*Running title:* Simvastatin antimicrobial action against *S. aureus*

TYPE OF ARTICLE:

ORIGINAL CONTRIBUTION

Title: Antimicrobial activity of simvastatin against CRS-related *Staphylococcus aureus*: an *in vitro* study.

Authors: Goldie SP 1,2, Lau LC 1, Jones HAS 2, Harries PG 2, \*Walls AF 1, \*Salib RJ 1,2

\*Walls AF and Salib RJ are joint senior authors.

Affiliation

1. School of Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, United Kingdom
2. Department of Otorhinolaryngology / Head & Neck Surgery, University Hospital Southampton NHS Foundation Trust, Southampton, United Kingdom

SUMMARY

Introduction: *Staphylococcus aureus (S. aureus)* in chronic rhinosinusitis (CRS)*,* particularly when localised intracellularly,is linked to disease recalcitrance and poor post-surgical outcomes. Antibiotics frequently fail to penetrate the mammalian cell membrane, resulting in an inability to address the intracellular component of *S. aureus.* This contributes to treatment failure and development of antimicrobial resistance. We investigated the antimicrobial effects of simvastatin, a widely used, inexpensive medication with extracellular and intracellular antimicrobial properties, against CRS-related *S. aureus*.

Methods: Simvastatin’s antimicrobial activity, in prodrug and hydroxylated forms, was assessed against *S. aureus* using the broth dilution method to determine the minimal inhibitory concentration (MIC). Intracellular activity of simvastatin was evaluated by pre-treating *S. aureus* -infected LAD2 mast cells with simvastatin and performing colony forming unit (CFU) enumeration and confocal microscopy. Cell viability was assessed using lactate dehydrogenase (LDH) assays.

Results: Simvastatin exhibited an extracellular MIC of 40 mmol/l against *S. aureus*. Intracellularly, it significantly reduced the bacterial burden by 46-fold in a dose-dependent manner between concentrations of 0.1-100 mmol/l. Toxicity to LAD2 cells was observed at 100 µmol/l. Confocal microscopy revealed a lower percentage of infected cells in the group pretreated with 30 µmol/l simvastatin (15.3%) compared to untreated cells (32.8%). Hydroxylated simvastatin demonstrated no antimicrobial activity against *S. aureus*.

Conclusions: Simvastatin demonstrates *in vitro* antimicrobial activity against CRS-related *S. aureus* with the potential for repurposing as a novel antibiotic-sparing topical agent for the treatment of refractory CRS. This could improve surgical outcomes and reduce the risk of antimicrobial resistance.

*Key words:* Drug Resistance, Bacterial; Drug Repositioning; Rhinosinusitis; Simvastatin; *Staphylococcus aureus*

INTRODUCTION

*Staphylococcus aureus (S. aureus)* colonises the nasal cavity in 64% of patients with chronic rhinosinusitis and nasal polyps (CRSwNP) compared with 33% of those without polyps (CRSsNP) and 20% in those without disease (1 ,2). Culture of *S. aureus* pre- and post-operatively in patients with chronic rhinosinusitis (CRS) is a poor prognostic indicator for disease recurrence and recalcitrance (3). *S. aureus* can persist in the nasal cavity of CRS patients, evading the immune system and the effects of antimicrobials (4). This is achieved through internalisation within host cells, sequestering it within the intracellular space or by creating extracellular biofilms (5 ,6). In 2015, our group made the novel observation that *S. aureus* internalises within mast cells in nasal polyps, serving as a reservoir of bacteria that seeds the extracellular space and perpetuates chronic inflammation in CRSwNP patients (7). Intracellular *S. aureus* is challenging to treat, as the mammalian cell wall prevents diffusion of many commonly used antibiotics (8). Furthermore, intracellular *S. aureus* often forms small colony variants (SCVs), which exhibit reduced metabolism and increased cell wall thickness (9). Consequently, anti-metabolic antibiotics have limited efficacy on these resistant variants.

In CRS, *S. aureus* appears to exist extracellularly and can transition phenotype into an intracellular SCV within epithelial and mast cells in the nasal mucosa (10 ,11). Interestingly, *S. aureus* cultured from antibiotic-treated tissue and nasal swabs of the middle meatus demonstrate identical genotypes, suggesting the extracellular bacteria can switch phenotype to thrive within cells (10). Furthermore, a significant association has been observed between the presence of intracellular *S. aureus* in the nasal mucosa and the need for revision endoscopic sinus surgery, with patients harbouring intracellular *S. aureus* at a higher risk of requiring additional surgery compared to those without(85% vs 33%, P=0.0083) (5). Consequently, intracellular *S. aureus* in CRS is commonly associated with refractory disease and antibiotic resistance often resulting in the need for multiple surgical procedures.

Given the challenges associated with intracellular S. aureus persistence and antibiotic resistance, alternative therapeutic strategies are being explored. Statins, widely used for their lipid-lowering effects, are now being investigated for diverse therapeutic applications, including cancer prevention (12), neuroprotection in Parkinson’s disease (13), treatment of chronic obstructive pulmonary disease (14), and as an antimicrobial-sparing therapy for tuberculosis (15). Notably, their potential role in *S. aureus*-related conditions is gaining interest with studies showing efficacy in treatment of *S. aureus* pneumonia, skin wound infections and biofilm formation on simulated joint implants in rats (16-19).

In relation to CRS, regular statin use has been associated with a reduced incidence in two large-scale studies. Gilani *et al* retrospectively analysed 10,965 patients and demonstrated a reduced odds ratio (OR) of being diagnosed with CRS (0.716; 95% CI, 0.612–0.838) among those taking statin medications 12. Similarly, Wilson *et al* demonstrated a reduced OR of CRS for patients taking statins on univariate (0.53; P<0.001) and multivariate (0.79; P=0.03) regression analyses using over 10 million records from the National Ambulatory Medical Survey of North America (20 ,21). Lipophilic statins including simvastatin, atorvastatin, lovastatin and fluvastatin have the capacity to cross cell membranes and have exhibited anti-bacterial properties both extra- and intracellularly (22). *In vitro* studies have shown these statins to be active against *S. aureus* at various concentrations, however simvastatin demonstrates particularly potent activity characterised by the lowest observed minimal inhibitory concentration (MIC) ranging between 16 to 63 mg/L (22). *In vivo* studies have demonstrated that topical treatment of MRSA-infected mice wounds with simvastatin reduces the bacterial load and significantly improves wound healing with reductions in pro-inflammatory cytokines IL-6, TNFα and IL-1β (23 ,24).

Lipophilic statins such as simvastatin are administered orally as an inactive prodrug which is metabolised in the liver into its active β-hydroxy acid form. Simvastatin is 95% protein bound and 5% is free in the serum and eliminated by hepatic metabolism (22 ,25). They mediate their effects through inhibition of the mevalonate pathway, which is essential for isoprenoid synthesis in humans and bacterial species, including *S. aureus.* By inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), statins reduce cholesterol and isoprenoid synthesis required for protein prenylation (26). In bacterial cells, lipophilic statins decrease cholesterol, directly affecting bacterial growth and protein production via a reduction in signalling protein prenylation (23 ,27). They also show broad antimicrobial effects when directly applied to bacteria including virulent strains of *S. aureus,* as well as methicillin and vancomycin resistant strains (23 ,26 ,28).

In mammalian cells, statins reduce cholesterol in lipid rafts, diminishing areas involved in bacterial translocation and intracellularisation, as well as the pro-inflammatory response associated with it. They also appear to modulate mast cell signalling, reducing degranulation in response to IgE-dependent stimulation and protecting cells from the effects of bacterial toxins (29-32).

Simvastatin is one of the most commonly used statins, well known to reduce the risk of

coronary deaths, myocardial infarctions, ischemic strokes, and coronary revascularisation procedures, in patients with elevated LDL cholesterol with infrequent adverse effects reported, including myalgia, new-onset type 2 diabetes, and haemorrhagic stroke (33).

Given these findings, we hypothesised that simvastatin, with its low MIC, well-characterised pharmacokinetics and low-cost, may reduce the burden of intracellular *S. aureus* in CRS. This represents an exciting opportunity to develop a novel targeted topical therapy for intracellular *S. aureus* in patients with refractory CRS, which could also reduce our dependence on antibiotics and the risk of antimicrobial resistance which has reached epidemic proportions worldwide.MATERIALS AND METHODS

***S. aureus* receipt and culture**

A well characterised strain of *S. aureus* (11 ,34-36), cultured from the intracellular space of polyp tissue from a patient with CRSwNP was used for further study. Ethical approval for the receipt of patient isolated strains of *S. aureus* was granted by Southampton and South-West Hampshire Research and Ethics committee (reference code: REC 09/HO501/74).

**Minimal inhibitory concentration of prodrug and activated simvastatin**

Prodrug simvastatin (Sigma-Aldrich) was dissolved in dimethylsulphoxide (DMSO) to create a 10mmol/l stock solution in 41.8% v/v DMSO. Activated simvastatin was prepared by dissolving 4mg of simvastatin in 100µL of ethanol and 150µl of 0.1M NaOH, followed by incubation at 50oC for 2 hours. The pH was adjusted to 7 and the total volume was made up to a 10mmol/l solution as described by McKay et al (37).

The MIC of prodrug and activated simvastatin against the CRSwNP strain of *S. aureus* was calculated using the international standard broth microdilution method (38). *S. aureus* was grown to the exponential growth phase, with absorbance at 600nm extrapolated using absorbance vs colony forming unit (CFU) enumeration graphs and diluted to create a stock concentration of 107 CFU/ml in Mueller Hinton broth, pH 7.0 (Sigma-Aldrich). Wells contained 90µl of Mueller Hinton broth (Sigma Aldrich) with serially decreasing concentrations of simvastatin. Each well was inoculated with 105 CFUs of CRSwNP *S. aureus* and incubated at 37oC in the presence of 5% CO2 for 16 hours. Absorbance was measured at 600nM using a microplate reader (Molecular Devices).

**Intracellular survival of *S. aureus* in LAD 2 cells with simvastatin treatment**

CRSwNP *S. aureus* was grown in RPMI 1640 (Fisher Scientific) at 37oC in the presence of 5% CO2 to the exponential growth phase. Absorbance at 600nm was calculated and extrapolated using absorbance vs CFU enumeration graphs.

Laboratory of Allergic Diseases 2 (LAD2) human mast cells were grown in antibiotic-free conditions in StemPro-34 media (Life Technologies) containing 0.1mM Stem Cell Factor (SCF; PeproTech). LAD2 cells (5x105 cells in 1 ml) were pre-incubated with simvastatin at concentrations ranging from 0 - 100µmol/l for 16 hours. Each condition was co-cultured with RPMI 1640 (control) or CRSwNP *S. aureus* (5x105 CFUs) and incubated for 6 hours. Cultures were centrifuged at 250g for 10 minutes and supernatants were collected for lactate dehydrogenase (LDH) assay.

Cell pellets were resuspended with 1ml 20µg/ml lysostaphin (Sigma-Aldrich) containing StemPro-34 media with 0.1mM SCF for 60 minutes. LAD2 cells were then centrifuged at 250g for 10 minutes and washed three times in antibiotic free media. Supernatants were streaked on Columbia blood agar (CBA) plates to ensure no growth. Pellets were resuspended in STEMPRO-34 media with SCF and 0.5% Triton-X100, vortexed for 10 minutes and used for serial CFU assessments using CBA plates.

**Lactate dehydrogenase assay**

A colorimetric LDH cytotoxicity assay (Sciencell Research Laboratories, USA) was performed per the manufacturer’s instructions. Control LAD2 cells (5x105 cells) were incubated in media alone for 6 hours and centrifuged at 250g for 10 minutes, removing the supernatant to calculate the spontaneous release. Maximal release was calculated by lysing cells after centrifugation in the presence of 0.5% Triton X-100 containing media with vortexing and rolling for 30 minutes. The subsequent lysate was centrifuged for 10 minutes at 250g and the supernatant was extracted to determine maximal release.

For the assay, 150µl of controls and culture supernatants were plated in flat-bottom 96-well plates (Greiner Bio-One, Austria) and 60uL of reaction mixture was added. The reaction was incubated at room temperature in the dark for 20 minutes and the reaction was stopped using 20µl of sodium oxamate per well. Absorbance was measured at 490nM using a spectrophotometer (Molecular Devices). Net release was calculated by subtracting the spontaneous release from each value and dividing by maximal release to determine percentage LDH release.

**Confocal microscopy**

LAD2 cells were preincubated with simvastatin at concentrations of 0, 1, 30 and 50µmol/l for 16 hours, then co-cultured with CRSwNP *S. aureus* for 6 hours. Cells were resuspended in 0.5ml 20 µg/ml lysostaphin for 60 minutes and washed three times in calcium and magnesium free phosphate-buffered saline (PBS).

Cells were resuspended in 15 µM Syto9 and 40µM propidium iodide in 1 ml PBS (Thermo-Fisher, UK). A 50µl aliquot of each suspension was placed on an Ibidi 8-well glass-bottom slide (Thistle Scientific, UK) and imaged using a Leica TCS SP5/8 inverted confocal microscope (Lecia Microsystems, UK) with a 63x glycerol immersion lens. Images were collected with Leica LAS-AF software and analysed using Fiji 2 (39).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software Inc, USA). Data was assessed for normality using histogram plots and normality tests. One-way ANOVA tests with Tukey’s multiple comparisons was used to compare data between experiments.

RESULTS

**Minimal inhibitory concentration of activated and prodrug simvastatin against *S. aureus***

To evaluate the concentration of simvastatin needed to inhibit the growth of CRS-related *S. aureus,* we performed MIC calculations using the internationally standardised broth dilution method (ISO 20776-1:2019) with a well-studied CRS strain of *S. aureus* (11 ,38). Our results demonstrated a MIC of between 25-50 μmol (Figure 1A), further refined to 40 μmol (Figure 1B).

As simvastatin was dissolved in DMSO, which has been shown to affect biofilm formation and bacterial growth, we performed a control experiment to rule out DMSO-related effects. The results indicated no significant bactericidal activity from DMSO alone at concentrations up to 0.418% v/v, corresponding to the 100 μmol/l simvastatin solution (Figure 1C).

Most simvastatin exists in its prodrug form (68-77%), with 95% bound to serum proteins (25). However, a small proportion is present in its hydroxylated, active form. As few studies have examined the antimicrobial activity of activated simvastatin, we converted simvastatin to its hydroxylated form and repeated the MIC calculation. Activated simvastatin demonstrated no antimicrobial activity against the CRSwNP strain of *S. aureus* (Figure 1D,E).

**Simvastatin at oral administration serum concentrations does not affect intracellular *S. aureus* survival**

The concentrations of simvastatin used for MIC determination were beyond those typically observed in human serum. Nevertheless, given the hydrophobic and lipophilic properties of simvastatin which may cause it to localise to the cell membrane, we hypothesised that it might still exert activity at these concentrations in co-culture.

To test this, LAD2 mast cells were pre-treated for 16 hours with simvastatin at serum concentrations typically observed in patients taking the drug orally (19-31 nmol) (25). The mast cells were then inoculated with S. aureus. Our results showed no significant effect on intracellular S. aureus survival at simvastatin concentrations of 0-40nmol/l (Figure 2A*).*

**Simvastatin at topical administration concentrations reduces intracellular *S. aureus* survival**

As simvastatin showed no effect at oral administration serum concentrations, we tested higher concentrations that could be achieved topically, as described by Horn *et al* and Thangamani *et al* to evaluate intracellular antimicrobial activity and cellular toxicity (23 ,40). A sequential reduction in intracellular survival was observed, ranging from 1.7x105 to 3.6x103 CFUs, between simvastatin concentrations of 0 to 100 μmol/l (Figure 2B).

**Cytotoxicity Assays**

To assess cytotoxicity, LDH assays were performed on supernatants. LAD2 cells treated with CRS *S. aureus* showed similar, non-significant changes in LDH release between 0-30 µmol/l concentrations of simvastatin (65.5-73.1%). However, at concentrations of 100 µmol/l, LDH release increased (98.4%; P≤0.0001) (Figure 2C). A parallel experiment using uninfected LAD2 cells treated with simvastatin for 6 hours confirmed no significant toxicity below 30 µmol/l with LDH release around 32.3-42.4% which rose to 92.3% at 100 µmol/l concentrations (P≤0.001). As the LDH levels were stable below 30 µmol/l, these findings suggest that the reduction in intracellular *S. aureus* survival was due to simvastatin reducing bacterial internalisation and intracellular survival rather than the number of viable host cells (Figure 2D). Furthermore, simvastatin appeared toxic to LAD2 cells at concentrations of 100µmol/l.

**Confocal microscopy demonstrates reduced LAD2 cell infection in simvastatin-treated LAD2 cells**

To validate these findings and determine whether the reduction in intracellular CFUs was due to there being fewer infected cells, we used confocal microscopy and BacLightTM LIVE/DEADTM imaging. LAD2 cells were pretreated with 0, 1, 30 and 50 µmol/l simvastatin and co-cultured with CRS-related *S. aureus*. At simvastatin concentrations of 0 and 1 μmol/l, 32.8-33.9% of LAD2 cells were infected, compared to 15.3-17.1% at 30, and 50 μmol/l (P≤0.01; Figure 3). These results confirmed a significant reduction in infection rate with increasing simvastatin concentrations.

DISCUSSION

Statins have been shown to possess significant anti-staphylococcal properties, with patients taking oral statins demonstrating a significantly reduced odds ratio of being diagnosed with CRS (20 ,21). Based on this, we hypothesised that statins could potentially be repurposed as a novel anti-staphylococcal treatment to reduce dependence on antibiotics in recalcitrant *S. aureus*-related CRS. To explore this possibility, we focused on simvastatin, a widely prescribed statin with a lower MIC against *S. aureus* compared to other statins (22 ,41).

In our study, we determined the MIC of prodrug simvastatin against a well-characterised, virulent CRSwNP isolate of *S. aureus* to be below 40µmol/l, consistent with previously reported MIC values ranging from 38.1-398 µmol/l (16-167mg/l) (22 ,23 ,26 ,28 ,42 ,43) . However, this concentration far exceeds the levels typically observed in the serum of patients taking oral simvastatin (19-31nmol/l) (25). We also found that the hydroxylated form of simvastatin exhibited no direct antimicrobial activity.

Previous studies have shown that simvastatin reduces intracellular translocation and survival of *S. aureus* in HEK293A epithelial cells at concentrations of 0.1-1 µmol/l, in a process that could be reversed by addition of HMG-CoA reductase products (40). Similarly, we found that simvastatin significantly reduced intracellular S. aureus in LAD2 mast cells at concentrations of 1-100 µmol/l. At 30 µmol/l, simvastatin reduced the percentage of infected cells from 32.8% vs 17.1%, highlighting its potential to inhibit bacterial internalisation and survival.

The mechanisms underlying this effect likely involve the inhibition of HMG-CoA reductase, which produces cholesterol and isoprenoids in mammalian and bacterial cells (22 ,40). Cholesterol is a major component of lipid raft domains, which act as docking sites for bacteria, and facilitate energy-efficient endocytosis (44 ,45). By reducing cholesterol, simvastatin may disrupt lipid raft domains, potentially impairing *S. aureus* internalisation*.* Furthermore, simvastatininhibits the prenylation of small GTPases including CDC42 and Rac preventing their localisation to the cell membrane and p85 and PI3K activation of actin-mediated caveolation and endocytosis (40 ,46 ,47).

*S. aureus* has an HMG-COA reductase enzyme (*mvaA*) which is essential for its survival and is inhibited by statins, such as fluvastatin (22). Statins reduce the production of isoprenoid intermediates involved in prenylation, a critical post-translational protein modification of bacterial toxins, antibiotic efflux pumps and cell wall components which are essential for bacterial survival, growth and antimicrobial resistance (12). Simvastatin has been shown to reduce *S. aureus* toxin production, including panton-valentine leukocidin and α-haemolysin at concentrations similar to those tested in our study (43). Alpha-haemolysin plays a critical role in intracellular translocation by assisting escape from phagosomes (48 ,49). Recent reports have shown that statins induce disassembly of functional membrane microdomains in MRSA which stabilise proteins during infection via recruitment of flotilin. This leads to denatured antimicrobial resistance proteins such as penicillin binding protein 2a and accumulation of unfolded proteins, which affect bacterial cell viability and induce penicillin susceptibility (50).

Given its well-established safety profile, affordability and ease of manufacture, these preliminary findings support the potential for simvastatin to be repurposed as a novel topical anti-staphylococcal agent for use in refractory *S. aureus*-related CRS. The accessibility of the nasal cavity to topical treatments such as creams, ointments, sprays and drops, further supports the feasibility of achieving the required concentrations for anti-staphylococcal effects.

This study has some limitations. Statins have been reported to reduce IgE-mediated signalling in RBL-2H3 cell lines, leading to reduced degranulation and potentially reduced bacterial entry via membrane recycling (30 ,51). We were unable to evaluate this mechanism due to a common loss-of-function mutation in the high affinity receptor for IgE in LAD2 cells. Nevertheless, we tested the MRGPRX2 receptor which uses similar signalling pathways and found no effect of simvastatin on degranulation (data not included). Whilst tissue-derived nasal polyp mast cells could have been used, these are notoriously difficult to isolate from nasal polyps with a typically low yield and inter-patient heterogeneity. This would have rendered this approach both costly and impractical. Delivering lipophilic simvastatin to the sinuses at therapeutically relevant concentrations is likely to be problematic. While simvastatin ointments at 1% and 3% have been formulated and tested on human skin in previous trials, delivering this at optimal antimicrobial concentrations in a high-volume nasal saline rinse may be more challenging (52).

Evidently clinical validation of these in vitro findings, including safety and tolerability profiles, will be required. Future in vivo antimicrobial efficacy clinical studies in patients with S. aureus-related CRS will be needed to prove effectiveness. Investigation of the absorption distribution and retention of topically applied simvastatin to the nasal mucosa will be also required to establish optimal dosing strategies. These studies are currently underway, and the results will be reported in due course.

CONCLUSIONS

At typical serum concentrations observed in patients taking oral simvastatin, neither the prodrug nor hydroxylated forms of simvastatin exhibited significant anti-staphylococcal effects. However, at concentrations achievable through the topical application route, simvastatin demonstrated a direct anti-staphylococcal effect. Treatment of mast cells with simvastatin significantly reduced both the *S. aureus* intracellular burden and the proportion of infected cells. Given the accessibility of the nasal cavity to topical treatments, topical simvastatin offers a promising approach for treating refractory *S. aureus*-related CRS, and could help reduce the need for revision sinus surgery and the risk of antimicrobial resistance which has reached epidemic proportions globally.

ACKNOWLEDGEMENTS

We are grateful for the assistance received from staff of the Biomedical Imaging Unit at the Faculty of Medicine, and in particular Mr David Johnston.

AUTHORSHIP CONTRIBUTION

SPG, AFW, RJS contributed to study conception and design. RJS, PGH, HASJ were responsible for retrieving bacterial samples. Sample preparation, data collection and analysis were performed by SPG, LCL. The first and final draft of the manuscript was written by SPG and all authors advised on previous drafts. All authors approved the final manuscript.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

FUNDING

This work was supported by the following: Royal College of Surgeons of England through the Dr Shapurji H Modi Memorial Research Fellowship, a pump priming grant provided by the Royal College of Surgeons Edinburgh, and a British Rhinological Society research grant.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated and analysed during the current study are available in the University of Southampton Institutional Repository, https://eprints.soton.ac.uk/490561/.

REFERENCES

1. Vickery TW, Ramakrishnan VR, Suh JD. The Role of Staphylococcus aureus in Patients with Chronic Sinusitis and Nasal Polyposis. Curr Allergy Asthma Rep. 2019;19(4):21.

2. Van Zele T, Gevaert P, Watelet JB, et al. Staphylococcus aureus colonization and IgE antibody formation to enterotoxins is increased in nasal polyposis. J Allergy Clin Immunol. 2004;114(4):981-3.

3. Maniakas A, Asmar MH, Renteria Flores AE, et al. Staphylococcus aureus on Sinus Culture Is Associated With Recurrence of Chronic Rhinosinusitis After Endoscopic Sinus Surgery. Front Cell Infect Microbiol. 2018;8:150.

4. Watkins KE, Unnikrishnan M. Evasion of host defenses by intracellular Staphylococcus aureus. Adv Appl Microbiol. 2020;112:105-141.

5. Tan NC, Foreman A, Jardeleza C, Douglas R, Vreugde S, Wormald PJ. Intracellular Staphylococcus aureus: the Trojan horse of recalcitrant chronic rhinosinusitis? Int Forum Allergy Rhinol. 2013;3(4):261-6.

6. Singhal D, Foreman A, Jervis-Bardy J, Wormald PJ. Staphylococcus aureus biofilms: Nemesis of endoscopic sinus surgery. Laryngoscope. 2011;121(7):1578-83.

7. Hayes SM, Howlin R, Johnston DA, et al. Intracellular residency of Staphylococcus aureus within mast cells in nasal polyps: A novel observation. J Allergy Clin Immunol. 2015;135(6):1648-51.

8. Rigaill J, Morgene MF, Gavid M, et al. Intracellular activity of antimicrobial compounds used for Staphylococcus aureus nasal decolonization. J Antimicrob Chemother. 2018;73(11):3044-3048.

9. Sendi P, Proctor RA. Staphylococcus aureus as an intracellular pathogen: the role of small colony variants. Trends Microbiol. 2009;17(2):54-8.

10. Tan NC, Cooksley CM, Roscioli E, et al. Small-colony variants and phenotype switching of intracellular Staphylococcus aureus in chronic rhinosinusitis. Allergy. 2014;69(10):1364-71.

11. Hayes SM, Biggs TC, Goldie SP, et al. Staphylococcus aureus internalization in mast cells in nasal polyps: Characterization of interactions and potential mechanisms. J Allergy Clin Immunol. 2020;145(1):147-159.

12. Schelz Z, Muddather HF, Zupko I. Repositioning of HMG-CoA Reductase Inhibitors as Adjuvants in the Modulation of Efflux Pump-Mediated Bacterial and Tumor Resistance. Antibiotics (Basel). 2023;12(9).

13. Dou L, Xu Z, Xu J, et al. A network-based systems genetics framework identifies pathobiology and drug repurposing in Parkinson's disease. NPJ Parkinsons Dis. 2025;11(1):22.

14. Schenk P, Spiel AO, Huttinger F, et al. Can simvastatin reduce COPD exacerbations? A randomised double-blind controlled study. Eur Respir J. 2021;58(1).

15. Dutta NK, Bruiners N, Pinn ML, et al. Statin adjunctive therapy shortens the duration of TB treatment in mice. J Antimicrob Chemother. 2016;71(6):1570-7.

16. McDowell SA, Ma Y, Kusano R, Akinbi HT. Simvastatin is protective during Staphylococcus aureus pneumonia. Curr Pharm Biotechnol. 2011;12(9):1455-62.

17. Abdelaziz AA, El-Barrawy MA, El-Nagar RAM. Potent synergistic combination of rosuvastatin and levofloxacin against Staphylococcus aureus: in vitro and in vivo study. J Appl Microbiol. 2021;131(1):182-196.

18. AlJunaydil NA, Lambarte RNA, Sumague TS, Alghamdi OG, Niazy AA. Lovastatin and Resveratrol Synergistically Improve Wound Healing and Inhibit Bacterial Growth. Int J Mol Sci. 2025;26(2).

19. Sun T, Huang J, Zhang W, et al. Simvastatin-hydroxyapatite coatings prevent biofilm formation and improve bone formation in implant-associated infections. Bioact Mater. 2023;21:44-56.

20. Gilani S, Bhattacharyya N. The Potential Protective Effects of Statins in Chronic Rhinosinusitis: A Case-Control Study. Laryngoscope. 2021;131(5):E1431-E1433.

21. Wilson JH, Payne SC, Fermin CR, Churnin I, Qazi J, Mattos JL. Statin use protective for chronic rhinosinusitis in a nationally representative sample of the United States. Laryngoscope. 2020;130(4):848-851.

22. Hennessy E, Adams C, Reen FJ, O'Gara F. Is There Potential for Repurposing Statins as Novel Antimicrobials? Antimicrob Agents Chemother. 2016;60(9):5111-21.

23. Thangamani S, Mohammad H, Abushahba MF, et al. Exploring simvastatin, an antihyperlipidemic drug, as a potential topical antibacterial agent. Sci Rep. 2015;5:16407.

24. Wang CC, Yang PW, Yang SF, Hsieh KP, Tseng SP, Lin YC. Topical simvastatin promotes healing of Staphylococcus aureus-contaminated cutaneous wounds. Int Wound J. 2016;13(6):1150-1157.

25. Bjorkhem-Bergman L, Lindh JD, Bergman P. What is a relevant statin concentration in cell experiments claiming pleiotropic effects? Br J Clin Pharmacol. 2011;72(1):164-5.

26. Masadeh M, Mhaidat N, Alzoubi K, Al-Azzam S, Alnasser Z. Antibacterial activity of statins: a comparative study of atorvastatin, simvastatin, and rosuvastatin. Ann Clin Microbiol Antimicrob. 2012;11:13.

27. Wilding EI, Kim DY, Bryant AP, et al. Essentiality, expression, and characterization of the class II 3-hydroxy-3-methylglutaryl coenzyme A reductase of Staphylococcus aureus. J Bacteriol. 2000;182(18):5147-52.

28. Jerwood S, Cohen J. Unexpected antimicrobial effect of statins. J Antimicrob Chemother. 2008;61(2):362-4.

29. Hansen GH, Niels-Christiansen LL, Thorsen E, Immerdal L, Danielsen EM. Cholesterol depletion of enterocytes. Effect on the Golgi complex and apical membrane trafficking. J Biol Chem. 2000;275(7):5136-42.

30. Kolawole EM, McLeod JJ, Ndaw V, et al. Fluvastatin Suppresses Mast Cell and Basophil IgE Responses: Genotype-Dependent Effects. J Immunol. 2016;196(4):1461-70.

31. Pruefer D, Makowski J, Schnell M, et al. Simvastatin inhibits inflammatory properties of Staphylococcus aureus alpha-toxin. Circulation. 2002;106(16):2104-10.

32. Tilahun ME, Kwan A, Natarajan K, et al. Chimeric anti-staphylococcal enterotoxin B antibodies and lovastatin act synergistically to provide in vivo protection against lethal doses of SEB. PLoS One. 2011;6(11):e27203.

33. Collins R, Reith C, Emberson J, et al. Interpretation of the evidence for the efficacy and safety of statin therapy. Lancet. 2016;388(10059):2532-2561.

34. Biggs TC. Characterising the role of staphylococcus aureus and its toxins in chronic rhinosinusitis: University of Southampton; 2018.

35. Biggs TC, Abadalkareem RS, Hayes SM, et al. Staphylococcus aureus internalisation enhances bacterial survival through modulation of host immune responses and mast cell activation. Allergy. 2021;76(6):1893-1896.

36. Biggs TC, Hayes SM, Harries PG, et al. Immunological profiling of key inflammatory drivers of nasal polyp formation and growth in chronic rhinosinusitis. Rhinology. 2019;57(5):336-342.

37. McKay A, Leung BP, McInnes IB, Thomson NC, Liew FY. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. J Immunol. 2004;172(5):2903-8.

38. International Organization for Standardisation. Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices — Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. ISO 20776-1 Geneva: ISO, 2019

39. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9(7):676-82.

40. Horn MP, Knecht SM, Rushing FL, et al. Simvastatin inhibits Staphylococcus aureus host cell invasion through modulation of isoprenoid intermediates. J Pharmacol Exp Ther. 2008;326(1):135-43.

41. Gu Q, Paulose-Ram R, Burt VL, Kit BK. Prescription cholesterol-lowering medication use in adults aged 40 and over: United States, 2003-2012. NCHS Data Brief. 2014(177):1-8.

42. Welsh AM, Kruger P, Faoagali J. Antimicrobial action of atorvastatin and rosuvastatin. Pathology. 2009;41(7):689-91.

43. Graziano TS, Cuzzullin MC, Franco GC, et al. Statins and Antimicrobial Effects: Simvastatin as a Potential Drug against Staphylococcus aureus Biofilm. PLoS One. 2015;10(5):e0128098.

44. Hastan D, Fokkens WJ, Bachert C, et al. Chronic rhinosinusitis in Europe--an underestimated disease. A GA(2)LEN study. Allergy. 2011;66(9):1216-23.

45. Riethmuller J, Riehle A, Grassme H, Gulbins E. Membrane rafts in host-pathogen interactions. Biochim Biophys Acta. 2006;1758(12):2139-47.

46. Arbibe L, Mira JP, Teusch N, et al. Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. Nat Immunol. 2000;1(6):533-40.

47. Cordle A, Koenigsknecht-Talboo J, Wilkinson B, Limpert A, Landreth G. Mechanisms of statin-mediated inhibition of small G-protein function. J Biol Chem. 2005;280(40):34202-9.

48. Kubica M, Guzik K, Koziel J, et al. A potential new pathway for Staphylococcus aureus dissemination: the silent survival of S. aureus phagocytosed by human monocyte-derived macrophages. PLoS One. 2008;3(1):e1409.

49. Giese B, Glowinski F, Paprotka K, et al. Expression of delta-toxin by Staphylococcus aureus mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of beta-toxin. Cell Microbiol. 2011;13(2):316-29.

50. Ukleja M, Kricks L, Torrens G, et al. Flotillin-mediated stabilization of unfolded proteins in bacterial membrane microdomains. Nat Commun. 2024;15(1):5583.

51. Fujimoto M, Oka T, Murata T, Hori M, Ozaki H. Fluvastatin inhibits mast cell degranulation without changing the cytoplasmic Ca2+ level. Eur J Pharmacol. 2009;602(2-3):432-8.

52. Adami M, Prudente Ada S, Mendes DA, Horinouchi CD, Cabrini DA, Otuki MF. Simvastatin ointment, a new treatment for skin inflammatory conditions. J Dermatol Sci. 2012;66(2):127-35.

CORRESPONDING AUTHOR

Mr Simon Patrick Goldie, Otorhinolaryngology/Head & Neck Surgery Registrar, School of Clinical and Experimental Sciences, Faculty of Medicine and Department of Otorhinolaryngology / Head and Neck Surgery, University Hospital Southampton NHS Foundation Trust, Tremona Road, Southampton SO16 6YD, United Kingdom, Telephone +44 23 8120 2928 E-mail [simongoldie.sg@googlemail.com](mailto:simongoldie.sg@googlemail.com)

FIGURES

**Figure 1: Minimal inhibitory concentration of prodrug and hydroxylated simvastatin**

Minimal inhibitory concentrations of simvastatin (**A+B**), DMSO (**C**) and hydroxylated simvastatin (**D+E**) was calculated against CRS *S. aureus*. Optical density at 600nM was used as a measure of bacterial density. DMSO concentrations of 0.013, 0.026, 0.052, 0.105, 0.209, 0.418 v/v correspond to that used to dissolve 3.375, 6.75, 15.5, 25, 50, 100 μmol/L simvastatin, respectively. Nine experimental repeats were completed for each variable with statistical analysis using one-way ANOVA and Tukey’s multiple comparisons test. Bars represent the mean of each experiment with dots showing the result of each experimental repeat (\*\*\* P≤0.001 \*\*\*\* P≤0.0001).

**Figure 2: Intracellular infection of simvastatin pretreated LAD2 cells with *S. aureus***

Mean intracellular survival of CRSwNP *S. aureus* in co-culture with LAD2 cells pretreated with simvastatin for 16 hrs at typical serum (**A**) and topical application (**B**) concentrations. Mean of nine experimental repeats are displayed showing *S. aureus* CFU/ 5x105 LAD2 cells represented by bars with each dot demonstrating the result of each experimental repeat. One-way ANOVA used with Tukey’s multiple comparisons test used to determine statistical significance (\*P≤0.05,\*\*P≤0.01, \*\*\*\*P≤0.0001).

Net LDH release of *S. aureus* infected cells was measured for topical application concentrations (**C**) and net LDH release for uninfected cells exposed to identical topical application concentrations of simvastatin are displayed (**D**). Mean of nine experimental repeats are displayed showing percentage net LDH release represented by bars with the result of each experimental repeat demonstrated by dots. One-way ANOVA used with Tukey’s multiple comparisons test used to determine statistical significance (\*\*\*\*P≤0.0001).

**Figure 3: LAD2 mast cell infection with *S. aureus* after pre-treatment with simvastatin**

LAD2 cells were pre-treated with simvastatin and subsequently cultured with CRS *S. aureus* for 6 hrs, followed by staining with BacLightTM LIVE/DEADTM staining. **A)** Representative confocal z-stacks of each experimental condition are displayed. The percentage of infected cells was calculated from six separate z-stack images, each containing between 74-152 cells. **Purple** arrows indicate infected cells, **blue** arrows highlight extracellular bacteria, **yellow** arrows denote apoptotic cells, and **red** arrows show infected apoptotic cell bodies. **B)** A graphical representation of the percentage of infected cells. Bars represent the mean number of cells infected, with each dot demonstrating the percentage of infected cells in each experimental repeat. Statistical analyses were performed using one-way ANOVA and Tukey’s multiple comparisons test (\*\*P≤0.01).