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1 **Proposed Title:** Statins as next generation anti-microbials: Is there potential for repurposing?

2 **Short Title:** Statins inhibit bacterial growth and virulence

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16

17 **Abstract**

18 Statins are a class of pharmaceutical widely used to treat high serum cholesterol. In addition,
19 statins have so-called “pleiotropic effects”, which include the reduction of inflammation,
20 immunomodulation, and anti-microbial effects. An increasing number of studies are emerging
21 which detail the attenuation of bacterial growth and *in vitro* and *in vivo* virulence by statin
22 treatment. In this review, we describe the current information available surrounding the effects of
23 statins on bacterial infections, and provide insight regarding the potential use of these
24 compounds as anti-microbial therapeutic agents.

25

26 Introduction

27 One of the major undisputed clinical breakthroughs of the 20th century was the discovery of the
28 statin family of drugs. These compounds are renowned for their ability to lower cholesterol
29 levels, and are used to treat approximately 40 million individuals with high cholesterol
30 worldwide. Since the discovery of mevastatin as a metabolic product of *Penicillium citrinum* in
31 1976 (1, 2) a total of nine statins have been characterized, seven of which are approved by the
32 FDA to treat patients with high cholesterol. Structurally, statins are characterized by the presence
33 of a conserved lactone ring (3). This structure is present as a hydrolyzed (active) form in all
34 statins except for mevastatin, lovastatin and simvastatin, where the lactone ring is hydrolyzed in
35 the liver (4). Statins can be divided into two broad classes (Figure 1). Type 1 statins are
36 lipophilic, and possess a butaryl side chain – they are said to structurally resemble mevastatin
37 (3). Lovastatin, pravastatin and simvastatin are type 1 statins. Type 2 statins are classically
38 lipophobic, and are distinguished from type 1 by the replacement of the butaryl side chain with a
39 fluorophenol group and typically possess larger side chains than type 1 statins (3). Atorvastatin,
40 cerivastatin, fluvastatin, pitavastatin and rosuvastatin are type 2 statins.

41 Statins exert their cholesterol lowering effect by binding to the active site of 3-hydroxy-3-
42 methylglutaryl-CoenzymeA reductase (HMGR), a rate-limiting enzyme involved in cholesterol
43 biosynthesis (3). HMGR is an integral part of the mevalonate pathway, which is not only
44 essential for cholesterol biosynthesis, but also contributes to the production of isoprenoids, lipid
45 compounds that are essential for cell signaling and structure. As well as the inhibition of
46 cholesterol, statins have also been found to have a number of cholesterol-independent, so-called
47 “pleiotropic” effects. Statins have been reported to confer anti-inflammatory,
48 immunomodulatory and anti-cancer effects on host cells, and these effects are well-characterized

49 (5–9). Furthermore, several studies have explored the pleiotropic effects of statins in combating
50 multi-system microbial infections, such as sepsis and pneumonia, and a growing number of
51 studies are demonstrating that statins can directly influence the growth and virulence of bacterial
52 pathogens. With the global increase in antibiotic resistance to existing antibiotics and the search
53 for new anti-microbial strategies reaching a critical stage, there is increasing interest in the
54 possibility of repurposing existing drugs that have already been approved to treat different
55 clinical conditions but that also possess antimicrobial activity. The repurposing of these drugs
56 would significantly reduce the lead-time from bench to bedside. Given their pleiotropic activities
57 statins are strong potential candidates to be repurposed as novel antimicrobial agents. However,
58 the evidence for this remains controversial owing to the number of apparently contradictory
59 studies. This review evaluates and discusses the effects of individual statins on bacterial growth
60 and virulence and bacterial infections in the context of pathogen-host interactomes (summarized
61 in Figure 2).

62

63 **Clinical evidence that statins influence morbidity and mortality of patients with microbial**
64 **infections.**

65 The clinical potential of statins as anti-microbial agents has been the subject of several studies
66 and reviews. A number of meta-analyses of cohort studies on the impact of overall statin use on
67 different infection outcomes showed positive findings, albeit while highlighting the limitations
68 and heterogeneity of the studies (10 - 13). These reviews included studies on infections such as
69 bacteraemia, pneumonia, sepsis and some acute infections and patient populations received
70 several different statins. For instance, two single centre retrospective studies showed that patients

71 with bacteraemia who have undergone prior statin treatment have a significant decreased risk of
72 in hospital mortality of 6% vs 28% ($p = 0.002$) and 13% vs 24% ($p = 0.001$) respectively (14,
73 15). The latter study also showed there was an inverse correlation between the length of statin
74 treatment and risk of mortality when they compared statin use ≥ 12 and < 12 weeks prior to
75 infection (11% vs 14%, $p = 0.04$) (15). A meta analysis of available published data found that
76 the use of statins was specifically associated with a reduced risk of morbidity and mortality
77 resulting from pneumonia (12). A retrospective study of patients in the UK found that current
78 statin treatment (within last 30 days) reduced pneumonia-associated mortality (adjusted OR 0.47,
79 95% CI 0.25-0.88) (16), while prior statin treatment also reduced mortality rates in patients in the
80 USA with community-acquired pneumonia (CAP) (adjusted OR 0.36, 95% CI 0.14–0.92) (17).
81 Furthermore, data from the Justification for the Use of Statin in Prevention: An Intervention
82 Trial Evaluating Rosuvastatin (JUPITER), which was initially undertaken to determine whether
83 rosuvastatin could reduce the risk of cardiac disease in people without hyperlipidemia (18) were
84 retrospectively analysed in 2012. This analysis suggested that rosuvastatin treatment may
85 decrease the occurrence of pneumonia before (HR 0.81, 95% CI 0.67–0.97) or after a cardiac
86 event (HR 0.83, 95% CI 0.69–1.00) (19). In contrast however, an earlier prospective cohort study
87 which examined adults in six Canadian hospitals had concluded that after adjusting for
88 confounding factors such as the ‘healthy user effect’ prior statin treatment does not yield reduced
89 mortality from pneumonia (20). This latter study encompassed 3415 patients > 17 yrs of age with
90 pneumonia admitted to hospital, while the JUPITER randomized, double-blind, placebo-
91 controlled trial of 17,802 healthy patients was restricted to men > 50 yrs and women > 60 yrs of
92 age. Indeed, the JUPITER study was designed to address the ‘healthy user effect’ suggesting that

93 study design in addition to age differences between the cohorts may underpin the contrasting
94 observations.

95 Sepsis is a serious infection-induced whole body inflammatory state, and due to the
96 immunomodulatory activity of statins, several studies have been carried out to evaluate the
97 benefit of statin therapy in the prevention or treatment of the disease. While the type, design, size
98 and measured outcomes of the studies have been varied and overall results conflicting, in recent
99 years extensive reviews evaluating these clinical studies have been published (21-27). The
100 majority of clinical studies to date have been retrospective cohort studies evaluating the impact
101 of prior treatment with statins on disease progression and mortality. Many of these, plus several
102 meta-analysis reviews, showed promising results whereby prior use of statins significantly
103 reduced disease progression and/or mortality associated with sepsis (25, 28 - 32). For instance,
104 studied by Almog et al. and Martin et al. demonstrated a reduced risk of developing severe sepsis
105 in patients pretreated with statins (2.4% vs 19%, $P<0.001$ and 56% vs 86%, $P<0.02$ respectively)
106 while Mortensen et al. showed a reduced risk of 30 day mortality in patients using statins (OR
107 0.48, 95% CI 0.36-0.64). One of the main limitations attributed to these studies was limited
108 sample size, and against this, a recent population-based, propensity score-matched analysis of the
109 effect of low and high doses of statins on sepsis outcomes involved a cohort of 27,792 statin
110 users compared with an equal number of non-users (33). This extensive study demonstrated a
111 significant reduction of 1-year mortality (HR 0.83, 95 % CI 0.81–0.85) and adverse
112 consequences of sepsis such as in-hospital death (OR 0.86, 95 % CI 0.83–0.89) and ICU
113 admission (OR 0.95, 95 % CI 0.92–0.98) in patients pretreated with statins. They also showed
114 that the benefits of pretreatment with statins increased significantly with higher doses.

115 Therefore, several studies have shown promising potential for the prior use of statins in the

116 prevention / progression of infections. Nevertheless, it is difficult to draw conclusions on
117 whether these statins effects were directly anti-bacterial / inflammatory or due to pleiotrophic
118 effects on co-morbidities associated with the infections. For example, it is estimated that
119 cardiovascular events account for up to 30% of deaths in patients with CAP and therefore it
120 could be argued that prior statin use could improve cardiovascular health and thus reduce
121 mortality rather than having any direct effect on the infection. Against this a study has reported
122 that while prior statin use was significantly associated with decreased 90-day mortality in CAP
123 patients, there was no significant association with cardiocascular events (34). In order to fully
124 understand the mechanistic effects of prior statin use on infections similar studies targeting for
125 example specific co-morbidities and/or inflammatory markers would be required.

126 In contrast to prior use of statins, however, studies investigating the benefits of de-novo
127 treatment of infections with statins have generally not shown favorable results. A recent
128 randomized control trial (RCT) investigating the effect of rosuvastatin on the clinical outcome of
129 patients with sepsis associated acute respiratory distress syndrome was discontinued because of
130 futility (35). Moreover, a number of recent meta-analyses of RCTs suggest that there is no
131 significant evidence to suggest that statin use improves the mortality outcome of patients with
132 sepsis (25 - 27).

133 Further large scale RCT research is also recommended to evaluate the efficacy of using de-novo
134 statin therapy to treat specific infections. Of particular note is that the majority of the studies
135 reviewed so far did not adjust for the type of statin used or the type of bacteria causing the
136 infection. An interesting study of the effect of prior statin use on mortality in patients with
137 bloodstream infections found a significant reduction in 90-day mortality in statin users with
138 Gram-negative infections (adjusted OR 0.38, 95 % CI 0.20–0.72, $P=0.003$) but no significant

139 difference in statin users with Gram-positive infections (adjusted OR 1.22, 95% CI 0.69–2.17,
140 $P=0.49$) (36), suggesting that the type of bacterial infection may be a significant factor.

141

142 **Effects of statins on *in vitro* bacterial growth.**

143 There is a large body of evidence demonstrating that statins have direct anti-bacterial effects on
144 the *in vitro* growth of both Gram-positive and Gram-negative bacterial pathogens responsible for
145 a wide range of infections (Table 1), although there have been conflicting reports on MICs
146 (ranging from 15 mg/l to 500 mg/l) and strain specificity may be a factor (Table 1). The growth
147 of the Gram-positive nosocomial pathogens *Staphylococcus aureus* and *Streptococcus*
148 *pneumoniae* has been shown to be inhibited by atorvastatin, rosuvastatin and simvastatin (37–
149 43), while fluvastatin has also been reported to inhibit the growth of *S. aureus* (37). In addition,
150 both type 1 (simvastatin) and type 2 (atorvastatin, fluvastatin and rosuvastatin) statins have also
151 demonstrated a bacteriostatic effect against other Gram-positive cocci, notably *Streptococcus*
152 *pyogenes*, *Staphylococcus epidermidis*, *Enterococcus* and *Bacillus* spp. (37, 39, 40, 42).
153 Promisingly, simvastatin, lovastatin and rosuvastatin have also been shown to have anti-bacterial
154 effects on the growth of antibiotic-resistant species such as methicillin-resistant *S. aureus*
155 (MRSA), vancomycin-resistant *S. aureus* (VRSA) and vancomycin-resistant enterococci (VRE),
156 although the MIC concentrations are typically higher than against antibiotic sensitive strains
157 (Table 1) (37, 39 - 43).

158 Both type 1 and type 2 statins have also been found to inhibit the growth of a number of
159 clinically important Gram-negative species including several respiratory pathogens. The growth
160 of the nosocomial respiratory pathogens *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and

161 *Klebsiella pneumoniae* is inhibited by atorvastatin, rosuvastatin and simvastatin (MICs ranging
162 from 15 - 333 mg/l) (39, 40) and simvastatin was reportedly bactericidal against *Moraxella*
163 *catarrhalis* (MIC 15 mg/l) (38). In addition to respiratory pathogens, statins have also been
164 reported to inhibit other Gram-negative nosocomial pathogens. Masadeh et al. reported that
165 atorvastatin, rosuvastatin and simvastatin have bacteriostatic effects against a range of pathogens
166 including *Citrobacter freundii*, *Enterobacter aerogenes*, *Haemophilus influenzae* and *Proteus*
167 *mirabilis* (MICs ranging from 15 - 166 mg/l) (39). Simvastatin and lovastatin (10 mg/l) are also
168 reportedly bactericidal against the spirochete *Borrelia burgdorferi* (the causative agent of Lyme
169 disease) (44) and atorvastatin, rosuvastatin and simvastatin were found to inhibit the growth of
170 *Escherichia coli*, a prominent cause of gastroenteritis and urinary tract infections (39). In
171 contrast, however, Bergman et al., using a maximum concentration of 250 mg/L observed that
172 simvastatin did not inhibit the growth of *H. influenzae* (38), while Graziano et al. found that
173 simvastatin, atorvastatin and pravastatin at concentrations up to 250 mg/l did not inhibit the
174 growth of *P. aeruginosa*, *E. coli* or *Enterococcus faecalis* (43). Furthermore, the study by
175 Thangamani et al. (42) reported that while the growth of Gram-positive species was inhibited by
176 statins, the growth of *P. aeruginosa* ATCC 15442 was not inhibited by the statins simvastatin,
177 atorvastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin or rosuvastatin. They
178 also reported that simvastatin did not inhibit the growth of a range of other Gram-negative
179 pathogens including different strains of *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, *E. coli* and
180 *Salmonella enterica* serovar Typhimurium. Interestingly, they did show that when combined
181 with sub-inhibitory concentrations of colistin, which compromises the outer membrane integrity,
182 simvastatin had anti-bacterial activity against the range of Gram-negative pathogens at MICs of
183 8 – 32 mg/l. While the activity shown by simvastatin against *E. coli* ATCC35218 (39) is in direct

184 contrast to the lack of activity by simvastatin against *E. coli* ATCC35150, ATCC 700728,
185 ATCC25922, and ATCC10536 (42-43), it is worth noting that the *E. coli* ATCC3218 assays
186 were performed on solid agar while the other studies were performed using the broth
187 microdilution method, perhaps explaining the apparent differences in activity.

188 Taken together, the data suggest that the anti-bacterial activity of statins may be both statin
189 specific and / or strain/species specific. Simvastatin and atorvastatin generally appear to be more
190 effective against *S. aureus*, *S. pneumoniae* and enterococci than other statins (37–40), while three
191 distinct simvastatin MICs were reported against *S. aureus* clinical isolates from the UK and
192 Jordan as well as typed reference strains (Table 1) (37, 39, 42, 43). It is also noteworthy that
193 while the MICs of statins varied according to statin and pathogen tested, the *in vitro* MICs
194 ranged from circa 15 to 400 mg/l, which far exceeds the typical peak plasma concentrations of
195 patients on oral statins, which generally ranges from circa 10 to 300 µg/l. Moreover, in the
196 majority of cases the *in vitro* statin MICs against multi-drug resistant pathogens were even
197 greater than those against equivalent antibiotic susceptible strains. As such, at these MIC
198 concentrations, it is unlikely that they would qualify as lead molecules in drug discovery
199 programs. This variability in MICs could be considered somewhat unexpected for what is
200 essentially a novel antibiotic compound being administered to a naive population. However,
201 recent studies have reported significant phenotypic and genotypic diversity within clinical
202 populations suggesting that adaptation to environmental or host related factors may be
203 widespread (45 - 47). While the mechanism of action of statin antimicrobial and anti-virulence
204 activity remains to be elucidated, some reports suggest the involvement of isoprenoids and
205 membrane integrity (48). Further deciphering the interaction between statins and the microbial

206 membrane may provide answers to this apparent heterogeneity, although other targets within the
207 microbial cell must also be considered.

208 However, two studies have recently demonstrated the *in vivo* clinical efficacy of locally high
209 concentrations of statins whereby topical applications of simvastatin at MIC / sub-MIC
210 concentrations significantly enhanced bacterial clearance and healing of MSSA and MRSA *S.*
211 *aureus*-contaminated wounds in mice wound models (41, 42). Wang et al. showed that
212 application of simvastatin (62.5 mg/l) reduced the MSSA wound size by over 50% at day seven
213 and significantly reduced (>60% reduction) the bacterial load visible in the wound histology
214 (41), while Thangamani et al. showed that topical simvastatin at concentrations of 1% and 3%
215 significantly reduced the bacterial load in MRSA wounds by 75% and 90% respectively (42).
216 The latter study also showed that this topical application of simvastatin had an additive healing
217 effect and it reduced the production of pro-inflammatory cytokines (IL-6, TNF- α and IL-1 β) in
218 MRSA infected wound lesions.

219 The mechanism by which statins inhibit bacterial growth is unclear. As previously described,
220 statins inhibit the mevalonate pathway in human cells. This pathway is present in higher
221 eukaryotes, as well as several bacterial species including staphylococci and streptococci.
222 However, not all bacteria possess a mevalonate pathway, and in these species (and in plants)
223 isoprenoid metabolism is mediated through the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-
224 xylulose 5-phosphate (MEP-DOXP) pathway, which is mevalonate-independent (49, 50). The
225 MEP-DOXP and mevalonate pathways both feed into the production of isoprenoid intermediates.
226 Generally, it appears that Gram-positive bacteria tend to possess a mevalonate pathway, while
227 Gram-negative species utilize mevalonate-independent isoprenoid biosynthesis, although there
228 are some exceptions to this observation. Statins have been shown to inhibit the growth of *S.*

229 *aureus* by binding to and inhibiting the activity of its HMGR enzyme (51) and this may to some
230 extent explain why Gram-positive bacteria tend to be more sensitive to statins. However, statins
231 can attenuate the growth of bacteria irrespective of the presence of HMGR, although the
232 mechanism is unknown and studies have reported equivalent statin MICs in species with and
233 without HMGR (39, 40) .

234

235 **Effects of statins on intracellular growth of bacteria**

236 The effect of statins on the intracellular growth of pathogens has also been studied and, at drug
237 concentrations closer to physiological levels, they have been shown to reduce the growth of
238 several obligate intracellular bacterial pathogens. Recent reports demonstrated that lovastatin at
239 0.4 mg/l (52) and both atorvastatin and simvastatin, in a dose-dependent fashion (0.08 – 0.8
240 mg/l), reduced the survival of the leprosy-causing species *Mycobacterium leprae* (by up to 90%
241 and 75% respectively) in *in vitro* macrophage models, but in a cholesterol-dependent manner
242 (53), suggesting an indirect effect on cholesterol levels as the intracellular growth of these
243 pathogens requires cholesterol. Prior but not concomitant treatment of murine fibroblast (L929)
244 cells with lovastatin at 0.4 mg/l also reduced both the intracellular growth of the respiratory
245 pathogen *Coxiella burnetii* (which causes Q fever) (by 43%, $P=0.064$) (54), and plaque
246 formation by the causative agent of Rocky mountain spotted fever, *Rickettsia conorii* (by 64%,
247 $P=0.003$) (55). Interestingly, in *in vivo* studies the hydrophobic statin, simvastatin, at a
248 physiological concentration (0.5mg/kg), but not the hydrophilic statin pravastatin significantly
249 decreased (up to 83%) the levels of the respiratory pathogen *Chlamydiae pneumoniae* in lung
250 cells of infected mice (56, 57). It was also found that cerivastatin (0.1 mg/l) reduced the cross
251 infection of VSMC (vascular smooth muscle cells) by *C. pneumoniae* infected macrophages (56,

58). In these studies the authors also suggest that the reduced growth may be an indirect effect due to cholesterol inhibition.

A number of studies report inhibition of the non-obligate intracellular growth of *Mycobacterium tuberculosis* in peripheral blood mononuclear cells (PBMCs) and macrophages. Parihar et al. demonstrated that *M. tuberculosis* growth was significantly reduced (circa 2-fold, $P<0.05$) in human mononuclear cells and macrophages taken from atorvastatin-treated patients with familial hypercholesterolemia compared with healthy donors while also showing that simvastatin (20.6 mg/l) significantly reduced (circa 3-fold, $P<0.01$) *M. tuberculosis* growth in murine macrophages and both simvastatin and rosuvastatin significantly decreased (circa 2 to 10 –fold $P<0.05$ /0.01) the bacterial load in the liver, spleen and lungs of infected mice (20 mg/kg) (59). The study further demonstrated that the simvastatin-mediated decrease in bacterial growth was reversed by mevalonate, the product of HMG-CoA reductase and suggested that statins control infection by phagolysosomal arrest of *M. tuberculosis*. These results were corroborated by the study by Lobato et al. whereby they showed that atorvastatin and simvastatin (2 μ M) significantly inhibited *M. tuberculosis* growth (circa 60% reduction) in macrophages and again this was reversed by mevalonate (53). A previous study by Parihar et al. also demonstrated that simvastatin treatment (20.6 mg/l) could significantly reduce, by up to 4-fold, ($P<0.001$) the ability of the food borne pathogen *Listeria monocytogenes* to grow inside mouse and primary macrophages, in a cholesterol dependent manner and significantly reduce the bacterial burden and dissemination (by 100-fold) to the liver ($P<0.001$) and spleen ($P<0.05$) in infected mice (60). The intracellular growth of another food borne bacteria, the gastroenteritis-causing *Salmonella enterica* serovar Typhimurium, was also attenuated more than 10-fold by lovastatin (50 nM & 30 μ M) treatment of murine macrophages, at least in part due to attenuation of the mevalonate

275 pathway (61). A key mechanism behind the attenuation of internalized bacterial infections by
276 statins appears to be the statin-mediated inhibition of lipid raft formation. Lipid rafts are
277 glycoprotein domains present in the cell membrane, which are formed as a result of cholesterol
278 spontaneously interacting with sphingoglycolipids. Bacteria can manipulate lipid rafts in order to
279 invade and survive within cells and induce apoptosis (62). However, statins are known to inhibit
280 the formation of lipid rafts due to inhibition of cholesterol biosynthesis (63). Two studies
281 investigating the effects of statins on the intracellular growth of *L. monocytogenes* and plaque
282 formation of *R. conorii* suggest their findings were due to the inhibition of lipid raft formation by
283 statins (55, 60).

284 As well as inhibiting intracellular growth, statin treatment at physiological concentrations also
285 promotes increased bacterial killing in host cells. Simvastatin significantly reduced the burden of
286 *S. pneumoniae* in the lungs of infected mice (dose = 1 / 10 mg/kg/day, 50/100-fold reduction,
287 $P=0.02$ / 0.002) (64) and significantly increased bacterial clearance (65% reduction, $P=0.01$) and
288 reduced dissemination (90% reduction, $P=0.01$) of *S. aureus* in a mouse model of pneumonia
289 (dose = 0.25 mg/kg/day) (65). Simvastatin (~ 41.7 mg/kg/day) also reduced *S. aureus* recovery
290 by circa 35 % from mouse peritoneal ($P<0.005$) and by 2-fold in lung cells ($P<0.05$) and
291 mevastatin (50 μM) significantly reduced (40% reduction, $P<0.005$) the amount of *S. aureus*
292 recovered from intracellular infection of human neutrophils and mouse macrophages (66). In this
293 latter study evidence suggests that there was no direct effect on bacterial viability but that statins
294 promoted bacterial killing by inducing the formation of phagocyte extracellular traps.

295 Therefore, evidence suggests that, while the mechanisms by which physiological concentrations
296 of statins influence intracellular or *in vivo* bacterial infections are not fully understood, most

297 studies suggest indirect action mainly due to pleiotropic effects of modulating the mevalonate
298 pathway in the host.

299

300 **Effects of statins on bacterial virulence**

301 An interesting development in the field of statins and bacterial infection is the discovery that sub
302 lethal doses of statins may influence bacterial virulence, raising the possibility that statins may
303 be repurposed as specific anti-virulence therapeutics. A number of studies have investigated the
304 impact of statin treatment on *in vitro* bacterial virulence (Table 2). Wang et al. and Graziano et
305 al. both showed that *S. aureus* biofilm formation is inhibited by simvastatin (41, 43) while
306 Hennessy et al. demonstrated that both the *in vitro* motility and early biofilm formation of the
307 predominant cystic fibrosis-associated pathogen *P. aeruginosa* are attenuated by statin
308 concentrations sub-inhibitory to growth (4 & 40 mg/l respectively) (67). Graziano and colleagues
309 also showed that simvastatin (4x MIC) could disrupt established *S. aureus* biofilms and
310 Thangamani et al. demonstrated that simvastatin at 2x and 4x MIC concentrations reduced
311 established biofilms of both *S. aureus* and *S. epidermidis* by approximately 40% (42, 43). This
312 latter study by Thangamani et al. also showed that simvastatin suppressed the production of the
313 *S. aureus* toxins Panton-Valentine leucocidin (PVL) and α -hemolysin (Hla) produced by MRSA.
314 They also showed that simvastatin inhibited bacterial protein synthesis and suggest that the
315 reduction in toxin production may be a reflection of this.

316 In cell culture studies, simvastatin (4 mg/l) significantly increased ($P \leq 0.05$) the adhesion of *P.*
317 *aeruginosa* to lung cells (68) but the translocation of *P. aeruginosa* across the apical membrane
318 of kidney cells was significantly inhibited ($P < 0.05$) by simvastatin treatment (5 μ M / 2 mg/l)
319 (69). Neither of these studies observed an alteration in the invasive potential of *P. aeruginosa* in

320 the presence of statin, however, the invasion of other pathogens is inhibited by statins. Horn et al.
321 demonstrated reduced invasion of *S. aureus* into vascular epithelial cells in the presence of
322 physiological concentrations of simvastatin (0.04 - 0.4 mg/l) (70), while mevastatin (4 mg/l)
323 completely inhibited the internalization of Group B *Streptococcus*, a common cause of
324 meningitis, into HeLa cells (71), and attenuated the invasion of *E. coli* into bladder epithelial
325 cells (72). In these latter studies inhibition of bacterial invasion was proposed to be due to the
326 ability of simvastatin and mevastatin to inhibit the activation of Rho GTPase proteins as a result
327 of the inhibition of the production of the isoprenoid intermediates farnesyl-pyrophosphate and
328 geranylgeranyl-pyrophosphate, which are required for the prenylation and activation of Rho
329 GTPases (73).

330 Therefore, there is promising evidence that statins may influence the invasiveness and/or biofilm
331 formation of some pathogens, however, a number of studies have observed the absence of statins
332 affecting other bacterial virulence factors (Table 2). Bacterial cell-cell communication may not
333 be impacted by statins as simvastatin, lovastatin and mevastatin failed to alter N-acyl-
334 homoserine lactone (AHL) or PQS quorum sensing by *P. aeruginosa* and mevastatin failed to
335 alter AHL signaling by *Burkholderia cenocepacia*, both prominent causes of respiratory
336 infections in cystic fibrosis patients (67, 74). In the same studies transcription of the *exoS* Type
337 Three Secretion toxin and protease production, respectively, were not altered by the statins
338 tested. Furthermore, an in-depth study carried out using *S. pneumoniae* demonstrated that sub-
339 inhibitory concentrations of simvastatin (1 mg/l) did not directly influence the activity of the
340 pneumolysin toxin against red blood cells (75). However, the same study showed that
341 simvastatin did protect vascular endothelial cells from pneumolysin-induced cytotoxicity *in*
342 *vitro*. This protective effect was reversed by mevalonate, again suggesting an indirect effect. The

343 protection was confirmed *in vivo* whereby it extended to reduced lung damage and increased
344 survival in a mouse model of infection.

345 Indeed, several studies have shown that statins can reduce the impact of bacterial toxins on host
346 cells. In a study that utilized *S. aureus* α -toxin, leukocyte recruitment and adhesion in mice was
347 attenuated by simvastatin pretreatment (100 μ g/kg) by >70% ($P<0.01$) (76). This finding is
348 significant as it suggests that statins may reduce α -toxin-mediated inflammation and
349 cardiovascular damage. In addition, lovastatin (1 mg/l) improved the survival of mice which
350 were exposed to another *S. aureus* toxin, enterotoxin B by 50% (77) and the cytotoxicity of
351 *Bacillus anthracis* lethal toxin against macrophages was reduced >60% by fluvastatin,
352 mevastatin, and simvastatin (78).

353 The protective mechanism(s) of statins against bacterial virulence has not been established,
354 however, the impact of statins on host cell isoprenoid metabolism appears to regulate at least
355 some of the effects observed on bacterial virulence in cell culture and infection models. Several
356 studies have shown that the observed statin effect on bacterial virulence can be reversed by the
357 addition of exogenous mevalonate (53, 58-60, 66, 70, 75, 77, 79), while statin-mediated
358 cholesterol depletion is protective against bacterial toxins (75, 60) and contributes to the killing
359 of intracellular bacteria (44, 53, 59 - 61, 66). In addition, the regulation of the inflammatory
360 response by statins may account for some of these protective effects. For instance, cerivastatin
361 treatment attenuated the production of pro-inflammatory mediators and superoxide in
362 macrophages infected with *C. pneumoniae*, and this was associated with a reduced bacterial
363 infection rate (79). The inflammatory response in lipopolysaccharide-treated mice was also
364 reduced by cerivastatin treatment, leading to improved survival (80), while simvastatin treatment

365 reduced both lung injury and the production of pro-inflammatory chemokines in a mouse sepsis
366 model (81).

367

368 **Co-prescription of statins with antibiotics**

369 It has been hypothesized that physiological or sub-inhibitory doses of statins could be used in
370 combination with antibiotics to increase the efficacy of treatment. Many researchers have
371 proposed dual action combinations that remove the virulence threat, either toxin or biofilm,
372 facilitating clearance by the antibiotic. Indeed, the growing evidence for the effectiveness of next
373 generation anti-virulence approaches has been tempered by a realization that conventional
374 antibiotics will still be required to clear the infecting pathogen and resolve the infection. Current
375 information on the synergistic relationship between statins and antibiotics is limited and
376 conflicting (Table 2). A significant synergistic effect resulting in increased bacterial lysis has
377 been reported with sub-lethal doses of penicillin and simvastatin (7.8 mg/l) against
378 pneumococcal growth *in vitro* (38), while atorvastatin and simvastatin (0.2 μ M) increased the
379 efficacy of rifampin against *M. tuberculosis* and *M. leprae* infection *in vitro* by approximately
380 50% (53). In addition, *in vivo* mice studies showed that atorvastatin (80 mg/kg/day) increased the
381 efficacy of rifampin against *M. leprae* infection ($P<0.05$) (53) and simvastatin (25 mg/kg)
382 increased the *in vivo* activity of first-line anti-TB antibiotics reducing the lung bacillary burden
383 by $>1 \log_{10}$ ($P<0.01$) (82). Thangamani and colleagues demonstrated a positive synergistic effect
384 of simvastatin on the anti-microbial effect of four topical antibiotics, mupirocin, fusidic acid,
385 retapamulin and daptomycin, against clinical isolates of multi-drug resistant *S. aureus*. However,
386 Graziano et al. showed there was no synergistic effect between simvastatin and vancomycin
387 against *S. aureus* (43). A recent study, which examined the *in vitro* effects of five statins, at

388 concentrations equivalent to recommended physiological doses (simvastatin, lovastatin,
389 atorvastatin, pravastatin = 0.01, 0.05, 0.1 mg/l; fluvastatin = 0.1, 0.2, 0.3 mg/l), on the MICs of
390 six antibiotics against four clinically important Gram-negative strains – *P. aeruginosa*, *A.*
391 *baumanii*, *E. coli* and *K. pneumoniae* – found that the statins did not significantly change the
392 susceptibility of any of these bacteria to any of the antibiotics tested (83). However, this *in vitro*
393 study may not reflect the true activity in an *in vivo* setting and therefore further *in vivo*
394 investigations are warranted. This is particularly relevant given that the majority of the studies
395 reviewed here that looked at the mechanism by which statins influence bacterial growth or
396 virulence *in vivo* suggest indirect effects as a result of interactions with host cells. In addition, the
397 anti-biofilm activity of statins towards Gram-negative pathogens, which would be expected to
398 reduce the MIC of antibiotics in biofilm forming populations (accounting for approximately 80%
399 of all infections), would not be reflected in the planktonic *in vitro* MIC assays performed.

400 It is important to note, however, that the repurposing of statins for use as combinatorial
401 antibiotics would rely on their compatibility with currently administered antibiotics. While data
402 in this aspect of antimicrobial therapy is limited, certain antibiotics may interfere with the
403 metabolism of statins which can lead to increased serum levels and thus an increased risk of
404 adverse effects (84). For instance, certain statins including simvastatin, lovastatin, and
405 atorvastatin are metabolised by cytochrome P450 3A4 (CYP3A4) isoenzymes and studies have
406 shown that co-prescription with drugs that inhibit CYP3A, such as macrolide antibiotics, can
407 lead to increased adverse effects including rhabdomyolysis in elderly patients (85-92). In light
408 of this the US FDA has stated that ‘caution should be exercised when prescribing clarithromycin
409 with statins’ and in particular ‘concomitant use of clarithromycin with lovastatin or simvastatin
410 is contraindicated’ (89). In contrast they suggest that the concomitant use of statins not

411 dependent on CYP3A metabolism (e.g. fluvastatin) could be considered. However, a recent study
412 by Li and colleagues demonstrated significantly increased adverse effects when clarithromycin
413 was co-prescribed with statins not metabolized by CYP3A4 (94), suggesting additional
414 mechanisms of drug interactions independent of the CYP3A4 pathway, possibly related to
415 impaired hepatic uptake of statins. In contrast to studies on macrolide-statin interactions, no
416 additive harmful effects have been attributed to the combined use of statins and the lipopeptide
417 antibiotic daptomycin, despite both agents being associated with muscle injury (95).

418

419 **Summary**

420 The repurposing of statins as anti-microbial agents held promising potential when clinical studies
421 revealed that patients on cholesterol lowering statins showed improved outcomes from bacterial
422 infections. However, as outlined in this review the most convincing evidence of significantly
423 improved infection outcomes is when patients are pretreated with statins and the anti-microbial
424 effect is probably indirect. There is little evidence of significantly improved outcomes when
425 infections are treated with de-novo statins. However, while the evidence for statin effectiveness
426 thus far has been provided from prophylactic studies, the anti-virulence activity emerging for
427 statins, whereby pathogens may be silenced rather than killed, offers an alternative perspective
428 on their potential clinical utility. In addition, statins may also offer selectivity in targeting
429 pathogenesis rather than the microbial population or microbiome as a whole, which is a major
430 factor in maintaining host homeostasis. This could have the added advantage of removing the
431 selective pressure that underpins the continued spread of antibiotic resistance among populations.
432 Thus, further RCTs and prospective studies have been recommended and based on this review
433 the design of these new studies will be crucial as *in vitro* and mouse studies clearly show that the

434 most gain may be achieved by matching particular statins with particular infecting pathogens.
435 Moreover, one of the most limiting factors is the concentration of statins required for the
436 inhibition of bacterial growth *in vitro*. In almost all cases cited the *in vitro* MICs far exceed the
437 general plasma levels found in patients receiving cholesterol-lowering statins and the feasibility
438 of raising the dose is questionable due to cytotoxicity and increased risk of debilitating side
439 effects. One area where specific targeted studies may be particularly beneficial is in the treatment
440 of infections caused by intracellular pathogens. Many *in vitro* cellular studies outlined here show
441 significant results when using statins at physiological concentrations, while again suggesting the
442 effect is indirect. It would be interesting to see if these beneficial effects could be mimicked in *in*
443 *vivo* clinical studies.

444 The effect of statins on *in vitro* virulence of some pathogens is interesting but again is hindered
445 by the high concentrations required for significant results. However, this may be overcome by
446 using sub-inhibitory concentrations of statins in combination with existing antibiotics. The
447 evidence presented here regarding the repurposing of statins in combination therapies is
448 promising but again may be statin / pathogen specific. While the most significant results have
449 again been against intracellular bacteria there are few *in vivo* / clinical studies available against
450 extracellular pathogens. When designing these studies however, the possibility of adverse effects
451 associated with drug-drug- interactions should be an important consideration.

452 Therefore, while overall clinical studies regarding the repurposing of statins as anti-microbials
453 are inconclusive, the evidence presented here suggests further prospective studies focusing on
454 statin and pathogen specificity, bacterial virulence, combinatorial therapy and/or means of drug
455 administration are warranted.

456

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740 **Figure Legends**

741 **Figure 1: Chemical structures of statins.** **A)** Type 1 statins are characterised by a conserved
742 lactone ring (blue), a decalin structure (black) and a butaryl side chain (red), which is different in
743 each statin. **B)** Type 2 statins differ from type 1 statins due to the replacement of the butaryl side
744 chain with a fluorenyl group (green), and although the lactone ring structure is conserved in all
745 statins, the decalin group of Type 1 statins is replaced by a longer distinct side chain. Statins
746 marked with an asterisk (*) are licensed to treat high cholesterol.

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748 **Figure 2: Statins modulate bacterial growth and virulence.** **A)** *In vitro* effects of statins on
749 bacterial species. Statins reduce *in vitro* bacterial growth, motility and attachment. **B)** Key anti-
750 virulence mechanisms of statins. At physiological concentrations statin treatment can reduce
751 bacterial invasion and translocation, in addition to inhibiting lipid raft production. The inhibition
752 of Rho GTPase activity and cholesterol production by statins contribute to reduced bacterial
753 virulence, decreased toxicity and impaired intracellular survival. **C)** At physiological
754 concentrations statin treatment can reduce bacterial load and dissemination and increase bacterial
755 clearance in mouse models of infection.

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762 **Table 1:** MIC of statins against Gram-positive and Gram-negative bacteria

Bacteria	Gram	Statin MIC mg/l					Ref
		Sim	Fluv	Ator	Ros	Prav	
<i>S. Aureus</i>	pos						
MSSA		16 - 63	~200	42 - >250	208 - 342	>250	37, 39-43
MSSA clinical isolate		60.42	nt	52.08	341.67	nt	39
MRSA		32 - 167	~250 - >1024	83 - >1024	100 - >1024	>250 - >1024	37, 39-40, 42-43
MRSA clinical isolate		116.67	nt	108.33	500	nt	39
VISA group of strains		32	nt	nt	nt	nt	42
VRSA group of strains		32 - 64	nt	nt	nt	nt	42
<i>S. epidermidis</i>	pos						
Type strains		26 - 32	nt	21	167	nt	39, 42
Clinical isolate		35	nt	20	233	nt	39
<i>S. Pneumoniae</i>	pos						
Type strains		16 - 167	>123	104	333	>50	38, 39, 42
Clinical isolate		292	nt	229	417	nt	39
<i>Enterococci</i>	pos						
VSE		50 - 52	300	83 - 250	100 - 333	nt	37, 39, 40
VSE clinical isolate		292	nt	96	333	nt	39
VRE		30 - 104	500	167 - 250	100 - 500	nt	37, 39, 40
VRE clinical isolate		292	nt	217	500	nt	39
<i>E. faecalis</i> group of strains		32	nt	nt	nt	nt	42

<i>S. pyogenes</i>	pos						
ATTC19615		62.5	nt	83.33	166.67	nt	39
Clinical isolate		146	nt	133.33	275	nt	39
<i>L. monocytogenes</i>	pos						
Group of strains		32	nt	nt	nt	nt	42
<i>B. anthracis</i>	pos						
Type strains		16	nt	nt	nt	nt	42
<i>H. influenza</i>	neg						
Clinical isolate		146 - >250	nt	104	367	nt	38, 39
ATTC29247		52	nt	83	167	nt	39
<i>Moraxella catarrhalis</i>	neg						
Clinical isolate		16	nt	nt	nt	nt	38
<i>E. coli</i>	neg						
Type strains		52 - >250	nt	26 - >250	104	>250	39, 40, 43
O157:H7 ATCC 700728		>256	nt	nt	nt	nt	42
Clinical isolate		112	nt	100	125	nt	39
<i>P. aeruginosa</i>	neg						
Type strains		166 - >1024	>1024	83 - >1024	100 - >1024	>250 - >1024	39, 40, 42, 43, 63
Clinical isolate		121	nt	96	292	nt	39
<i>K. pneumoniae</i>	neg						
Type strains		167 - >256	nt	167	333	nt	39, 42
Clinical isolate		242	nt	217	258	nt	39

<i>A. baumannii</i>	neg						
Type strains		104 - >256	nt	16	333	nt	39, 42
Clinical isolate		32	nt	22	300	nt	39
<i>C. freundii</i>	neg						
ATTC 8090		52	nt	83	167	nt	39
Clinical isolate		133	nt	108	333	nt	39
<i>E. aerogenes</i>	neg						
ATTC 29751		26	nt	16	104	nt	39
Clinical isolate		33	nt	20	183	nt	39
<i>P. mirabilis</i>	neg						
ATTC 12459		167	nt	63	250	nt	39
Clinical isolate		146	nt	133	275	nt	39
<i>S. Tphimurium</i>	neg						
ATCC 700720		>256	nt	nt	nt	nt	42

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764 Key: Sim, Simvastatin; Fluv, Fluvastatin; Ator, Atorvastatin; Ros, Rosuvastatin; Prav,

765 Pravastatin; pos, Gram-positive; neg, Gram-negative; nt, not tested.

766 Table 2. Effect of statins on bacterial virulence and antibiotic activity

Bacteria	Virulence trait										Ref	
	Reduced biofilm formation	Disrupt established biofilm	Motility	QS	Protease	T3SS ExoS	Increased adhesion to human cells	Reduced Invasion	Trans- location	Toxin prodn		
Statin (mg/l)												
Sim	Sim	Sim/Lov/Mev				Sim	Sim	Mev	Sim	Sim		
<i>S. aureus</i>	0.98 - 62.5	62.5	-				-	-	-	-	-	43
	62.5	-	-				-	-	-	-	-	41
	-	64	-				-	-	-	↑ 40	40	42
	-	-	-				-	0.04 - 0.4	-	-	-	66
<i>S.epidermitis</i>	-	128	-				-	-	-	-	-	42
<i>P. aeruginosa</i>	4 & 40	-	40	NC		NC	4	NC	-	-	-	63,64
	-	-	-				-	NC	-	↓ 2	-	65
<i>Streptococcus</i>	-	-	-				-	-	4	-	-	67
<i>E.coli</i>	-	-	-				-	-	4	-	-	68
<i>B. cepacia</i>	-	-	-	NC	NC		-	-	-	-	-	70

Antibiotic synergy							
Bacteria	<i>In vitro</i>			<i>In vivo</i> (mice)			Ref
	Antibiotic + statin	Statin conc.	Effect	Antibiotic + statin	Statin conc.	Effect	
Pneumococci	Pen + Sim	7.8	↑ Autolysis	-	-	-	38
MRSA/VRSA	Mup/Fus/Dap + Sim	<32	↓ Growth	-	-	-	42
<i>S. aureus</i>	Van + Sim	?	NC	-	-	-	43
<i>M. tuberculosis</i>	Rif + Sim/Ator	0.2microM	↓ Viability	-	-	-	49
				Rif, Pyr, iso + sim	25 mg/kg/d	↑ bacillary killing	78
<i>M. leprae</i>	Rif + Ator	0.2microM	↓ Viability	Rif + Ator	80 mg/kg/d	↓ Viability	49
<i>A. baumannii</i> ,	Ami/Imi/Min + Prav/Sim/Ator/Fluv	-	NC	-	-	-	79
<i>P. aeruginosa</i>	Cip/Cep/Pip + Ator/Fluv	-	NC	-	-	-	79
<i>K. pneumoniae</i>	Cip/Cep/Pip + Ator/Fluv	-	NC	-	-	-	79
<i>E. coli</i>	Cip/Cep/Pip + Ator/Fluv	-	NC	-	-	-	79

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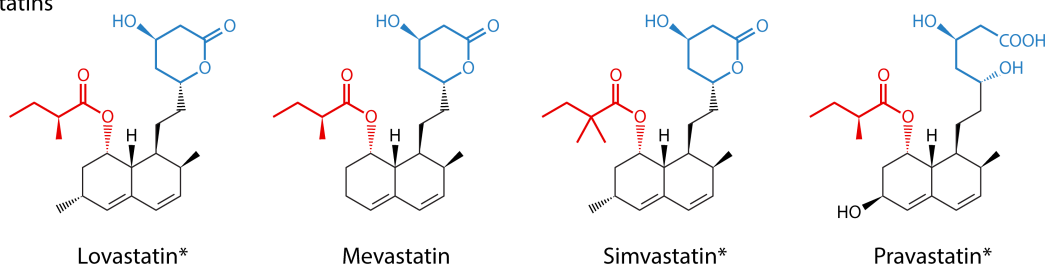
768 Key: Statins: Sim, Simvastatin; Ator, Atorvastatin; Prav, Pravastatin; Fluv, Fluvastatin. Antibiotics: Pen, penicillin; Mup, mupirocin;

769 Fus, fusidic acid; Dap, daptomycin; Van, vancomycin; Rif, rifampicin; Pyr, pyrazinamide; Iso, isoniazid; Ami, amikacin; Imi,

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770 imipenem; Min, minocycline; Cip, ciprofloxacin; Cep, cefepime ; Pip, piperacillin

A. Type 1 statins



B. Type 2 statins

