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

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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.

1. 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

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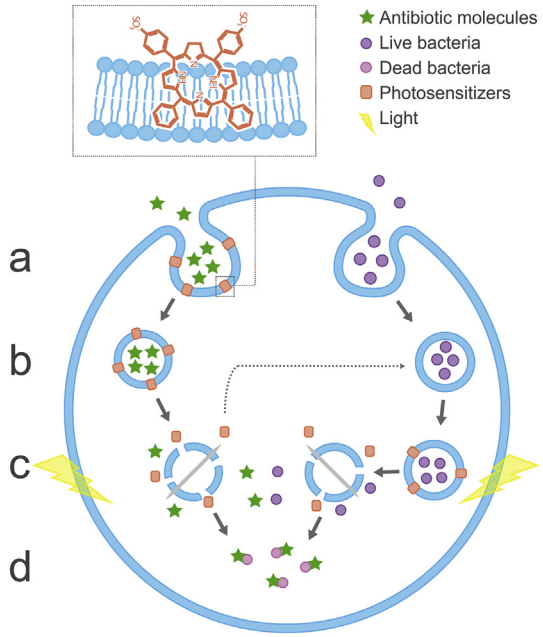
types of antibiotics, and modifications of the molecular structures of antibiotics may reduce their activity [21]. Moreover, most of these approaches will hardly mediate efficient release of the cargos from endocytic vesicles into the cytosol [22, 23]. Endosomal entrapment of many therapeutics is known 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 ($TPCS_{2a}$) (Fig. 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



Scheme 1. Proposed mechanism of photochemical internalization (PCI) of antibiotics combatting intracellular bacteria. a) Cellular uptake of antibiotics and bacteria; amphiphilic photosensitizers (PS) are administered together with antibiotics and dock into the plasma membrane prior to the formation of endosomes (insertion of $TPCS_{2a}$ in magnification); b) Entrapment of antibiotics and bacteria in endosomes/phagosomes; c) PCI–induced cytosolic release of antibiotics by disrupting the membranes of endosomes upon illumination and concomitant dissociation of PS; dashed arrow indicates re-location [29] of liberated PS to the membranes of phagosomes containing bacteria during illumination, causing PCI-induced cytosolic release of bacteria. d) Contact of antibiotics with bacteria within the cytosol allowing antimicrobial action. Of note, the sizes of the symbol of antibiotics, bacteria and photosensitizers are schematic, not proportional to their actual molecular/cell sizes. Live bacteria and dead bacteria share the same symbol. (For interpretation of the references to the color of the symbols in this scheme, the reader is referred to the web version.)

[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 intracellular efficacy of antibiotics, and to show proof of concept of this novel approach to treat intracellular infections.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

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

2.2. Culturing condition of RAW 264. 7 cells

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

2.3. Cytotoxicity for RAW cells

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

2.4. In vitro phagocytosis assay

After culturing in Tryptic Soy Broth (TSB) medium, *S. epidermidis* bacteria were pelleted by centrifugation (208,000 ×*g*, 2 min), re-suspended in 1.5 ml of PBS mixed with 0.5 ml of human serum (H1 serum, Bio Whittaker, The Netherlands) and incubated for 20 min for opsonization. The inoculum was adjusted to 1×10^8 CFU/ml with RPMI. The

cells were seeded as described above and medium of cells was replaced by 40 µl of the bacterial inoculum (bacteria to cell ratio of 40:1) and phagocytosis was allowed to proceed for 45 min. RAW cells were then gently washed four times with 60 µl, and with a final wash with 200 µl of PBS to prevent carry-over of planktonic *S. epidermidis,* which was always < 0.5% of the numbers of retrieved intracellular bacteria after these washing steps. Cells were lysed with 100 µl of 1% saponine. After lysis, the PBS containing lysed cells and bacteria was transferred into a vial and centrifuged (208,000 ×*g*, 2 min). The pelleted bacteria were washed and re-suspended in fresh PBS before quantitative culture of serial 10-fold dilutions [31]. Intracellular surviving *S. epidermidis* in RAW cells were expressed as numbers of CFU per well. The phagocytosis assay is schematically depicted in Fig. S2 (Supplementary data).

2.5. Bactericidal activity assay

To test whether photosensitizer $TPPS_{2a}$ has bactericidal activity against *S. epidermidis*, we performed a 99.9% lethal concentration assay. After pre-culture in TSB medium, an *S. epidermidis* inoculum was prepared of 1×10^6 CFU/ml with refresh TSB medium. One hundred µl of the inoculum was added to 100 µl of TSB medium containing different concentrations of $TPPS_{2a}$ (final concentrations of 0.005 to 0.5 µg/ml) in a 96 wells plate. After overnight incubation with $TPPS_{2a}$, the bacteria were illuminated for 6 min using the LumiSource device, and quantitatively cultured immediately and at 3 and 24 h after illumination, as described earlier. Bacteria incubated in TSB medium without $TPPS_{2a}$ served as controls. The concentration of $TPPS_{2a}$ eliminating 99.9% of the numbers of CFU relative to the inoculum was defined as the 99.9% lethal concentration ($LC_{99.9}$).

2.6. Intracellular antimicrobial activity assay

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

2.7. Preparation of fluorescently labeled gentamicin

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

2.8. Confocal fluorescence microscopy

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

2.9. Zebrafish husbandry and maintenance

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

2.10. Injection into zebrafish embryos

Injections of antibiotic solution (alone or with photosensitizers) or bacterial inoculum into the blood circulation of zebrafish embryos *via* either the blood island or the duct of Cuvier was performed as described [34]. An injection volume of 1 nl was used for all injections performed in the present study. The needles were pulled from a glass capillary (Harvard apparatus) and the tip was broken at an outer diameter of approximately 15 µm using a microscope with a scale bar (Leica M20) [34]. Pressure and injection time of the FemtoJet microinjector (Eppendorf) were subsequently adjusted to deliver liquid droplets with a diameter of 125 µm, corresponding to a calculated volume of 1 nl.

2.11. Dose finding of S. aureus for zebrafish embryo infection

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

2.12. Visualization of co-localization of phagocytes and bacteria in zebrafish embryos

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

2.13. Toxicity for zebrafish embryos

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

2.14. Treatment of S. aureus-infected zebrafish embryos

Wild type zebrafish embryos were injected with 3000 CFU of *S aureus* ATCC#49230 at 30 hpf, and randomly divided into groups for different treatments. At 32 h post fertilization 1 nl of PBS solution containing gentamicin alone (0.05, 0.1 or 0.4 µg/ml) or combined with 0.25 µg/ml $TPCS_{2a}$ was injected. Control embryos received PBS injections. The embryos were protected from light except during illumination for 10 min with the LumiSource, at 34 h post fertilization. They were separately maintained in E3 medium which was refreshed daily. Survival was monitored until 6 days post fertilization. The blue light LumiSource lamp was used to illuminate zebrafish embryos for two reasons: 1) zebrafish embryos are transparent and thin, deep tissue

penetration of light is therefore not needed and the blue light ($\lambda_{max} \approx 420$ nm) is capable of penetrating the embryos for *in vivo* light-activation of $TPCS_{2a}$; 2) The LumiSource lamp is a practical way to simultaneously illuminate multiple zebrafish embryos.

2.15. Statistical analysis

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

3. Results

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

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

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

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

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

To investigate whether $TPPS_{2a}$-PCI enhanced the efficacy of gentamicin against intracellular *S. epidermidis in vitro*, we exposed *S. epidermidis*-infected RAW cells to $TPPS_{2a}$ only (0.25 μg/ml), to gentamicin only (1, 10 or 30 μg/ml) or to the respective gentamicin-$TPPS_{2a}$ combinations (Fig. 2a). Since with 5 min of illumination no effect of the gentamicin-$TPPS_{2a}$ combinations was observed (data not shown), cells were illuminated for 10 or 15 min. Treated but non-illuminated cells and cells only illuminated served as controls. Without illumination, none of the treatments caused reduction of the numbers of intracellular bacteria in RAW cells. Treatment with $TPPS_{2a}$-illumination or only with illumination did not affect intracellular survival of *S. epidermidis*. None of the treatments with gentamicin only, with or without illumination for 10 or 15 min, showed significant reduction in numbers of CFU of

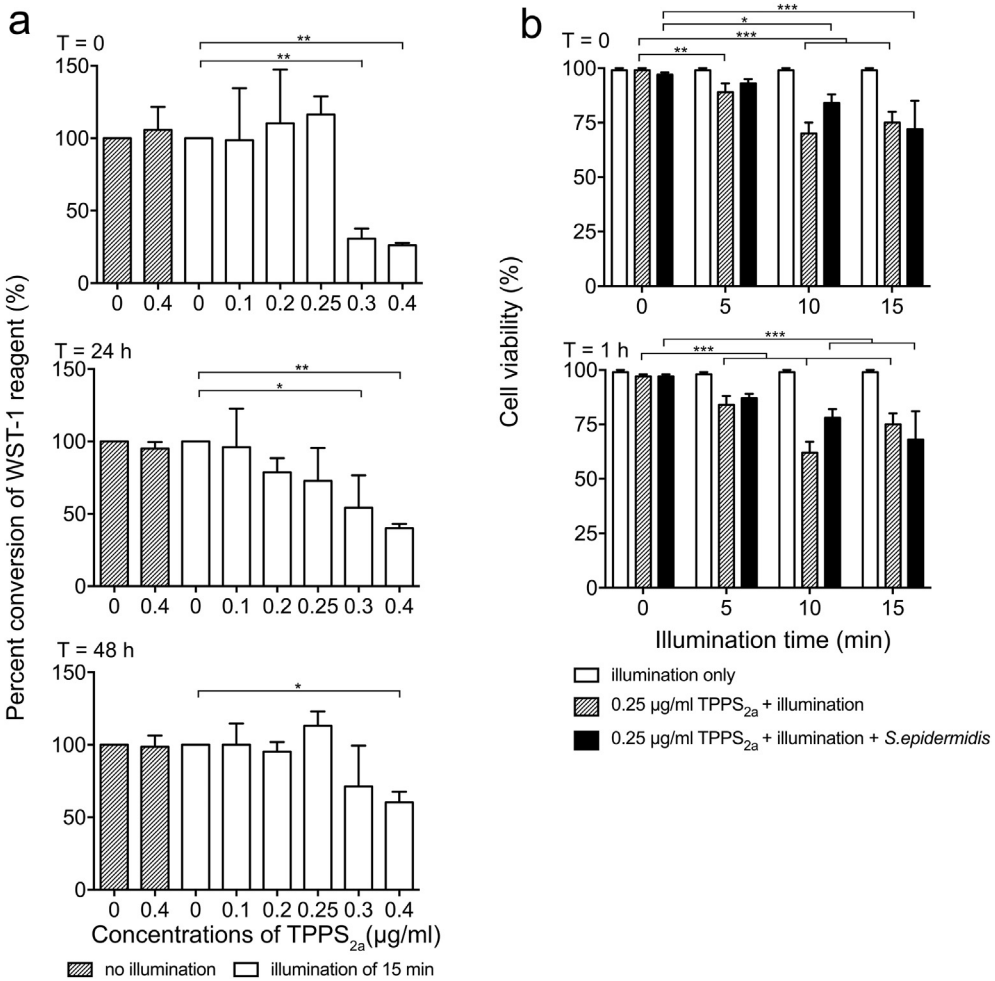


Fig. 1. Effect of $TPPS_{2a}$ and $TPPS_{2a}$ combined with *S. epidermidis* on metabolic activity and viability of RAW cells. a) Metabolic activity of RAW cells expressed as percent conversion of WST-1 reagent relative to that of non-treated cells (0 μg/ml). Differences between the $TPPS_{2a}$-treated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

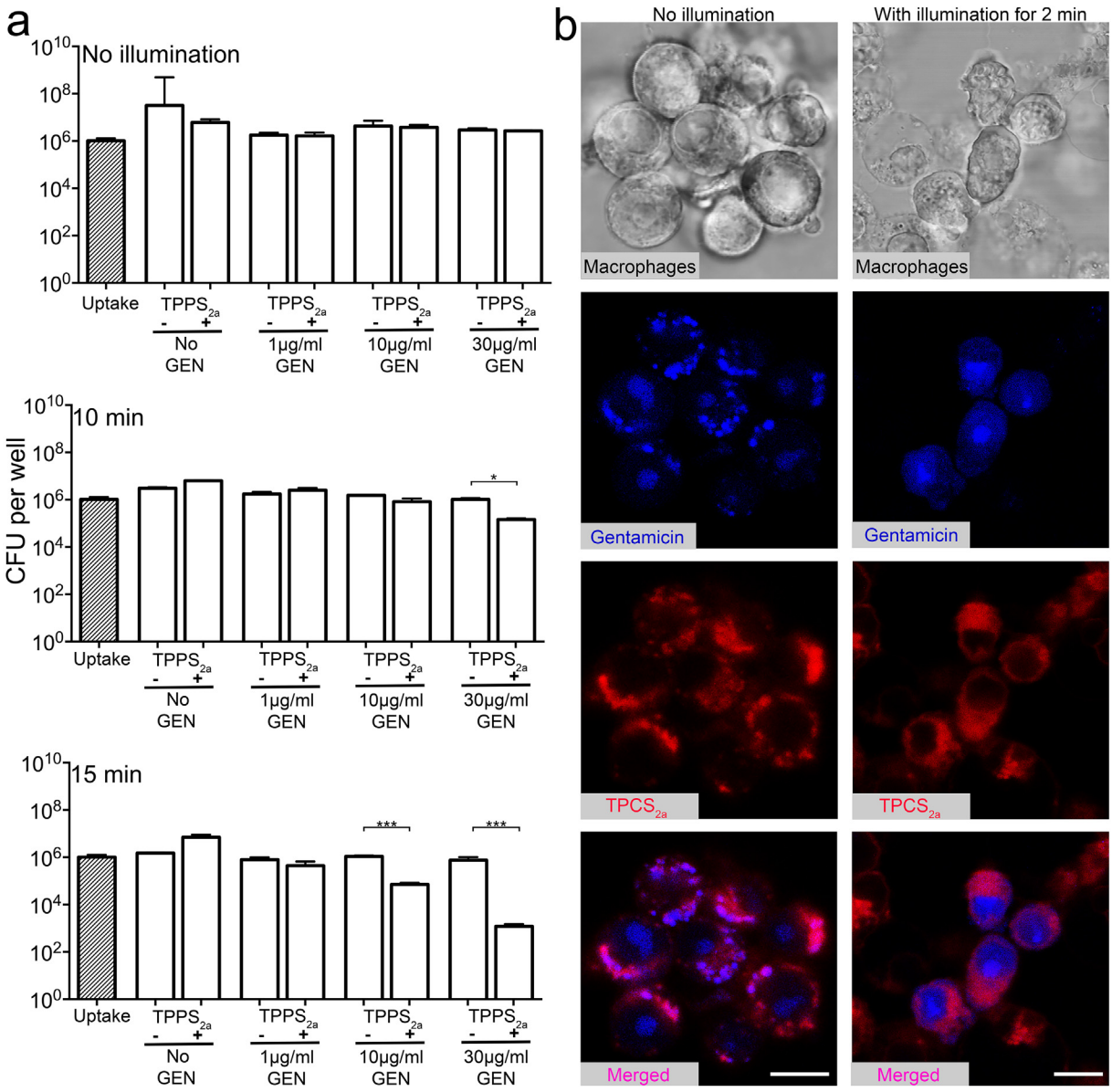


Fig. 2. PCI-enhanced efficacy of gentamicin against intracellular *S. epidermidis* in RAW cells. a) Reduction of numbers of CFU of intracellular *S. epidermidis* by $TPPS_{2a}$-PCI of gentamicin. Cells containing *S. epidermidis* were illuminated only, treated with 0.25 µg/ml $TPPS_{2a}$ or gentamicin (GEN) only or with GEN-$TPPS_{2a}$ combinations. Cells subsequently were illuminated for 0, 10 or 15 min. Differences between GEN alone and respective GEN-$TPPS_{2a}$ treatments were analyzed using Sidak's multiple comparisons test. Data represent mean ± standard deviation (n = 3). *, $P \leqq 0.05$, ***, $P < 0.001$; b) PCI-induced cytosolic release of gentamicin in RAW cells upon illumination. Gentamicin was labeled with Alexa Fluor 405 (blue) and $TPCS_{2a}$ was observed in the red channel. Intracellular co-localization of gentamicin and $TPCS_{2a}$ is shown as magenta color in the merged images. Scale bars = 10 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intracellular *S. epidermidis*. Treatment with $TPPS_{2a}$ and 30 µg/ml gentamicin with illumination for 10 min significantly enhanced killing of intracellular bacteria (1 log reduction). With illumination for 15 min, combination of $TPPS_{2a}$ and either 10 or 30 µg/ml gentamicin significantly increased killing of intracellular *S. epidermidis* to levels of 1 and 3 log reduction, respectively. A repetition experiment showed highly similar results (Fig. S3, Supplementary data).

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

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

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

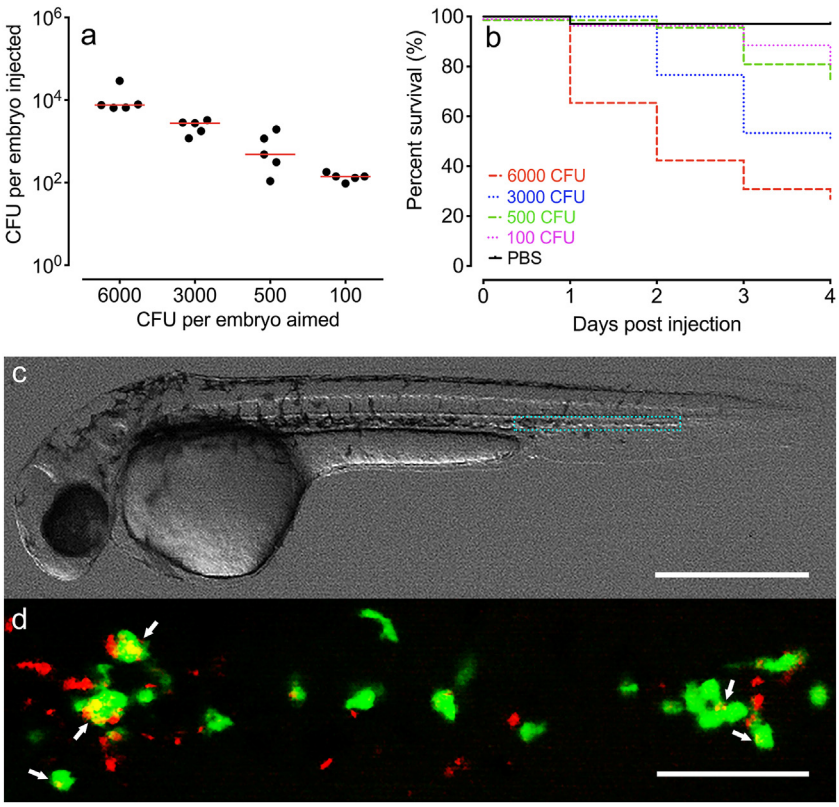


Fig. 3. Determination of *S. aureus* challenge doses for zebrafish embryo infection and co-localization of *S. aureus* and zebrafish macrophages. a) CFU numbers of *S. aureus* cultured from crushed embryos injected with inocula of 100 to 6000 CFU in 1 nl PBS. The red lines represent the median numbers of CFU. b) Effect of different inocula of *S. aureus* on survival of embryos. PBS injections served as controls. Initial group sizes ranged from 26 to 38 embryos. c) Bright field image of a representative 1 day old zebrafish embryo at 2 h post *S. aureus* injection. Scale bar = 500 μm. The blue box indicates the area shown in d) at high magnification with co-localization of *S. aureus*-mCherry (red) and zebrafish macrophages (green) as co-localization in yellow (arrows). Scale bars = 100 μm in d). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

antibiotic treatment.

In order to investigate whether *S. aureus* was phagocytosed shortly after injection, we challenged 1 day old transgenic zebrafish embryos expressing Kaede green fluorescent protein in their macrophages (Fig. 3c and d), with 3000 CFU of mCherry red fluorescent protein-expressing *S. aureus*. We chose the time point of 2 h post injection to assess whether the injected *S. aureus* were phagocytosed by zebrafish macrophages since the majority of *S. aureus* are taken up by zebrafish macrophages and/or neutrophils with 2 h post injection, and these cells containing bacteria are important niches for *S. aureus* infection in the embryos [4, 37]. In the present study co-localization of *S. aureus* and macrophages was observed in the blood circulation at 2 h post injection (Fig. 3d). The bacteria not associated with labeled phagocytes seemed to be clustered (Fig. 3d), suggesting that they were phagocytosed by the non-labeled phagocyte type of the embryos such as neutrophils. This shows that (a portion of) *S. aureus* had been phagocytosed *in vivo* at 2 h post injection, which is in line with the results reported in the previous studies [4, 37].

3.3. Enhanced survival of S. aureus-infected embryos treated with gentamicin combined with PCI

To test their toxicity for zebrafish embryos, the effect of injection of graded doses of gentamicin, $TPCS_{2a}$ and gentamicin-$TPCS_{2a}$ combinations on survival was assessed. $TPCS_{2a}$ and gentamicin both showed a dose-dependent toxicity, with maximal non-toxic concentrations of 2.5×10^{-3} and 2 ng/embryo, respectively (Fig. S4a and b, Supplementary data). Combinations of 1.6 or 0.8 ng/embryo gentamicin with 2.5×10^{-3} ng/embryo $TPCS_{2a}$ did not significantly reduce survival of embryos (Fig. 4a).

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

4. Discussion

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

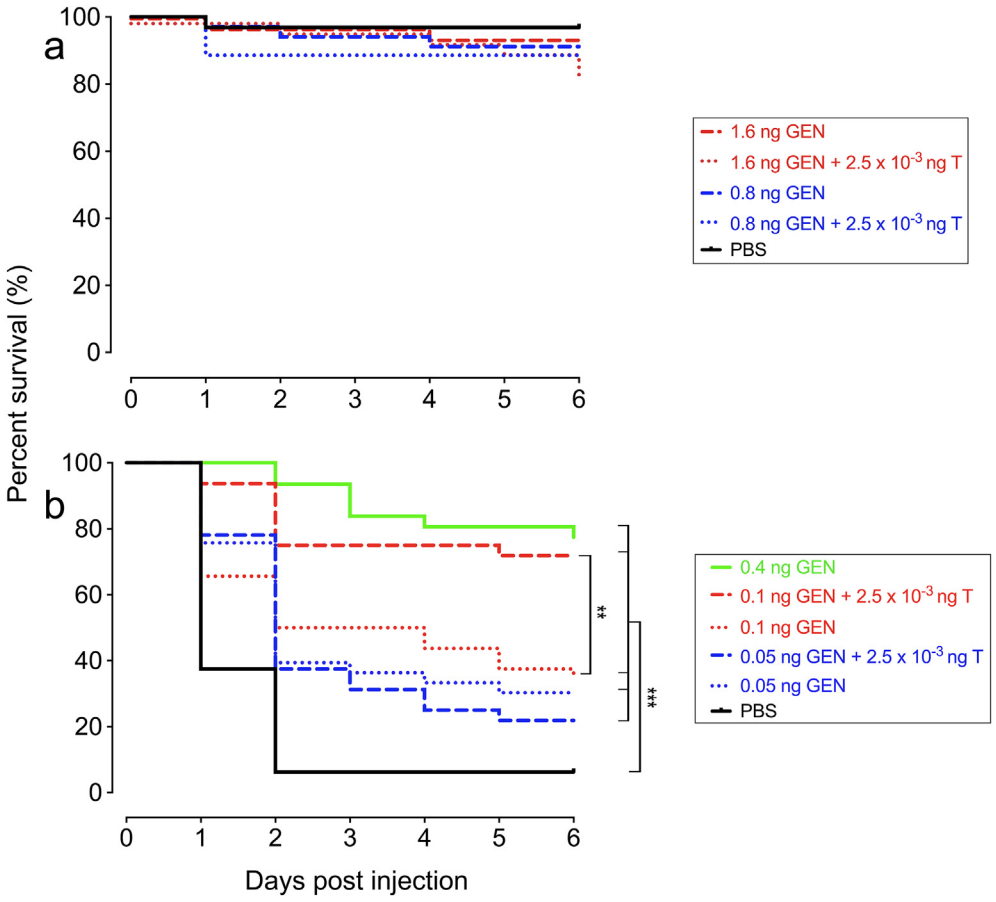


Fig. 4. Survival of non-infected and *S. aureus*-infected zebrafish embryos treated with gentamicin only or gentamicin-$TPCS_{2a}$ combinations after illumination for 10 min. a) Effect of gentamicin alone (GEN) or combined with $TPCS_{2a}$ (T) (in 1 nl of PBS) on non-infected embryos. Embryos injected with PBS served as controls. Initial group sizes ranged from 31 to 35 embryos; b) Survival of embryos infected with 3000 CFU of *S. aureus* treated with gentamicin only or combined with $TPCS_{2a}$. PBS mock treatment served as control. Initial group size ranged from 31 to 33 embryos. Differences between survival of each of the treated groups *versus* the PBS control group, as well as between survival of the gentamicin only group and the respective gentamicin-$TPCS_{2a}$ treatment group were analyzed using Log-rank test. Survival curves share the same line style. (For interpretation of the references to the color of survival curves in this figure, the reader is referred to the web version of this article.) **, $p < 0.01$. ***, $p < 0.001$.

infections.

Photosensitizers such as $TPPS_{2a}$ and $TCPS_{2a}$ have been developed for treatment of tumors by enhancing delivery of cytotoxic chemotherapeutics [24, 25, 38]. According to the principle of PCI, the doses of photosensitizer and light required to disrupt the endosomal/lysosomal membranes are likely sublethal [39]. In our study, although PCI had slight to moderate levels of cytotoxicity for RAW cells *in vitro* (Fig. 1b), the concentrations required to enhance the efficacy of gentamicin *in vivo* did not significantly reduce survival of the zebrafish embryos (Fig. S4). Similarly, in a recent clinical phase I trial for delivery of the antitumor drug bleomycin $TPCS_{2a}$-PCI was shown to be safe and tolerable for human patients receiving infusions of $TPCS_{2a}$ solution [38].$TPPS_{2a}$ and $TPCS_{2a}$ molecules tend to preferentially accumulate in diseased tissues and inflamed areas [24, 25], and other similar types of photosensitizers were shown to be internalized by local highly active cells in inflamed areas, such as macrophages and neutrophils [40, 41]. Therefore, treatment of infected areas by PCI combined with antibiotic therapy will likely preferentially target phagocytic cells containing bacteria. Moreover, any cytotoxicity of photosensitizers will only be induced when illumination is applied. Therefore, application of site-specific illumination to diseased tissues/inflamed areas will minimize the potential side effects of PCI on healthy tissues and normal cells which are not exposed to illumination. Compared to chemical endosomal disruption agents such as chloroquine, ammonium chloride and methylamine which have relatively high toxicity and low cell/tissue specificity [42, 43], PCI provides temporally and spatially controlled cytosolic release of therapeutics from endocytic vesicles with potentially less side effects *in vivo* [24, 44].

Treatment of *S. epidermidis*-infected RAW cells by gentamicin alone, even with relatively high concentrations (10 and 30 μg/ml), did not remarkably reduce the numbers of the intracellular bacteria. Combining the treatment with PCI however significantly improved the efficacy (Fig. 2a). A similar efficacy-enhancing effect of PCI was observed *in vivo* in our zebrafish embryo *S. aureus* infection model (Fig. 4b). PCI did however not increase the efficacy of the lowest dose of gentamicin, neither *in vitro* nor *in vivo*. Possibly the amount of intracellular gentamicin was too low to be efficacious even after cytosolic release following PCI treatment. Interestingly, in our experiments with RAW cells the liberated gentamicin molecules seemed to accumulate at the nuclei after illumination. This is in line with the observation of gentamicin binding to the nuclei of kidney cells [45]. Although theoretically such binding may reduce the amount of free gentamicin in the cytosol, enhanced efficacy of gentamicin by PCI (4-fold) was still observed in our study. This suggests that intracellular activity of antibiotics which do not show nuclear binding, might be even more strongly enhanced by PCI. Our results, showing an enhancing effect of PCI on antibiotic efficacy in the zebrafish embryo infection model, offer prospects for further *in vivo* studies in larger mammalian animal models. *In vivo* studies with PCI in mouse models have already been performed extensively for cancer treatment [46, 47]. The available relevant information on PCI modality from these studies supports further investigation on PCI-antibiotic treatment of intracellular infection *in vivo*. Moreover, since the photosensitizer $TPCS_{2a}$ has passed clinical phase I trials for safety testing in human patients [38] and many antibiotics to be combined with PCI are available, there is the possibility to relatively rapidly progress towards clinical studies of PCI-antibiotic treatment of diseases associated with intracellular infections.

Eradication of intracellular bacteria by antibiotics may be impeded by their different subcellular localization inside cells [13, 27]. Even when endosomes containing antibiotics and photosensitizers would be ruptured after illumination, bacteria might still be safely shielded within phagosomes, which would not necessarily contain photosensitizers. Our results however did show increase of killing of the intracellular bacteria. This may be explained in two ways. After rupture of

the endosomes containing antibiotics, photosensitizer molecules dissociated from the lysed endosomal membranes may intracytoplasmically re-localize to the membranes of phagosomes containing the bacteria, and also rupture these membranes (Scheme 1). As a result, bacteria are released into the cytosol and are intracellularly killed by gentamicin. In addition, during PCI partially ruptured vesicles are suggested to fuse with still intact intracellular vesicles causing them to also become leaky/ruptured, even without additional illumination [29]. Such fusion therefore may also (partially) contribute to the cytosolic release of both antibiotics and bacteria, facilitating the intracellular antimicrobial action. Since intracellular delivery of bioactive molecules using PCI generally does not rely on particular properties of the molecules to be delivered [24, 25], PCI can likely also improve the intracellular efficacy of other antibiotics than gentamicin *via* controlled release of drugs into the cytosol of cells. Such antibiotics may be other aminoglycosides, glycopeptides and macrolides, whose activity likely is limited by their inability of endosomal escape [13, 26, 27]. PCI thus has a strong potential to increase the numbers of antibiotics to be effective in treatment of intracellular infection and may increase their therapeutic window, since PCI will lower the effective antibiotic dose owing to enhanced intracellular delivery. Because PCI can in principle enhance intracellular delivery of different antibiotics, it has the potential to enhance efficacy of antibiotic treatment of infections caused by a broad range of intracellular bacterial pathogens such as *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Salmonella typhi* [11, 12]. As a result, PCI may also help reduce the rate of resistance development which might occur intracellularly due to the low, permissive concentration of antibiotics.

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

5. Conclusions

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

Declarations of interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2018.06.004.

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