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Innovative approach in *Legionella* water treatment with photodynamic cationic amphiphilic porphyrin

A. Lesar, G. Begić, N. Malatesti and I. Gobin

ABSTRACT

Legionella is an opportunistic premise plumbing pathogen that can be present in municipal and other water supplies. Building water systems may provide conditions (such as low flow, water hardness, low disinfectant residual levels and optimal temperature) that accelerate *Legionella* growth to levels that may result in an increased risk to public health. The standard disinfection of water systems (periodic overheating of water and chlorination) in the interest of prevention of Legionnaires' disease have often proved to be inefficient. It is therefore necessary to develop new approaches for removing *Legionella* from water systems. One of the new methods is antimicrobial photodynamic therapy (aPDT), which includes the combined activity of a photosensitizer (PS), molecular oxygen and visible light of appropriate wavelength to create singlet oxygen ($^1O_2$) and other oxygen reactive species (ROS) leading to the oxidation of numerous cellular components and cell death. In this study, a newly synthesized cationic, amphiphilic porphyrin TMPyP3-$C_{17}H_{35}$, was tested against *Legionella* in tap water. The minimal effective concentration (MEC) of PS photoinactivation test and PS uptake assay in sterile tap water were explored to determine the anti-*Legionella* activity. The complete inactivation of *Legionella* in sterile tap water was achieved with 0.024 μM of the PS. Also, the tested PS was found to be very effective in reducing *Legionella* growth in the sterile tap water and photoinactivation was dose-dependent. The tested PS binds well to the bacterial cell, after only 10 minutes of incubation in the dark. In conclusion, these studies indicate that TMPyP3-$C_{17}H_{35}$ is highly efficient in aPDT which leads to reducing *Legionella* growth in sterile tap water, and these results suggest that cationic amphiphilic photosensitizers may have a broader application in the photoinactivation of bacterial cells implicated in water disinfection.

Key words | antimicrobial photodynamic inactivation, *Legionella*, opportunistic premise plumbing pathogen (OPPP), water disinfection

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INTRODUCTION

Populations of microbes have been treated with different types of antimicrobial agents (antibiotics, preservatives, antiseptics, etc.) over many years, all for the purpose of preventing the spread of contagious diseases. However, this has resulted in the rise of antimicrobial resistance, which is currently a very serious worldwide problem (Walsh 2000). Therefore, it is important to explore new types of antimicrobial agents with different mechanisms of action against microbial cells, which will find not only use for human medicine purposes, but also widespread use in protection of environmental health (such as production of foodstuff, distribution of drinking water, waste water treatment, personal hygiene, wellness and spa, Heating, Ventilation and Air Conditioning (HVAC) systems, animal breeding, etc.).

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Legionella is a waterborne microorganism, also known as an opportunistic premise plumbing pathogen (OPPP) that is a normal inhabitant of premise plumbing and which causes infections in individuals with predisposing conditions, such as advanced age (>70 years), cancer, or immunodeficiency (Cooper & Hanlon 2010; Williams *et al.* 2013; Phin *et al.* 2014). *Legionella* and other OPPP, like *Pseudomonas* and *Mycobacterium*, are ideally adapted to survival, growth, and persistence in drinking water distribution systems and premise plumbing (Mullis & Falkinham 2013; Edwards *et al.* 2014; Falkinham *et al.* 2015). Furthermore, these pathogens are inherently disinfectant-resistant. Thus, standard disinfectant dosages for drinking water treatment will essentially kill off all microbes apart from these opportunistic pathogens.

Even though it was initially discovered on a microorganism, photodynamic therapy (PDT) has been explored and developed mostly as an anticancer therapy for a long time. However, in the last two decades PDT has been put forward for its significant antimicrobial effect (Sharma *et al.* 2011).

The antibiotic function can generally be explained using the principle of 'key-and-lock', while antimicrobial photodynamic therapy (aPDT), also known as antimicrobial photodynamic inactivation (APDI), is based on a very different principle and mechanism of action. The APDI procedure combines a photosensitizer (PS), light and oxygen leading to the formation of cytotoxic species ($^1O_2$ and ROS) that destroy the microorganisms. This approach has proven to be effective *in vitro* against bacteria (including drug-resistant strains), yeasts, viruses and protozoa (Alves *et al.* 2009). The $^1O_2$ and/or ROS generated by photoactivation of the PS react with microbial biomolecules such as proteins, lipids and/or nucleic acids, which can lead to killing, rather than just inhibiting growth, of the microorganism. Due to such mechanism and multiple targets, the chances that microbes can develop tolerance or resistance to aPDT are considered unlikely (Tavares *et al.* 2010; Tavares *et al.* 2011; Hamblin 2016; Kashef & Hamblin 2017).

Cationic amphiphilic porphyrin TMPyP3-$C_{17}H_{35}$, which we used in this work, was recently shown to be the most PDT efficient, having the lowest IC_{50} values measured against several cancer cell lines, in the series of several pyridylporphyrins we were investigating (Jelovica *et al.* 2018). Previous to this work, the presence of a long alkyl chain was shown to be important for high PDT efficiency of the amphiphilic tripyridyl porphyrins (Malatesti *et al.* 2016).

Our aim in this work was to investigate the anti-*Legionella* potential of the tricationic amphiphilic porphyrin (TMPyP3-$C_{17}H_{35}$) in tap water. Therefore, we have developed a new method of water microdilution in order to determine the minimal effective concentrations (MEC).

MATERIALS AND METHODS

Bacterial culture conditions and photosensitizer (PS)

A clinical isolate of *Legionella pneumophila* serogroup 1, strain 130b, also known as ATCC BAA-74, Wadsworth strain or AA100, provided by Prof. Elizabeth L. Hartland, University of Melbourne in Australia, was used. Bacteria were routinely cultured on buffered charcoal yeast extract agar (BCYE) (Oxoid, UK) plates at 35 ± 2 °C for 3–5 days. The bacteria were stored at −80 °C in glycerol broth (10% glycerol) (Biolife, Italy). To determine bacterial numbers, *L. pneumophila* were re-suspended in sterile tap water and the optical density at 600 nm (OD600) was determined, whereby an OD600 of 1 equaled 10^9 bacteria/mL.

The 5-(4-octadecanamidophenyl)-10,15,20-tris(*N*-methylpyridinium-4-yl)porphyrin trichloride (TMPyP3-$C_{17}H_{35}$) (Figure 1) was prepared and characterized as previously

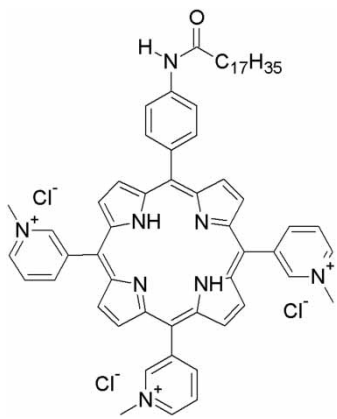

Figure 1 | 5-(4-Octadecanamidophenyl)-10,15,20-tris(*N*-methylpyridinium-3-yl)porphyrin trichloride, (TMPyP3-$C_{17}H_{35}$).

described (Malatesti *et al.* 2016). The porphyrin is completely water soluble and stable if kept in the dark as a solid.

Stock solutions (200 mM) of TMPyP3-$C_{17}H_{35}$ (M_r = 1054.816 g/mol) in deionized water were freshly prepared each time.

Tap water samples

For the experiments, tap water was used from the public water supply of the city of Rijeka. Chemical properties of the used tap water were: salinity 0, pH 7.5–8.0, and conductivity 216–300 μS cm^{-1}. The tap water sample in a glass bottle was left at RT to dechlorinate for 2 days and was sterilized by autoclaving at 121 °C for 15 min, cooled to RT and stored at 4 °C until use.

Determination of the anti-*Legionella* activity

Minimum bactericidal concentrations (MBC) of PS were determined using a microdilution technique in AYE broth. Series of twofold dilutions ranging from 50 μM up to 0.043 μM in sterile tap water were performed in sterile 96-well microtiter plates (Synthesis, Italy). Each dilution was mixed with an equal volume of bacterial suspension 1.0×10^5 CFU per well. After 30 min of mixing the bacteria with the two-fold dilution of PS in the dark and a 10 min treatment with total dose of 12 J/cm^2 violet light, the plates were incubated for 24 h, at 35± °C. MEC was determined by inoculating the samples onto BCYE agar and incubating further for 3–5 days. MEC was defined as the lowest concentration of PS yielding negative subcultures on the solid medium. For control purposes, bacteria and series of two-fold dilution were incubated in the dark for 24 h, at 35± °C. To detect toxicity of the PS to *Legionella* cells without irradiation, the samples were inoculated onto BCYE agar and incubated further for 3–5 days. MEC without light was the lowest concentration of PS yielding negative subcultures on the solid medium.

Photosensitizer uptake assay

Legionella cell suspensions (2 mL, ~10^9 CFU/mL) in sterile tap water were incubated with MEC value of the PS for 30 min. in the dark. PS was added from a stock solution (~200 mM) in sterile deionized water. At different time points the cell suspensions were centrifuged (4,000 rpm for 10 min). Pellets were washed 1× with sterile tap water and re-suspended in 2 mL of 2% SDS and incubated overnight at 4 °C. Then, the cells were sonicated for 30 min. The concentration of porphyrin in the supernatant was determined by spectrofluorimetry (TMPyP3-$C_{17}H_{35}$: $\lambda_{exc} = 422$ nm, $\lambda_{em} = 651$ nm). The fluorescence intensities of each sample referred to the total number of cells. The concentration of the porphyrin in the solution was calculated by comparison with a calibration curve obtained with standard solutions of the photosensitizer in 2% SDS.

Photoinactivation test in sterile tap water

In the experiment, 200 μL of the cell suspensions (~10^8 CFU/mL) and test compound (conc. 2 × MEC, MEC and 0.5MEC) were mixed in tap water in 96-well microtiter plates (Syntesis, Italy). The cells were incubated with stirring at RT in the dark for 30 min and the samples were exposed to a total dose of 12 J/cm^2 of violet light. At different time intervals, the number of bacteria was determinated by plating ten-fold dilutions on the BCYE agar and incubating at 35 ± 2 °C for 3–5 days.

Controls and statistical analysis

Control experiments were performed in the presence and absence of porphyrin in the dark and in the absence of porphyrin with cells irradiated. Three values were obtained per each experiment and were repeated for two separate times. The unpaired *t*-test was used to establish the significance of differences between groups. Differences were considered statistically significant with a confidence level of 95% ($p < 0.05$). Data were represented as the mean ± standard deviation of each group.

RESULTS

The amphiphilic porphyrin (TMPyP3-$C_{17}H_{35}$) was used in photoinactivation of pure cultures of *L. pneumophila* in sterile tap water. The photoinactivation experiments were performed using bacterial density of 10^6 CFU/mL and the

irradiation was carried out in a static model at RT under artificial violet light (394 nm) with a fluence of 20 mWcm^{-2}. The viability of *Legionella* cells was monitored using the plate count method as described above. The complete inactivation of *Legionella* in sterile tap water was achieved with 0.024 μM of PS with 10 min irradiation (Figure 2). This dose presented the MEC with irradiation in sterile tap water. The dose of 1.56 μM of PS was effective against *Legionella* in tap water without irradiation and it was annotated as MEC without

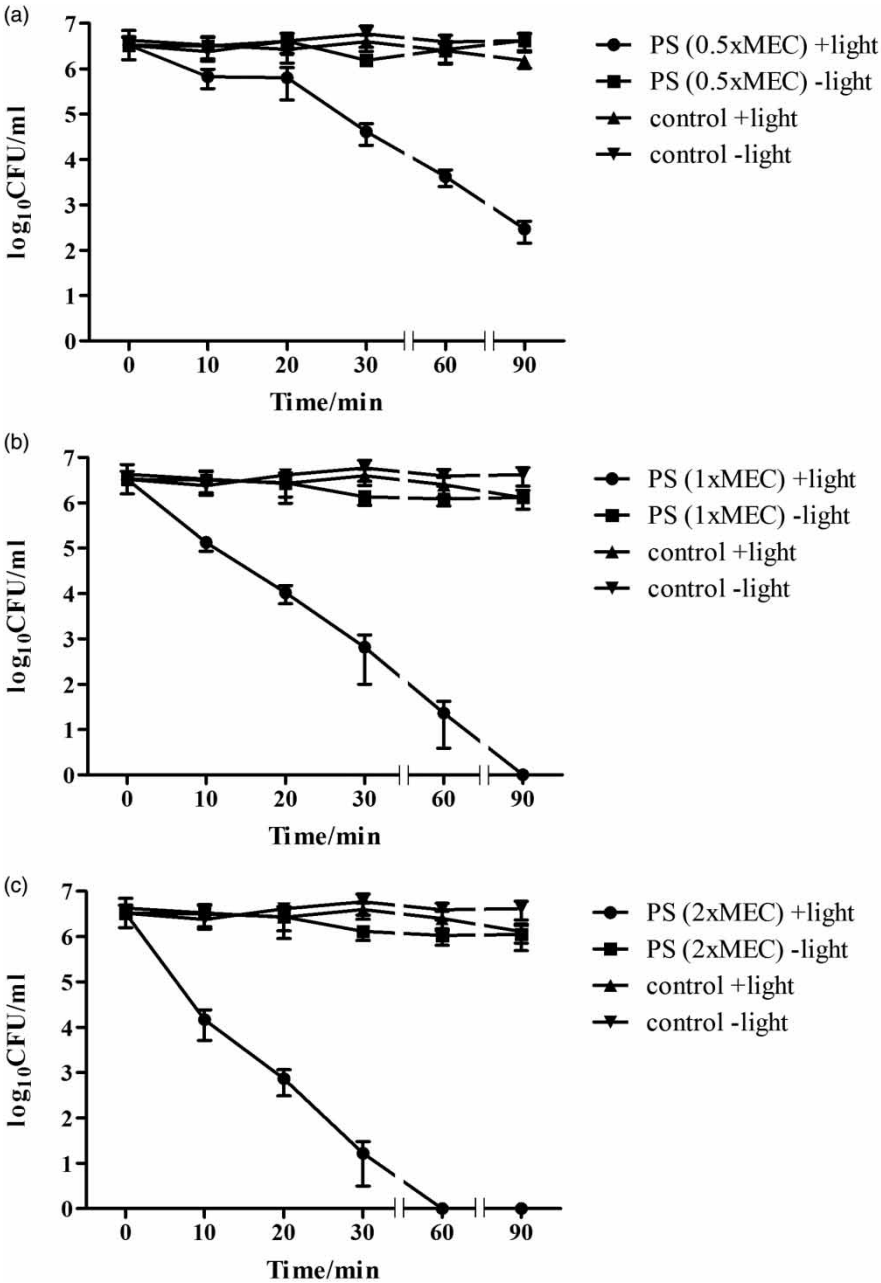

Figure 2 | Photoinactivation of *L. pneumophila* cells incubated with PS in the dark and after 10 min treatment with total dose of 12 J cm^{-2} of violet light: (a) treatment with 0.5 × MEC value; (b) treatment with 1 × MEC value; (c) treatment with 2 × MEC value; control presents *Legionella* cells exposed to violet light in the absence of the PS and *Legionella* cells in the dark. Data are the mean of three independent experiments ± SD. *$p < 0.05$ for PS + light vs PS − light samples.

irradiation. The photokilling efficacy of PS (0.5 × MEC, 1 × MEC and 2 × MEC) against *Legionella* was also evaluated. The tested amphiphilic PS was found to be very effective in reducing *Legionella* in the sterile tap water and the photoinactivation was dose-dependent. PS showed great photokilling efficacy; already 10 min after the treatment with 2 × MEC 99.53% of the bacteria were inactivated and 99.99% of the bacteria were inactivated after 30 min. Doses of 0.5 × MEC and 1 × MEC of PS, showed 99.62% bacterial killing with 1 × MEC after 20 min., and 99.85% with 0.5 × MEC after 60 min (Figure 3). With all three doses, the bacterial were non-cultivable 24 hours after the photodynamic inactivation (data not shown). Irradiation of the bacterium without the presence of the PS did not affect *Legionella* viability during 90 min and after 24 hours. The tested PS binds well to the bacterial cell, after only 10 min of incubation in the dark and there were no differences of PS uptake on bacteria incubated for 30 min (Figure 4).

DISCUSSION

Thermal water treatment with temperatures higher than 50 °C, UV radiation, use of disinfectants, and treatment with chlorine, ozone or chlorodium oxide are the basic techniques for the removal of *Legionella* from water systems (Kuchta *et al.* 1985; Bédard *et al.* 2016). Despite the implementation of all these methods, legionellosis is becoming more frequent, so it is necessary to use new approaches in water disinfection. One of the new approaches is the use of antimicrobial photodynamic therapy (aPDT) as a disinfection method of tap water. The effect of the recently described cationic porphyrin with lipophilic moiety was investigated to determine its antibacterial activity against the opportunistic plumbing pathogen *Legionella* in its natural environment, tap water. A recent overview of the development and use of amphiphilic porphyrins in PDT emphasized the need for further investigation of cationic porphyrins with different lipophilic moieties as this class of compounds has shown a promising photodynamic activity in anticancer and antimicrobial PDT (Shaker *et al.* 2016). In our previous study, we prepared amphiphilic porphyrin 5-(4-octadecanamidophenyl)-10,15,20-tris(*N*-methylpyridinium-4-yl) porphyrin trichloride (TMPyP3-$C_{17}H_{35}$) and showed that the lipophilic moiety significantly improved PDT efficiency compared with the hydrophilic analogue that lacked the long alkyl chain. The potent antiproliferative action against different cell lines (HeLa, HepG2, MCF-7, HCT116, u87MG and human foreskin fibroblasts) was achieved with TMPyP3-$C_{17}H_{35}$ and a low fluence (2 mWcm^{-2}) of red light (643 nm). Amphiphilic porphyrin (TMPyP3-$C_{17}H_{35}$) caused a strong cytotoxic effect in all tested cell lines, with LC_{50} ranging from 0.39 μM in u87MG cells to 3.47 μM in MCF-7 cells (Jelovica *et al.* 2018). Since high PDT efficiency of TMPyP3-$C_{17}H_{35}$ was demonstrated against eukaryotic cells, we were interested in its potential antimicrobial use such as in water disinfection. *Legionella* as a target organism is especially

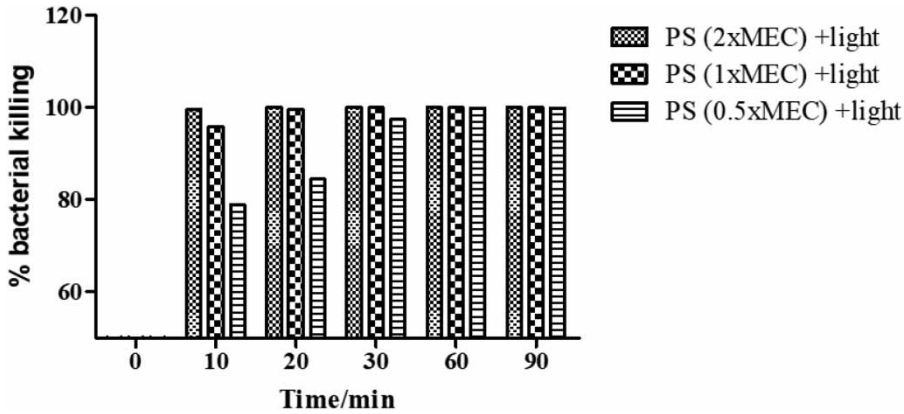

Figure 3 | Percentage of bacterial killing after photoinactivation with PS with and without light. *L. pneumophila* cells were incubated with PS in the dark and then treated for 10 min with a total dose of 12 J cm^{-2} violet light or without light. Data are the mean of three independent experiments.

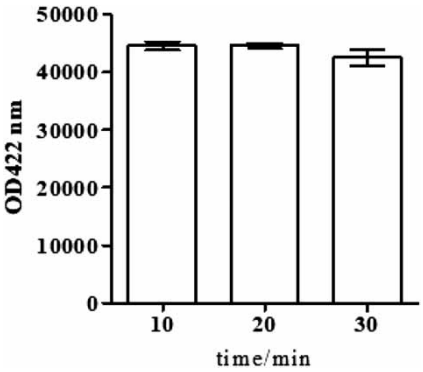

Figure 4 | Cellular uptake of PS (0.024 μM) by *L. pneumophila* cells (10^8 CFU/mL) during 30 min. incubation in the dark. Data are the mean of three independent experiments ± SD.

interesting as there are very few data in the literature available on aPDT activity against this bacterium, and *Legionella* still poses a significant health risk in various water systems (Parr *et al.* 2015). In the aforementioned cell culture assays, a low fluence (2 $mWcm^{-2}$) of red light (643 nm) was used, however, this was not efficient in inhibition of *Legionella* in tap water (data not shown). The violet and blue lights have the strongest penetration through water (Chiang *et al.* 2011), therefore, the study continued using the violet light (12 J/cm^2) for the activation of the PS in water samples. The irradiation was carried out in a static model at RT for 10 min, after 30 min of incubation of the PS and bacteria in the dark. In order to test the bactericidal concentration of PS in tap water, we used a modification of the method by Peruč *et al.* (2018) in which instead of broth, sterile tap water was used. In our experiment we did not use the oxidation–reduction indicator resazurin because there were no visible reactions after 24 h. Instead, treated bacteria were inoculated on BCYE agar and incubated at 37 °C for 5 days. The lowest dose that completely inhibited the growth of bacteria was the minimal effective dose (MEC) and it was 0.024 μM of PS. We also tested the dark toxicity of PS, and serial dilutions of tested PS and bacteria were kept in the dark for 24 hours. PS was effective against *Legionella* in tap water without irradiation and 1.56 μM of PS efficiently inhibited *Legionella*. These results suggest there is a certain dark toxicity caused by the PS on *Legionella*, however, photoinactivation is significantly more efficient and very low doses of the PS could be used for successful water treatment against *Legionella*. In the literature, we did not find data for the photodynamic activity of porphyrin on *Legionella*. The concept of disinfection of wastewaters with photodynamic activation of synthetic dyes is described even though the light source refers to sunlight (Bonnetta *et al.* 2006; Kuznetsova & Kaliya 2015). Furthermore, the photokilling efficacy of PS against *Legionella* was also evaluated in time–kill curves. The photoinactivation was dose-dependent and the PS showed great photokilling efficacy only 10 min after treatment with 2 × MEC, after 20 min with 1 × MEC and after 60 min with 0.5 × MEC of PSs. It seems that the compound is very effective in the inactivation of *Legionella*, which could be explained by the fact that it rapidly adheres to the bacterial cell (10 min). Preliminary experiments showed that no leakage of cellular proteins and DNA occurs (data not shown). Previous investigations of the mechanism of action showed that the photodynamic activity of cationic porphyrins produces DNA photodamage after a long period of irradiation, and an interference with membrane functions could be the main cause of Gram-negative bacterial photoinactivation upon short PDI treatments (Caminos *et al.* 2008).

In conclusion, high aPDT activity was achieved using tricationic amphiphilic porphyrin, TMPyP3-$C_{17}H_{35}$ against *Legionella* in water. There is a certain dark toxicity of the PS and this mechanism will be investigated further. Nevertheless, the PS is significantly more efficient in photodynamic conditions, which suggests there is a great potential of this methodology for applications in water disinfection. Application of photodynamic action to inactivation and decontamination of water certainly seems to be a promising alternative to the conventional chemical methods of treatment.

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