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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.

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Graphical abstract

Keywords

Antimicrobial Photodynamic Inactivation; Efflux Pump Inhibitor; MethyleneBlue; *Escherichia coli*; *Acinetobacter baumannii*

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) (Figure 1) are illuminated with red light to produce reactive oxygen species (ROS) (e.g. singlet oxygen, ¹O₂ 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*.

***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 μ M and illuminated with red light (652 nm) at 6 J/cm². CFUs were counted from serially diluted aliquots and the results plotted as survival fractions versus compound concentration (Figure 2). MB and the hybrids showed no killing effect against either strain in the dark (Supplementary Data Figure S1 and S2). For the wild-type strain, illumination in the presence of MB produced a 2log₁₀ kill at 10 μ M, which increased to 2.5log₁₀ at 20 μ M. MB showed similar killing at 10 μ M against the TolC mutant strain with higher killing (3.5 log₁₀) at 20 μ M. The increased susceptibility of the TolC- mutant was consistent with MB serving as a TolC efflux substrate.³⁰ Hybrid **1** produced a 2log₁₀ kill against the wild-type strain at 10 μ M and a 4log₁₀ kill at 20 μ M. Against the TolC- strain, hybrid **1** produced a 2log₁₀ kill at 10 μ M that increased to 7log₁₀ at 20 μ M. For hybrid **2**, a 4log₁₀ kill was observed against the wild-type strain at 10 μ M, which increased to 6log₁₀ at 20 μ M. Exceptional potency was seen with **2** against the TolC- strain, where a 6log₁₀ kill was observed at 10 μ M and almost complete eradication was achieved at 20 μ M. Hybrid **3** produced a 3log₁₀ kill at the highest concentration (20 μ M) against the wild-type strain and 4.5log₁₀ against the TolC- mutant. The increased activity of all three hybrids against the TolC- strain relative to the wild-type suggests they may be substrates for this pump.

aPDI of *A. baumannii* was examined *in vitro* using the wild-type strain AB007. MB and the three hybrids showed no killing of AB007 in the dark over the concentration range 1-20 μ M (Figure 3). Following illumination, hybrids **2** and **3** showed similar aPDI potency to MB at 20 μ M (~4log₁₀ kill), with hybrid **1** producing an extra log₁₀ kill at this concentration.

***In vivo* aPDI of *E. coli* with hybrid 2**

Having shown the highest aPDI potency against the two *E. coli* strains *in vitro*, hybrid **2** was evaluated alongside MB for *in vivo* aPDI efficacy using a mouse full-thickness third-degree burn *E. coli* infection model.³¹ A pathogenic variant of bioluminescent enteropathogenic *E. coli* (EPEC, WS2572) was inoculated into burns on the shaved dorsal surfaces of mice and the infected areas were treated with solutions containing MB or hybrid **2**. The areas were illuminated with 652 nm red light and luminescence images captured. The normalized luminescence emanating from wounds (relative luminescence units, RLU) during the 'light treatment' phase of the experiment was plotted as a function of applied light fluence (Figure 4(a)).

The light only control cohort (Group A) showed a slight (~10%) reduction in luminescence following application of the highest fluence (120 J/cm²). Application of MB in the absence of light (Group B) produced a ~25% reduction in luminescence at the same fluence, while

aPDI with MB (Group C) resulted in a ~90% reduction in luminescence. In the absence of light, hybrid **2** (Group D) showed a slightly higher killing than MB (~40% reduction in luminescence), while aPDI with hybrid **2** (Group E) produced a remarkable ~80% reduction in bacterial luminescence at low fluence (36 J/cm²) and total loss of the luminescence signal at 84 J/cm².

Post-treatment monitoring of infection sites by capturing daily bioluminescence images for 14 days (Figure 4(b)) revealed a slight rebound in bacterial load for the controls (Group A) and MB treated groups (Groups B and C) 1 day after infection/treatment. Infections in the control groups A, B and D were all resolved (i.e. no luminescence detected) within 13-14 days, while for the MB aPDI cohort (Group C) infections were resolved after 12 days. aPDI with hybrid **2** (Group E) produced a lower bacterial burden compared to all other groups throughout the entire monitoring period and the infections were resolved more rapidly (10 days).

***In vivo* aPDI of *A. baumannii* with hybrid 1**

Hybrid **1** was tested for *in vivo* aPDI efficacy alongside MB using a mouse needle back-scratch wound abrasion *A. baumannii* infection model.³² Bioluminescent *A. baumannii* (strain AB Iraqi 007) was inoculated into needle-scratch wounds on the shaved dorsal surfaces of mice and the infected areas were treated with solutions containing MB or hybrid **1**. Control and aPDI treatment groups (Groups A-E) and infection site monitoring were as for the *E. coli* burn model.

The light only controls (Group A) showed no reduction in the luminescence signal over the 20 min 'light-treatment' phase of the experiment, confirming that the *A. baumannii* infection was stable over this period (Figure 5(a)). The MB (Group B) and hybrid **1** (Group D) dark controls produced slight reductions (<25%) in luminescence during this period. A 50% reduction in the luminescence signal was seen following aPDI with MB (Group C) at 36 J/cm² and total loss of the signal occurred at 108 J/cm². aPDI with hybrid **1** (Group E) showed an impressive 95% reduction in luminescence at low fluence (36 J/cm²) and complete loss of the signal at 84 J/cm².

A rebound in bacterial load (50% or greater) was observed 1 day after infection/treatment in all cohorts except Group E (aPDI with **1**) (Figure 5(b)). Low level luminescence remained in control Group A at Day 14, while the signal disappeared after 12 days in the presence of MB in the dark (Group B). aPDI with MB (Group C) produced slightly lower bacterial loads throughout the monitoring period and the luminescence signal had disappeared after 10 days. Treatment with hybrid **1** in the dark (Group D) reduced the bacterial load at a rate that paralleled the MB dark control, with infections resolving within 12 days. aPDI with **1** (Group E) showed lower bacterial loads than all other cohorts throughout the entire monitoring period and the infections were resolved within 6 days. Bioluminescence images captured from representative animals in Groups A-E during the first 6 days of the monitoring period are provided in the Supplementary Data (Figure S3).

Previous work by Tegos *et al.* showed that phenothiazinium-based photosensitizers such as MB are substrates for bacterial MDR efflux pumps and that these pumps can expel photosensitizers from cells leading to reduced aPDI effectiveness.²⁷ They also showed that aPDI with MB against the Gram-positive bacterium *S. aureus* is enhanced by the co-presence of inhibitors of the major facilitator efflux pump NorA.²⁷ We recently reported that synthetic hybrids formed by covalently attaching NorA pump inhibitors to MB also enhance aPDI of *S. aureus*,²⁹ with three leading hybrids **1-3** showing significant ROS generation upon illumination, potent aPDI of *S. aureus in vitro* and higher intracellular accumulation in *S. aureus* cells than MB. When hybrid **2** (INF55-(Ac)en-MB) was advanced to *in vivo* aPDI studies it outperformed MB by all measures in a murine back-scratch *S. aureus* infection model.

Buoyed by these findings, we chose to study the aPDI effects of **1-3** against two representative Gram-negative bacteria, i.e. *E. coli* and *A. baumannii*, despite neither having previously been shown to express membrane efflux pumps that are inhibited by INF55 or INF271. Indeed these two EPIs to date have only been shown to inhibit the NorA pump in *S. aureus*.³³ Nevertheless, Gram-negative bacteria are known to be less susceptible to extracellular singlet oxygen than Gram-positive species,^{34,35} suggesting that a hybrid approach that could increase intracellular ROS was worth investigating.

The major efflux pump in *E. coli* comprises the outer membrane channel protein TolC and two other proteins AcrA and AcrB, which together form the tripartite efflux system AcrAB-TolC; a member of the resistance nodulation division (RND) superfamily.³⁶ Several reports have shown that phenothiazinium salts are efflux substrates in *E. coli*.^{27,37,38} When tested against *E. coli* wild-type cells and a TolC knockout mutant, all three hybrids showed greater aPDI than MB against both strains. When the most potent hybrid **2** was evaluated for aPDI efficacy in a murine *E. coli* burn infection model it showed greater aPDI than MB during the 'light-treatment' phase of the experiment, lower bacterial counts throughout the post-treatment monitoring period and more rapid resolution of the infection. These results suggest that hybrid **2** is either a poorer substrate for *E. coli* pumps than MB or it acts directly as a pump inhibitor, both of which would lead to higher intracellular concentrations of the hybrid and higher intracellular ROS during aPDI. Further experiments are ultimately required, however, to confirm which (if any) *E. coli* pumps are targeted by **2**, how they are affected and whether these effects play a dominant role in the enhanced *E. coli* aPDI seen with **2**.

The most prevalent pumps in *A. baumannii* are members of the RND superfamily and include the AdeABC two-component regulatory system AdeIJK and AdeFGH. Non-RND efflux systems have also been characterised in *A. baumannii*.³⁹ When tested *in vitro* for aPDI of *A. baumannii* AB007, hybrids **2** and **3** showed no increase in activity relative to MB. However, hybrid **1** showed 1log10 greater killing than MB at the highest concentration (20 µM) and was subsequently found to outperform MB in a murine aPDI *A. baumannii* infection model. These results are consistent with **1** (but not **2** or **3**) being a poorer efflux substrate than MB in *A. baumannii* and possibly a pump inhibitor, although alternative explanations are plausible. For example, the physicochemical properties of **1** may engender higher affinity for *A. baumannii* cell surface components relative to MB and **2/3**, leading to higher cell-localized concentrations of ROS during aPDI and greater lethality. The

relationship between higher cell surface affinity of photosensitizers and increased aPDI has been noted.⁴⁰

In conclusion, this study demonstrates that attaching NorA EPIs to MB can increase aPDI effectiveness against the Gram-negative pathogens *E. coli* and *A. baumannii* *in vitro* and *in vivo*. Further experiments to establish whether INF55, INF271 and the hybrids are inhibitors of efflux pumps in Gram-negative bacteria will shed light on the underlying mechanisms responsible for the observed increases in aPDI efficacy relative to MB. Hybrids containing MB attached to known inhibitors of Gram-negative efflux pumps (e.g. Phenyl-arginine-beta-naphthylamide, PaßN)⁴¹ would be of interest in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

aPDI	Antimicrobial photodynamic inactivation
MB	methylene blue
EPI-MB	efflux pump inhibitor-methylene blue hybrid
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
aPDI	antimicrobial photodynamic inactivation
PS	photosensitizing dye
ROS	reactive oxygen species
•OH	hydroxyl radicals
EPEC	bioluminescent enteropathogenic <i>E. coli</i>
BHI	brain heart infusion
CrEL	Cremophor EL
CFU	colony forming units
NIH	national institutes of health
RLU	relative luminescence units
RND	resistance nodulation division

Figure 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 INF271¹³ are also shown.

Figure 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 $\pm$ SEM from three independent experiments.

Figure 3.
aPDI of *Acinetobacter baumannii* AB007 (and dark controls) using: (a) MB, (b) **1**, (c) **2** and (d) **3** over the concentration range 1-20 μM against. Data represent the mean $\pm$ SEM from three independent experiments.

Figure 4.

(a) Bioluminescence of *E. coli*-infected mouse burn wounds during initial 'light-treatment' phase of experiment: Group A - light controls (no compound), Group B - MB dark control, Group C - MB with aPDI, Group D - hybrid 2 dark control and Group E - hybrid 2 with aPDI. MB or hybrid (40 μL of 250 μM stock solution) was applied to infection sites. Data represent the mean ($\pm$ SEM) normalized relative luminescence units (RLU) emanating from the burn wounds of 4-6 mice in each group during application of 0, 12, 36, 84, 108 and 120 J/cm^2 red light (652 nm). (b) Fourteen day post-treatment monitoring of burn wound infection site luminescence.

Figure 5.

(a) Bioluminescence of *A. baumannii*-infected mouse back scratch abrasion wounds during initial 'light-treatment' phase of experiment: Group A - light control (no compound), Group B - MB dark control, Group C - MB with aPDI, Group D - hybrid **1** dark control and Group E - hybrid **1** with aPDI. MB or hybrid (40 μL of a 250 μM stock solution) was applied to infection sites. Data represent the mean ($\pm$ SEM) normalized relative luminescence units (RLU) emanating from the scratch wounds of 4-6 mice in each group after application of 0, 12, 36, 84, 108 and 120 J/cm^2 red light (652 nm). (b) Fourteen day post-treatment monitoring of wound infection site luminescence.