A Novel Dicationic Boron Dipyrrromethene-based Photosensitizer for Antimicrobial Photodynamic Therapy against Methicillin-Resistant Staphylococcus aureus

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Abstract: Background: We report herein the synthesis of a novel dicationic boron dipyrrromethene derivative (compound 3) which is symmetrically substituted with two trimethylammonium styryl groups.

Methods: The antibacterial photodynamic activity of compound 3 was determined against sixteen methicillin-resistant Staphylococcus aureus (MRSA) strains, including four ATCC type strains (ATCC 43300, ATCC BAA-42, ATCC BAA-43, and ATCC BAA-44), two mutant strains [AAC(6')]-APH(2") and RN4220/pUL5054], and ten non-duplicate clinical strains of hospital- and community-associated MRSA. Upon light irradiation, the minimum bactericidal concentrations of compound 3 were in the range of 1.56-50 μM against all the sixteen MRSA strains. Interestingly, compound 3 was not only more active than an analogue in which the ammonium groups are not directly connected to the n-conjugated system (compound 4), but also showed significantly higher (p < 0.05) antibacterial potency than the clinically approved photosensitizer methylene blue. The skin irritation of compound 3 during topical application was tested on human 3-D skin constructs and proven to be non-irritant in vivo at concentrations below 1.250 mM. In the murine MRSA infected wound study, the colony forming unit reduction of compound 3 + PDT group showed significantly (p < 0.05) higher value (>2.5 log10) compared to other test groups except for the positive control.

Conclusion: In conclusion, the present study provides a scientific basis for future development of compound 3 as a potent photosensitizer for photodynamic therapy for MRSA wound infection.

Keywords: Methicillin resistant Staphylococcus aureus, antimicrobial photodynamic therapy, boron dipyrrromethene, murine wound infection model, photosensitizer, antibacterial potency.

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1. INTRODUCTION

Photodynamic therapy (PDT) could be used to treat site-confined diseases via administration of a non-toxic photosensitizer (PS), followed by light irradiation of the site with an appropriate wavelength, usually in the visible region in the presence of molecular oxygen [1]. Combination of PS, light and molecular oxygen can generate highly reactive oxygen species (ROS) such as singlet oxygen \((^1\text{O}_2)\), leading to non-selective cell destruction [1, 2]. PDT is proven as a successful treatment modality for many cell proliferative diseases such as localized solid tumours. It is particularly effective in treating skin cancers and other premalignant and non-malignant dermal abnormalities [2, 3]. Moreover, its application has been extended in treating cutaneous lesions caused by burns or wounds and for the disinfection of dental caries [2].

Besides the proven efficacy against eukaryotic cells [4], PDT is effective in killing pathogenic bacteria (prokaryotes). It is especially attractive because the technique has some unique advantages over conventional antibiotic treatments. For example, antibacterial PDT (aPDT) has an ability to inhibit both drug-resistant and drug-sensitive strains [5], and sometimes, it is even more potent against the former [6-8]. Furthermore, the absence of dark toxicity [9], fast antibacterial action [10], as well as no apparent drug resistance, are also its potential advantages [11, 12].

PSs commonly used in aPDT are organic molecules, with an extended \(\pi\)-bond conjugation that gives them a unique characteristic of harvesting long-wavelength visible light from electromagnetic radiation. Apart from the presence of an extended \(\pi\)-conjugated system, it is worth noting that the PSs suitable for aPDT usually require cationic moieties in their molecular structure to ascertain an effective interaction with the negatively charged bacterial cell wall, leading consequently to improved bacterial uptake of the PS [13]. Indeed, a range of cationic dyes such as cationic porphyrins (e.g. gallium(III) protoporphyrin IX [14] and 5,10,15,20-tetraakis(1-methylpyridinium-4-yl)porphyrin (TMPyP) [15]), polycationic phthalocyanines (e.g. zinc(II) phthalocyanine (ZnPc) ZnPcE [16], ZnPc with oligolysine chains [17] and tetracationic ZnPc chloride RLP068/C1 [18]) as well as some commercial dyes (e.g. methylene blue (MB) [19] and toluidine blue O [20]) were found to be able to eradicate different classes of bacteria.

Recently, a new class of PSs, the 4, 4-difluoro-4-bora-3a,4a diaza-s-indacenes (known as boron dipyrromethene or simply the acronym BDP), have emerged to play a role in aPDT [21, 22]. These compounds, however, have some unfavourable features that may hinder their clinical applications. Owing to their high lipophilicity, aggregates may form in aqueous solutions. Besides, the \(\lambda_{\text{max}}\) of the \(\pi-\pi\) transition of these compounds appears at ca. 500 nm and the strong fluorescence does not favour the singlet oxygen formation. Therefore, structure modification should be warranted to circumvent the adverse effects of BDPs, making them effective for aPDT [13].

We report herein the synthesis of a novel dicationic and diiodinated BDP (compound 3) which has good solubility in DMSO/aqueous solutions (<1:10 \(v/v\)), a high singlet oxygen quantum yield and a strong absorption at 600-700 nm. Although the aPDT activity of several BDPs against a few clinically relevant bacteria has been reported recently [21-24], the compatibility of BDPs in mammalian systems is poorly understood. As far as we know, the present study reported for the first time the photodynamic efficacy of cationic BDPs against a broad spectrum of clinically relevant methicillin-resistant Staphylococcus aureus (MRSA) strains. Following a systematic study on the in vitro efficacy of compound 3 using a previously reported structural analogue (compound 4) for comparison, the highly potent compound 3 was thoroughly investigated in a murine MRSA wound infection model to determine the treatment efficacy in vivo. To our surprise, despite the structural and photophysical similarities of compound 3 and 4, they possessed very different aPDT potencies. The difference in potency may be due to the different positively-charged functional groups. Although the effect of varying the number of positive charges on a photosensitizer has been reported [25], the effect of different kinds of positively-charged functional groups has not been studied in detail. The difference in potency between trialkylaminium and tetraalkylammonium BDPs could also give clues in choosing the most effective charged functional groups when designing potent PSs. Further, we used the human three-dimensional skin constructs EpiDerm™ to predict the in vivo skin irritation of compound 3. Overall, we provide a comprehensive experimental evidence on compound 3 as a promising BDP derivative against MRSA in vitro and in vivo.

2. MATERIALS AND METHODS

2.1. General

All the reactions were performed under a nitrogen atmosphere. N,N-dimethylformamide (DMF) was purified with an INERT solvent purification system (P-S-MD-5, Amesbury, MA, USA). All other solvents...
Fig. (1). Timeline for in vivo aPDT study. Four treatment cycles were performed at Day 2, 3, 5 and 9. After the 4th treatment, the mice were sacrificed for the CFU count.

and reagents were of the reagent grade and used as received. The course of reactions was monitored by thin layer chromatography (TLC) with Merck pre-coated silica gel 60 F254 plates (Merck Millipore, Darmstadt, Germany). Chromatographic purification was performed on silica gel (Macherey-Nagel, Düren, Germany; 230–400 mesh) with the indicated eluents. Compound 1 was prepared as described [37].

1H and 13C{1H} NMR spectra were recorded on a Bruker AVANCE III 400 spectrometer (Billerica, MA, USA) (1H, 400 MHz; 13C, 100.6 MHz) in CDCl3 or DMSO-d6. Spectra were referenced internally by using the residual solvent [1H, δ = 7.26 (for CDCl3), δ = 2.50 (for DMSO-d6)] or solvent [13C, δ = 77.2 (for CDCl3), δ = 39.5 (for DMSO-d6)] resonances relative to SiMe4. Electrospray ionization (ESI) mass spectra were recorded on a Thermo QEF MS mass spectrometer (Waltham, MA, USA). Electronic absorption and steady-state fluorescence spectra were taken on a Shimazu UV-1800 UV-Vis spectrophotometer (Tokyo, Tokyo Prefecture, Japan) and a Horiba FluoroMax spectrofluorometer (Kyoto, Kyoto Prefecture, Japan), respectively.

The bacterial strains, MRSA (ATCC 43300, ATCC BAA-42, ATCC BAA-43, ATCC BAA-44) and two mutant SA strains, AAC(6’)-APH(2”) and RN4220/pUL5054, were included for testing. The AAC(6’)-APH(2”) strain expresses the bi-functional enzyme AAC(6’)-APH(2”), which is an aminoglycoside-modifying enzyme with the minimum inhibitory concentration (MIC): >128 μg/mL for gentamicin. The RN4220/pUL5054 strain shows a high level of erythromycin (MIC: 128 μg/mL) resistance due to the over-expression of msr(A) gene encoding for an ATP-binding cassette (ABC) transporter [27]. Five each from (non-duplicate) hospital (HA)- and community-associated (CA)-MRSA were also included. These ten strains (clonal types ST239, ST30 and ST59) were specifically chosen due to their high prevalence rates in Hong Kong and neighbouring countries [28-30]. MR-SAs were cultured in Mueller-Hinton Broth (MHB) for 18h at 37°C under an aerobic condition. The overnight culture was adjusted to 0.5 McFarland turbidity and diluted until the final concentration was 1.0×10^6 CFU/mL. Altogether, 16 MRSAs were included for in vitro aPDT studies.

2.2. Synthesis

2.2.1. Synthesis of Compound 2

A mixture of compound 1 (100 mg, 135 μmol), 4-(dimethylamino)benzaldehyde (60.6 mg, 406 μmol), acetic acid (0.3 mL), piperidine (0.4 mL), and a catalytic amount of Mg(ClO4)2 in toluene (50 mL) was stirred under reflux with a Dean-Stark trap for 2 h, and then concentrated. The residue was purified by column chromatography to give compound 2 (52 mg, 38%) as a dark green solid. 1H NMR (400 MHz, CDCl3) δ 8.16 (d, J = 16.4 Hz, 2 H, C=CH), 7.53-7.58 (m, 6 H, ArH and C=CH), 7.15 (d, J = 8.4 Hz, 2 H, ArH), 7.04 (d, J = 8.8 Hz, 4 H, ArH), 6.74 (d, J = 8.8 Hz, 2H, ArH), 4.21 (t, J = 4.4 Hz, 2 H, CH2), 3.93 (t, J = 4.8 Hz, 2 H, CH2), 3.78-3.80 (m, 2 H, CH2), 3.71-3.74 (m, 2 H, CH2), 3.68-3.70 (m, 2 H, CH2), 3.57-3.59 (m, 2 H, CH2), 3.40 (s, 3 H, CH3), 3.04 (s, 12 H, NCH3), 1.48 (s,
6 H, CH3). 13C 1H] NMR (CDCl3) δ 17.7, 40.3, 59.1, 67.6, 69.7, 70.6, 70.7, 70.9, 72.0, 111.0, 112.1, 114.5, 115.3, 125.2, 127.9, 129.3, 129.9, 133.1, 136.5, 139.5, 144.8, 150.2, 151.2, 159.6 (Section 1 Supporting information, Fig. S1). HRMS (ESI) Calcd. for C46H55BF2I2N4O4 [M]+ 1000.1906, found 1000.1898.

2.2.2. Synthesis of Compound 3

A mixture of compound 2 (25 mg, 25 μmol) and an excess amount of CH3I (709 mg, 2.5 mmol) in DMF (10 mL) was stirred at 45-50 °C for 24 h. The mixture was then poured into excess ether to induce precipitation. The precipitate formed was collected by filtration and washed by ether to give compound 3 (24 mg, 95%) as a green solid. 1H NMR (400 MHz, DMSO-d6) δ 8.02-8.09 (m, 6 H, ArH and C=CH), 7.87 (d, J = 8.4 Hz, 2 H, ArH), 7.18 (d, J = 8.4 Hz, 2 H, ArH), 4.19 (pseudo s, 2 H, CH2), 3.80 (pseudo s, 2 H, CH2), 3.66 (s, 18 H, NCH3), 3.60-3.63 (m, 2 H, CH2), 3.52-3.57 (m, 4 H, CH2), 3.43-3.45 (m, 2 H, CH2), 3.25 (s, 3 H, CH3), 1.49 (s, 6 H, CH3). 13C [H] NMR (DMF-d6) δ 17.4, 30.8, 34.4, 35.8, 56.5, 58.1, 67.4, 68.9, 69.6, 69.8, 70.0, 71.3, 85.7, 115.6, 120.8, 121.5, 125.8, 128.4, 129.5, 132.5, 136.8, 137.5, 141.8, 146.5, 147.4, 149.8, 159.7, 162.3 (Section 1 Supporting information, Fig. S2). HRMS (ESI) Calcd. for C44H49BF2I2N4O4 [M]+ 515.1185, found 515.1181.

2.3. Determination of Fluorescence Quantum Yield (ΦF)

The values of ΦF were determined by the equation

\[
Φ_F(s) = \frac{(F_s/F_{ref})(A_{ref}/A_s)(n_s/n_{ref})}{Φ_F(ref)}
\]

where subscripts s refers to the sample solution while ref stands for the reference. F, A, and n are the measured fluorescence (area under the emission peak), the absorbance for the reference, and the index of the solvent, respectively [25]. Unsubstituted ZnPc in DMF was used as the reference (ΦF = 0.28).

2.4. Determination of Singlet Oxygen Quantum Yield (ΦD)

A solution of 1,3-diphenylisobenzofuran (DPBF, 30 μM) and different BDPs (1 μM) in DMF was illuminated with light from a 100 W halogen lamp under a color filter with a cut-on wavelength at 610 nm (65CGA-610, Newport, Franklin, MA, 176 USA) upon passing through a water tank for cooling. The absorption maximum of DPBF at 415 nm was monitored along with the irradiation time. The values of ΦD of the samples were determined by the equation

\[
Φ_D(sample) = \frac{(W_{sample}/W_{ref})(I_{ref}/I_{sample})Φ_D(ref)}{W}
\]

where W and I are the DPBF photobleaching rate and the rate of light absorption, respectively [26]. Unsubstituted ZnPc in DMF was used as the reference (ΦD = 0.56).

2.5. Assessment of in vitro PD-MBC

Minimum bactericidal concentrations (MBCs) of compounds 3, 4, and methylene blue (MB) were determined according to the method described previously [31]. Sample of each bacterial suspensions (100 μL) underwent a pre-irradiation step for 120 min with 100 μL of compounds 3 and 4 [Tween 80 and dimethyl sulfoxide (DMSO) concentrations were maintained ≤ 0.1% and 1% (v/v), respectively] in dark. Thereafter, the selected plates were illuminated with light intensity at 40 mW/cm2 using a 300 W quartz-halogen lamp attenuated by a 5 cm layer of water (heat buffer) and a color glass filter with a cut-on wavelength of 610 nm (65CGA-610, Newport, Franklin, MA, 176 USA), as shown in Section 2 in Supporting information, Fig. S3. The illumination was carried out for 20 min, corresponding to a fluence (λ > 610 nm) of 48 J/cm2 for PDT [16, 17, 31]. The dark toxicity of compounds 3 and 4 was evaluated by incubating the PSs along with the bacteria in dark. The effect of PDT alone was verified by replacing PSs with PBS and illuminating it for 20 min (48 J/cm2).

The negative control did not receive any PSs nor light. Solvent toxicity (blank control) was also evaluated with 0.1% Tween 80 and 1% DMSO (v/v) to mimic the in vitro aPDT assay. MB was used as the positive control group, following the same protocol as that for the test (compounds 3 and 4) groups. After an overnight incubation of the treated plates in dark, the MBC was determined using the method described previously [31].

2.6. In vitro EpiDerm™ Skin Irritation Test (EPI-200-SIT)

Reconstructed human three-dimensional skin constructs EpiDerm™ (EPI-200, MatTek Cor, Ashland, MA) were used for the toxicity test. The chemical exposure (compound 3 and 4) followed by the tissue viability assay was executed exactly according to the manufacturer’s instruction manual; the detailed method has been given in our previous publication [32].

2.7. Murine MRSA-infected Wound Model

The induction of MRSA infected murine skin wound model, PDT and enumeration of the CFU load was carried out according to the exact procedure de-
scribed in our previous report [32]. All animal experiments conformed to the university guidelines and approved by the Animal Experimentation Ethics Committee (Ref. no.16/176/MIS-5) of The Chinese University of Hong Kong. 4-6 weeks old male BALB/c mice (17-21 g) were supplied by Laboratory Animal Services Centre (LASEC), The Chinese University of Hong Kong. On Day 0, mice were anaesthetized intraperitoneally (i.p.) by ketamine (40 mg/kg) and xylazine (8 mg/kg), with the hair of the back shaved, and the skin cleansed with 10% povidone-iodine solution. A 4-5 mm diameter, full-thickness skin punch wound was established on subcutaneous tissue of the back of each mice. Buprenorphine, commercially available as Temgesic®, was administered (0.05 mg/kg) subcutaneously to the mice twice in the first 24 hours after wound induction to relieve pain.

Table 1. Conditions of the five groups of mice in the murine MRSA wound infection model.

| Group                     | No. of Animals (n) | Treatment                  |
|---------------------------|--------------------|----------------------------|
| A. Negative control group (NC) | 7                  | 50 μL of distilled water alone |
| B. Positive control group (PC)  | 7                  | 50 μL of Fucidin® cream (containing fusidic acid 20 mg/g) alone |
| C. Light control group (LC)   | 7                  | 50 μL of distilled water + PDT |
| D. Dark control group (DC)    | 7                  | 50 μL of Compound 3 (1250 μM) alone |
| E. Test group (T)            | 7                  | 50 μL of Compound 3 (1250 μM) + PDT |

On Day 2, mice were re-anaesthetized and 20 μL aliquots of 1 x 10⁸ CFU/mL suspension of MRSA RN4220/pUL5054 in MHB were spread evenly over the wound area using a micropipette. A dressing (Tegaderm™ film, 3M, USA) was applied to cover the wound immediately. The mice with infected wound were randomly divided into 5 cohorts (n=7 each group), as listed in Table 1.

First treatment was carried out 30 min after MRSA inoculation on Day 2. A 50 μL of 1250 μM compound 3 solution (to represent 100 x times of MBC value against MRSA RN4220/pUL5054 during in vitro studies), Fucidin® cream or distilled water was injected under the dressing (Tegaderm™) by syringe and allowed to spread over the wound.

For the Groups C and E, irradiation (Biolitec group, Bonn, Germany) was initiated immediately after compound inoculation with a single dosage of the laser at 1 W for 60 s, corresponding to 60 J/wound. After each treatment, the mice returned to individual ventilated cages (IVC) and Groups D and E were placed in the dark. The subsequent treatments were carried out on Day 3, Day 5 and Day 9, respectively, to cover 4 similar treatment cycles (Fig. 1).

On Day 9, the mice were sacrificed by the administration of an intravenous dose of pentobarbital solution (Dorminal) after the last treatment (4th treatment). The wound (5 x 10 mm) was aseptically excised and the skin sample was homogenized in PBS (0.5 mL) for bacterial viability counts. Quantification of viable bacteria was performed by culturing serial dilutions (10 μL) of the homogenized suspension on blood agar plates and incubated at 37 °C for 24 h, and the CFU enumerated.

Fig. (2). Synthetic scheme of Compound 3.
3. RESULTS AND DISCUSSION

3.1. Design and Synthesis

The synthetic scheme used to prepare compound 3 is given in Fig. (2). The dicationic diiodinated BDP was synthesized using a triethylene glycol-conjugated diiodinated BDP (compound 1) as a starting material. It underwent Knoevenagel condensation with 4-(dimethylamino)benzaldehyde in the presence of a catalytic amount of magnesium perchlorate to form the distyryl BDP 2. It was then methylated with methyl iodide in DMF to yield the dicationic compound 3. Both compounds 2 and 3 were purified readily by column chromatography and were characterized by various spectroscopic methods. Compound 3 was an analogue of the previously reported dicationic compound 4 (Fig. 3) [31]. Both BDPs consist of two cationic moieties at the distyryl positions while the cationic groups in compound 4 are not directly connected to the π-conjugated system as in compound 3.

![Fig. (3). Structure of Compound 4.](image)

The electronic absorption and photophysical data of compound 3 were recorded in DMF and compared with that of compound 4. The data are summarized in (Table 2). The Q-band absorption was 31 nm blue-shifted for compound 3 when compared with that for compound 4 due to the difference in electronic effect between the trimethylammonium and alkoxyl substituents at the styryl positions in compound 3 and 4, respectively. The fluorescence emission of compound 3 also appeared at a shorter wavelength (by 42 nm) than that of compound 4, with a lower fluorescence quantum yield (0.14 versus 0.21 for compound 4). Despite the difference in electronic absorption and fluorescence properties, the singlet oxygen generation efficiencies, as determined by the rate of decay of the singlet oxygen quencher DPBF and sensitized by the two BDPs in DMF, were very similar (0.52 and 0.54 for compounds 3 and 4, respectively). The results suggested that the diiodo distyryl BDP core of the two compounds possessed a similar intrinsic photosensitizing ability.

3.2. In vitro photodynamic minimal bactericidal concentration (PD-MBC) studies

In (Table 2), the MBCs of compounds 3 and 4 against a panel of reference strains are presented. The aPDT of compound 3 against MRSAs showed bactericidal activities at concentrations ranging from 1.56 to 50 μM (Table 3). In contrast, compound 3 exhibited only a mild bactericidal activity in the absence of light (MBC = 100 to >100 μM) (Table 3). The PDT effect thus increased the potency of compound 3 against clinically relevant MRSAs by 2 to > 64 times of magnitude when compared with its bactericidal activity in the dark (Table 3).

The aPDT of compound 3 was compared with another PS (compound 4) with similar structural moieties and λ\text{max} value under identical conditions. The MBC values for compound 4 were >100 μM, indicating its significantly lower (p < 0.05) aPDT activity against

Table 2. Electronic absorption and photophysical data for compounds 3 and 4 in DMF.

| Compound | λ\text{max} /nm (log ε) | λ\text{em} /nm | Φ\text{F}\text{a,b} | Φ\text{Δ}\text{c,d} |
|----------|--------------------------|----------------|------------------|------------------|
| 3        | 349 (4.63), 425 (3.93), 630 (4.72) | 648 | 0.14 | 0.52 |
| 4        | 316 (4.28), 379 (4.40), 446 (4.03), 661 (4.60) | 690 | 0.21 | 0.54 |

\text{Excited at } λ = 610 \text{ nm.} \text{ Relative to unsubstituted ZnPc (Φ_\text{F} = 0.28).} \text{ Excited at } λ > 610 \text{ nm; Relative to unsubstituted ZnPc (Φ_\text{Δ} = 0.56).}
Table 3. The PD-MBC values for compounds 3, 4, and MB against 16 MRSA strains.

| MRSA Type | MRSA Strain | Compound 3 | Compound 4 | MB |
|-----------|-------------|------------|------------|----|
|           |             | PDT (μM)   | No PDT (μM) | aPDT potency | PDT (μM) | No PDT (μM) | aPDT potency |
| ATCC      | 43300       | 12.5       | 1000 - 100 | 8 - >8      | >100      | >100        | >4          |
| ATCC      | BAA 42      | 12.5 - 25  | >100       | 4 - 8       | >100      | >100        | >4          |
| ATCC      | BAA 43      | 25-50      | >100       | 2 - >4      | >100      | >100        | 4->8        |
| ATCC      | BAA 44      | 12.5 - 25  | 100        | 4 - 8       | >100      | >100        | 4->8        |
| Mutant    | AAC(6")-APH(2") | 6.25 | 100        | 16         | >100      | >100        | 2->4        |
| Mutant    | RN4220/pUL5054 | 12.5 - 25 | 1000 - 100 | 4 - >8      | >100      | >100        | 2->4        |
| CA'       | W44         | 6.25 - 12.5 | 1000 - 100 | 8 - >16     | >100      | >100        | >1          |
| CA        | W45         | 12.5 - 50  | >100       | 2 - >8      | >100      | >100        | 1->2        |
| CA        | W46         | 25 - 50    | >100       | 2 - >4      | >100      | >100        | >1          |
| CA        | W47         | 3.125 - 6.25 | >100      | 16 - >32    | >100      | >100        | >1          |
| CA        | W48         | 3.125 - 12.5 | >100     | 8 - >32     | >100      | >100        | >1          |
| HA'       | W231        | 1.56 - 6.25 | >100     | 16 - >64    | >100      | >100        | >1          |
| HA        | W232        | 3.125 - 6.25 | >100     | 16 - >32    | >100      | >100        | >1          |
| HA        | W233        | 6.25       | >100       | >16         | >100      | >100        | >1          |
| HA        | W234        | 12.5       | >100       | >8          | >100      | >100        | >1          |
| HA        | W235        | 1.56 - 3.125 | >100    | 32 - >64    | >100      | >100        | >1          |

*Fold reduction in compound 3, 4 and MB concentration, relative to dark toxicity (no light exposure); *Highest concentration tested for compound 3 and 4; &Highest concentration tested for MB; *Community-associated; *Hospital-associated.

MRSAs when compared with compound 3. The aPDT of MB was also tested and included as a control for comparison. It is a mono-cationic phenothiazine that has long been studied and already in clinical use. In vitro aPDT studies using a λ > 610 nm filter cut-on light revealed that MB possessed aPDT activity at significantly (p < 0.05) higher concentrations than compound 3.

These results were unexpected because the structural and photophysical characteristics of compounds 3 and 4 were similar. The similar singlet oxygen quantum yields of compounds 3 and 4 suggested that the difference in potency should not be inflicted by the intrinsic efficiency of the PSs to produce ROS. Instead, the triakylanilium and tetraakylammonium groups responsible for the cationic charges in photoinactivation, where the symmetry of the cationic groups has a significant impact on the antibacterial potency [33]. Since their finding could not exactly explain the effect difference observed in this paper, further study on these charge-bearing amine groups needs to be done to clarify their contribution in the antibacterial potency, whether it is from the position of the charges, the flexibility of the charges, or the specific type of cationic charges.

The in vitro studies (Table 3) showed that compound 4 has (> 100 μM) significantly (p < 0.05) higher MBC values than compound 3 against the whole panel of bacteria. Therefore, we have refrained from further investigation of compound 4 on human 3-D skin constructs to determine cytotoxicity on in vivo MRSA infected wound model in mice due to their intrinsic toxicity at very high concentrations.

3.3. In vitro EpiDerm™ Skin Irritation Test

The toxicity of compound 3 was determined by a skin irritation test as applied on human 3-D skin constructs (EpiDerm™). As shown in (Fig. 4), the cell viability of EPI-200 cells was >50% at 1250 μM (100 x times of MBC value against MRSA RN4220/pUL5054). This indicates that compound 3 did not manifest toxicity (according to the EU and GHS classi-
fication) on human 3-D skin constructs EpiDerm™, at ≤1250 μM concentration (Section 3 supporting information). This finding supports the safe use of compound 3 topically for aPDT.

Fig. (4). Cell viability of EPI-200 cells under different treatments. They were treated with DPBS [Negative control (NC)], 5% SDS [Positive control (PC)], 12.5 μM of Compound 3 [equivalent to MBC against MRSA RN 4220/pUL5054 (Compound 3 (MBC)], 125 μM of Compound 3 [equivalent to 10 x MBC against MRSA RN 4220/pUL5054 (Compound 3 (10 x MBC)] or 1250 μM of Compound 3 [equivalent to 100 x MBC against MRSA RN 4220/pUL5054 (Compound 3 (100 x MBC)]. Experimental data are expressed as mean ± SD (n=3). Means that do not share a letter are significantly different. Mean cell viability > 50% for the Compound 3 (MBC, 10 x MBC or 100 x MBC) implies that Compound 3 did not pose any skin irritation on human 3-D skin constructs at concentrations below 1250 μM. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

3.4. Murine MRSA Wound Infection Model (in vivo aPDT)

Compounds 3 (1250 μM) were directly applied topically to the infection wounds for each treatment and NIR (λ > 610 nm) was applied in 4 doses over 9 days with 60 J/wound. The bacterial load at Day 9 is given in Fig. (5). PDT alone (Group C) shows just 0.67 ± 0.21 log_{10} CFU reduction Fig. (5), indicating that the effect of PDT alone does not have a direct impact on the reduction of bacterial load. We compared the wound appearances before and after the PDT cycles and noticed there was no photothermal damage or skin irritation on or around the wounds (Supporting information, Section 4, Fig. S4). For compound 3, dark control group (Group D), showed a 0.75 ± 0.36 log_{10} CFU reduction Fig. (5) and the value is comparable with Group C (PDT alone). Irradiation of compound 3-treated cohort (Group E) ascertained 2.85 ± 0.32 log_{10} CFU reduction and the value is significantly higher (p < 0.05) than all other treated groups, except for Group B (Positive control, Fucidin® cream) that attained complete eradication of bacteria Fig. (5). Here we used Fucidin® cream as a positive control instead of MB because it is currently used topically in the clinical setting for the treatment of S. aureus skin and soft tissue infections. Hence, we wanted to compare the effect and activity of our PDT therapy (as an alternative approach) with the conventional treatment modality. A similar observation has been made by other investigators [34-37], where PDT-treated infected wounds had significantly (P < 0.05) lower bacterial counts at all time points than irradiation alone or PS alone-treated groups.

Fig. (5). In vivo aPDT efficiency against MRSA RN4220/pUL5054 infected wound mediated by 1250 μM concentration of Compound 3. Experimental data are expressed as mean ± SD (n=7). Means that do not share a letter are significantly different. Compound 3 + PDT cohort showed significantly lower (p < 0.05) bacterial load after 4 treatment cycles, compared to all other treated groups, except positive control (2% Fusidic cream). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

In the animal study, we inoculated the bacteria to the wounds 2 days after wound induction. It mimicked the clinical situation of postoperative infection. Unlike many other in vivo aPDT studies [36-39], we identified that multiple treatment cycles (4 cycles) rather than a single high dosage of light were more effective in our
preliminary studies (data not shown). Furthermore, we found that continuous treatment was also inappropriate due to the possible photothermal effect. Therefore, we optimized the light treatment protocol so that more treatments were given early in the infection (Fig. 1) in order to reduce the bacterial load, while minimizing delay in healing. The regimen of repeated treatment was supported by Lambrechts et al. [39] in their discussion as an improving step to decrease the bacterial load in S. aureus-infected burn wounds in mice.

DMSO is a polar aprotic solvent that enhances drug penetration through the skin and it is incorporated into an idoxuridine-based commercial preparation to treat herpetic infections of the skin [40]. Several studies [40-42] have used 10-25% DMSO/PBS mixture to dissolve PS and observed a light dose-dependent bacterial load reduction by increasing permeability towards deeper infected tissues. However, in the current investigation, <1% (v/v) DMSO was used and a higher percentage DMSO may be considered to improve its permeability towards deeper infected tissues and induce a subsequent increase in aPDT potency in full thick wound in vivo.

The post-treatment monitoring of infection sites was performed after the final treatment by measuring the wound size and change in body weight of animals. The size of wounds in the Negative Control Group (Group A) was slightly larger but there was no significant difference in wound size among all groups (data not shown).

Furthermore, there occurred a progressive body weight increase in all groups over the treatment period (9 days, Section 5 Supporting information, Fig. S5), suggesting that PDT did not have any observable side effects. Our observation was in line with that of Mai et al. [43], where they found that mice with untreated infected wounds exhibited progressive increases in body weight over the 5-day treatment cycle and mice with PDT-treated infected wounds exhibited a similar pattern, regardless of the concentration of the photosensitizer.

CONCLUSION

In summary, a novel symmetric dicationic boron dipyromethene with increased hydrophilic properties was synthesized. The photophysical characteristics of this compound 3 and its structural analogue compound 4 were comparable, suggesting their similar intrinsic photosensitizing ability. However, compound 3 ascertained significantly higher (p < 0.05) in vitro aPDT efficacy against MRSA than its structural analogue (compound 4), implying other factors such as the type of cationic charge (triakylanilium and tetraakylammonium) and their positions to also alter the activities. The encouraging results obtained using a dicationic compound 3 as an antimicrobial photosensitizer combined with NIR (λ > 610 nm) both in vitro and in vivo warrant further investigations on their mechanistic action, aPDT potency as a function of positive charge carrying group, and selectivity towards MRSA.

AUTHORS’ CONTRIBUTIONS

Priyanga Dharmaratne: Completed in vitro aPDT studies, completed skin irritation test using EpiDerm™, helped to perform in vivo aPDT studies and drafted the manuscript.

Ligang Yu and Roy Chi-Hang Wong: Synthesized Compounds 3 and 4, evaluated the photophysical data and revised the manuscript.

Ben Chun-Lap Chan: Designed and performed in vivo aPDT studies, and revised the manuscript.

Kit-Man Lau: Designed and performed in vivo aPDT studies, and revised the manuscript.

Baiyan Wang: Revised the manuscript.

Clara Bik San Lau: Commented on the study and revised the manuscript.

Dennis Kee-Pui Ng: Advised on the synthesis of compounds 3 and 4 and provided critical feedback on the manuscript.

Dennis Kee-Pui Ng and Margaret Ip: Conceptualized, initiated, and oversaw the project and provided critical feedback on the manuscript.

LIST OF ABBREVIATIONS

aPDT = Antimicrobial Photodynamic Therapy
ATCC = American Type Culture Collection
BDP = Boron Dipyromethene
CA = Community Associated
CFU = Colony Forming Unit
DMEM = Dulbecco’s Minimum Essential Medium
DMF = Dimethylformamide
DMSO = Dimethyl Sulfoxide
DPBF = Diphenylisobenzofuran
ESBL = Extended Spectrum β-lactamase
HA = Hospital Associated
MB = Methylene Blue
MBC = Minimum Bactericidal Concentration
MDR  = Multi-drug Resistant  
MHB  = Mueller Hinton Broth  
MIC  = Minimum Inhibitory Concentration  
MRSA  = Methicillin-resistant Staphylococcus aureus  
MSSA  = Methicillin-sensitive Staphylococcus aureus  
NIR  = Near Infrared  
PS  = Photosensitizer  
PBS  = Phosphate Buffer Saline  
ROS  = Reactive Oxygen Species  
ST  = Sequential Type  
VRSA  = Vancomycin-resistant S. aureus  
ZnPc  = Zn(II) phthlocyanines  
TMPyP  = 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)-porphyrin.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study has been approved by the University Animal Experimentation Ethics Committee (AEEC; Reference no.:16-176-MIS) and conducted at the Laboratory Animal Services Centre at the Chinese University of Hong Kong.

HUMAN AND ANIMAL RIGHTS

The use of animals is in compliance with International Guiding Principles for Biomedical Research involving animals and The Hong Kong Code of Practice for care and use of animals for experimental purposes.

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher’s web site along with the published article.

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