In-vitro Examination of The Potential Antibacterial Activity of Simvastatin

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ABSTRACT

Background: Statins are group of medicines that block mevalonate pathway by competitive inhibition of the rate limiting enzyme hydroxy-methylglutaryl-CoA reductase (HMG-CoA). Statins additionally inhibit the biosynthesis of important isoprenoid intermediates which have a role in peptidoglycan synthesis and cell growth. Several in-vivo and in-vitro studies have shown that certain type of statin family possess antibacterial activity in bacteraemia and sepsis.

Method: Two gram-positive pathogenic bacterial strain Staphylococcus aureus and Bacillus species were used for examining the antimicrobial activity of the lipophilic simvastatin using nutrient agar and nutrient broth and results were calculated by measuring the clear zone around the paper disk and compared with those obtained by the antibiotics, amoxycillin and ceftriaxone.

Results: Data have shown that simvastatin 1, 3, and 10 and 30 μM inhibited both Staphylococcus aureus and bacillus species, it exhibited inhibitory zone of (17.9 ± 0.6 mm) and (16.9 ± 0.3 mm), respectively.

Conclusion: the lipid soluble simvastatin, with relatively higher concentration than those obtained in-vivo, caused a significant inhibition of both Staphylococcus aureus bacteria and bacillus species.

KEYWORDS: Simvastatin, antibacterial, Staphylococcus aureus.

الخلاصة:

مجموعة ادوية الستاتيي هي ادوية تعمل عن طريق تثبيط الانزيم المحدد لتفاعل الميفالونيت والمسؤول عن انتاج الكوليسترول؛ تثبيط هذا التفاعل يؤدي أيضا إلى تثبيط تصنيع عدة مركبات وسطية لها دور بنمو الخلية وانقسامها. عدة دراسات اثبتت ان لهذه المجموعة من الادوية تأثير مثبط على نمو البكتريا في حالات تسمم الدم بالبكتريا وأيضا تثبيط البكتريا بالأوساط الزيترية المختبرة. وعليه الدراسة صممت لدراسة تأثير السمفستاتين ذو الخلاصة الدهنية على نمو بكتريا المكورات العنقودية Staphylococcus aureus. 

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INTRODUCTION

One of the achievements in pharmacology was the discovery of statin group as a metabolite isolated from the culture of the fungus Penicillium citinum in 1970s (1), the isolated metabolite markedly reduce cholesterol in rats and dogs, since then statins family broadly utilized for the treatment of million people with hypercholesterolemia around the globe. Statins are group of medicines act by competitive inhibition of the rate limiting enzyme in hepatic cholesterol biosynthesis 3-hydroxy-3-methylglutaryl-CoenzymeA reductase (HMG-CoA) (1, 2). Accordingly, they block an essential limiting stage in mevalonate pathway causing reduction in the synthesis of the malignant cholesterol i.e., LDL-cholesterol while increased catabolism of cholesterol in the body circulation (3, 4). Accordingly, statins reduce the rate of morbidity and mortality occurred as a consequence of cardiovascular complications (5-7). In general, these agents have relatively good safety profile with few adverse effects allowing their increased indications in the treatment of most patients with hyperlipidemia (8). In fact, by the inhibition of mevalonate pathway, statins additionally inhibit the biosynthesis of important isoprenoid intermediates like geranyl-geranyl diphosphate (GGPP) and farnesyl-diphosphate (FPP) followed by reduction of an important signaling molecules like Rho, Ras, and Rac (9). The latter result in many extrahepatic, cholesterol-independent effects collectively known as statins-pleiotropic effects improvement of endothelial function (10, 11), anti-inflammatory effect (12, 13), antioxidant effect(14), antiplatelet effect (15). Moreover, multiple clinical results have shown that statins decreased the rate of mortality secondary to bacterial infection, they even have recorded role in the prophylaxis and even treatment of different bacterial infections (16, 17). These results suggest that statins have the ability to prevent the initiation of
bacterial infections and inhibit bacterial growth by reduction of cholesterol biosynthesis (18). In fact, several in vivo and in vitro studies have shown that certain type of statin family possess antibacterial activity in bacteremia and sepsis (19) for example, Streptococcus, Enterococcus and Moraxella spp Staphylococcus. Aureus, S. pneumoniae (20) Helicobacter pylori (21). Lovastatin inhibits the intracellular growth of Salmonella typhimurium (22). In addition, simvastatin combination with the first line TB treatment had shorten the duration of TB treatment in laboratory mice (23, 24), they even reduce the incidence of tuberculin infection in diabetic patients (25). In fact, simvastatin and atorvastatin increased the mycobactericidal effect of rifampicin in both vivo and vitro experiments (26). With limited information about the mechanism of the antibacterial effect of statin (22).

Given the fact of increased resistance of bacteria to the available antibacterial drugs because of the inappropriate prescription of the antibiotics, which is indeed considered as a serious issue in therapy (27, 28), therefore there is an urgent and continuous need to search for new agents against the resistant strains of bacteria (29). The use of the existing medicines primarily approved for the treatment of certain medical condition with additional antimicrobial effect has the potential to accelerate the research to discover new antimicrobial drugs since their safety have been already examined with a detailed data about their pharmacokinetics and pharmacodynamics (20). Statins were originally known as a fungal metabolite produced to inhibit bacteria by HMG-CoA reductase inhibition (1), due their significant cholesterol reducing efficacy, they commonly used for cardiovascular diseases (30), however as part of their pleiotropic effect statins also investigated for their efficacy to inhibit bacteria (31), fungi (32), parasite (33) and even virus infection (34-36). This study was designed to address in vitro effect of the lipophilic simvastatin on Staphylococcus bacteria and bacillus species, and also to determine the minimal inhibitory concentration of simvastatin on these species.

MATERIALS AND METHODS

Bacterial isolates
Two isolates of pathogenic bacteria: gram- positive cocci including Methicillin-resistant Staphylococcus aureus (MRSA) (29) the main cause of nosocomial infection i.e acquired in
hospital, they have ability of biofilm formation which render the bacteria resistant to several types of antibiotics. The second bacteria examined was bacillus species which also resist many chemicals and antibiotics by spore formation. Gram negative bacteria like *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Escherichia coli* also examined for comparison. Those bacterial isolates were obtained from Microbiology Lab/ College of Sciences at the University of Mosul. Bacterial isolates were kept in a nutrient agar according to their growth requirement for about 24 hr. at 37°C. growth medium containing bacterial isolates were also prepared in 5ml nutrient broth.

**Chemicals**

Simvastatin tablets 20mg (AstraZeneca) were provided from a local pharmacy, dissolved in the suitable organic solvent DMSO, dimethyl sulfoxide, was used as a the drug solvent, which already examined in previous studies and have shown no antibacterial activity with concentration not exceeding 1% (31, 37). Amoxycillin and ceftriaxone disc (Bio-Rad) obtained from local market.

**Antibacterial activity**

The activity of simvastatin as antibacterial agent was estimated by the method of disc diffusion as described before (38).The method is basically done by diffusion of agent to be tested on a solid media, a sterile petri dish was filled by the agar Mueller–Hinton (Difco™) (about 100 mm in diameter), bacteria were recovered from a frozen stock by seeding on the agar with concentration about (1× 10⁸), the plates were inspected for any contamination, paper disc prepared in the lab by dispersion of the paper (6 mm diameter) in the drug solution with stock concentrations 1, 3, and 10 and 30μM. Ceftriaxone and amoxicillin disc was used as appositive control while DMSO disk was used as a negative control. The dishes were prepared and left for a bout 1hr. at room temperature then bacterial smear was done with the drug simvastatin, then the petri dishes were incubated overnight at 37°C Petri. The effect antibacterial effect of simvastatin was measured as a clear zone around the paper disk which correspond to inhibition of bacterial growth and compared with those obtained by the amoxycillin and ceftriaxone (31). Then bacterial strain was smeared on the agar media in each plate. DMSO-containing plate was used as a control to exclude diluent
effect on bacterial growth. The prepared plates were incubated for about 24 hr. at 37°C. minimum concentration that inhibit bacterial growth was considered the minimal inhibitory concentration (MIC), where no visible bacterial growth was seen on the agar, usually the method was repeated twice for each concentration and the average calculated (figure 1).

**Calculation of the minimum inhibitory concentration (MIC)**

Dilutions were done serially according to standards of the National Committee for clinical laboratories (39). Tubes containing a stock solution of 1mM, 10mM and 30mM were prepared by dissolving simvastatin in 1 mL DMSO. From each dilution, simvastatin was added to the nutrient broth (Difco™) to get final concentrations of 1μM, 10μM and 30μM. The tubes divided to three groups, control negative (C–) (blank) for standardization, control positive (C+) contain bacteria without simvastatin and the test tube contain bacteria with simvastatin with different concentration 1, 10 and 30μM. Bacteria incubated with each tube except the control taking into consideration the number in comparision with MaFarland tube number 0.5 with the standard turbidity. Tubes then incubated at 37°C for 24 hr. the degree of turbidity then measured with Reader Eliza at wave length of 630 nm.

**RESULTS**

The current data demonstrated that supratherapeutic concentration of simvastatin have a significant antimicrobial effect against *Staphylococcus* gram positive bacterial strain. In vitro antibacterial activity of simvastatin 30μM caused bacterial growth inhibition of *Staphylococcus* reflected by the inhibitory zone of (13 mm) on the agar media (figure 1, table 1), it also produced inhibition of *bacillus* bacteria with inhibition zone of (12mm), ceftriaxone (CRO) and amoxycillin (Ax) induced a clear inhibitory zone for both strains.
Figure 1. Antibacterial activity (measured zones of inhibition) of simvastatin (1 µM, 3 µM, 10 µM, 30µM) on A-Staphylococcus aureus and B-bacillus species in a direct contact method, amoxycillin and ceftriaxone were used as a positive control.

However, results have shown that simvastatin with concentration up to 30µM had no effect on the gram-negative bacteria examined in our study, Pseudomonas aeruginosa, Klebsiella pneumonia and Escherichia coli, (data not shown). Results also have shown that MIC is around 10 µM.
Table 1. Activity of Simvastatin (sim) 1 µM, 10 µM and 30 µM against Staph. aureus and Bacillus spp. bacteria in a nutrient agar measured as inhibitory zone around each drug concentration, results compared with those obtained by the amoxycillin (Ax) and ceftriaxone (CRO).

| Drug     | Staph. aureus | Bacillus spp. |
|----------|---------------|---------------|
| Sim 1µM  | 10            | 10            |
| Sim 10µM | 12            | 12            |
| Sim 30µM | 13            | 12            |
| Ax 25 µM | 20            | 14            |
| CRO10µM  | 18            | 16            |

Table 2. Activity of Simvastatin (Sim) 1 µM, 10 µM and 30 µM against Staph. aureus and Bacillus spp. bacteria in nutrient broth, C- is control negative tube used as a blank, C+ is a positive control without Sim. Activity measured as by turbidity with Reader Eliza at wave length 630nm.

| Drug     | Bacillus spp. | Staph. aureus |
|----------|---------------|---------------|
| C-       | 0.015         | 0.012         |
| C+       | 0.505         | 0.294         |
| Sim 1µM  | 0.424         | 0.250         |
| Sim 10µM | 0.438         | 0.210         |
| Sim 30µM | 0.476         | 2.215         |
| Stock solution | 0.246 | 0.129 |
DISCUSSION

The use of antibiotics has long been considered as the cornerstone in the management of bacterial infections. However, the appearance of microorganism (in particular MRSA) showing resistance to many antibacterial groups even those reserved as a last choice, like meropenem (40), giving the worrying impression that the currently available antibiotics could be ineffective in the future (29). Accordingly, there is a continuous need to focus more research experiments to search for a new antibacterial in order to overcome such health challenge. The ordinary methods used to investigate novel agents are possibly unlikely to keep steps from the developed resistance by the infectious pathogens and usually such methods cost the pharmaceutical companies billions of dollars to get successfully approved antibiotic (success rate about 3.5%). Another plan to speed up the investigation process is reusing an old agent for different use, moreover certain non-antibiotic may synergize the action of the antibiotics and thus acting as antibiotic resistance breakers (41), for example statins have a good safety profile and are well tolerated by most people, they commonly used to treat hypercholesterolemia (42), impressively, clinical and laboratory data suggested that statins may have additive antibacterial action and thus may be used as a novel antimicrobial agent to inhibit pathogenic multidrug-resistant bacteria (43). Results of the current study suggest antibacterial effect of the lipophilic simvastatin against methicillin-resistant S. aureus (Gram-positive bacterial species). The concentrations of simvastatin examined in the study are higher than the therapeutic concentration measured in blood of patient on simvastatin treatment around from about 10nM to 1μM in those treated with a daily dose of 20 -60 mg (44). Thus, simvastatin with this concentration do not have antibacterial effect in normal daily dosing. However, multiple dosing of simvastatin may result in drug accumulation in vivo at certain tissues with resultant inhibition of bacterial growth (45, 46), Current study supports previous data published by Jerwood et al, (47) which states that simvastatin has antimicrobial effect, similarly rosuvastatin and atorvastatin have antibacterial activity against both gram positive and gram negative bacteria. Another study by Masadeh et al (2012) revealed that simvastatin and atorvastatin both have antibacterial
activity against *staphylococcus aureus*, *Methicillin-sensitive and methicillin-resistant strains*, MSSA and MRSA respectively (43). Moreover, Peter et al, (2011) showed that in-vitro incubation with lipophilic Simvastatin was effective against *S pneumoniae* at concentration about 36 µmol/L, which is indeed similar to the concentration examined in our study (31). By contrast pravastatin with concentration up to 100 µg/mL showed no antibacterial action. On the other hand, statins inhibit the intracellular growth of *Mycobacterium leprae* with concentrations approximate to those physiological concentrations suggesting indirect effect of statin secondary to cholesterol depletion (48). Statin, especially the lipophilic group, have been shown in vitro studies to have cytotoxic effect on many cell types like endothelial cells (49, 50), vascular smooth muscle (51-53), skeletal muscle (54), cardiac muscle (55) pancreatic cells (56) and hepatic cells (57). Previous studies suggested that by lowering cholesterol levels in the host cells statin may inhibit production of membrane protective layer produced from mevalonate pathway with resultant induction of bacterial cytotoxic effect (58, 59). However, this inconsistent with the results obtained from Haeri et al study which stated that lovastatin with the potent inhibitory effect on mevalonate pathway, did not have bactericidal effect on gram-positive bacteria. thus, this mechanism cannot explain statin cytotoxicity (18). Research suggested that the gram-negative bacteria synthesize isoprenoid by mevalonate-independent pathway, in addition the outer layer of the gram-negative bacteria works as barrier against statin penetration, however, data also reported that water soluble rosuvastatin, which has less ability to penetrate cell membrane, inhibited *E. coli* (18).

Previous results showed additive effect of statin especially of the lipophilic nature like simvastatin with beta-lactam antibiotics against certain types of bacteria like *K. pneumoniae*, by contrast, pravastatin had no additive effect with penicillin however, the mechanism of the additive effect is still not clearly defined (31). Statin group induce hypolipidemic effect via inhibition of the reductase enzyme as mentioned before, the role of HMG-CoA reductase activity in bacterial cells is essential for isoprenoids and peptidoglycan synthesis and cell growth (59, 60) (figure 2), however, the reductase enzyme in bacteria is of
different structure with about 1000 times less affinity towards statins (58).

![Diagram]

**Figure 2.** Representative scheme demonstrates that mevalonate contribution for the synthesis of peptidoglycan and its role in cell wall synthesis and bacterial growth.

Thus, the possibility of simvastatin to act as antibacterial agent via inhibition of mevalonate pathway is less likely, accordingly simvastatin acts in *S. aureus* by different mechanism to the mechanism known in human beings since they lack class I HMG-CoA reductase enzyme (61). On the other hand, results suggested that simvastatin had no effect on membrane integrity and thus exclude the direct cytotoxic effect of simvastatin (31). Experiments also demonstrated that simvastatin inhibited protein synthesis in bacterial species (62), other antibiotics that interferes with protein synthesis like linezolid, tetracyclines and chloramphenicol was found to inhibit to inhibit protein synthesis both in human and bacterial cells (63), the latter is due to mitochondrial inhibition in mammalian cells owing to the similarity between human and bacterial cells (64), by contrast, simvastatin have shown to be a selective inhibitor of bacterial protein synthesis, Additionally, simvastatin interferes with other biosynthetic pathways involved in energy production like glycolysis and deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis (62), thus the lipophilic statin like
Simvastatin, could suppress bacterial growth by acting on multiple targets with a complex mechanism of action. To sum up, our data agree with previous experiments that have shown the antibacterial effect of the lipid soluble simvastatin with special regards to the gram-positive bacteria\textsuperscript{11}. More studies are advised to investigate the mechanism(s) by which simvastatin is acting as antimicrobial agent.

CONCLUSION

Simvastatin had in-vitro antibacterial action against \textit{staphylococcus aureus} and \textit{bacillus species} with concentration above those measured in vivo.

CONFLICT OF INTEREST:

All authors declare that there is no conflict of interests.

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