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Stable synthetic mono-substituted cationic bacteriochlorins mediate selective broad-spectrum photoinactivation of drug-resistant pathogens at nanomolar concentrations

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ABSTRACT

Three stable synthetic mono-substituted cationic bacteriochlorins (BC37, BC38 and BC39) were recently reported to show exceptional activity (low nanomolar) in mediating photodynamic killing of human cancer cells after a 24 h incubation upon excitation with near-infrared light (730 nm). The presence of cationic quaternary ammonium groups in each compound suggested likely activity as antimicrobial photosensitizers. Herein this hypothesis was tested against a panel of pathogenic microorganisms that have all recently drawn attention due to increased drug-resistance (Gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis*; Gram-negative bacteria, *Escherichia coli* and *Acinetobacter baumannii;* and fungal yeasts, *Candida albicans* and *Cryptococcus neoformans*). All three bacteriochlorins were highly effective against both Gram-positive species (>6 logs of eradication at $\leqslant$200 nM and 10 J/cm^2). The dicationic bacteriochlorin (BC38) was best against the Gram-negative species (>6 logs at 1–2 μM) whereas the lipophilic monocationic bacteriochlorin (BC39) was best against the fungi (>6 logs at 1 μM). The bacteriochlorins produced substantial singlet oxygen (and apparently less Type-1 reactive-oxygen species such as hydroxyl radical) as judged by activation of fluorescent probes and comparison with 1H-phenalen-1-one-2-sulfonic acid; the order of activity was BC37 > BC38 > BC39. A short incubation time (30 min) resulted in selectivity for microbial cells over HeLa human cells. The highly active photodynamic inactivation of microbial cells may stem from the amphiphilic and cationic features of the bacteriochlorins.

1. Introduction

Bacteriochlorins (and related bacteriopheophorbides) are highly attractive as photosensitizers to mediate photodynamic therapy (PDT) of cancer, and/or photodynamic inactivation (PDI) of microbial cells due to the characteristic strong long wavelength absorption in the near-infrared spectral region (700–900 nm) [1–4]. We have reported a synthetic route to bacteriochlorins that are stabilized against adventitious oxidation by introduction of a geminal dimethyl group in each reduced pyrrole ring [5,6]. The synthetic route also allows peripheral substituents to be tailored to vary the lipophilicity, polarity and overall charge (cationic, anionic or neutral) borne by the molecule [7]. The synthetic route to such bacteriochlorins as well as semisynthetic methods enable the synthesis of amphiphilic bacteriochlorins [8]. This synthetic versatility has enabled the design of photosensitizers that are able to kill cancer cells at extremely low concentrations upon activation by near-infrared light [9].

A bacteriochlorin photosensitizer was able to effectively reverse the resistance to PDT exhibited by pigmented melanoma both *in vitro* and in a mouse model *in vivo* [10]. The chelation of a palladium atom in the bacteriochlorin macrocycle increased the PDT potency, possibly due to increased probability of electron-transfer to produce Type 1 reactive-oxygen species such as hydroxyl radicals [11,12]. Type 1 activity was likely further enhanced by the

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addition of two electron-withdrawing cyano groups symmetrically substituted at the 3- and 13-positions, which also improved the photostability [11,12]. Four members of a set of symmetrically di-substituted bacteriochlorins (bearing two tertiary amines, or two, four, or six quaternary cationic groups; structures given below) were investigated as broad-spectrum antimicrobial photosensitizers [13]. The optimum photosensitizer structure was found to be different for each class of microbial cells. The di-tertiary amine substituted bacteriochlorin was best for killing fungal cells, the di-quaternized analogue was best for killing Gram-positive bacteria, and the hexa-quaternized analogue was best for killing Gram-negative bacteria [13].

Very recently, three mono-substituted cationic bacteriochlorins (BC37, BC38, BC39; Fig. 1) were synthesized and tested for PDT-mediated killing of human cancer cells [14]. These compounds exhibited exceptional activity, with substantial cancer cell killing at concentrations as low as 6 nM excited with modest fluences of light (5 J/cm^2) after 24-h incubation. The fact that these three compounds possessed cationic (quaternary ammonium) groups suggested the likelihood of good activity as antimicrobial photosensitizers. Antimicrobial PDI is attracting increasing interest as an alternative antimicrobial technique as the inexorable rise of drug-resistance in pathogens continues unabated [15–18].

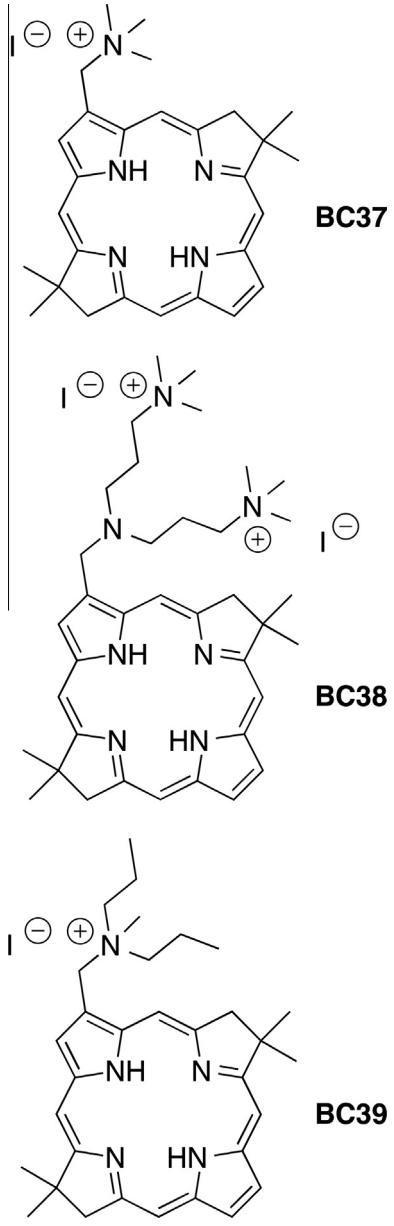

Fig. 1. Structures of the three bacteriochlorin photosensitizers.

Herein we tested this hypothesis against a panel of pathogenic microorganisms that have all recently drawn attention due to their increased drug resistance. The microorganisms included Gram-positive bacteria, *Staphylococcus aureus* [19] and *Enterococcus faecalis* [20]; Gram-negative bacteria, *Escherichia coli* [21] and *Acinetobacter baumannii* [22]; and fungal yeasts *Candida albicans* [23] and *Cryptococcus neoformans* [24]. PDI is postulated to have the greatest clinical impact against drug-resistant organisms that cause localized infections in wounds, burns and surgical sites [25] For this anti-infective approach to advance to clinical trials, it is necessary to demonstrate that the new bacteriochlorin photosensitizers do not mediate PDT damage to host human cells at the short (<30 min) incubation times generally used in PDI of microbial cells.

2. Materials and methods

2.1. Bacteriochlorins

The bacteriochlorins were designed and synthesized as described by Sharma et al. [14]. BC37 and BC39 each bears a single quaternized ammonium group and hence is monocationic. BC38 bears two quaternized ammonium groups and one trialkylamine. The latter is basic and is expected to be predominantly in the protonated state at neutral pH in aqueous solution. Nonetheless, given the presence of two permanent charges, BC38 is referred to as dicationic. All three bacteriochlorins are mono-substituted at the 3-position. The presence of a geminal dimethyl group at position 8 and at position 18 blocks the reduced (pyrroline) rings from undergoing adventitious dehydrogenation and thereby affords a robust bacteriochlorin chromophore.

2.2. Studies with fluorescence probes

Black-sided 96-well plates were used for experiments that employ fluorescence probes. Singlet oxygen sensor green reagent (SOSG), 3′-(*p*-aminophenyl)fluorescein (APF) or 3′-(*p*-hydroxyphenyl)fluorescein (HPF) (all from Molecular Probes, Life Technologies, Grand Island, NY) at a final concentration of 10 μM was added to a 10 μM solution of bacteriochlorin in 200 μL of phosphate-buffered saline per well. 1H-Phenalen-1-one-2-sulfonic acid (PN, a kind gift from Dr. Santi Nonell at IQS Barcelona, Spain) was dissolved in water to give a 2 mM stock solution and was also used at 10 μM concentration. Fluorescence spectrometry (SpectraMax M5 plate reader, Molecular Devices, Sunnyvale, CA) used excitation/emission at 504/525 nm for SOSG and 490/515 nm for APF and HPF. Increasing fluences (J/cm^2) were delivered using near-infrared light (700–850 nm band pass filter, Lumacare, Newport Beach, CA) for bacteriochlorins, and violet light from a 415 ± 15 nm LED array (Omnilux Clear-U, Photomedex, Horsham, PA) for PN. Light was delivered at an irradiance of 100 mW/cm^2 as measured with a power meter (model DMM 199 with 201 standard head; Coherent, Santa Clara, CA). The plate fluorescence was measured after each increment in the fluence was delivered.

2.3. Bacterial strain and culture conditions

The bacteria used in this study were as follows: Gram-positive bacteria *S. aureus* 8325-4 and *E. fecalis* ATCC29212 (ATCC, Manassas, VA; Gram-negative bacteria *E. coli* K12 and *A. baumannii* ATCC BAA747; fungal yeasts *C. albicans* DAY286 and *C. neoformans*