the drugs. Plates were incubated at 37°C for 24 h.

### Statistical analysis

Student t-test was performed to compare treatments with growth control using Excel software. P value <0.05 was considered to be statistically significant. The results were expressed as Mean ± SE.

### Results

After 24 h of incubation under aerobic conditions, simvastatin showed inhibitory effects on gram-positive bacteria. MIC and MBC for *Staphylococcus aureus* were 0.5 ± 0 mg/mL and for *Enterococcus faecalis* was 1 ± 0 mg/mL (*P* = 0.0001). However, simvastatin had no inhibitory effect on gram-negative bacteria [Table 1].

Alendronate could inhibit the growth of *Pseudomonas aeruginosa* in a concentration higher than 6 mg/mL but had no inhibitory effect on other bacteria. In this case, MIC was 6.66 ± 2.3 (*P* = 0.0001) and MBC was 8 ± 0 mg/mL. Terbinafine in a concentration equal to 8 ± 0 mg/mL could inhibit the growth of *Staphylococcus aureus* (MIC was equal to MBC = 8 ± 0 mg/mL).

Lowest minimum inhibitory and bactericidal concentration of ketoconazole was seen against *Staphylococcus aureus*,

| Organism          | MIC (mg/mL) | MBC (mg/mL) |
|-------------------|-------------|-------------|
| *Staphylococcus aureus* | 0.5 ± 0     | 8 ± 0     |
| *Enterococcus faecalis* | 1 ± 0     | 8 ± 0     |
| *Pseudomonas aeruginosa* | 6.66 ± 2.3 | 8 ± 0 |
in which MIC = 0.166 ± 0.07 (P = 0.001) and MBC = 0.416 ± 0.1 mg/mL (P = 0.018). Furthermore, ketoconazole could reduce the growth of Enterococcus faecalis, MIC = 1 ± 0 mg/mL and MBC = 2 ± 0 mg/mL (P = 0.0001). Predictably, ketoconazole had no inhibitory effect on gram-negative bacteria.

**Discussion**

Our results show that tested drugs, which are known as sterol biosynthesis inhibitors, had an inhibitory effect mainly on gram-positive bacteria. Action sites of the drugs on the mevalonate pathway are shown in Figure 1.[25]

Simvastatin has proven an ability to inhibit HMG-CoA reductase.[4] Enterococcus faecalis and Staphylococcus aureus have close relationships in the phylogenetic tree because of having mevalonate pathway and its rate-limiting enzyme HMG-CoA reductase.[26] Therefore, it has a great probability that simvastatin could affect the growth of two-gram-positive bacteria through inhibition of HMG-CoA reductase.

Two classes of HMG-CoA reductase have been identified. The class I genes are present in all eukaryotes, some archaea, and Streptomyces, and class II genes are present in some Eubacteria[22] such as Staphylococcus aureus.[12] These two classes of HMG-CoA reductase are structurally different.[27] Therefore, it is obvious that a higher concentration of simvastatin, a class I inhibitor, is needed to inhibit class II that is present in bacteria. This means simvastatin is less specific for class I HMG-CoA reductase than class II. This nonspecificity is seen for all drugs used in this study.

_Pseudomonas aeruginosa_ is a gram-negative bacteria independent of the mevalonate pathway but has farnesyl pyrophosphate synthase as a part of the isoprenoid biosynthesis pathway [Figure 1]. Farnesyl pyrophosphate synthase is inhibited _in vitro_ by the bisphosphonate class, which includes alendronate.[28] Our results show that alendronate inhibited the growth of _Pseudomonas aeruginosa_ at a concentration not lower than 8 mg/mL. Alendronate is a highly negatively charged bisphosphonate that made it difficult to pass through the bacterial membrane. This effect can be seen in other bacteria with different cell wall compositions. As seen in Table 1, alendronate had no significant inhibitory effect on _Staphylococcus aureus_. Therefore, there is a need to modify the chemical structure of bisphosphonates to overcome this obstacle.

Terbinafine and ketoconazole exert their antifungal activity, respectively, through inhibiting squalene epoxidase[29] and lanosterol demethylase.[22] Squalene epoxidase and lanosterol demethylase are two enzymes in the final steps of steroid biosynthesis [Figure 1] that are not found in all kinds of bacteria and archaea.[30,31] Accordingly, squalene epoxidase and lanosterol demethylase inhibitors did not affect the growth of _Pseudomonas aeruginosa_ and _E. coli_. However, if this reason is plausible for all of the bacteria, we should have seen no effect of terbinafine and ketoconazole on _Staphylococcus aureus_ and _Enterococcus faecalis_, whereas ketoconazole showed the most potent effect (with the lowest MBC) seen in this study on the growth of _Staphylococcus aureus_ and with the lesser extent on _Enterococcus faecalis_ [Table 1]. Moreover, terbinafine showed little effect only on _Staphylococcus aureus_. McLean and his colleagues reported the potent inhibitory effect of azole antifungals (sterol demethylases inhibitors) against Mycobacterium that previously was considered to be devoid of the enzyme.[32] These data indicate that terbinafine and ketoconazole have found targets in bacteria that were not previously known. One other possible mechanism for ketoconazole could be changing membrane permeability for potassium.[33] However, further research is needed to understand the exact mechanisms of action of ketoconazole, especially in _Staphylococcus aureus_.

Some steps of steroid synthesis have recently been discovered in some bacteria.[34] Moreover, cephalosporin P1, helvolic acid, and their derivatives, which have close structural similarity to sterol backbone, show strong bactericidal activity against _Staphylococcus aureus_[35] indicating the possibilities of sterol synthesis in _S. aureus_. Therefore, there is a need to find the exact mechanisms of action of ketoconazole in _Staphylococcus aureus_ in future studies.

**Conclusion**

Finding new targets to kill antibiotic-resistant bacteria is now necessarily important. In the present study, we used the drugs that are known as the inhibitors of the enzymes of the eukaryotic isoprenoid biosynthesis pathway, to target bacterial enzymes. Our results show that farnesyl pyrophosphate synthase and class II HMG-CoA reductases that are, respectively, targeted...
by ketoconazole and simvastatin represent promising points for new antibacterial agents. Among the drugs tested, ketoconazole and simvastatin can be considered as drug candidates against some pathogenic bacteria.

Acknowledgment
We sincerely thank Mrs. Rezaei for her technical assistance.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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