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NOTE

Statins inhibit *in vitro* virulence phenotypes of *Pseudomonas aeruginosa*

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Statins are a family of drugs that lower cholesterol levels by inhibiting 3-hydroxy-3-methylglutaryl-CoA-reductase, a rate-limiting enzyme in the human mevalonate pathway of which cholesterol is the biosynthetic end product.[1] Statins also have a range of cholesterol-independent effects, including anti-inflammatory functions and antimicrobial activity. These pleiotropic effects are thought to account for the improved survival observed in statin-treated patients suffering from severe bacterial infections, such as sepsis and pneumonia.[2–4] In order to identify the mechanism involved in the protective effects of statins against infection, research studies focused on the direct effect of statins on bacteria. These studies suggest that statins have bacteriostatic effects on the *in vitro* growth of clinically important bacterial species, including *Staphylococcus aureus* and Enterococci,[5] *Streptococcus pneumoniae* and *Moraxella catarrhalis*,[6] and *Escherichia coli* and *Pseudomonas aeruginosa*.[7] However, the concentrations used in these *in vitro* studies exceed the concentration detected in human serum during statin therapy,[6] suggesting the *in vitro* bacteriostatic effects of statins are not likely to account for the beneficial outcome of patients suffering from severe bacterial infections.

To date, a single study has examined the effects of statins on bacterial virulence traits: Rosch *et al.* reported that simvastatin (SIM) could reduce the *in vivo* attachment of *S. pneumoniae* to the lung and vascular tissue, but did not affect bacterial toxin production.[8] Therefore, our objective was to investigate whether statins could modulate virulence factor behaviour in the human opportunistic pathogen *P. aeruginosa*. This significant nosocomial pathogen is the most important microorganism associated with chronic respiratory disease in cystic fibrosis (CF) patients.[9] *P. aeruginosa* is also capable of causing serious infections in other sites in the body, including burn wounds, the cornea and urinary tract. The ability of *P. aeruginosa* to establish such infections is owing to its ability to utilize a range of virulence traits to colonize its host and evade the immune response.[10]

In this study, *P. aeruginosa* model strains PAO1 (Holloway *et al.*[11]) and PA14 (Liberati *et al.*[12]) were cultured at 37 °C in Luria–Bertani broth unless otherwise specified. SIM and lovastatin (LOV) were obtained from Sigma-Aldrich, Dorset, UK, and mevastatin (MEV) was obtained from Calbiochem, Darmstadt, Germany. All statins were resuspended in dimethyl sulfoxide. The inactive prodrug form of each compound, where the lactone ring is intact, was used in all experiments.

To ensure that any effects on virulence factor behaviour occurred independently of growth inhibition, growth in the presence and absence of statins was measured. Bacteria were cultured in 96-well microtitre plates in Mueller–Hinton broth containing a 10-fold dilution series (1 mM–10 nM) of each statin and optical density[5] at 600 nm was measured after 24 h incubation. No statin was found to have a significant inhibitory effect on bacterial growth (data not shown). This correlates with a previous observation that statins only decrease the growth of *P. aeruginosa* at high concentrations.[7]

The influence of statins on motility, a key factor intrinsically linked to other traits, including biofilm formation[13] and quorum sensing,[14] was examined. Swarming motility of *P. aeruginosa* was tested using 0.6% (w/v) Eiken agar (Eiken Chemical, Tokyo, Japan) supplemented with 0.5% (w/v) glucose, while swimming and twitching motility were measured on 0.3% (w/v) and 1% (w/v) agar, respectively. Interestingly, swarming motility of both model strains was decreased by 100 μM concentrations of SIM, LOV and MEV compared with a dimethyl sulfoxide vehicle control (Figure 1a). However, swimming and twitching motility of these strains were not affected by these statin concentrations (data not shown). To further substantiate our finding, the effect of swarming motility of isolates from CF and non-CF patients was investigated. Hundred micromolar of all three statins inhibited swarming motility of both these isolates, indicating the statin effect was not strain-specific (Figure 1b).

Swarming motility is often associated with biofilm formation, a key pathogenic trait in many microorganisms, but particularly in *P. aeruginosa* as it is associated with chronic infections. Therefore, the effect of statins on attachment, an early stage of biofilm formation, was investigated using 10 and 100 μM SIM. For this, PA14 was incubated at 37 °C for 2 h in 24-well microtitre plates with

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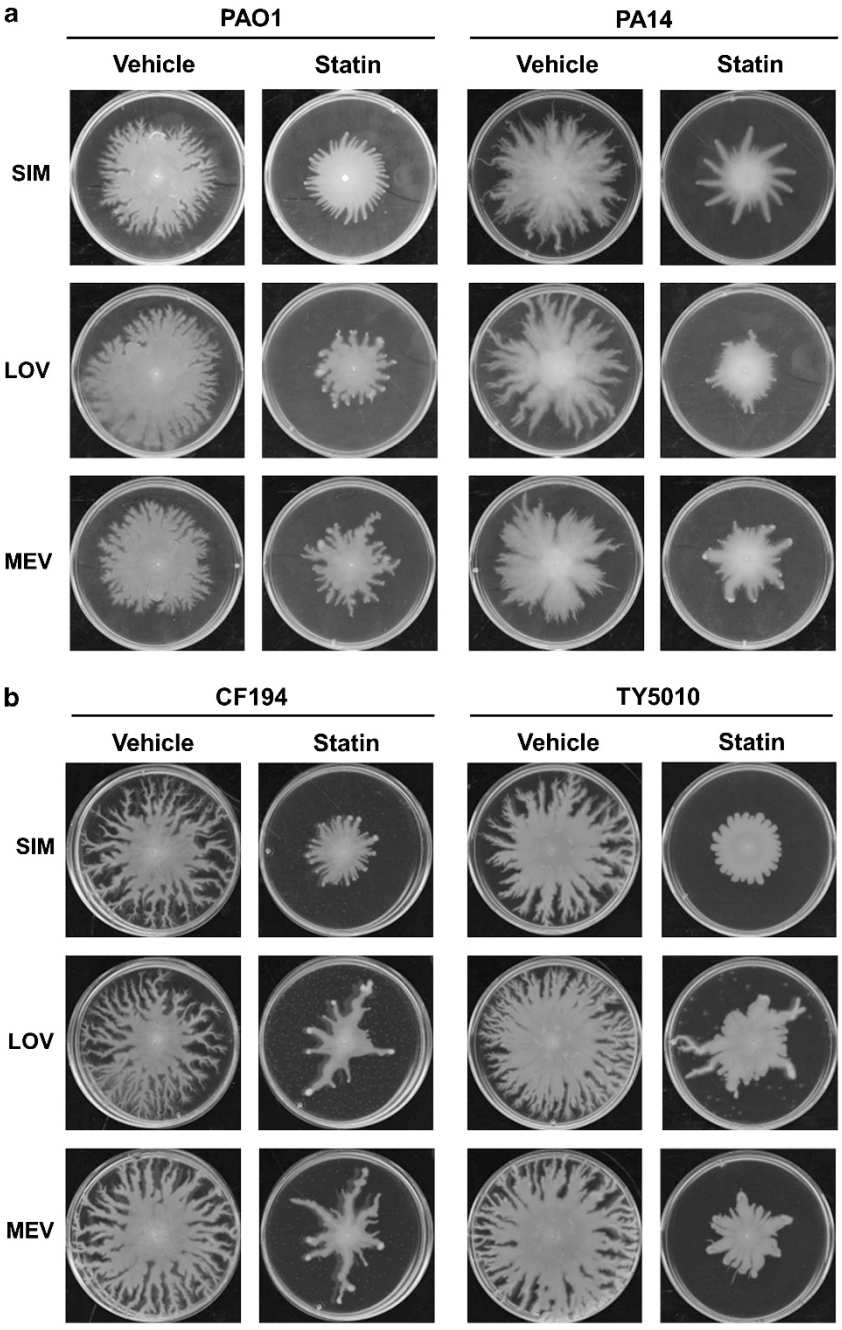

Figure 1 Statins modulate swarming motility of *P. aeruginosa*. Strains were cultured on 0.6% (w/v) Eiken agar in the presence of 100 μM SIM, LOV and MEV or an equivalent vehicle control. Statins attenuated swarming motility of (**a**) the model strains PAO1 and PA14, and (**b**) the clinical isolates CF194 (CF) and TY5010 (non-CF). Data shown are representative of three biological replicates. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

and without SIM. Plates were washed three times, and attached bacteria were stained using 0.1% (w/v) crystal violet. Excess dye was removed by washing and remaining dye was resuspended in 96% (v/v) ethanol, after which the absorbance at 570 nm was measured to quantify attachment. Hundred micromolar SIM significantly attenuated the attachment of PA14 (Figure 2). Although 10 μM SIM was also found to reduce attachment, it did not have a significant effect. To examine if statins could cause disruption of attached bacteria, PA14 was cultured in 96-well microtitre plates at 37 °C for 8 h, following which unattached cells were removed and fresh media containing 100 μM of SIM was added and re-incubated for 12 h. Attachment was measured as described above. Bacterial attachment was not disrupted by the addition of SIM (data not shown).

In addition to the motility and biofilm virulence assays, the effects of SIM, LOV and MEV on type 3 toxin secretion and quorum-sensing signaling of *P. aeruginosa* were also examined. Expression of the Type 3 toxin ExoS was measured using a PAO1 *exoS-lacZ* transcriptional fusion. Production of acylated homoserine lactones by PAO1 and PA14 was examined using the indicator strain *Chromobacterium violaceum* CV026,[15] and expression of the PQS biosynthetic operon

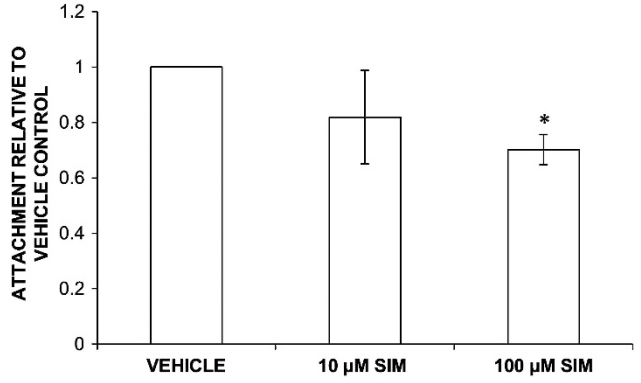

Figure 2 Effect of statins on *P. aeruginosa* attachment. PA14 was cultured in the presence of 10 and 100 μM SIM for 2 h. Attached cells were stained with 0.1% (w/v) crystal violet, and the $OD_{570\,nm}$ of resuspended dye was used to measure attachment. Hundred micromolar SIM was capable of significantly reducing bacterial attachment (*$P<0.05$). Data represent three biological replicates.

in response to statins was examined using a P_{pqsA}-*lacZ* reporter fusion. However, neither of these virulence factors were altered by 100 μM of SIM, LOV and MEV as all fold changes were lower than 1.1 fold compared with the vehicle control.

Motility and biofilm formation are crucial bacterial virulence factors linked to the establishment of chronic infections and are associated with successful colonization of the lungs of CF patients.[16] As well as attenuating these traits, statins have also been shown to lower the levels of *P. aeruginosa*-induced pro-inflammatory cytokines, such as IL-8[17] and TNFα,[18] in the host and to reduce mucin production *in vivo*.[18] Therefore, the efficacy of using statins in the treatment of chronic *P. aeruginosa* infection may warrant further investigation. Although circulating statin concentrations are typically lower than the concentrations used in this study, an alternative administrative route, such as inhalation, could lead to higher local concentrations in the lungs and may provide a novel treatment option for the use of statins, particularly in the case of chronic respiratory infection in people with CF. Statins are often administered in the form of a prodrug and are hydrolyzed to an active form in the liver. Interestingly, the prodrug form of each statin yielded the effects described in this study, further supporting inhalation of statins as a potential administrative route. This strategy has recently been used for the antibiotic tobramycin, where inhaled tobramycin powder led to improved lung function in *P. aeruginosa*-infected CF patients.[19,20] Furthermore, statins were capable of attenuating clinical isolates from CF and non-CF patients to a similar extent, suggesting that statins could also be used in the treatment of non-CF infections.

The mechanism by which statins modulate *P. aeruginosa* virulence traits remains to be elucidated. While a number of bacteria possess orthologues of the human 3-hydroxy-3-methylglutaryl-CoA-reductase enzyme[21] on which statins exert their inhibitory effect, *P. aeruginosa* does not possess a 3-hydroxy-3-methylglutaryl-CoA-reductase homologous protein. This suggests that the effects of statins on *P. aeruginosa* is mediated through an alternative novel mechanism and warrants further investigation to elucidate the components involved in this process.

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1 Istvan, E. S. & Deisenhofer, J. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* **292,** 1160–1164 (2001).
2 Almog, Y. *et al.* Prior statin therapy is associated with a decreased rate of severe sepsis. *Circulation* **110,** 880–885 (2004).
3 Schlienger, R. G., Fedson, D. S., Jick, S. S., Jick, H. & Meier, C. R. Statins and the risk of pneumonia: a population-based, nested case-control study. *Pharmacotherapy* **27,** 325–332 (2007).
4 Mortensen, E. M., Restrepo, M. I., Anzueto, A. & Pugh, J. The effect of prior statin use on 30-day mortality for patients hospitalized with community-acquired pneumonia. *Respir. Res.* **6,** 82 (2005).
5 Jerwood, S. & Cohen, J. Unexpected antimicrobial effect of statins. *J. Antimicrob. Chemother.* **61,** 362–364 (2008).
6 Bergman, P. *et al.* Studies on the antibacterial effects of statins—in vitro and in vivo. *Plos One* **6,** e24394 (2011).
7 Welsh, A. M., Kruger, P. & Faoagali, J. Antimicrobial action of atorvastatin and rosuvastatin. *Pathology* **41,** 689–691 (2009).
8 Rosch, J. W. *et al.* Statins protect against fulminant pneumococcal infection and cytolysin toxicity in a mouse model of sickle cell disease. *J. Clin. Invest.* **120,** 627–635 (2010).
9 Govan, J. R. & Deretic, V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and Burkholderia cepacia. *Microbiol. Rev.* **60,** 539–574 (1996).
10 Williams, B. J., Dehnbostel, J. & Blackwell, T. S. *Pseudomonas aeruginosa*: host defence in lung diseases. *Respirology* **15,** 1037–1056 (2010).
11 Holloway, B. W., Krishnapillai, V. & Morgan, A. F. Chromosomal genetics of Pseudomonas. *Microbiol. Rev.* **43,** 73–102 (1979).
12 Liberati, N. T. *et al.* An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc. Natl. Acad. Sci. USA* **103,** 2833–2838 (2006).
13 O'Toole, G. A. & Kolter, R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol. Microbiol.* **30,** 295–304 (1998).
14 Glessner, A., Smith, R. S., Iglewski, B. H. & Robinson, J. B. Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of twitching motility. *J. Bacteriol.* **181,** 1623–1629 (1999).
15 Ravn, L., Christensen, A. B., Molin, S., Givskov, M. & Gram, L. Methods for detecting acylated homoserine lactones produced by Gram-negative bacteria and their application in studies of AHL-production kinetics. *J. Microbiol. Methods* **44,** 239–251 (2001).
16 Breidenstein, E. B., de la Fuente-Nunez, C. & Hancock, R. E. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol.* **19,** 419–426 (2011).
17 Jouneau, S. *et al.* Anti-inflammatory effect of fluvastatin on IL-8 production induced by *Pseudomonas aeruginosa* and *Aspergillus fumigatus* antigens in cystic fibrosis. *Plos One* **6,** e22655 (2011).
18 Chen, Y. J. *et al.* Simvastatin attenuates acrolein-induced mucin production in rats: involvement of the Ras/extracellular signal-regulated kinase pathway. *Int. Immunopharmacol.* **10,** 685–693 (2010).
19 Konstan, M. W. *et al.* Tobramycin inhalation powder for *P. aeruginosa* infection in cystic fibrosis: The EVOLVE trial. *Pediat. Pulm.* **46,** 230–238 (2010).
20 Konstan, M. W. *et al.* Safety, efficacy and convenience of tobramycin inhalation powder in cystic fibrosis patients: The EAGER trial. *J. Cyst. Fibros.* **10,** 54–61 (2011).
21 Feng, L. *et al.* Specific inhibitions of annonaceous acetogenins on class II 3-hydroxy-3-methylglutaryl coenzyme A reductase from *Streptococcus pneumoniae. Bioorg. Med. Chem.* **19,** 3512–3519 (2011).