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1 **Lysosomal alkalization to potentiate eradication of intraosteoblastic *Staphylococcus aureus* in the**
2 **bone and joint infection setting**

3

4 Lélia ABAD¹⁻³, Pierre CHAUVELOT^{4,5}, Estelle AUDOUX⁶, Camille ANDRE^{2,3}, Jérôme JOSSE^{1,2}, Céline
5 DUPIEUX¹⁻⁴, Sébastien LUSTIG^{2,4,7}, Tristan FERRY^{1,2,4,5}, Paul VERHOEVEN⁶, Alan DIOT¹, Frédéric
6 LAURENT^{1-4,#}, Florent VALOUR^{1,2,4,5#*}

7

8 ¹CIRI – Centre International de Recherche en Infectiologie, Inserm, U1111, Université Claude Bernard
9 Lyon 1, CNRS, UMR5308, Ecole Normale Supérieure de Lyon, Univ Lyon, F-69007, Lyon, France

10 ²Université Claude Bernard Lyon 1, Lyon, France

11 ³Laboratoire de bactériologie, Institut des Agents Infectieux, French National Reference Center for
12 Staphylococci, Hospices Civils de Lyon, Lyon, France

13 ⁴Centre de Référence pour la prise en charge des Infections ostéo-articulaires complexes (CRIOAc
14 Lyon ; www.crioac-lyon.fr), Hospices Civils de Lyon, Lyon, France

15 ⁵Service des maladies infectieuses et tropicales, Hospices Civils de Lyon, Lyon, France

16 ⁶Groupe Immunité Muqueuse et Agents Pathogènes, EA 3064, PRES Lyon, Université Jean Monnet et
17 CHU de Saint-Etienne, Saint-Etienne, France

18 ⁷Service de chirurgie orthopédique, Hospices Civils de Lyon, Lyon, France

19

20 **Key words:** Antimicrobial; Bone and joint infection; Intracellular activity; lysosomal alkalization;
21 *Staphylococcus aureus*.

22

23 **Running title:** Lysosomal alkalization during BJI

24

25 *Corresponding author: Florent Valour, Centre International de Recherche en Infectiologie, Croix-
26 Rouse Hospital, 103 Grande Rue de la Croix-Rousse, 69004 Lyon, France; Tel. +33 4 72 07 11 07; Fax
27 +33 4 72 07 17 50; florent.valour@chu-lyon.fr.

28

29 #These authors contributed equally to this work.

30

31 **Main points:** The intraosteoblastic reservoir of *S. aureus* involved in BJI chronicity and the impact of
32 pH on antimicrobial activity advocate for lysosomal alkalization to potentiate *S. aureus* intracellular
33 eradication. In an *in vitro* model of osteoblast infection, hydroxychloroquine enhanced the ability of
34 clindamycin, cotrimoxazole, daptomycin and levofloxacin to eradicate intracellular *S. aureus*
35 reservoir by both restoring antimicrobial activity through lysosomal alkalization and modulating
36 autophagy flux.

37

38 **ABSTRACT**

39 **Objectives:** Beyond intracellular penetration, acidic lysosomal pH might affect the intracellular
40 activity of some antimicrobials. This study evaluated the ability of lysosomotropic alkalizing agents to
41 potentiate antimicrobial eradication of intraosteoblastic *S. aureus* reservoir in the bone and joint
42 infection (BJI) setting.

43 **Methods:** MICs of sixteen anti-staphylococcal molecules against MSSA were evaluated at pH 5 and 7.
44 Additionally, lysosomal alkalinizing potential (spectrofluorometry) and cytotoxicity (MTT assay) of
45 hydroxychloroquine, amantadine and NH₄Cl were assessed. Results led to further investigate
46 clindamycin, cotrimoxazole, daptomycin and levofloxacin in an *in vitro* model of osteoblast infection,
47 alone or in combination with hydroxychloroquine. The impact of hydroxychloroquine on autophagy
48 was finally investigated, using Western blot detection of two autophagic flux indicators, the LC3
49 membrane protein and the SQSTM1 cargo protein.

50 **Results:** Daptomycin, cotrimoxazole, clindamycin and levofloxacin alone decreased significantly the
51 intracellular staphylococcal reservoir (5.12 log₁₀CFU/100,000 cells) by 0.14 (95%CI, 0.01-0.34), 0.25
52 (95%CI, 0.12-0.43), 0.16 (95%CI, 0.004-0.39) and 1.18 (95%CI,1.04-1.38) log₁₀CFU/100,000 cells,
53 respectively ($p < 10^{-3}$). Adding hydroxychloroquine (20 mg/L) increased intralysosomal pH from 4.8 to
54 7, and concomitantly the inoculum reduction of each antimicrobials by 0.50 (95%CI,0.30-0.84), 0.73
55 (95%CI,0.59-0.96), 0.59 (95%CI,0.46-0.78) and 1.8 (95%CI,1.66-2.1) log₁₀CFU/100,000 cells,
56 respectively ($p < 10^{-4}$). Cellular levels of LC3II and SQSTM1 showed that hydroxychloroquine has a
57 direct activity on the autophagic flux, fostering intracellular *S. aureus* eradication by antimicrobials.

58 **Conclusion:** At high concentrations, hydroxychloroquine used as an adjuvant to antimicrobials
59 improves eradication of *S. aureus* intraosteoblastic reservoir in our *in vitro* cell infection model.
60 These findings advocate for further *in vivo* evaluation of alkalization efficacy and tolerance in *S.*
61 *aureus* BJI.

62 **INTRODUCTION**

63

64 *Staphylococcus aureus* represents the leading cause of bone and joint infection (BJI), and is
65 responsible for difficult-to-treat infections related to several persistence mechanisms (1–3). In
66 particular, its ability to persist within non-professional phagocytic bone cells subverts the host
67 immune system and the action of most antimicrobials (4,5). Consequently, the ability of
68 antimicrobials to eradicate the intracellular *S. aureus* reservoir may have a significant impact on the
69 risk of BJI chronicity and relapse.

70 *S. aureus* has been shown to persist within phagocytes through phagolysosomal replication and
71 escape to gain the cytoplasm (6–8). It also notably affects autophagy, a part of the innate immune
72 response against bacterial infections corresponding to a catabolic process leading to autophagosome
73 formation, a compartment that encloses intracytoplasmic bacteria. It involves SQSTM1/p62
74 (sequestosome 1), a ubiquitin receptor essential to this selective degradation, and LC3, a membrane
75 protein participating to phagosomal elongation and maturation. To eradicate intracellular bacteria,
76 the autophagosome and lysosome merge, generating the autolysosome, a digestive hybrid organelle
77 with an acidic environment (9,10). Interestingly, the impaired intracellular antimicrobial activity relies
78 on the poor cellular penetration of some molecules, their low distribution in the subcellular location
79 of *S. aureus* and the intracellular emergence of small colony variants (SCVs), but also on drug
80 inactivation by the acidic pH of intracellular organelles (11,12). Consequently, some studies have
81 considered lysosomal alkalization to restore intracellular antibiotic activity (13–15). Alkalization also
82 appeared to prevent SCV emergence (15,16). Thus, lysosomotropic alkalinizing agents might help
83 targeting *S. aureus* intraosteoblastic reservoir in the BJI setting. The aim of this study was to compare
84 the intra-osteoblastic activity of anti-staphylococcal agents alone or in combination with
85 lysosomotropic alkalinizing agents in an *in vitro* model of osteoblast infection.

86

87 **MATERIAL AND METHODS**

88

89 **Study drugs.** All antimicrobials and the three lysosomotropic agents (i.e., amantadine, ammonium
90 chloride [NH₄Cl] and hydroxychloroquine) were used after dilution in NaCl 0.9% (Table 1).

91

92 **Bacterial strains.** All experiments were performed using three MSSA strains: i) 6850 (ATCC 53657),
93 highly virulent strain isolated from patient with osteomyelitis described by Balwit *et al.* (17); and ii)
94 clinical isolates n°1 and n°2 collected from patients with recurrent osteomyelitis in our institution.

95

96 **MICs at pH5 and pH7 determination.** MICs of drugs included in the study were determined by broth
97 microdilution following the recommendation of the French Committee for Antimicrobial
98 Susceptibility Testing (CA-SFM). Of note, pH recommended for routine MIC determination is 7.2-7.4
99 (CA-SFM). Here, Mueller-Hinton broths (MHB, Sigma-Aldrich, Saint-Louis, USA) were adjusted to pH 5
100 and 7 to mimic cytosolic and lysosomal compartments.

101

102 **Lysosomal pH measurement from MG63 cells.** Measurement of lysosomal pH from human
103 osteoblastic cell line MG63 (CRL-1427) (18) was performed using LysoSensor Yellow/Blue DND-160
104 (Invitrogen, Carlsbad, USA), a lysosomotropic pH indicator dye, exhibiting a pH-dependent excitation
105 at 329 nm and 384 nm and allowing the ratiometric assessment of pH changes in acidic organelles
106 (19). Uninfected MG63 cells were seeded at 10,000 cells per well into 96-well tissue culture plates in
107 100 µL of growth medium (DMEM + 10 % fetal calf serum) and cultured for 24 h. Cells were
108 incubated with increasing concentrations (2.5, 5, 10 and 20 µg/mL) of lysosomotropic agents for
109 various incubation time (0.5h, 2h, 4h, 24h, 48h and 72h). When incubation exceeded 24 h, medium
110 was changed daily. Then, medium was removed and 50 µL of LysoSensor (dilution 2:1000 in NaCl
111 0.9%) was added in each well for a 3-min incubation. The fluorescence was quantified using a
112 microplate reader (TECAN Infinite® 200 PRO) to determine lysosomal pH. A calibration curve was

113 performed in duplicate with uninfected cells incubated with a dedicated buffer (KCl 100mM, 20mM
114 NaCl, 20mM Mes, 20µM monensin, 10µM nigericin) at pH 4.5, 5.0, 5.5, 6.0 and 6.5.

115

116 **Cytotoxicity measurement.** A colorimetric assay based on the enzymatic reduction of 3-[4,5-
117 dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan was used to
118 determine the cells viability as previously described (20). For each condition, the absorbance was
119 normalized on “untreated cells (0 mg/L)” corresponding to 100%.

120

121 **Intracellular activity of antibiotics and impact on infection-induced cytotoxicity.** Intracellular
122 activities of levofloxacin, clindamycin, cotrimoxazole and daptomycin with or without
123 hydroxychloroquine were assessed in an *in vitro* model of osteoblast infection, as previously
124 described (20).

125 Bacterial strains were cultured in tryptic soy broth at 37°C with shaking for 6 h before storage at -
126 80°C in individual cryovials containing 1×10^{10} CFU/mL in PBS (Gibco) containing 10 % glycerol (21).

127 For each experiment, one cryovial was used to prepare standardized bacterial suspension in DMEM +
128 10 % fetal calf serum added to osteoblasts for 2 h at a multiplicity of infection (MOI) of 100
129 bacteria/cell. Cells were then incubated for 1 h with growth medium supplemented with 10 mg/L of
130 lysostaphin (Sigma-Aldrich) to kill the remaining extracellular bacteria. Infected cells were then
131 incubated for 24h with growth medium containing the tested antimicrobials with or without
132 hydroxychloroquine at 20 mg/L. Antimicrobials were used at the bone concentrations reached during
133 standard human therapy (22) : daptomycin (20 mg/L), clindamycin (7 mg/L), cotrimoxazole (40/8
134 mg/L) and levofloxacin (10 mg/L). For each condition, lysostaphin at 10 mg/L was added to rapidly kill
135 the bacteria released upon host cell lysis. Cells were lysed by a 10-min incubation with 1 mL of sterile
136 water, then serial dilutions of lysates were spiral-plated (Spiral®, Interscience, St Nom la Bretèche,
137 France) on TSA (Trypcase Soy Agar, Biomérieux, Marcy-l'Étoile, France) to quantify intracellular

138 bacteria. The impact on infection-induced cytotoxicity was determined with the MTT assay, as
139 described above.

140

141 ***Impact of hydroxychloroquine on the autophagic flux.*** MG63 cells were infected with the 6850
142 strain and exposed to hydroxychloroquine at 2.5, 5, 10 and 20 mg/L for 24h. Cell lysates were
143 centrifuged at 10,000 rpm for 10 min at 4°C. Proteins concentrations of the supernatants were
144 determined by BCA assay (Kit Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific). Proteins
145 lysates were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
146 PAGE) and transferred to polyvinylidene fluoride membrane by electroblotting (BioRad, Hercules,
147 USA). Membranes were blocked with 5 % non-fat milk in PBS-Tris buffer for 1 h, followed by
148 incubation with rabbit primary antibody targeting LC3 (Cell Signaling, Danvers, USA), SQSTM1 (Cell
149 Signaling) and actin (used as reference protein, Cell Signaling) at 4°C overnight. After three steps of
150 washing, membranes were incubated with secondary anti-rabbit goat antibody (HRP, Thermo Fisher
151 Scientific) at room temperature for 1 h. Clarity Western Blot ECL substrate (BioRad) was used for
152 protein detection.

153

154 ***Statistical methods.*** Data were summarized as means and their 95% confidence intervals (95%CI).
155 Conditions were compared using a nonparametric two-sided Mann-Whitney U test. A *p*-value < 0.05
156 was considered significant. All analyses were performed using Prism software (GraphPad, San Diego,
157 USA).

158

159 **RESULTS**

160

161 ***Susceptibility studies and choice of the tested antimicrobials.*** MICs of the three *S. aureus* strains
162 (6850, clinical isolates n°1 and 2) are presented in Table 1. As shown by significant increased MICs

163 (i.e., at least 2 dilutions) at pH 5 compared to pH 7, acidic pH impaired the activity of clindamycin,
164 cotrimoxazole, levofloxacin, ofloxacin, rifabutin, daptomycin, fosfomycin and tigecyclin.

165

166 ***Alkalization kinetics and cytotoxicity of the lysosomotropic agents.*** Lysosomal pH of uninfected-
167 untreated cells was 4.8 (95%CI, 4.5-5.0). Alkalization kinetics (0-72 h) for increasing concentrations of
168 NH₄Cl, hydroxychloroquine and amantadine are reported in Figure 1.B, 1.C and 1.D respectively.
169 Regardless of the tested concentration and exposition time, amantadine induced a slight lysosomal
170 pH rise, reaching a maximum of 6.0 (95%CI, 5.3-6.6) at 10 mg/L after 72 h of incubation. NH₄Cl at 20
171 mg/L allowed to reach a maximal pH of 8.1 (95%CI, 7.4-8.8) after 2 h, but with high variations
172 observed over time. For hydroxychloroquine, a reproducible dose-effect was observed at all time-
173 points, with a maximal effect reached after 2 h and remaining stable over time. At the maximal
174 concentration of 20 mg/L, the lysosomal pH rose to 7.0 (95%CI, 6.8-7.3) after 2 h.

175 In parallel, cytotoxicity kinetics of the three lysosomotropic agents were explored (Figure 2 and
176 supplementary data table 2). Amantadine and NH₄Cl decreased cell viability from and over 10 mg/L
177 for all incubation times. Hydroxychloroquine was significantly cytotoxic at 20 mg/L only after 72 h.

178 The alkalinizing and cytotoxicity profiles of hydroxychloroquine led us to use this drug at 20 mg/L for
179 24 h when tested in combination with antimicrobials in our *in vitro* osteoblast infection model.

180

181 ***Intraosteoblastic activity of antimicrobials with or without hydroxychloroquine and impact on***
182 ***infection-induced cytotoxicity.*** At the tested concentrations, daptomycin, cotrimoxazole, clindamycin
183 and levofloxacin with or without hydroxychloroquine (20 mg/L) had no significant cytotoxicity on
184 uninfected cells (supplementary Figure 1).

185

186

187

188 After the 3h infection step, the intracellular inoculum was 2.5×10^5 CFU/ 100,000 cells (95%CI, $1.6 \times$
189 10^5 - 3.5×10^5). Without treatment, the number of intraosteoblastic bacteria was 1.2×10^5 CFU/
190 100,000 cells (95%CI, 7.6×10^4 - 1.6×10^5) after 24h (Figure 3A), and the infected cell viability was
191 reduced by 55.7% (95%CI, 48.7-62.7) after 24 h in comparison with uninfected cells ($p < 10^{-4}$) (Figure
192 3B).

193 Hydroxychloroquine alone (20 mg/L) did not reduce infection-induced cytotoxicity but significantly
194 decreased the intracellular inoculum by 0.36 (95%CI, 0.18-0.66; $p < 10^{-4}$) \log_{10} CFU/100,000 cells.
195 Daptomycin, cotrimoxazole, clindamycin and levofloxacin alone reduced *S. aureus* intraosteoblastic
196 inoculum by 0.14 (95%CI, 0.01-0.34; $p < 10^{-3}$), 0.25 (95%CI, 0.12-0.43; $p < 10^{-4}$), 0.16 (95%CI, 0.004-0.39;
197 $p < 10^{-4}$) and 1.18 (95%CI, 1.04-1.38; $p < 10^{-4}$) \log_{10} CFU/100,000 cells, respectively. Adding
198 hydroxychloroquine (20 mg/L) led to multiply bactericidal effect of antimicrobial alone by 2.7 for
199 daptomycin, cotrimoxazole and clindamycin, and 6.5 for levofloxacin, corresponding to an inoculum
200 reduction of 0.50 (95%CI, 0.30-0.84), 0.73 (95%CI, 0.59-0.96), 0.59 (95%CI, 0.46-0.78) and 1.8 (95%CI,
201 1.66-2.1) \log_{10} CFU/100,000 cells, ($p < 10^{-4}$), respectively (Figure 3A).

202 Compared to untreated cells, daptomycin, cotrimoxazole, clindamycin and levofloxacin alone
203 increased significantly cell viability by 28.0% (95%CI, 9.0-46.9; $p = 0.021$), 42.0% (95%CI, 26.0-58.0;
204 $p = 0.002$); 83.1% (95%CI, 66.4-99.5; $p < 10^{-4}$) and 50.6% (95%CI, 29.6-71.6; $p < 10^{-3}$), respectively.
205 Compared to antibiotics alone, the adjuvant uses of hydroxychloroquine did not significantly increase
206 the cell viability (Figure 3B).

207 Additional experiments were designed to evaluate the combined dose-effect of hydroxychloroquine
208 and antimicrobials using increasing concentrations of hydroxychloroquine and one antimicrobial.
209 Levofloxacin was chosen because it was the most potent antimicrobials against intracellular *S. aureus*
210 based on previous results. From 2.5 μ g/mL, hydroxychloroquine alone reduced the intracellular
211 inoculum -0.35 \log_{10} CFU/100,000 cells (95%CI, - 0.22 to - 0.55; $p = 0.002$) with no significant difference
212 until 10 mg/L. Levofloxacin alone decreased - 1.3 \log_{10} CFU/100,000 cells (95%CI; -1.1 to -1.8;
213 $p = 0.002$) the intracellular inoculum. Potentiation by hydroxychloroquine was significant from 10

214 mg/L, increasing the bactericidal effect of levofloxacin to $-1.8 \log_{10}\text{CFU}/100,000$ cells (95%CI; -1.6 to
215 -2.8; $p=0.015$) (Figure 4B).

216

217 **Impact of hydroxychloroquine on autophagy.** Compared to untreated infected cells (mean LC3-
218 II/actin ratio, 0.15), the relative level of LC3-II trended to increase until 10 mg/L of
219 hydroxychloroquine, with a mean ratio 0.53 for 2.5 mg/L, 0.62 for 5 mg/L, 1.14 for 10 mg/L and 0.44
220 for 20 mg/L (Figure 4A). While less pronounced, SQSTM1/actin ratios followed the same trend,
221 measured at 0.47, 0.57, 0.66, 0.65 and 0.43 when hydroxychloroquine concentration increased from
222 0 to 20 mg/L (Figure 4A).

223

224 **DISCUSSION**

225

226 The present study represents the first evaluation of various lysosomotropic alkalinizing agents used
227 as adjuvants to conventional antistaphylococcal drugs in an *in vitro* model of osteoblast infection.

228 The preliminary experiments performed allowed us to set the best conditions to establish the proof
229 of concept of alkalization in our model. First, MIC determinations at pH 5 and 7 confirmed that low
230 lysosomal pH might impair the activity of many antimicrobials. Impacted by acid pH and being the
231 most antimicrobial prescribed in BJIs, daptomycin, cotrimoxazole, clindamycin and levofloxacin were
232 chosen for further evaluation in the osteoblast infection model. Second, based on its high alkalinizing
233 potential with low cytotoxicity at the concentration and time of exposition tested,
234 hydroxychloroquine was chosen as the better adjuvant to be tested in combination with antibiotics.
235 Indeed, after 24 hours of incubation at 20 mg/L, hydroxychloroquine allowed the lysosomal pH to be
236 increased from 4.8 to 7.0. Similarly, Maurin *et al.* reported an increase of lysosomal pH from 4.8 to
237 5.3, 5.7 and 6.8 in a murine macrophage model using amantadine (1 mg/L), chloroquine (1 mg/L) and
238 ammonium chloride (1 mg/mL) (13).

239 Regarding the impact of lysosomal alkalization, the addition of hydroxychloroquine to antimicrobials
240 allowed a significant increase of their bactericidal effects, without inter-strain variability. Data are
241 scarce in the literature regarding antibiotic potentiation by alkalinizing agents toward intracellular *S.*
242 *aureus* killing. Our results complete the preliminary results, showing that chloroquine (1 mg/L)
243 associated with amikacin increased intramacrophagic *S. aureus* inoculum reduction (14). Additionally,
244 using human lung epithelial cells, Leimer *et al.* showed that flucloxacillin associated with chloroquine,
245 decreased significantly the proportion of intracellular SCVs (15). Together, these data support the
246 use of this adjuvant therapy against intracellular *S. aureus*.

247 On the other hand, some antimicrobials with increased activity at acidic pH could be impeded by
248 alkalinizing therapy. In particular, a 8 to 42-fold increase in rifampin MICs at pH 7 compared to pH 5
249 was observed, which can constitute an significant limitation of the use of alkalinizing strategies
250 according to the crucial role of rifampin in orthopedic device-associated infection outcome (23,24).
251 However, rifampin MICs evaluated at neutral pH remains far below the intraosseous concentrations
252 reached with standard therapeutic dosages. Additionally, other rifamycins could be proposed as an
253 alternative, and especially rifabutin, that could theoretically benefit from alkalization and present
254 higher antibiofilm and intracellular activities (20).

255 Finally, we highlighted that the antimicrobial activity potentiation related to lysosomal alkalization
256 was not the only mechanism of hydroxychloroquine on staphylococcal intracellular reservoir. Indeed,
257 and despite the absence of intrinsic antibacterial activity, hydroxychloroquine alone was able to
258 significantly reduce *S. aureus* intracellular inoculum. Interestingly, hydroxychloroquine has been
259 reported to interact with autophagic pathways, mostly in the oncology setting (25–27). The observed
260 increase in LC3-II starting at the lowest concentrations of hydroxychloroquine are in favor of an
261 induction of autophagy. In addition a weak SQSTM1 accumulation advocate for a simultaneous
262 inhibition of phagolysosome formation, as described for other cell types (27,28). As
263 hydroxychloroquine improved intracellular bacteria eradication starting at 2.5 mg/L but fostered the
264 antimicrobials activity from 10 mg/L only, we therefore assumed a combined effect of the induction

265 of the autophagic flux at low concentrations, followed by antimicrobial potentiation by lysosomal
266 alkalization at higher concentrations. Of note, if hydroxychloroquine alone induced a significant
267 reduction of intracellular inoculum at low concentrations, it could not prevent infection-induced
268 cytotoxicity. This paradoxical result might rely on the absence of intrinsic effect of
269 hydroxychloroquine on bacterial growth and toxin secretion. Finally, the usual posology of
270 hydroxychloroquine as lifelong treatment for rheumatoid arthritis and lupus erythematosus is 400-
271 800 mg/day, providing blood concentrations between 1 and 2.5 mg/L (29,30). The use of higher daily
272 doses – even for shorter duration – during BJI treatment to improve eradication of bacteria
273 intracellular is likely not be possible due to tolerance issues. Nevertheless, the present data
274 represents a proof of concept in cellular model and other lysosomotropic agents such as Lys05, a
275 novel 4-aminoquinoline with an improved ability to accumulate in the lysosomes (31), may have
276 more potent alkalinizing effect and could be an alternative to hydroxychloroquine.

277 To conclude, hydroxychloroquine as adjuvant to antimicrobials may improve the eradication of the
278 intraosteoblastic *S. aureus* reservoir by both restoring intracellular activity of some antimicrobial and
279 interfering with the bacterial subversion of autophagy. This new clinical perspective requires now *in*
280 *vivo* confirmation of its effectiveness and toxicity.

281

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284

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289

290 **TRANSPARENCY DECLARATIONS**

291 None to declare.

292

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391 **TABLES**

392

393 **Table 1.** *In vitro* MICs of the study drugs at pH 5 and 7.

MICs (mg/L)	6850		Clinical isolate n°1		Clinical isolate n°2	
	pH 7	pH 5	pH 7	pH 5	pH 7	pH 5
Antimicrobials						
Cefazolin ¹	0.125	0.125	0.0625	0.03	0.5	0.03
Clindamycin ²	8	>32	4	>32	4	32
Cotrimoxazole ³	0.5	2	1	4	1	4
Daptomycin ⁴	1	4	2	4	2	4
Doxycycline ⁵	0.125	0.0625	0.5	0.125	0.5	0.125
Fosfomycin ⁶	0.25	2	0.5	1	8	16
Levofloxacin ⁷	0.25	2	0.125	0.5	0.125	1
Linezolid ²	4	4	2	2	2	2
Ofloxacin ⁸	0.5	2	0.25	2	0.5	2
Oxacillin ⁹	0.0625	0.0625	0.0625	0.03	<0.015	<0.015
Rifabutin ⁸	<0.0035	0.125	0.007	0.5	0.007	0.5
Rifampin ⁶	0.0075	0.0009	0.03	0.007	0.015	0.0015
Rifapentine ⁸	0.007	<0.0035	0.015	0.007	0.015	0.0035
Teicoplanin ⁸	0.5	0.0625	0.25	2	0.5	1
Tigecycline ⁸	0.25	1	0.0625	1	0.0625	1
Vancomycin ¹⁰	2	1	1	0,5	0.25	0.125
Lysosomotropic agents						
Amantadine ⁸	>100	>100	>100	>100	>100	>100
Hydroxychloroquine ⁸	>100	>100	>100	>100	>100	>100
NH ₄ Cl ⁸	>100	>100	>100	>100	>100	>100

394 *Grey boxes: increased MICs at pH 5 by at least 2 dilutions.*

395 *Providers: ¹Mylan, Canonsburg, USA; ²Pfizer, New York, USA; ³Roche, Bâle, Switzerland; ⁴Novartis,*
 396 *Bâle, Switzerland; ⁵Sandoz, Bâle, Switzerland; ⁶Sanofi, Paris, France; ⁷Fresenius Kabir, Bad Homburg*
 397 *vor der Höhe, Germany; ⁸Sigma–Aldrich, Saint-Quantin Fallavier, France; ⁹Bristol-Myrs-Squibb, New*
 398 *York, USA; ¹⁰Sandoz, Holzkirchen, Germany.*

399

400

401

402 **FIGURE LEGENDS**

403

404 **Figure 1.** Modulation of lysosomal pH by various lysosomotropic agents using increasing
405 concentrations and exposition times.

406 Calibration curve for determination of lysosomal pH from fluorescence intensity ratio measurements
407 (A); Alkalization kinetics of ammonium chloride (B), hydroxychloroquine (C) and amantadine (D).

408 Lysosomal pH was determined by spectrofluorimetry with LysoSensor Yellow/Blue. Each point
409 represents the mean of three independent measures. Standard deviation bars and significance
410 markers were omitted to focus on comparison of the trends among each dose groups.

411

412 **Figure 2.** Cytotoxicity-induced kinetics for ammonium chloride (A), hydroxychloroquine (B) and
413 amantadine (C) after exposition at various concentration in the osteoblast model using uninfected
414 cells. Each point represents the mean of three independent measures. Standard deviation bars and
415 significance markers were omitted to foster comparison of the trends among each dose groups. The
416 horizontal dotted lines corresponds to "untreated cells" used as controls (100%).

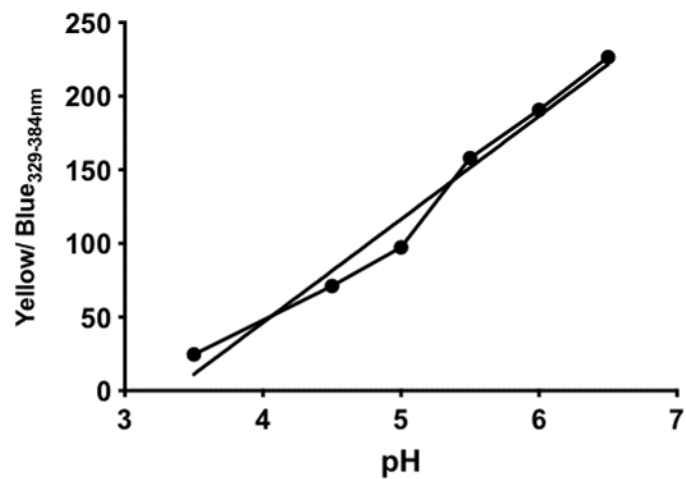
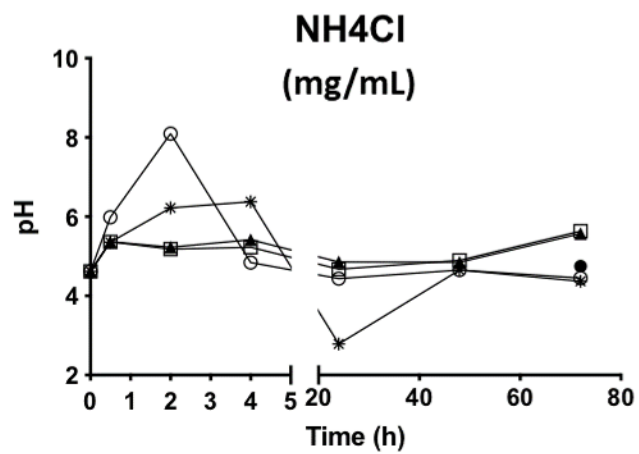
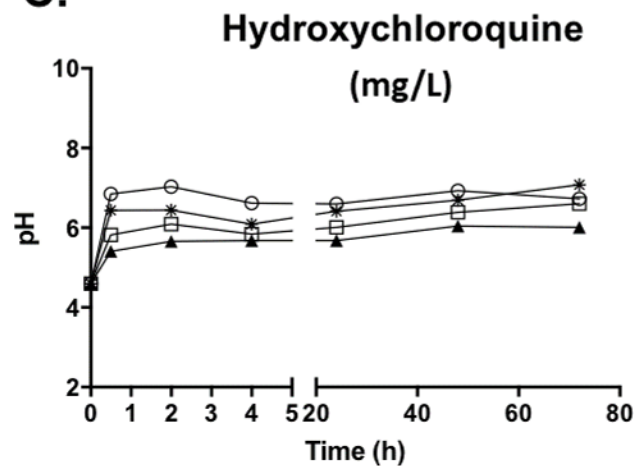
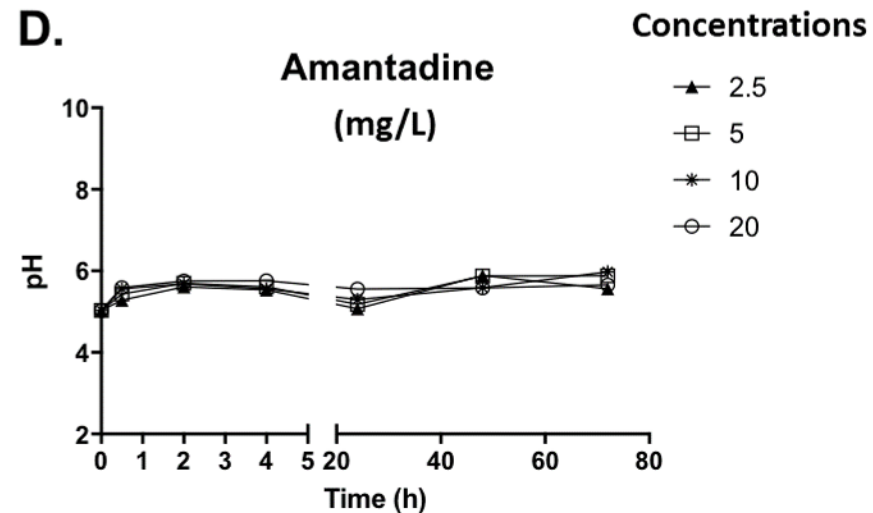
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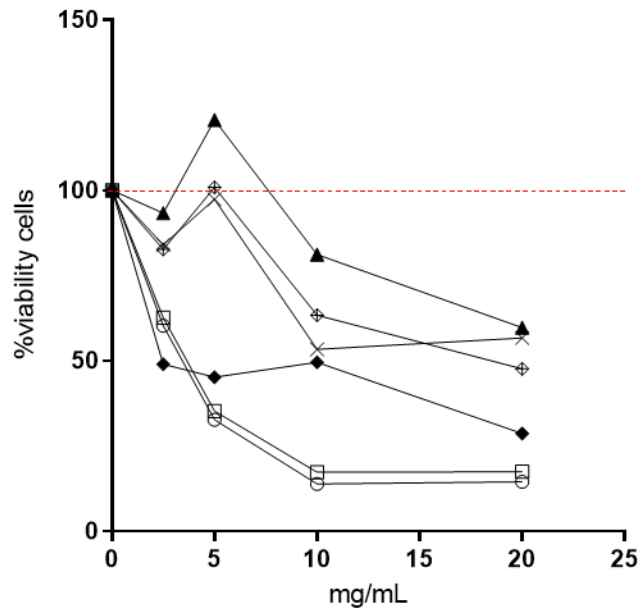
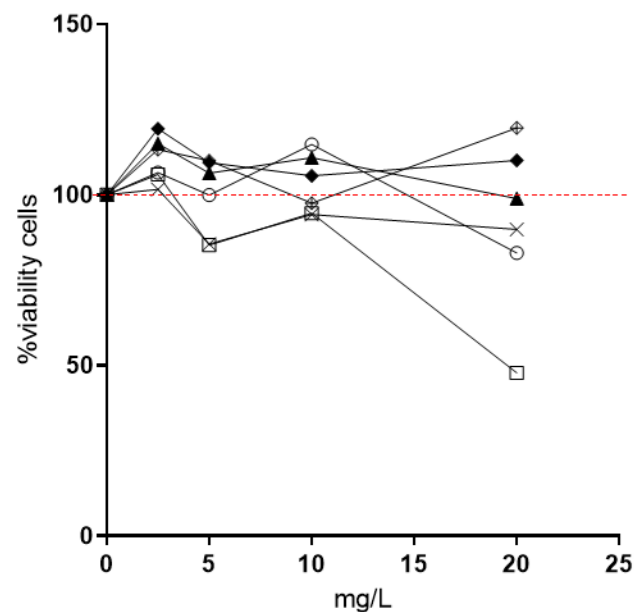
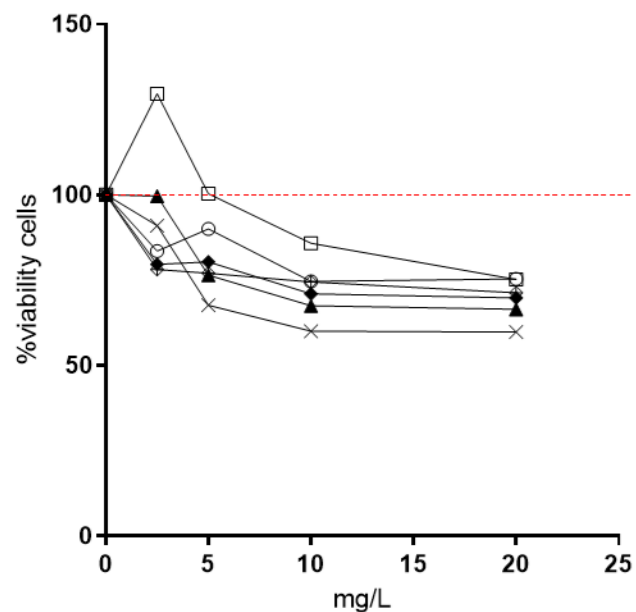
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419 **Figure 3.** Impact of daptomycin (20 mg/L), clindamycin (7 mg/L), cotrimoxazole (40/8 mg/L) and
420 levofloxacin (10 mg/L) with or without hydroxychloroquine (20 mg/L) on *S. aureus* intraosteoblastic
421 inoculum (A) and infection-induced cytotoxicity (B). Each histogram and error bar represent the
422 mean and its 95%CI from 27 estimates (pooled data obtained with the three tested isolates, each of
423 them being evaluated in triplicate in three independent experiments). Each condition was
424 normalized to 'untreated uninfected cells' (horizontal dotted lines, 100%) and compared to
425 "untreated infected cells" using a Mann–Whitney U-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not
426 significant).

427

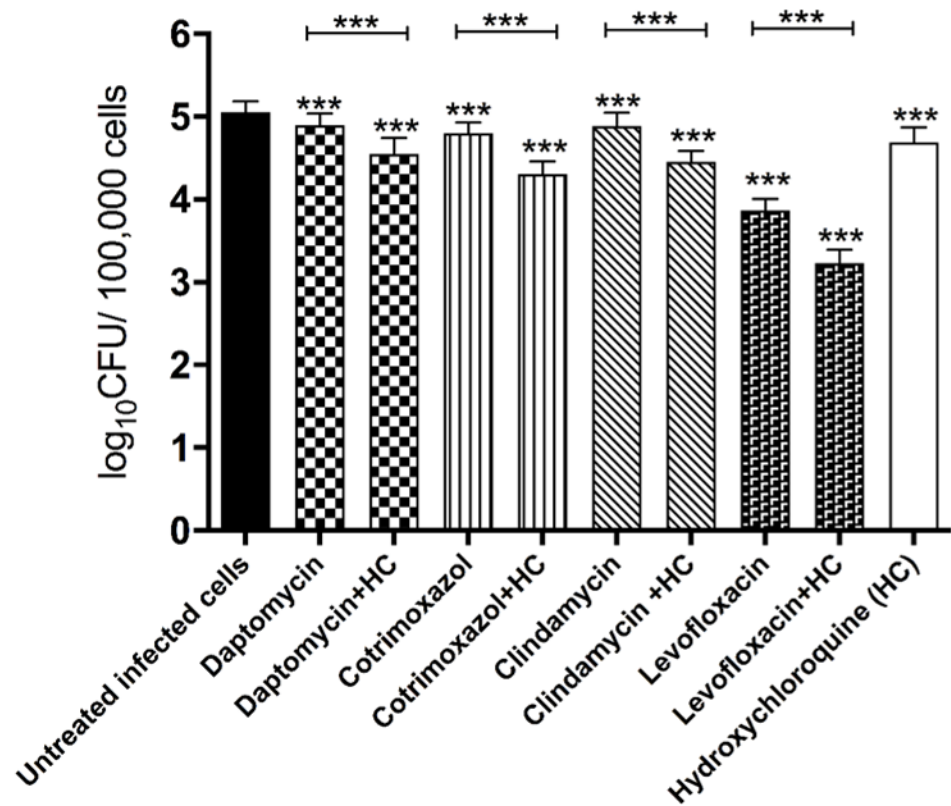
428 **Figure 4.** Impact of increasing concentration of hydroxychloroquine on autophagy markers, and
429 correlation with intracellular inoculum when used with or without levofloxacin at 10 mg/L.
430 Western blot of actin, LC3, SQSTM1 expressed by MG-63 infected cells exposed to
431 hydroxychloroquine at 2.5, 5, 10, 20 mg/L (A). Ability of hydroxychloroquine at 2.5, 5, 10, 20 mg/L
432 with or without levofloxacin to eradicate *S. aureus* intracellular inoculum (B). Each histogram and
433 error bar represent the mean and its 95%CI from 6 estimates (triplicate in two independent
434 experiments) and a Mann–Whitney U-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant) was
435 used.
436

A.**B.****C.****D.**

NH4Cl**Hydroxychloroquine****Amantadine**

Time (h)

- ▲ 0.5
- ◇ 2
- ◆ 4
- × 24
- 48
- 72

A.**B.**