1 # Photochemical internalization enhances cytosolic release of
2 antibiotic and increases its efficacy against staphylococcal infection

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22

Abstract

Bacterial pathogens such as *Staphylococcus aureus* and *Staphylococcus epidermidis* can survive in different types of cells including professional phagocytes, causing intracellular infections. Antibiotic treatment of intracellular infections is often unsuccessful due to the low efficacy of most antibiotics inside cells. Therefore, novel techniques which can improve intracellular activity of antibiotics are urgently needed. We aimed to use photochemical internalization (PCI) to enhance cytosolic release of antibiotics from endocytic vesicles after internalization. Our results show that PCI indeed caused cytosolic release of gentamicin and significantly increased its efficacy against *S. epidermidis in vitro* in mouse macrophages. Upon illumination for 15 min, the killing of intracellular *S. epidermidis* in RAW 264.7 cells by 10 or 30 μg/ml gentamicin was increased to 1 or 3 CFU log, respectively, owing to the use of PCI, whereas no killing by gentamicin only without PCI was observed. Moreover, survival of *S. aureus*-infected zebrafish embryos was significantly improved by treatment with PCI-gentamicin. PCI improved the therapeutic efficacy of gentamicin at a dose of 0.1 ng per embryo to a level similar to that of a dose of 0.4 ng per embryo, indicating that PCI can lower the antibiotic dose required for treating (intracellular) staphylococcal infection. Thus, the present study shows that PCI is a promising novel approach to enhance the intracellular efficacy of antibiotics via cytosolic release, allowing them to reach intracellular bacteria. This will expand their therapeutic window and will increase the numbers of antibiotics which can be used for treatment of intracellular infections.

Keywords

Photochemical internalization (PCI); cytosolic release; intracellular antimicrobial efficacy; gentamicin; (intracellular) staphylococcal infection

Introduction

As an opportunistic intracellular pathogen, *Staphylococcus aureus* can survive in several types of cells including professional phagocytes such as macrophages and neutrophils, resulting in high frequencies of occurrence of intracellular infections, possibly leading to life-threatening infectious diseases such as biomaterial associated infection, endocarditis and sepsis [1-6]. The closely related commensal *Staphylococcus epidermidis* also can colonize healthy tissues and persist intracellularly in macrophages after implantation of biomaterials [7-10]. Like staphylococci, important bacterial pathogens such as *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Salmonella typhi* can survive intracellularly and cause tuberculosis, meningitis and typhoid fever, respectively [11, 12].

Intracellular infections are very difficult to treat since most antibiotics have limited activity against intracellular bacteria [11-14], because of low penetration of eukaryotic cells [13], low intracellular retention [13], or high frequencies of resistance development [15]. Resistance may develop since the low, permissive intracellular concentrations of antibiotics provide a selective advantage for bacteria with reduced susceptibility [16]. Moreover, some pathogens such as *S. aureus* may undergo structural changes inside the host cells, resulting in reduction of sensitivity to antibiotics [2]. Thus, techniques for improving intracellular activity of antibiotics are urgently required. Cellular internalization of antibiotics and other biomolecules can be enhanced by using liposomes, polymeric micro-/nanoparticles and (nano-)biomimetic as carriers [11, 12, 14, 17], conjugation to specific antibodies, provoking receptor-mediated uptake [18], or conjugation to cell penetrating peptides [19, 20]. However, development of delivery

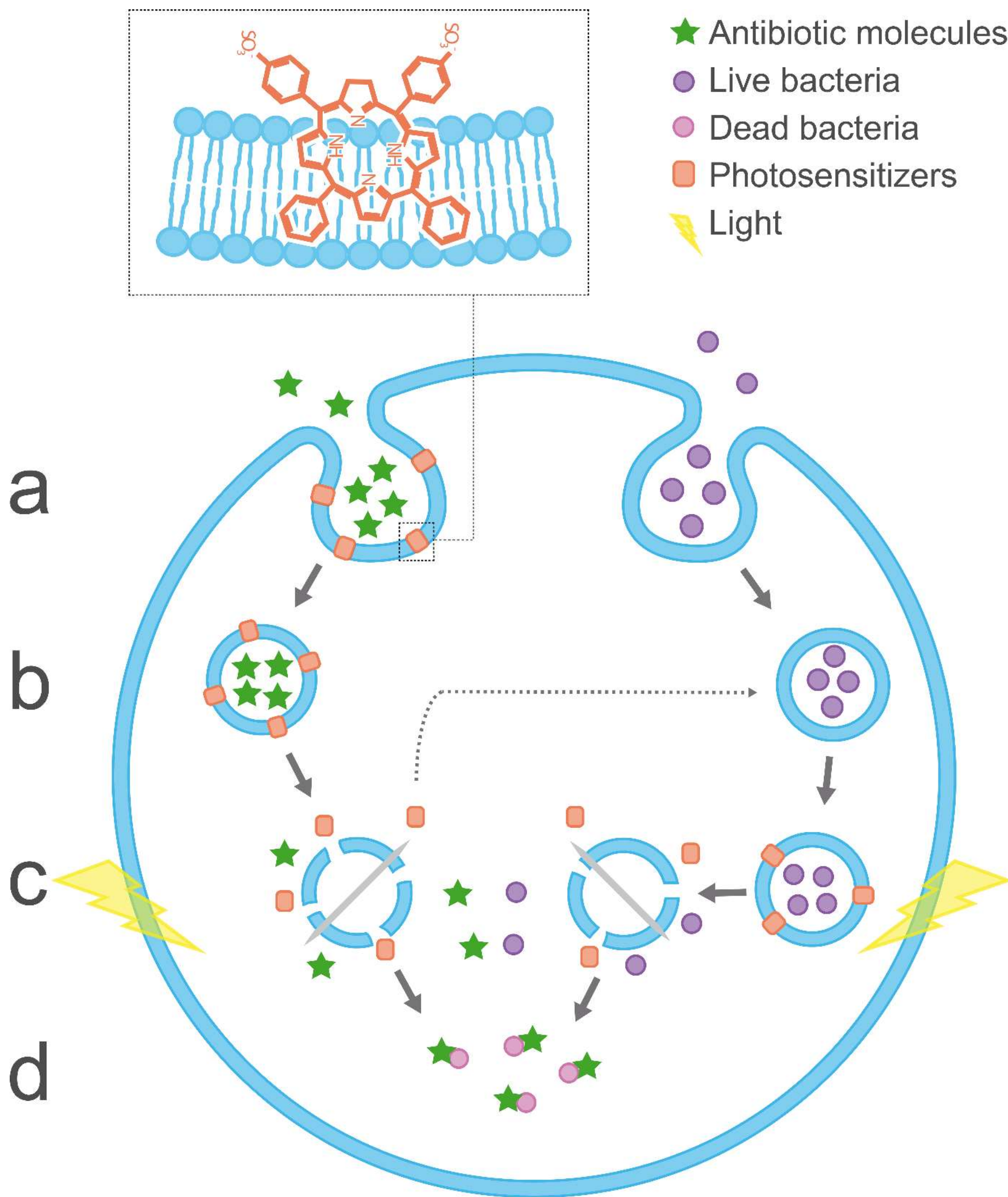
systems or conjugation systems is complex and/or often targeted to single types of antibiotics, and modifications of the molecular structures of antibiotics may reduce their activity [21]. Moreover, most of these approaches will hardly mediate efficient release of the cargos from endocytic vesicles into the cytosol [22, 23]. Endosomal entrapment of many therapeutics is known to hinder them from reaching their intracellular site of action and will eventually result in degradation of the entrapped drugs in lysosomes [22, 23].

To solve this problem, photochemical internalization (PCI) would be a promising method to improve cytosolic release of therapeutics and as a result enhance their intracellular efficacy. PCI has recently been developed to improve intracellular efficacy of drugs for tumor treatment using amphiphilic photosensitizers e.g. tetraphenyl phorphyrin disulphonate ($TPPS_{2a}$) and tetraphenyl chlorin disulphonate ($TPCS_{2a}$) (Figure S1, Supplementary data) [24, 25]. In PCI, photosensitizers localize to the membranes of endocytic vesicles in which drugs may be sequestered within cells. Upon illumination, these photosensitizer-bound membranes are disrupted, causing cytosolic release of the drugs from the vesicles allowing them to reach their intracellular targets [24, 25].

In the present study, we therefore assessed whether PCI combined with antibiotics can combat intracellular bacterial infection by enhancing cytosolic release of the antibiotics. Different from the application of PCI for tumor treatment which aims for an effect on the entire target cancer cells, we used PCI to deliver antibiotics intracellularly to target another organism, i.e. the intracellular bacteria. This novel concept is depicted in Scheme 1. Gentamicin was selected as the antibiotic since it has low intracellular activity due to its inability of endosomal escape [26, 27]. The efficacy of gentamicin against intracellular staphylococci with and without PCI was evaluated *in vitro* in RAW 264.7 mouse macrophages and *in vivo* using a zebrafish embryo staphylococcal infection model [4, 28]. To the best of our knowledge, our study is the first to demonstrate this potential of PCI in an entirely new application field, i.e. to improve

94 intracellular efficacy of antibiotics, and to show proof of concept of this novel approach to treat

95 intracellular infections.



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98 Scheme 1. Proposed mechanism of photochemical internalization (PCI) of antibiotics

99 combatting intracellular bacteria. **a**) Cellular uptake of antibiotics and bacteria; amphiphilic

100 photosensitizers (PS) are administered together with antibiotics and dock into the plasma
101 membrane prior to the formation of endosomes (insertion of $TPCS_{2a}$ in magnification); **b**)
102 Entrapment of antibiotics and bacteria in endosomes/phagosomes; **c**) PCI–induced cytosolic
103 release of antibiotics by disrupting the membranes of endosomes upon illumination and
104 concomitant dissociation of PS; dashed arrow indicates re-location [29] of liberated PS to the
105 membranes of phagosomes containing bacteria during illumination, causing PCI-induced
106 cytosolic release of bacteria. **d**) Contact of antibiotics with bacteria within the cytosol allowing
107 antimicrobial action. Of note, the sizes of the symbol of antibiotics, bacteria and
108 photosensitizers are schematic, not proportional to their actual molecular/cell sizes. (in color)
109

110 Materials and methods

111 *Bacterial strains and inoculum preparation*

112 *S. epidermidis* strain O-47 [7] was used for *in vitro* studies with RAW 264.7 mouse
113 macrophages (indicated as RAW cells in the manuscript, #TIB-71 ATCC, U.S) [30]. The
114 minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) [27] of
115 gentamicin (Centrafarm B.V, The Netherlands) for *S. epidermidis* strain O-47 in RPMI medium
116 (Gibco, ThermoFisher Scientific) were 0.04 and 0.33 µg/ml, respectively. *S. aureus* strain
117 ATCC#49230 was used for zebrafish embryo infection. *S. aureus* strain RN4220 expressing
118 mCherry fluorescent protein (designated as *S. aureus*-mCherry in the manuscript) was
119 constructed as described [7, 31] and used for *in vivo* visualization of cell-bacteria interaction in
120 zebrafish embryos. Bacterial inocula were prepared as described [7, 31].
121

122 *Culturing condition of RAW 264. 7 cells*

123 RAW cells were seeded in 96-well plates (Greiner bio-one) at a concentration of 1 x 10^5
124 cells/well and incubated overnight in RPMI medium supplemented with 5 % fetal calf serum
125 (RPMI) (Gibco, ThermoFisher Scientific) at 37 °C in a humidified atmosphere containing 5 %
126 CO_2.

127

128 *Cytotoxicity for RAW cells*

129 Cultured RAW cells were incubated overnight in 200 µl of RPMI containing gentamicin (15.6
130 to 1000 µg/ml), or incubated for 2 hours in RPMI containing the photosensitizer $TPPS_{2a}$ (0.1 to
131 0.4 µg/ml) (PCI Biotech AS, Norway). The $TPPS_{2a}$-treated cells were incubated for another 2
132 hours in fresh RPMI in order to remove excess $TPPS_{2a}$ from cytoplasma membranes. RAW
133 cells incubated in RPMI alone served as controls. Cells were protected from light except during
134 illumination for 15 minutes using the LumiSource device (a broad-band blue light source, λ_{max}
135 ≈ 420 nm; PCI Biotech AS). After illumination, cells were incubated in fresh RPMI for 24
136 hours. The effect of gentamicin and of $TPPS_{2a}$ on the metabolic activity of RAW cells was
137 tested using MTT assay at 24 hours after incubation or using WST-1 assay directly and at 24
138 hours after illumination, respectively, according to the manufacturer's instruction (Sigma-
139 Aldrich). In order to test the effect of $TPPS_{2a}$ alone or combined with *S. epidermidis* on the
140 viability of RAW cells, cells were either allowed to phagocytose bacteria for 45 minutes (assay
141 described below) or incubated in bacteria-free medium. After phagocytosis, the cells were
142 incubated in 200 µl of RPMI containing 0.25 µg/ml $TPPS_{2a}$ for 2 hours, and then incubated in
143 fresh RPMI for another 2 hours to remove excess cell membrane-bound $TPPS_{2a}$ and
144 subsequently illuminated for 0, 5, 10 or 15 minutes. Cells only illuminated served as controls.
145 The influx of propidium iodide was measured to quantify the loss of cell viability directly or at
146 24 hours after illumination.

147

148 *In vitro phagocytosis assay*

149 After culturing in Tryptic Soy Broth (TSB) medium, *S. epidermidis* bacteria were pelleted by
150 centrifugation (208,000 x *g*, 2 minutes), re-suspended in 1.5 ml of PBS mixed with 0.5 ml of
151 human serum (H1 serum, Bio Whittaker, The Netherlands) and incubated for 20 minutes for
152 opsonization. The inoculum was adjusted to 1 x 10^8 CFU/ml with RPMI. The cells were seeded
153 as described above and medium of cells was replaced by 40 µl of the bacterial inoculum
154 (bacteria to cell ratio of 40:1) and phagocytosis was allowed to proceed for 45 minutes. RAW
155 cells were then gently washed four times with 60 µl, and with a final wash with 200 µl of PBS
156 to prevent carry-over of planktonic *S. epidermidis,* which was always less than 0.5 % of the
157 numbers of retrieved intracellular bacteria after these washing steps. Cells were lysed with 100
158 µl of 1 % saponine. After lysis, the PBS containing lysed cells and bacteria was transferred into
159 a vial and centrifuged (208,000 x *g*, 2 minutes). The pelleted bacteria were washed and re-
160 suspended in fresh PBS before quantitative culture of serial 10-fold dilutions[31]. Intracellular
161 surviving *S. epidermidis* in RAW cells were expressed as numbers of CFU per well. The
162 phagocytosis assay is schematically depicted in Figure S2 (Supplementary data).

163

164 *Bactericidal activity assay*

165 To test whether photosensitizer $TPPS_{2a}$ has bactericidal activity against *S. epidermidis*, we
166 performed a 99.9% lethal concentration assay. After pre-culture in TSB medium, an *S.*
167 *epidermidis* inoculum was prepared of 1 x 10^6 CFU/ml with refresh TSB medium. One hundred
168 µl of the inoculum was added to 100 µl of TSB medium containing different concentrations of
169 $TPPS_{2a}$ (final concentrations of 0.005 to 0.5 µg/ml) in a 96 wells plate. After overnight

170 incubation with $TPPS_{2a}$, the bacteria were illuminated for 6 minutes using the LumiSource
171 device, and quantitatively cultured immediately and at 3 and 24 hours after illumination, as
172 described earlier. Bacteria incubated in TSB medium without $TPPS_{2a}$ served as controls. The
173 concentration of $TPPS_{2a}$ eliminating 99.9% of the numbers of CFU relative to the inoculum
174 was defined as the 99.9% lethal concentration ($LC_{99.9}$).

175

176 *Intracellular antimicrobial activity assay*

177 RAW cells were allowed to phagocytose *S. epidermidis.* This bacterial species was chosen for
178 these experiments since the bacteria survive inside the macrophage *in vitro* without killing them
179 [32]. Cells were then washed to remove extracellular bacteria as described earlier, and treated
180 for 2 hours with gentamicin (1, 10 or 30 μg/ml) with or without $TPPS_{2a}$ (0.25 μg/ml) (Figure
181 S2, Supplementary data). Cells incubated in RPMI or in RPMI containing $TPPS_{2a}$ served as
182 controls. The medium was then changed for fresh RPMI containing gentamicin in the identical
183 concentrations but without $TPPS_{2a}$, and cells were incubated for 2 hours to remove excess cell
184 membrane-bound $TPPS_{2a}$. Medium was then replaced by RPMI containing 1 μg/ml gentamicin
185 in order to prevent growth of extracellular bacteria in the subsequent steps, and cells were
186 illuminated for 10 or 15 minutes. Non-illuminated cells served as controls. After illumination
187 cells were incubated overnight, lysed, and intracellular surviving bacteria were quantitatively
188 cultured as described earlier.

189

190 *Preparation of fluorescently labeled gentamicin*

191 Gentamicin (Sigma-Aldrich) was labelled with Alexa Fluor 405 succinimidyl ester (Life
192 Technologies) (ratio of 1:1), purified by C-18 reversed phase chromatography, aliquoted,
193 lyophilized and stored in the dark at -20°C.

194

195 *Confocal fluorescence microscopy*

196 After culturing, RAW cells were seeded in a culture dish at 3×10^5 cells/dish (MatTek Glass
197 Bottom Culture Dish, U.S) and incubated overnight in 1 ml of RPMI containing 10 µg/ml
198 fluorescently labeled gentamicin alone or combined with 1 µg/ml $TPCS_{2a}$ (PCI Biotech AS).
199 The cells were then incubated in fresh RPMI for 4 hours to remove excess cell membrane-
200 bound $TPCS_{2a}$, illuminated for 2 minutes and covered with Prolong® Gold antifade reagent (Life
201 Technologies) for confocal microscopy (Leica).

202

203 *Zebrafish husbandry and maintenance*

204 The zebrafish embryo experiments were performed according to the EU Animal Protection
205 Directive 2010/63/EU. Adult wild type (WT) or transgenic (Tg) zebrafish and embryos were
206 maintained as described [33] and handled in compliance with animal welfare regulations, as
207 approved by the local animal welfare committee (DEC).

208

209 *Injection into zebrafish embryos*

210 Injections of antibiotic solution (alone or with photosensitizers) or bacterial inoculum into the
211 blood circulation of zebrafish embryos via either the blood island or the duct of Cuvier was
212 performed as described [34]. An injection volume of 1 nl was used for all injections performed

213 in the present study. The needles were pulled from a glass capillary (Harvard apparatus) and
214 the tip was broken at an outer diameter of approximately 15 µm using a microscope with a scale
215 bar (Leica M20) [34]. Pressure and injection time of the FemtoJet microinjector (Eppendorf)
216 were subsequently adjusted to deliver liquid droplets with a diameter of 125 µm, corresponding
217 to a calculated volume of 1 nl.

218

219 *Dose finding of S. aureus for zebrafish embryo infection*

220 Using graded inocula of *S. aureus* (ATCC#49230 strain; 6000, 3000, 500 and 100 CFU per
221 embryos), we assessed the lethal challenge dose for zebrafish embryos. Embryos were injected
222 at 30 hours post fertilization, and individually maintained in 200 µl of E3 medium as described
223 [33]. Medium was refreshed daily. The injected doses were checked by quantitative culture of
224 5-6 embryos per group, crushed using a MagNA lyser (Roche). Survival was monitored daily
225 until 4 days post injection.

226

227 *Visualization of co-localization of phagocytes and bacteria in zebrafish embryos*

228 At 30 hours post fertilization, inocula of *S. aureus*-mCherry were injected into zebrafish
229 embryos of the Tg line (mpeg1: Gal4/UAS: Kaede) featuring macrophages expressing Kaede
230 green fluorescent protein [35]. The injected doses were checked as described above. At 32 hours
231 post fertilization, so 2 hours post injection, images were recorded under bright field as well as
232 with the FITC and mCherry filters, using a fluorescence microscope (LM 80, Leica).

233

234 *Toxicity for zebrafish embryos*

235 Gentamicin (0.16 to 16 mg/ml) or $TPCS_{2a}$ (0.25 to 25 μg/ml) solutions (both in PBS) or
236 mixtures were injected into WT zebrafish embryos at 32 hours post fertilization. Control
237 embryos received PBS injections. The embryos were group-wise maintained in petri-dishes,
238 and protected from light except during illumination for 10 minutes with the LumiSource to
239 activate the $TPCS_{2a}$ photosensitizer, at 34 hours post fertilization. Survival of embryos was
240 monitored daily until 6 dpi based on the observation of movement and heartbeat of the embryos.

241

242 *Treatment of S. aureus-infected zebrafish embryos*

243 Wild type zebrafish embryos were injected with 3000 CFU of *S aureus* ATCC#49230 at 30 hpf,
244 and randomly divided into groups for different treatments. At 32 hours post fertilization 1 nl of
245 PBS solution containing gentamicin alone (0.05, 0.1 or 0.4 μg/ml) or combined with 0.25 μg/ml
246 $TPCS_{2a}$ was injected. Control embryos received PBS injections. The embryos were protected
247 from light except during illumination for 10 minutes with the LumiSource, at 34 hours post
248 fertilization. They were separately maintained in E3 medium which was refreshed daily.
249 Survival was monitored until 6 days post fertilization. The blue light LumiSource lamp was
250 used to illuminate zebrafish embryos for two reasons: 1) Since zebrafish embryos are
251 transparent and thin, deep tissue penetration of light is therefore not needed and the blue light
252 ($\lambda_{max} \approx 420$ nm) is capable of penetrating the embryos for *in vivo* light-activation of $TPCS_{2a}$; 2)
253 The LumiSource lamp is a practical way to simultaneously illuminate multiple zebrafish
254 embryos since it has a light emission surface area of 765 cm^2.

255

256 *Statistical analysis*

257 For *in vitro* studies with RAW cells, data were analyzed by one-way ANOVA, and
258 subsequently groups were compared pairwise by either Dunnett's or Sidak's multiple
259 comparisons tests, depending on the experimental setup. Percent survival of embryos and
260 differences between pairs of survival curves were analyzed using the Kaplan-Meier method and
261 log rank test, respectively. Differences were considered significant for P values $\leqq 0.05$. All
262 analyses were performed using GraphPad Prism 7.0.

263

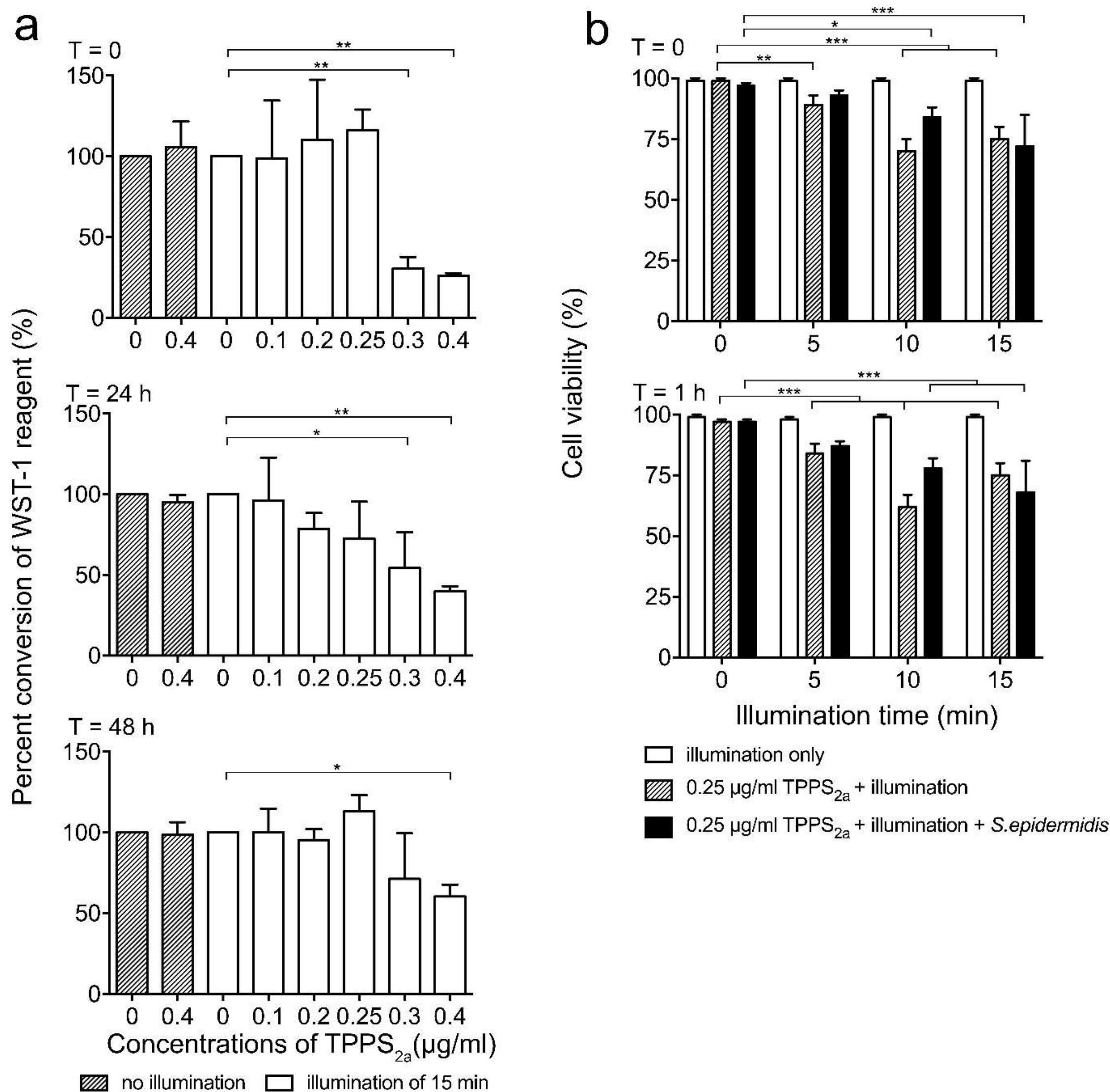
264 # Results

265 ### *Effect of gentamicin, $TPPS_{2a}$ and $TPPS_{2a}$-S. epidermidis combination on*
266 ### *metabolic activity and viability of RAW cells*

267 Exposure to up to 250 µg/ml of gentamicin for 24 hours did not reduce the metabolic activity
268 of RAW cells. Without illumination, 0.4 µg/ml of $TPPS_{2a}$ did not reduce the metabolic activity.
269 With illumination for 15 minutes, concentrations of $TPPS_{2a}$ up to 0.25 µg/ml did not reduce the
270 metabolic activity, neither immediately after illumination (T = 0) nor after 24 (T = 24) or 48
271 hours (T = 48) (Figure 1a). Hence, we chose 250 µg/ml of gentamicin and 0.25 µg/ml of $TPPS_{2a}$
272 as the maximum concentrations for further experiments.

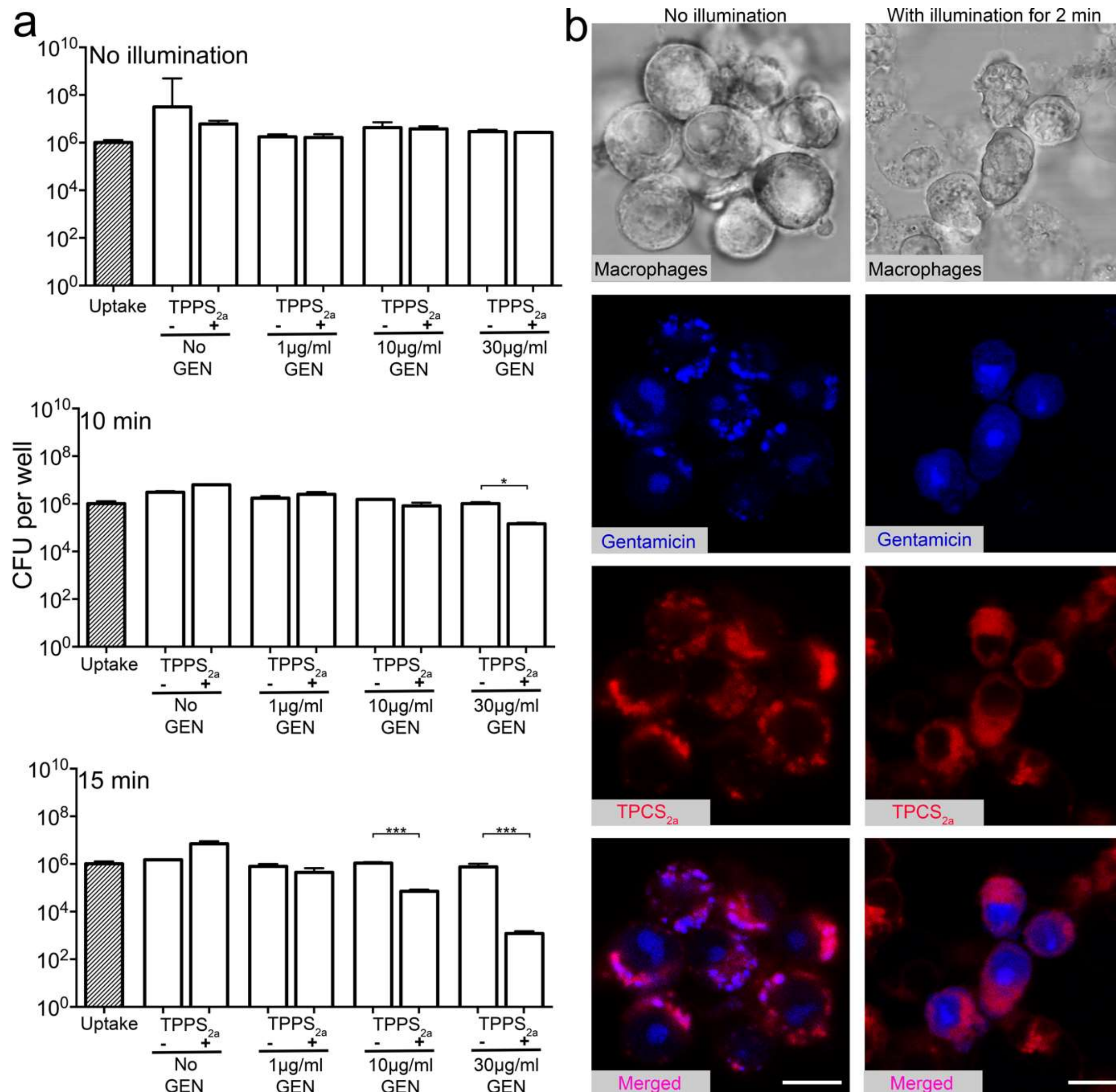
273 The effect of $TPPS_{2a}$-PCI treatment alone or in presence of *S. epidermidis* on viability of RAW
274 cells was assessed immediately (T = 0) and at 1 hour after illumination for 0, 5, 10 or 15 minutes
275 (T = 1) (Figure 1b). Cells illuminated in absence of $TPPS_{2a}$ served as controls. Illumination as
276 such did not influence cell viability. Cells exposed to $TPPS_{2a}$ alone or combined with *S.*
277 *epidermidis* showed significant reduction of viability when illuminated for 5-15 minutes and
278 for 10-15 minutes, respectively (Figure 1b).

279



280 Figure 1. Effect of $TPPS_{2a}$ and $TPPS_{2a}$ combined with *S. epidermidis* on metabolic activity and
281 viability of RAW cells. **a**) Metabolic activity of RAW cells expressed as percent conversion of
282 WST-1 reagent relative to that of non-treated cells (0 µg/ml). Differences between the $TPPS_{2a}$-
283 treated groups and the non-treated group were analyzed using Dunnett's multiple comparisons
284 test; **b**) Viability of RAW cells recorded as percentage of cells not permeable to propidium
285 iodide. The cells were treated with illumination only, with $TPPS_{2a}$ and illumination, or with
286 $TPPS_{2a}$ combined with *S. epidermidis* and illumination. Differences between indicated groups
287 were analyzed using Dunnett's multiple comparisons test; Data represent mean ± standard
288 deviation (n=3) in Panel a and b, *, $P \leqq 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

289 *PCI-induced cytosolic release of gentamicin enhances efficacy against*
290 *intracellular S. epidermidis in RAW cells*

291 

292 Figure 2. PCI-enhanced efficacy of gentamicin against intracellular *S. epidermidis* in RAW
293 cells. **a**) Reduction of numbers of CFU of intracellular *S. epidermidis* by $TPPS_{2a}$-PCI of
294 gentamicin. Cells containing *S. epidermidis* were illuminated only, treated with 0.25 µg/ml
295 $TPPS_{2a}$ or gentamicin (GEN) only or with GEN-$TPPS_{2a}$ combinations. Cells subsequently were
296 illuminated for 0, 10 or 15 minutes. Differences between GEN alone and respective GEN-
297 $TPPS_{2a}$ treatments were analyzed using Sidak's multiple comparisons test. Data represent mean

298 ± standard deviation (n=3). *, P ≦ 0.05, ***, $P < 0.001$; **b**) PCI-induced cytosolic release of
299 gentamicin in RAW cells upon illumination. Gentamicin was labeled with Alexa Fluor 405
300 (blue) and $TPCS_{2a}$ was observed in the red channel. Intracellular co-localization of gentamicin
301 and $TPCS_{2a}$ is shown as magenta color in the merged images. Scale bars = 10 µm. (in color)

302

303 To study whether $TPPS_{2a}$ with illumination itself would kill bacteria, we exposed *S. epidermidis*
304 bacteria to $TPPS_{2a}$ at concentrations of up to 0.5 µg/ml and illuminated the bacteria. The
305 numbers of CFU of *S. epidermidis* were not reduced after incubation with $TPPS_{2a}$ and
306 illumination, showing that $TPPS_{2a}$ with illumination has no inhibitory or cidal effect on the
307 bacteria.

308 To investigate whether $TPPS_{2a}$-PCI enhanced the efficacy of gentamicin against intracellular *S.*
309 *epidermidis in vitro*, we exposed *S. epidermidis*-infected RAW cells to $TPPS_{2a}$ only (0.25
310 µg/ml), to gentamicin only (1, 10 or 30 µg/ml) or to the respective gentamicin-$TPPS_{2a}$
311 combinations (Figure 2a). Since with 5 minutes of illumination no effect of the gentamicin-
312 $TPPS_{2a}$ combinations was observed (data not shown), cells were illuminated for 10 or 15
313 minutes. Treated but non-illuminated cells and cells only illuminated served as controls.
314 Without illumination, none of the treatments caused reduction of the numbers of intracellular
315 bacteria in RAW cells. Treatment with $TPPS_{2a}$-illumination or only with illumination did not
316 affect intracellular survival of *S. epidermidis.* None of the treatments with gentamicin only,
317 with or without illumination for 10 or 15 minutes, showed significant reduction in numbers of
318 CFU of intracellular *S. epidermidis*. Treatment with $TPPS_{2a}$ and 30 µg/ml gentamicin with
319 illumination for 10 minutes significantly enhanced killing of intracellular bacteria (1 log
320 reduction). With illumination for 15 minutes, combination of $TPPS_{2a}$ and either 10 or 30 µg/ml
321 gentamicin significantly increased killing of intracellular *S. epidermidis* to levels of 1 and 3 log

322 reduction, respectively. A repetition experiment showed highly similar results (Figure S3,
323 Supplementary data).

324 To investigate whether PCI induced cytosolic release of gentamicin, intracellular distribution
325 of gentamicin and photosensitizer in RAW cells with and without illumination was visualized
326 (Figure 2b). For these and subsequent *in vivo* studies with zebrafish embryos we selected
327 $TPCS_{2a}$. This photosensitizer absorbs red light which has a favorable tissue penetration, and
328 therefore is more suitable for applications *in vivo* than $TPPS_{2a}$ [36]. Without illumination, both
329 gentamicin and $TPCS_{2a}$ localized within intracellular compartments in the periphery of the cells,
330 likely endocytic vesicles. After illumination both gentamicin and $TPCS_{2a}$ were released into the
331 cytosol. Gentamicin seemed to accumulate at the nuclei of the RAW cells.

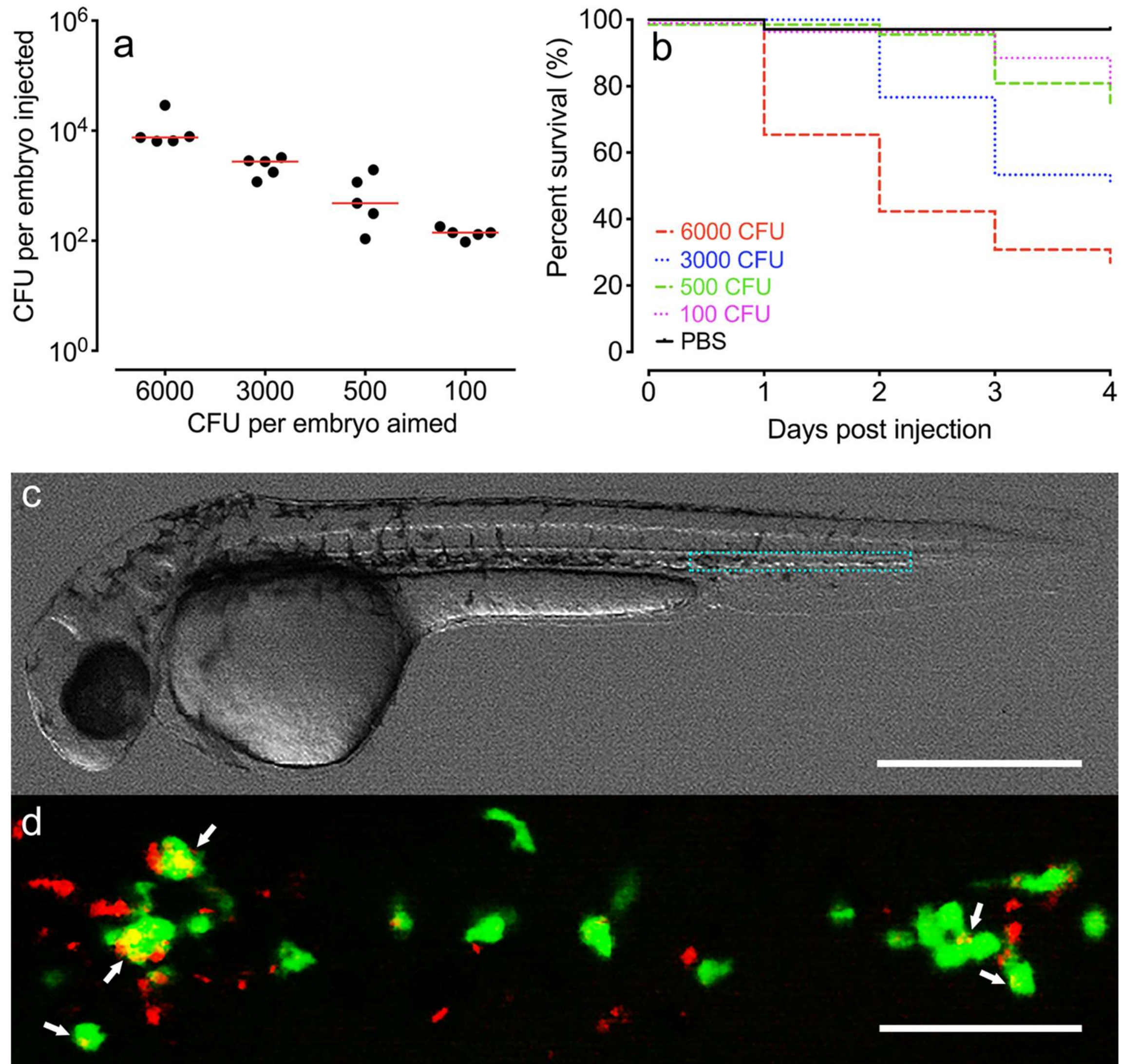
332

333 *Dose finding of S. aureus for zebrafish embryo infection and visualization of*
334 *cell-pathogen interaction in vivo*

335 To assess suitable doses of *S. aureus* for zebrafish embryo infection, we injected graded inocula
336 with doses of 6000, 3000, 500 or 100 CFU per embryos into the blood circulation at 30 hours
337 post fertilization. The actual doses of bacteria injected were close to the aimed doses with minor
338 variations in each group (Figure 3a). Death rate of *S. aureus*-infected embryos was proportional
339 to the inoculum dose (Figure 3b). The dose of 3000 CFU/embryo caused approximately 50%
340 of the embryos to die at 4 days post injection (Figure 3b), which is suitable to assess the efficacy
341 of antibiotic treatment.

342 In order to investigate whether *S. aureus* was phagocytosed shortly after injection, we
343 challenged 1 day old transgenic zebrafish embryos expressing Kaede green fluorescent protein
344 in their macrophages (Figure 4d), with 3000 CFU of mCherry red fluorescent protein-

345 expressing *S. aureus*. We chose the time point of 2 hours post injection to assess whether the
346 injected *S. aureus* were phagocytosed by zebrafish macrophages since the majority of *S. aureus*
347 are taken up by zebrafish macrophages and/or neutrophils with 2 hours post injection, and these
348 cells containing bacteria are important niches for *S. aureus* infection in the embryos [4, 37]. In
349 the present study co-localization of *S. aureus* and macrophages was observed in the blood
350 circulation at 2 hours post injection (Figure 3d). The bacteria not associated with labelled
351 phagocytes seemed to be clustered (Figure 3d), suggesting that they were phagocytosed by the
352 non-labelled phagocyte type of the embryos such as neutrophils. This shows that (a portion of)
353 *S. aureus* had been phagocytosed *in vivo* at 2 hours post injection, which is in line with the
354 results reported in the previous studies [4, 37].



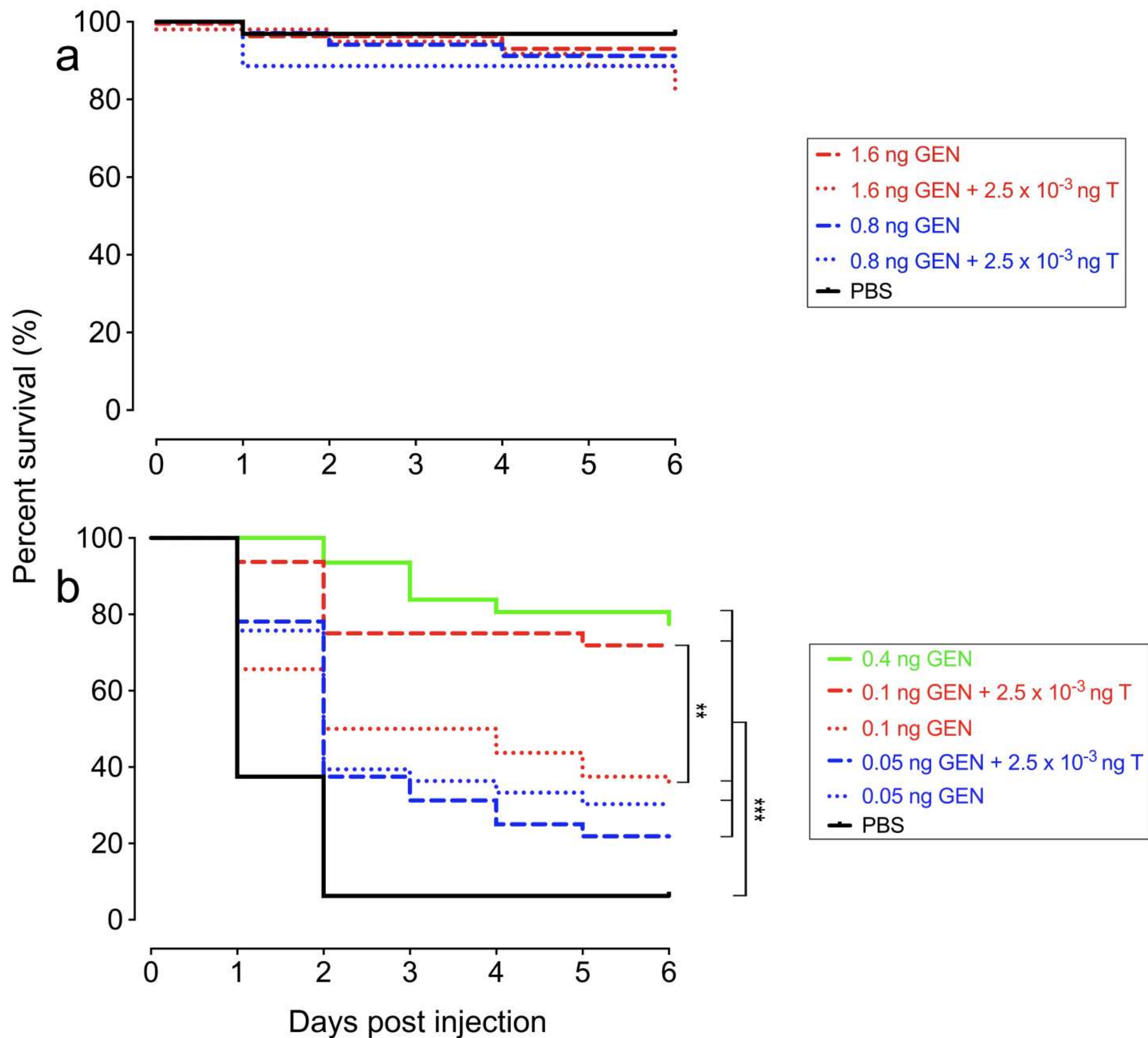
355

356 Figure 3. Determination of *S. aureus* challenge doses for zebrafish embryo infection and co-
357 localization of *S. aureus* and zebrafish macrophages. **a**) CFU numbers of *S. aureus* cultured
358 from crushed embryos injected with inocula of 100 to 6000 CFU in 1 nl PBS. The red lines
359 represent the median numbers of CFU. **b**) Effect of different inocula of *S. aureus* on survival
360 of embryos. PBS injections served as controls. Initial group sizes ranged from 26 to 38 embryos.
361 **c**) Bright field image of a representative 1 day old zebrafish embryo at 2 hours post *S. aureus*
362 injection. Scale bar = 500 µm. The blue box indicates the area shown in **d**) at high magnification
363 with co-localization of *S. aureus*-mCherry (red) and zebrafish macrophages (green) as co-
364 localization in yellow (arrows). Scale bars = 100 µm in **d**). (in color)

365

366 *Enhanced survival of S. aureus-infected embryos treated with gentamicin*

367 *combined with PCI*



368

369 Figure 4. Survival of non-infected and *S. aureus*-infected zebrafish embryos treated with
370 gentamicin only or gentamicin-$TPCS_{2a}$ combinations after illumination for 10 min. **a**) Effect of
371 gentamicin alone (GEN) or combined with $TPCS_{2a}$ (T) (in 1 nl of PBS) on non-infected embryos.
372 Embryos injected with PBS served as controls. Initial group sizes ranged from 31 to 35 embryos;
373 **b**) Survival of embryos infected with 3000 CFU of *S. aureus* treated with gentamicin only or

374 combined with $TPCS_{2a}$. PBS mock treatment served as control. Initial group size ranged from
375 31 to 33 embryos. Differences between survival of each of the treated groups versus the PBS
376 control group, as well as between survival of the gentamicin only group and the respective
377 gentamicin-$TPCS_{2a}$ treatment group were analyzed using Log-rank test. **, $p < 0.01$. ***, $p <$
378 0.001. (in color)

379

380 To test their toxicity for zebrafish embryos, the effect of injection of graded doses of gentamicin,
381 $TPCS_{2a}$ and gentamicin-$TPCS_{2a}$ combinations on survival was assessed. $TPCS_{2a}$ and
382 gentamicin both showed a dose-dependent toxicity, with maximal non-toxic concentrations of
383 2.5×10^{-3} and 2 ng/embryo, respectively (Figure S4 a and b, Supplementary data).
384 Combinations of 1.6 or 0.8 ng/embryo gentamicin with 2.5×10^{-3} ng/embryo $TPCS_{2a}$ did not
385 significantly reduce survival of embryos (Figure 4a).

386 To investigate whether PCI enhanced the efficacy of gentamicin against staphylococcal
387 infection *in vivo*, we treated *S. aureus*-infected zebrafish embryos (3000 CFU/embryo) with
388 gentamicin alone or combined with $TPCS_{2a}$ (Figure 4b). All treatments significantly improved
389 survival as compared to the PBS mock treatment. Addition of $TPCS_{2a}$ significantly improved
390 the treatment efficacy of 0.1 ng gentamicin, resulting in levels of survival similar to those
391 obtained with treatment of 0.4 ng gentamicin alone. This shows that PCI enhances the efficacy
392 of gentamicin against *S. aureus* infection *in vivo* in zebrafish embryos and lowers the required
393 dose for efficacy. However, a minimal gentamicin dosing is necessary to observe the enhancing
394 effect of $TPCS_{2a}$, since $TPCS_{2a}$ did not improve the efficacy of 0.05 ng gentamicin.

395

396 # Discussion

397 Intracellular niches are considered a ”safe haven” for intracellular bacterial pathogens such as
398 staphylococci, where they are protected from clearance by the host [1-3, 8, 10]. Intracellular
399 infections are very difficult to treat with most conventional antibiotics, and even are considered
400 part of the cause of antibiotic resistance development [2, 11, 16]. Although some approaches
401 such as using micro-/nano-sized vehicles as carriers for delivery of antibiotics [11, 12, 14, 17]
402 and conjugation of antibiotics to cell penetrating peptides or specific antibodies [18-20] are
403 reported to improve their cell penetration, endosomal entrapment of antibiotics after
404 endocytosis still remains a major problem, resulting in low bioavailability of the drugs in the
405 cytosol [22, 23]. Therefore, novel approaches to enhance intracellular activity of antibiotics are
406 urgently needed. In our study, we have devised and applied a novel use of photochemical
407 internalization (PCI) as a means of controlled release of antibiotics into the cytosol, targeting
408 intracellular bacteria. We have applied PCI to enhance intracellular activity of gentamicin, an
409 antibiotic with limited efficacy inside cells, against staphylococci both *in vitro* and *in vivo*. In
410 RAW cells, PCI induced cytosolic release of gentamicin and increased eradication of
411 phagocytosed *S. epidermidis*. *In vivo,* in a zebrafish embryo model with *S. aureus* internalized
412 by phagocytes, PCI enhanced efficacy of gentamicin against *S. aureus* infection and lowered
413 the required dose of the antibiotic. To the best of our knowledge, our study is the first to
414 demonstrate the potential of PCI to enhance antimicrobial efficacy of an antibiotics inside cells
415 and thus provides a new concept for treating intracellular infections.

416 Photosensitizers such as $TPPS_{2a}$ and $TCPS_{2a}$ have been developed for treatment of tumors by
417 enhancing delivery of cytotoxic chemotherapeutics [24, 25, 38]. According to the principle of
418 PCI, the doses of photosensitizer and light required to disrupt the endosomal/lysosomal
419 membranes are likely sublethal [39]. In our study, although PCI had slight to moderate levels
420 of cytotoxicity for RAW cells *in vitro* (Figure 1b), the concentrations required to enhance the
421 efficacy of gentamicin *in vivo* did not significantly reduce survival of the zebrafish embryos

422 (Figure S4). Similarly, in a recent clinical phase I trial for delivery of the antitumor drug
423 bleomycin $TPCS_{2a}$-PCI was shown to be safe and tolerable for human patients receiving
424 infusions of $TPCS_{2a}$ solution [38].$TPPS_{2a}$ and $TPCS_{2a}$ molecules tend to preferentially
425 accumulate in diseased tissues and inflamed areas [24, 25], and other similar types of
426 photosensitizers were shown to be internalized by local highly active cells in inflamed areas,
427 such as macrophages and neutrophils [40, 41]. Therefore, treatment of infected areas by PCI
428 combined with antibiotic therapy will likely preferentially target phagocytic cells containing
429 bacteria. Moreover, any cytotoxicity of photosensitizers will only be induced when illumination
430 is applied. Therefore, application of site-specific illumination to diseased tissues/inflamed areas
431 will minimize the potential side effects of PCI on healthy tissues and normal cells which are
432 not exposed to illumination. Compared to chemical endosomal disruption agents such as
433 chloroquine, ammonium chloride and methylamine which have relatively high toxicity and low
434 cell/tissue specificity [42, 43], PCI provides temporally and spatially controlled cytosolic
435 release of therapeutics from endocytic vesicles with potentially less side effects *in vivo* [24, 44].

436 Treatment of *S. epidermidis*-infected RAW cells by gentamicin alone, even with relatively high
437 concentrations (10 and 30 μg/ml), did not remarkably reduce the numbers of the intracellular
438 bacteria. Combining the treatment with PCI however significantly improved the efficacy
439 (Figure 2). A similar efficacy-enhancing effect of PCI was observed *in vivo* in our zebrafish
440 embryo *S. aureus* infection model (Figure 4). PCI did however not increase the efficacy of the
441 lowest dose of gentamicin, neither *in vitro* nor *in vivo*. Possibly the amount of intracellular
442 gentamicin was too low to be efficacious even after cytosolic release following PCI treatment.
443 Interestingly, in our experiments with RAW cells the liberated gentamicin molecules seemed
444 to accumulate at the nuclei after illumination. This is in line with the observation of gentamicin
445 binding to the nuclei of kidney cells [45]. Although theoretically such binding may reduce the
446 amount of free gentamicin in the cytosol, enhanced efficacy of gentamicin by PCI (4-fold) was

447 still observed in our study. This suggests that intracellular activity of antibiotics which do not
448 show nuclear binding, might be even more strongly enhanced by PCI. Our results, showing an
449 enhancing effect of PCI on antibiotic efficacy in the zebrafish embryo infection model, offer
450 prospects for further *in vivo* studies in larger mammalian animal models. *In vivo* studies with
451 PCI in mouse models have already been performed extensively for cancer treatment[46, 47].
452 The available relevant information on PCI modality from these studies supports further
453 investigation on PCI-antibiotic treatment of intracellular infection *in vivo*. Moreover, since the
454 photosensitizer $TPCS_{2a}$ has passed clinical phase I trials for safety testing in human patients [38]
455 and many antibiotics to be combined with PCI are available, there is the possibility to relatively
456 rapidly progress towards clinical studies of PCI-antibiotic treatment of diseases associated with
457 intracellular infections.

458 Eradication of intracellular bacteria by antibiotics may be impeded by their different subcellular
459 localization inside cells [13, 27]. Even when endosomes containing antibiotics and
460 photosensitizers would be ruptured after illumination, bacteria might still be safely shielded
461 within phagosomes, which would not necessarily contain photosensitizers. Our results however
462 did show increase of killing of the intracellular bacteria. This may be explained in two ways.
463 After rupture of the endosomes containing antibiotics, photosensitizer molecules dissociated
464 from the lysed endosomal membranes may intracytoplasmically re-localize to the membranes
465 of phagosomes containing the bacteria, and also rupture these membranes (Scheme 1). As a
466 result, bacteria are released into the cytosol and are intracellularly killed by gentamicin. In
467 addition, during PCI partially ruptured vesicles are suggested to fuse with still intact
468 intracellular vesicles causing them to also become leaky/ruptured, even without additional
469 illumination [29]. Such fusion therefore may also (partially) contribute to the cytosolic release
470 of both antibiotics and bacteria, facilitating the intracellular antimicrobial action. Since
471 intracellular delivery of bioactive molecules using PCI generally does not rely on particular

472 properties of the molecules to be delivered [24, 25], PCI can likely also improve the intracellular
473 efficacy of other antibiotics than gentamicin via controlled release of drugs into the cytosol of
474 cells. Such antibiotics may be other aminoglycosides, glycopeptides and macrolides, whose
475 activity likely is limited by their inability of endosomal escape [13, 26, 27]. PCI thus has a
476 strong potential to increase the numbers of antibiotics to be effective in treatment of
477 intracellular infection and may increase their therapeutic window, since PCI will lower the
478 effective antibiotic dose owing to enhanced intracellular delivery. Because PCI can in principle
479 enhance intracellular delivery of different antibiotics, it has the potential to enhance efficacy of
480 antibiotic treatment of infections caused by a broad range of intracellular bacterial pathogens
481 such as *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Salmonella typhi* [11, 12].
482 As a result, PCI may also help reduce the rate of resistance development which might occur
483 intracellularly due to the low, permissive concentration of antibiotics.

484 Infectious diseases involving intracellular bacteria can occur or relapse at different sites of the
485 human body (e.g. skin, deep tissues, urinary tract and lung). In addition to professional
486 phagocytes, non-professional phagocytic cells (e.g. epithelial cells, osteoblasts) can be niches
487 for intracellular bacteria [1, 2, 11]. The potential of PCI-antibiotic combinations to treat such
488 local infections is dependent on whether light can be applied to the site of infection with
489 intracellular bacteria. Similar to antibacterial photodynamic therapy (aPDT) [48-50], PCI can
490 be considered for local treatment of (sub)cutaneous skin or mucosal infections such as infected
491 chronic wounds, ulcers, abscesses and diabetic foot infection as well as for nasal and oral
492 infections (e.g. chronic rhinosinusitis and periodontal infections), where the site of infection is
493 accessible for light required for the controlled release of antibiotics. The PCI-antibiotic
494 treatment of infections of internal organs, deep tissue or bone is more challenging, but certainly
495 not impossible. Techniques such as those developed for clinical applications of PDT in the
496 treatment of tumors in bile duct, lung, brain and bladder [51] offer a good toolset to develop

497 PCI-enhanced treatment for deep infections. Consequently, PCI has strong potential to improve
498 antibiotic treatment of intracellular infections in a broad spectrum of clinically challenging
499 infectious diseases.

500

501 Conclusions

502 In our *in vitro* mouse macrophage as well as *in vivo* zebrafish embryo studies, we demonstrate
503 that photochemical internalization (PCI) can significantly enhance the antimicrobial efficacy of
504 an antibiotic with limited activity (e.g. gentamicin) against (intracellular) staphylococcal
505 infection, likely owing to the cytosolic release of the antibiotic. To the best of our knowledge
506 we are the first to report an entirely novel application of PCI, i.e. to specifically enhance the
507 efficacy of antibiotics against intracellular infections. This opens new avenues to improve the
508 antibiotic treatment of infections associated with intracellular survival of bacteria and may also
509 help prevent resistance development.

510

511 Acknowledgements

512 This work was supported by the IBIZA project of the BioMedical Material (BMM) program,
513 co-funded by the Dutch Ministry of Economic affairs. We thank Ing. R. van Leeuwen for
514 assistance and Mr. Wing Ho Man from Department of Pediatric Immunology and Infectious
515 Diseases, Utrecht University Medical Centre for his assistance in preparing Scheme 1. The
516 authors would also like to thank Prof. Dr. Graham Lieschke from Monash University, Australia
517 for providing the zebrafish transgenic line (mpeg1:Gal4/UAS:Kaede).

518

519 # Declarations of interest

520 Dr. Anders Høgset holds stock shares of PCI Biotech AS.

521

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