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Research Article

In-Vitro Effect of Statins on *Enterococcus Faecalis*

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Abstract

Objective: The aim of this study was to assess the *in-vitro* efficacy of statin medications on putative *Enterococcus faecaelis*, as determined by minimum inhibitory concentration.

Methods: *Enterococcus faecalis* 47077 was grown in the presence of simvastatin lactone (prodrug), simvastatin carboxylate (active metabolite), rosuvastatin, pravastatin and fluvastatin. Minimum Inhibitory Concentrations (MICs) were determined by serial broth dilution assays and bacteriostatic activity by observing the effect of statin on growth curves.

Results: MICs against *E. faecalis* were simvastatin lactone (26.1 μg/ml) and fluvastatin (249 μg/ml). The antibacterial effect of simvastatin lactone and fluvastatin was determined to be bacteriostatic. Simvastatin carboxylate, rosuvastatin and pravastatin did not inhibit bacterial growth.

Conclusion: The lipophilic statins simvastatin and fluvastatin act as *in-vitro* bacteriostatic antimicrobial agents against *E. faecalis*, whereas the hydrophilic statins simvastatin carboxylate, rosuvastatin and pravastatin did not inhibit bacterial growth *in-vitro*. The suppression of this pathogen may contribute to the known pleiotropic effect of certain statins, in particular simvastatin.

Keywords: Statins; Bacteriostatic; Pleiotropy

Introduction

Statins are competitive inhibitors of 3-Hydroxy-3-Methylglutaryl- coenzyme A (HMG-CoA) reductase, the enzyme that produces mevalonate, the precursor of cholesterol. As a group, they lower cholesterol and because of their high tolerance, are widely prescribed for the treatment of hypercholesterolemia [1].

A number of beneficial side effects of statin intake have also been observed in humans [2]. While not typically thought of as antimicrobial agents, there are multiple studies that have demonstrated *in-vitro* antibacterial effects of statin medications [3]. Additionally, a study published in 2018 demonstrated an increased rate of healing of periapical lesions after root canal therapy in a cohort of patients who receive long-term statin therapy, most of whom used simvastatin [4]. Since *Enterococcus faecalis* is associated with persistent endodontic infections, the aim of this study was to assess the *in-vitro* efficacy of both lipophilic and hydrophilic lipids on *E. faecalis* as determined by minimum inhibitory concentrations [5].

Material and Methods

Approval

This research was approved by the Temple University Kornberg School of Dentistry Research Committee. The committee deemed Ethics Committee approval unnecessary as it does not involve human subjects or use of animals. This study was conducted in accordance with the Declaration of Helsinki.

Statistics

All experiments were performed in triplicate. Descriptive statistical values are presented as means as calculated on Microsoft Excel 2010 spreadsheet software (Microsoft Corporation Redmond, WA, USA).

Bacterial Strains

Enterococcus faecalis (19433) cells and genomic DNA (700802DQ) were purchased from the American Type Culture Collection (Manassas, VA). *E. faecalis* was inoculated in brain heart infusion (BHI) broth and allowed to grow for 24 hours at 37°C. Bacterial cells were enumerated by real time quantitative polymerase chain reaction (qPCR). Quantitative genomic DNA from *E. faecalis* (105 copies/μl) was used to generate an absolute copy number standard curve and compare to target DNA extracted from *E. faecalis.* Primers were synthesized by Sigma Chemical Co. (St. Louis Mo.). Primer mixes included the following oligonucleotides: forward primer 5'- TGTTCGGTGTTGGTG-3' and backward primer 5'-ACTGCTGCCGCTTGT-3' [6]. To 10 μl of each primer (2 μM) was added 20 μl of Chai Master Mix and 10 μl standard or target DNA. Chai Master Mix contains Taq DNA polymerase and Chai green fluorescent dye, which binds to amplified double stranded DNA and fluoresces. Amplifications were performed in a single channel thermocycler (Chai Biotek, Santa Clara, Ca) with cycling as follows: initial denaturation at 95°C for 3 min., followed by 40 cycles at 95 μC for 3 min., 62°C for 30 sec. and 72°C for 30 sec. The terminal denaturation was performed at 72°C for 5 min. PCR products were detected by monitoring the increase in fluorescence. The most probable number of cells was estimated by comparing dilutions of DNA extracted from cells to standard copy number assuming one copy per cell. Cell numbers were reported as number of cells per μl.

Preparation of Statin Medications

The lipophilic statins simvastatin lactone (prodrug) and fluvastatin and the hydrophilic statins simvastatin sodium carboxylate salt (active metablolite), rosu-vastatin and pravastatin were purchased from Sigma Chemical Co. (St. Louis Mo). Statins were solubilized and diluted in either molecular grade water or 100% dimethylsulfoxide (DMSO) according to manufacturer's directions to make stock solutions ranging from 0.3 mM to 20 mM.

Determination of the Minimum Inhibitory Concentration (MIC)

MIC values for the statins were measured in accordance with Clinical Laboratory Standards Institute (CLSI) procedures (NCCLS document OSO. 20776-1;2019). A broth dilution assay was prepared to query if each statin medication had an antibacterial effect and if an antibacterial effect is present, to determine the minimum inhibitory concentration (MIC) of the statin medication. Tubes were prepared with 2.85 ml of brain heart infusion (BHI) broth. To each tube, one 75 μl sample of a statin dilution was added (either simvastatin/DMSO, simvastatin carboxylate/H2O, rosuvastatin/DMSO, fluvastatin/H2O or pravastatin/H2O). All statin dilutions were included. Then, a fixed culture of bacteria (75 μl cell suspension, 105cells/ μl, of *E. faecalis*) was added to each tube. The final volume of each tube was 3 ml. DMSO or sterile H2O alone (75 μl), added to bacterial suspension (75 μl) and media (2.85 ml), was used as a positive control. As a negative control, 75 μl of the final dilution of each statin solution was added to a tube of 2.85 ml of media, followed by an additional 75 μl of media (without bacteria). The tubes were then incubated at 37°C for 24 hours, after which they were observed for turbidity. The MIC was considered to be the lowest concentration of statin medication that prevented bacterial growth, i.e. a clear test tube. Each clear experimental tube was then subcultured onto BHI agar plates and incubated at 37°C for 24 hours to determine if the effect is bactericidal or bacteriostatic. Positive growth was considered indicative of bacteriostatic effect and negative growth was considered indicative of bactericidal effect. These experiments were carried out in independent triplicates to validate the results.

E. faecalis Growth Ability at Different Statin Concentrations

A time-kill test was performed to further evaluate the antimicrobial effect of simvastatin lactone and fluvastatin on *E. faecalis* [7]. Tubes of growth media (2.95 ml) were inoculated with *E. faecalis* (50 μl cell suspension, 105cells/ μl) and allowed to grow for 2 hours at 37°C. At the two-hour point, either simvastatin lactone or fluvastatin was added to separate tubes at 1 x MIC, 0.5 x MIC and 0.25 x MIC concentrations, with one tube without statin to serve as a control. At the 2-hour, 4 hour and 11-hour time points after the addition of the statins, 100μl aliquots were taken from each tube for the purpose of counting bacterial cell numbers by q PCR.

Results

Determination of MIC by Broth Dilution Assay

The MIC of simvastatin lactone (prodrug) was determined to be 0.0625 mM (26.1 μg/ml) against *E. faecalis* (Fig. 1). The MIC of fluvastatin was determined to be 0.575 mM (249 μg/ml). No growth was found in any of the negative control tubes, while growth was observed within the positive control tubes. When samples with inhibited *E. faecalis* growth were plated onto BHI agar plates and incubated for 24 hours, bacterial growth was observed, implying that the effect of the statin medications is bacteriostatic. No antibacterial activity was observed for simvastatin carboxylate, rosuvastatin nor pravastatin at any of the tested concentrations.

E. faecalis Growth Ability at Different Statin Concentrations

To growing cells, either simvastatin lactone or fluvastatin were added at several concentrations (1 x MIC, 0.5 x MIC, 0.125 x MIC). At the 2,4- and 11-hour time points, tubes with the MIC concentrations of statins had significantly fewer cell counts than the control, representing arrested growth. Tubes with two-fold dilutions of MIC, ie, 0.5 x MIC and 0.125 x MIC, also displayed diminished growth over the entire time course (Fig. 2).

Figure 1: Example of MIC calculation of simvastatin lactone against *E. faecaliis*. Tubes containing *E. faecalis* and sequential dilutions of simvastatin were incubated at 37°C for 24 hrs. From left: Negative control, positive control, S3 (MIC), S4 (0.5 x MIC), S5 (.25 x MIC) and S6 (0.125 x MIC). S3 (26.1 μg/ml) was determined to be the MIC based on its lack of turbidity. (Identical results for three replicates).

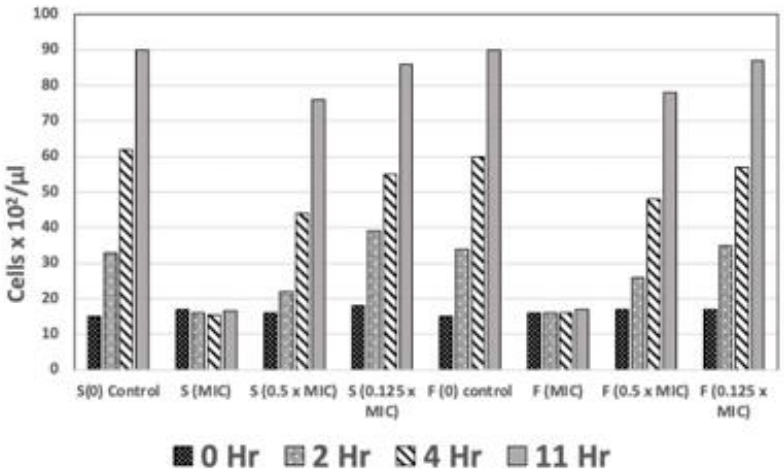

Figure 2: *E. faecalis* growth ability at different statin concentrations. MIC concentrations and two-fold dilutions, of simvastatin lactone (S) and fluvastatin (F) were added to growing cells and growth was monitored at 2,4 and 11 hrs by counting cells in aliquots by qPCR. Results are reported as cells/µl and are an average of three experiments. Compared to controls, MIC concentrations arrested growth and sub-MIC levels diminished growth of *E. faecalis*.

Discussion

In humans, statins are used to lower cholesterol by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the liver, which leads to a decreased synthesis of endogenous cholesterol [8]. In addition to reducing cholesterol, statins have been shown to have pleiotropic characteristics including immunodilatory, anti-inflammatory as well as antibacterial activity [9-11]. Of particular interest is a clinical study that demonstrated an increased rate of healing of periapical pathology after root canal treatment for patients who routinely ingest simvastatin, compared to controls [4]. This work focused on *E. faecalis*, as it has been implicated particularly in persistent endodontic infections [5].

HMG-CoA reductase is important to *E. faecalis* metabolism and survival [12]. The experiments performed demonstrate the *in-vitro* bacteriostatic effect of simvastatin lactone and fluvastatin on *E. faecalis*. These are findings consistent with the effect of simvastatin lactone on primary pathogens of the respiratory tract [13]. No antibacterial activity was observed with rosuvastatin, pravastatin, nor simvastatin carboxylate, the active metabolite of simvastatin.

While inhibiting HMG-CoA reductase may be a potential mechanism by which statin medications inhibit bacterial growth, the question remains why some statins (simvastatin lactone and fluvastatin) exhibited *in-vitro* antibacterial activity, while others (simvastatin carboxylate, rosuvastatin and pravastatin) did not. Compared to other statins, simvastatin acid, rosuvastatin and pravastatin have relatively low lipophilicity [14]. Simvastatin lactone, in particular has good liposolubility, enabling passive diffusion though the cell membrane. Simvastatin differs from the other statins in that it is supplied as an enzyme-inactive lactone that is converted to active metabolite, simvastatin carboxylate. However, in tissues, significant amounts of most statins are converted back to their lactone forms. Since both lactone and acid forms are important in statin effects, both simvastatin prodrug and the active metabolite were used in this study. It is possible that, unlike simvastatin lactone and fluvastatin, simvastatin carboxylate, rosuvastatin and pravastatin may not be able to penetrate the bacterial membrane, therefore rendering them unable to access the HMG-CoA reductase enzyme. Another mechanism that has been proposed is that stains inhibit bacteria in a soap-like manner by disrupting cell membranes. This also would account for growth inhibition by the lipophilic simvastatin and fluvastatin and not by the more hydrophilic simvastatin carboxylate, rosuvastatin and pravastatin. Initial studies are underway to identify the mechanism.

Simvastatin lactone demonstrated an antibacterial effect in a concentration as low as 0.0625 mM (26.1 µg/ml) against *E. faecalis*. Fluvastatin had an antibacterial effect at 0.575 mM (249 µg/ml). These are similar to MIC values reported for statins against other bacteria [15]. Both simvastatin lactone and fluvastatin were found to bacteriostatic since growth resumed when aliquots from MIC tubes were streaked onto fresh nutrient plates.

The results of *in-vitro* experiments are difficult to translate directly into clinical practice. Minimum inhibitory concentrations are laboratory measures of a fixed concentration of an antibacterial agent tested against an initially fixed concentration of bacteria. These values do not necessarily correspond to drug and bacterial densities at a site of infection as the way statins are metabolized by the body is complex. Nevertheless, the MIC of statins are far less potent than traditional antibiotics, e.g. penicillin (MIC 0.03-0.06 μg/ml) [17]. However, the MIC of statins against oral bacteria compares favorably with topical agents such as essential oil (MIC 512 μg/ml), chlorhexidine gluconate (MIC 1-2 μg/ml) and triclosan (MIC 7.8 μg/ml) [18,19]. While *in-vitro* antibacterial effects were seen with simvastatin lactone and fluvastatin in this study, it is not clear whether similar effects would be seen in vivo because laboratory studies cannot replicate the complex conditions inside a human being.

In theory, it may be possible that the antibacterial effect of orally-ingested statins contributes to endodontic healing, such that a persistent low-grade infection may be met by a persistent low concentration of statin. Routinely, bacteriostatic agents have *in-vitro* efficacy against bacteria several-fold lower than the observed MICs; i.e. in our time-kill test, slowed growth was observed in tubes 8-fold less concentrated than MIC values (as low as 3.3 μg/ml). Upon ingestion, statins target the liver, such that simvastatin and fluvastatin have 5% and 30% bioavailability respectively, i.e. 5% - 30%of the ingested dose circulates in plasma (even simvastatin pro-drug) [20,21]. Thus, for the average adult who has 5 liters of blood, ingestion of one single statin dose of 80 mg reaches a peak plasma concentration of 0.08-5 μg/ml. By these calculations, statin concentrations in plasma may not reach levels needed for antimicrobial inhibition, but may slow bacterial growth. However, bacteria do not persist in plasma but may persist in latent periapical lesions. Furthermore, the concentration of bacteria and statin in tissues, e.g. bone and the intracellular concentration of bioavailable statin in endothelial cells is unknown and may be higher than plasma values as the lipophilic statins diffuse into tissues very well. Given the widespread use of these medications, further studies of the clinical effect and *in-vitro* mechanism of statin-bacterial interactions are warranted.

Conclusion

The lipophilic statins simvastatin and fluvastatin act as *in-vitro* bacteriostatic antimicrobial agents against *E. faecalis*, whereas the hydrophilic statins simvastatin carboxylate, rosuvastatin and pravastatin did not inhibit bacterial growth *in-vitro*. The suppression of this pathogen may contribute to the known pleiotropic effect of certain statins.

Conflict of Interests

The authors have no conflict of interest to declare.

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