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Attaching NorA efflux pump inhibitors to methylene blue enhances antimicrobial photodynamic inactivation of *Escherichia coli* and *Acinetobacter baumannii in vitro* and *in vivo*

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ABSTRACT

Resistance of bacteria to antibiotics is a public health concern worldwide due to the increasing failure of standard antibiotic therapies. Antimicrobial photodynamic inactivation (aPDI) is a promising non-antibiotic alternative for treating localized bacterial infections that uses non-toxic photosensitizers and harmless visible light to produce reactive oxygen species and kill microbes. Phenothiazinium photosensitizers like methylene blue (MB) and toluidine blue O are hydrophobic cations that are naturally expelled from bacterial cells by multidrug efflux pumps, which reduces their effectiveness. We recently reported the discovery of a NorA efflux pump inhibitor-methylene blue (EPI-MB) hybrid compound INF55-(Ac)en–MB that shows enhanced photodynamic inactivation of the Gram-positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA) relative to MB, both *in vitro* and *in vivo*. Here, we report the surprising observation that INF55-(Ac)en–MB and two related hybrids bearing the NorA efflux pump inhibitors INF55 and INF271 also show enhanced aPDI activity *in vitro* (relative to MB) against the Gram-negative bacteria *Escherichia coli* and *Acinetobacter baumannii*, despite neither species expressing the NorA pump. Two of the hybrids showed superior effects to MB in murine aPDI infection models. The findings motivate wider exploration of aPDI with EPI-MB hybrids against Gram-negative pathogens and more detailed studies into the molecular mechanisms underpinning their activity.

Antibiotic resistance has emerged as a significant threat to global public health,[1–3] with the diminishing treatment options for several infections leading to commentary that we are approaching the end of the 'golden-age' of antibiotics.[4–6] Resistance in the Gram-positive bacteria methicillin resistant *Staphylococcus aureus* (MRSA) is extensive in US hospitals and healthcare facilities,[7] where it accounts for more than 60% of *S. aureus* isolates and kills 23,000 patients each year.[8]

Drug resistant Gram-negative bacteria like *Escherichia coli* and *Acinetobacter baumannii* are increasingly causing life-threatening infections in hospitals,[6,9,10] with an estimated 12% of critical infections caused by *E. coli* alone.[11] Data from the Centres for Disease Control and Prevention (CDC) shows that *Acinetobacter baumannii* causes 2% of all nosocomial infections and 7% of infections in critically ill patients on mechanical ventilators.[12] It has been estimated that 63% of the 12,000 annual *Acinetobacter* infections are multidrug resistant and cause 500 deaths annually.

Antimicrobial photodynamic inactivation (aPDI) is an emerging non-antibiotic alternative for treating localized infections and countering microbial resistance.[14,15] In this approach, photosensitizing dyes (PS) like methylene blue (MB) and toluidine blue O (TBO) (Fig. 1) are illuminated with red light to produce reactive

Abbreviations: aPDI, antimicrobial photodynamic inactivation; MB, methylene blue; EPI-MB, efflux pump inhibitor-methylene blue hybrid; MRSA, methicillin-resistant *Staphylococcus aureus*; PS, photosensitizing dye; ROS, reactive oxygen species; ·OH, hydroxyl radicals; EPEC, bioluminescent enteropathogenic *E. coli*; BHI, brain heart infusion; CrEL, Cremophor EL; CFU, colony forming units; NIH, National Institutes of Health; RLU, relative luminescence units; RND, resistance nodulation division.

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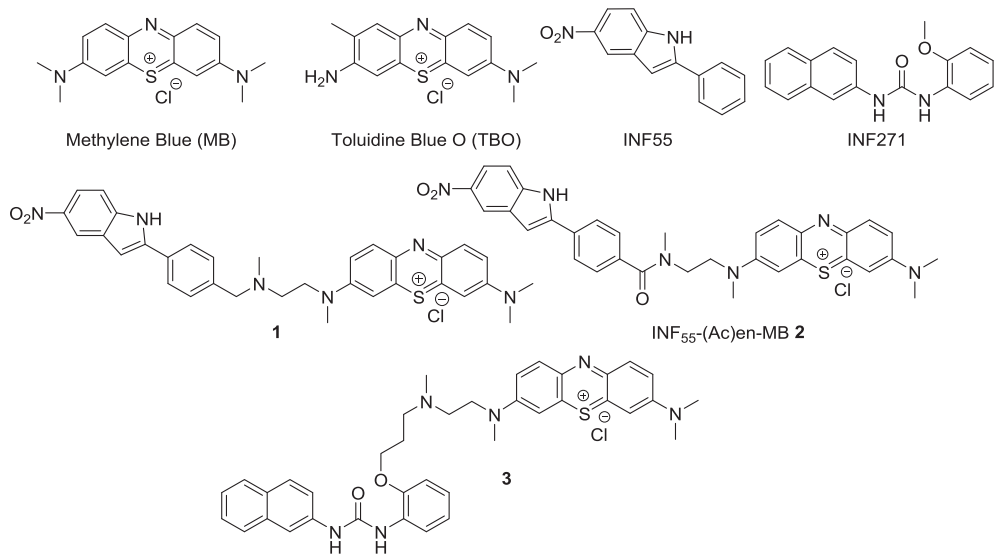

Fig. 1. Structures of phenothiazinium photosensitisers methylene blue (MB) and toluidine blue O (TBO) and efflux pump inhibitor-MB hybrids **1**–**3**. Structures of the NorA efflux pump inhibitors INF55 and INF271[13] are also shown.

oxygen species (ROS) (e.g., singlet oxygen, $^1O_2$ and hydroxyl radicals, ·OH) that kill microbes.[16,17] The approach is used routinely in dentistry[18,19] and in some dermatological treatments.[20,21]

Over the past ten years the powerful killing effect of aPDI has been demonstrated against a wide variety of Gram-positive and Gram-negative bacteria,[22,23] with MRSA being the focus of several studies.[24–26] One of the limitations when using phenothiazinium salts in aPDI is that as hydrophobic cations, these photosensitizers are natural substrates for bacterial multi-drug efflux pumps, which serve to rapidly expel the compounds from cells and reduce aPDI effectiveness,[27] presumably by lowering the concentration of intracellular ROS. It was shown that aPDI with phenothiazinium salts can be enhanced in *S. aureus* when used in combination with NorA efflux pump inhibitors (EPI).[28] Based on these observations, we postulated that covalently linking NorA inhibitors to a phenothiazinium PS to form a single EPI-MB hybrid compound might have similar effects, and we recently prepared sixteen such hybrids and reported their aPDI activities against *S. aureus*.[29] Two of the hybrids incorporating the NorA EPI INF55 (**1** and **2**) and one containing the NorA EPI INF271 **3** showed the highest *in vitro* aPDI of MRSA *in vitro*. The most potent hybrid **2** (denoted INF55-(Ac)en–MB) showed enhanced aPDI activity and wound healing effects (relative to MB) in a murine MRSA wound infection model. In the current study, we examined the *in vitro* and *in vivo* aPDI activities of EPI-MB hybrids **1**–**3** against two representative Gram-negative bacteria, *E. coli* and *A. baumannii*.

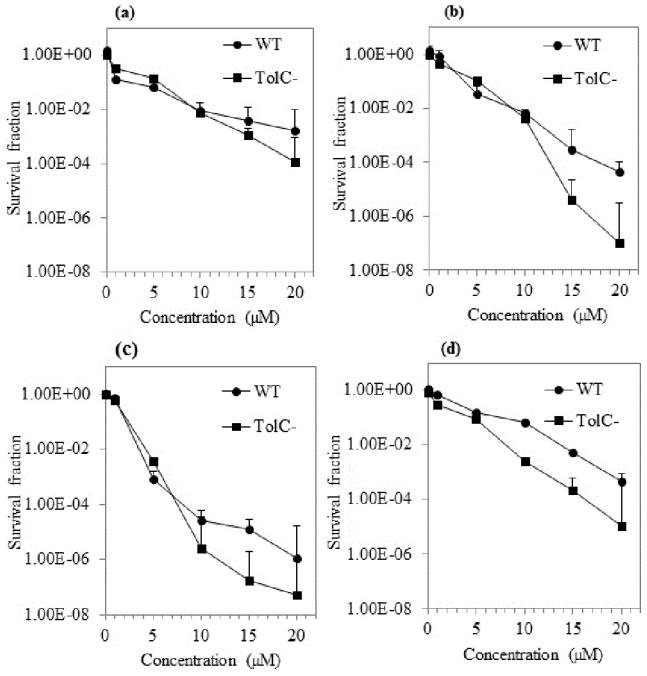

Fig. 2. aPDI of *E. coli* wild-type (WT, K-12) and TolC knockout (TolC−, JW5503-1) strains using: (a) MB, (b) **1**, (c) **2** and (d) **3**. Cells were illuminated with 100 mW/cm^2 red light (652 nm, 6 J/cm^2) and survival fractions determined. Data represent the mean ± SEM from three independent experiments.

In vitro aPDI

E. coli wild-type (K-12) cells and an isogenic TolC efflux pump knock-out strain JW5503-1 (TolC−) were incubated with MB and hybrids **1**–**3** over the concentration range 1–20 μM and illuminated with red light (652 nm) at 6 J/cm^2. CFUs were counted from serially diluted aliquots and the results plotted as survival fractions verses compound concentration (Fig. 2). MB and the hybrids showed no killing effect against either strain in the dark (Supplementary data Figs. S1 and S2). For the wild-type strain, illumination in the presence of MB produced a 2 $\log_{10}$ kill at 10 μM, which increased to 2.5 $\log_{10}$ at 20 μM. MB showed similar killing at 10 μM against the TolC mutant strain with higher killing (3.5 $\log_{10}$) at 20 μM. The increased susceptibility of the TolC− mutant was consistent with MB serving as a TolC efflux substrate.[30] Hybrid **1** produced a 2$\log_{10}$ kill against the wild-type strain at 10 μM and a 4$\log_{10}$ kill at 20 μM. Against the TolC− strain, hybrid **1** produced a 2$\log_{10}$ kill at 10 μM that increased to 7$\log_{10}$ at 20 μM. For hybrid **2**, a 4$\log_{10}$ kill was observed against the wild-type strain at 10 μM, which increased to 6$\log_{10}$ at 20 μM. Exceptional potency was seen with **2** against the TolC− strain, where a 6$\log_{10}$ kill was observed at 10 μM and almost complete eradication was achieved at 20 μM. Hybrid **3** produced a 3$\log_{10}$ kill at the highest concentration (20 μM) against the wild-type strain and 4.5$\log_{10}$ against the TolC− mutant. The increased activity of all three hybrids against the TolC− strain relative to the wild-type suggests they may be