Journal of Antimicrobial Chemotherapy (2009) **64**, 782–785
doi:10.1093/jac/dkp278
Advance Access publication 6 August 2009

JAC

Highly efficient *in vitro* photodynamic inactivation of *Mycobacterium smegmatis*

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Received 7 February 2009; returned 21 April 2009; revised 8 July 2009; accepted 9 July 2009

***Objectives*: Efforts to control tuberculosis (TB) have been hampered by the emergence of multiple-drug resistant strains, necessitating pursuit of alternative approaches to the current antibiotic-based treatments. Herein, we explore the feasibility of photodynamic inactivation (PDI) of mycobacteria.**

***Methods*: *In vitro* PDI studies employing *Mycobacterium smegmatis* as a surrogate for *Mycobacterium tuberculosis* were performed examining photosensitizer (PS) type, concentration and light dose. *M. smegmatis* was grown to a concentration of 10^8 colony forming units (cfu) per mL, resuspended in PBS–0.5% Tween-80-containing buffer, incubated with the PS for 5 min and subsequently illuminated with white light (400–700 nm) at a fluence rate of 60 mW/cm^2 for 1, 5, 15 or 30 min (equivalent to 3.4, 18, 54 or 108 J/cm^2). The percentage survival was determined by the ratio of the colony count from illuminated and non-illuminated control cell suspensions. The PSs examined were 5,10,15,20-tetrakis(1-methyl-4-pyridinyl)porphyrin tetratosylate (TMPyP), 5,10,15,20-tetrakis(4-*N,N,N*-trimethylanilinium)porphyrin tetrachloride (TNMAP), methylene blue (MB), 5,10,15,20-tetrakis(4-sulphonatophenyl)porphyrin (TSPP), 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin-Pd(II) (TCPP-Pd) and phthalocyanine tetrasulphonic acid (PhCS).**

***Results*: Our best results demonstrate that PDI of *M. smegmatis* can achieve a noteworthy 5–6 log unit reduction in cfu (99.999%+ viable cell eradication) when cationic PSs are employed in the nanomolar concentration range. Anionic PSs did not effectively mediate PDI of mycobacteria due to their inability to associate with the negatively charged mycobacterial cell membrane.**

***Conclusions*: PDI of *M. smegmatis* was found to be highly efficient in reducing the number of viable cells *in vitro* when cationic PSs were employed.**

Keywords: multidrug resistant, TB, photosensitizers

Introduction

Tuberculosis (TB) is a significant health threat in the world today, with approximately one-third of the world's population currently infected, resulting in 9.2 million new infections and 1.7 million deaths reported for 2006 alone.[1] While a number of antibiotics (isoniazid, rifampicin, ethionamide) are effective against the causative agent *Mycobacterium tuberculosis*, a Gram-positive bacterium, the rise in multidrug-resistant (MDR) strains due to improper or incomplete drug therapy has increased dramatically. For 2006, the WHO reported the highest ever recorded global average of MDR-TB among newly detected TB cases at 5%, with areas in the former Soviet Union peaking at >20%.[1] These alarming numbers have sparked a new round of interest in the search for better and alternative anti-TB treatments.

Significant advances made in the past decade in treating several forms of cancer with photodynamic therapy (PDT) suggest that photodynamic inactivation (PDI) of localized bacterial infections such as TB might also be feasible, thus allowing for the development of a valuable alternative or supplemental option to the current antibiotic-based treatments.[2] PDI employs a localized, light-activated photosensitizer (PS) that generates cytotoxic species, particularly singlet oxygen, upon illumination with visible light.[3] Development of bacterial resistance to PDI is believed to be unlikely due to the non-specific damage caused by $^1O_2$.[4] The efficacy of PDI against *Escherichia coli*, *Staphylococcus aureus* (including methicillin-resistant strains), *Acinetobacter baumannii* and *Pseudomonas aeruginosa* has been discussed widely in the literature,[2] but fundamental *in vitro* studies on mycobacteria are still lacking. In light of this, we

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have examined the drug and light dose dependencies of several PSs on mycobacterial survival using *Mycobacterium smegmatis* mc^{2}155, a widely employed non-pathogenic surrogate for *M. tuberculosis*.

Methods

M. smegmatis was grown in 5 mL cultures in Middlebrook 7H9 media with ADS and 100 μg/mL cycloheximide as the antibiotic. The cells were incubated at 37°C on an orbital shaker until an optical density of 0.6–0.7 at 600 nm, corresponding to a concentration of $1–3\times10^8$ cfu/mL, was reached. The cultures were then pelleted by centrifugation (10 min, 3716 **g**), the supernatant discarded, and the cells were resuspended in a total volume of 5 mL PBS (170 mM NaCl, 3.4 mM KCl, 10.0 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) containing 0.5% Tween-80 and the appropriate concentration of the PS. The cells were incubated for 5 min in the dark at 37°C under agitation.

The PSs (see Figure 1) 5,10,15,20-tetrakis(1-methyl-4-pyridinyl)porphyrin tetratosylate (**1**, TMPyP), 5,10,15,20-tetrakis(4-*N*,*N*,*N*-trimethylanilinium)porphyrin tetrachloride (**2**, TNMAP), 5,10,15,20-tetrakis(4-sulphonatophenyl)porphyrin (**4**, TSPP), 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin-Pd(II) (**5**, TCPP-Pd) and phthalocyanine tetrasulphonic acid (**6**, PhCS) were obtained from Frontier Scientific (USA), and methylene blue (**3**, MB) was purchased from Fisher Scientific (USA). All photosensitization experiments were performed using a non-coherent light source, PDT light model LC122 (LumaCare, USA), and the fluence rate was measured with an Orion power meter (Orphir Optronics Ltd, Israel). Aliquots (1 mL) of the cell suspension in PBS were added to a sterile 24-well plate (BD Falcon, flat bottom) and illuminated with visible light (400–700 nm) with a fluence rate of 60 mW/cm^2 for 1, 5, 15 or 30 min (corresponding to fluences of 3.6, 18, 54 and 108 J/cm^2) while magnetically stirred.

After illumination, 100 μL of the cell suspension was 1:10 serially diluted six times, plated on square plates (Middlebrook 7H10-ADS) and incubated at 37°C in the dark for 40 h. The survival rate was determined from the ratio of cfu/mL of the illuminated solution and the dark control. Variations in the bacterial concentration of the starter cultures ($1–4\times10^8$ cfu/mL) resulted in a variation of the detection limit spanning the region of 0.001–0.0001% survival. Samples with PS present but kept in the dark (dark control) and illuminated samples without PS (light control) served as controls. All experiments were conducted in triplicate at a minimum, and statistical significance was assessed via a two-tailed, unpaired Student's *t*-test.

Results

The greatest extent of mycobacterial PDI was observed when employing cationic porphyrins **1** and **2** as the PSs (Figure 1). In the low micromolar concentration range, TMPyP (**1**) (Figure 2a) reduced bacterial survival by 5–6 log units (99.999% + viable cell eradication, $P<0.001$), even after illumination times as short as 60 s. Excellent antimycobacterial activity was also observed for the pharmaceutically relevant concentration of 150 nM: 3, 4 and 5–6 log unit reduction in viable cells after 5, 15 or 30 min illumination, respectively ($P<0.001$). With TNMAP (**2**) (Figure 2b), a maximum viable cell reduction of 3.5 log units ($P<0.001$) occurred after 30 min illumination at 7.5 μM (~300-fold less efficient than with **1**). However, **2** exhibited the lowest PS concentration (100 nM) for which antimycobacterial PDI was achievable (14% survival, 30 min illumination, $P=0.001$). The results obtained here for **1** and **2** are significantly better than the only other report of *in vitro* mycobacterial PDI (<2 log units reduction of *Mycobacterium bovis* BCG, 5 μM verteporfin as PS, 60 J/cm^2, 690 nm).[5] In the presence of 2–20 mM of the singlet oxygen quencher NaN_3, a statistically significant increase in mycobacterial survival was observed for **1** [see Table S1 available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)], suggesting that PDI of *M. smegmatis* is mediated in part by singlet oxygen production.

MB (**3**), a cationic phenothiazine, was effective only at the highest concentrations tested (Figure 2c): a 4 log unit reduction in viable cells was observed after 30 min illumination at 75 μM ($P<0.001$), but this decreased to a 2 log unit reduction at 7.5 μM ($P<0.001$). In general, the anionic PSs TSPP (**4**), TCPP-Pd (**5**) and PhCS (**6**) did not mediate any statistically significant ($P>0.05$) PDI efficiently in the concentration range tested (Figure 2d–f), with the only exception being a 50% reduction in viable cells for TSPP at the highest concentration tested ($P=0.02$).

The degree of PS association (binding or uptake) to *M. smegmatis* was evaluated by fluorescence spectroscopy (Table 1).[2] Consistent with its efficient PDI at 100 nM (Figure 2b), TNMAP (**2**) exhibited the highest amount of PS recovered from lysed cells, suggesting a strong association with *M. smegmatis*. TMPyP (**1**) was found to bind to *M. smegmatis* to a lesser degree than **2**, but a number of factors not related to binding, such as differences in photophysical properties, dye aggregation, localization and/or uptake, are likely reasons why **1** is overall the more efficient PS. MB (**3**) is the least able of the cationic PS to bind to *M. smegmatis*, which helps to rationalize the large difference in PDI mediated by **3** compared with **2** despite their similar quantum yields of singlet oxygen production [Φ_Δ; see Table S2 available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. PS **4** associates poorly with *M. smegmatis*, explaining its inability to mediate PDI despite possessing a favourable Φ_Δ. Binding of PS **5** could not be examined by fluorescence spectroscopy (fluorescence quantum yield of $<10^{-4}$). Decreased emission and high background fluorescence did not allow for quantification of PS binding of **6** in solutions containing lysed *M. smegmatis* [see Figure S1 and additional explanation available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)].

Discussion

Our results demonstrate that cationic PSs can be potent photodynamic inactivators of mycobacteria, whereas anionic PSs exhibit little effect. Comparison of the PSs' photophysical properties alone, however, cannot be used to rationalize the observed differences in the extent of PDI mediated by each, as such properties may be significantly different within the mycobacterium cytoplasm, or at the cell surface where the PS may bind. A table of the photophysical properties of **1**–**6**, where known, is provided in the Supplementary data at *JAC* Online along with their relevant literature citations (Table S2). For example, PS **1** possesses very favourable photophysical properties, with a quantum yield of singlet oxygen production (Φ_Δ) of 0.74, whereas PSs **2** and **3** exhibit a Φ_Δ of ~0.5, although our study shows that **2**

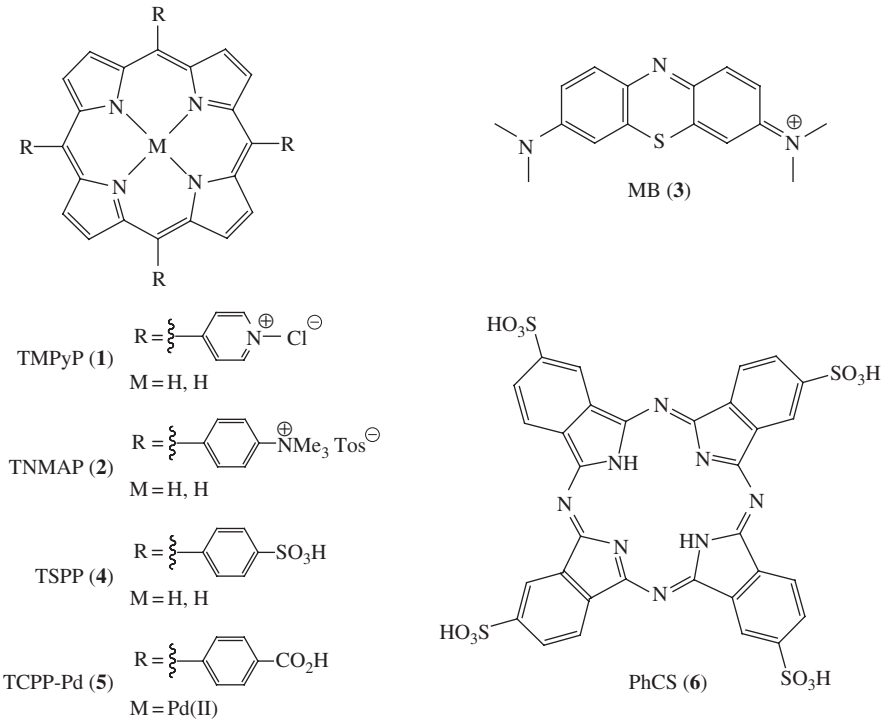

Figure 1. PSs employed in this study.

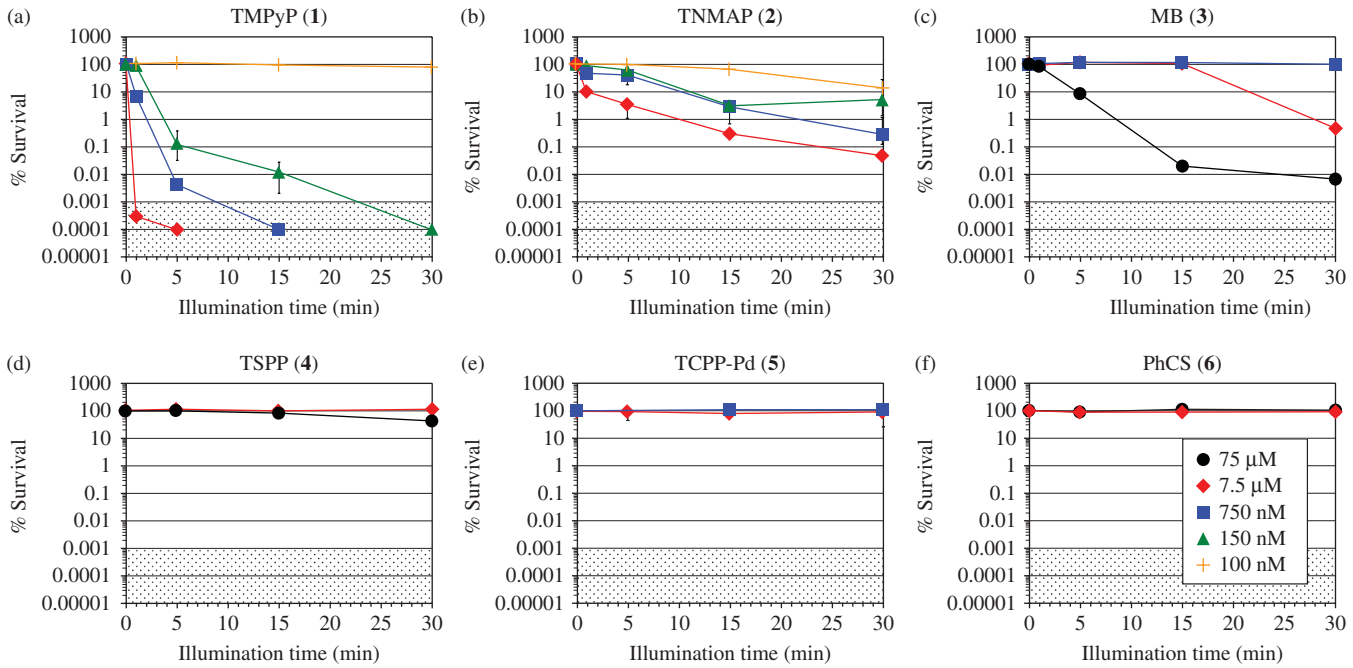

Figure 2. PDI of *M. smegmatis* using PSs **1**–**6**. The illumination time was varied over 1, 5, 15 and 30 min, corresponding to total fluences of 3.6, 18, 54 and 108 J/cm^2, respectively, at 60 mW/cm^2. Variations in the bacterial concentration of the starter cultures (1×10^8–4×10^8 cfu/mL) resulted in a variation of the detection limit spanning the region of 0.001–0.0001% survival (shaded). As the plating technique employed did not allow for detection of survival rates of <0.0001%, data points below the detection limit are set to 0.0001% survival for graphing purposes. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

appears to be significantly more effective against *M. smegmatis*. Among the anionic PSs, TSPP (**4**) also shows a favourable Φ_Δ of 0.75, yet efficient inactivation of *M. smegmatis* was not observed with any anionic PS (**4**–**6**).

Looking beyond photophysical properties as the primary explanation for the observed mycobactericidal PDI effects, we hypothesize that PS binding may be the critical factor. Interestingly, cationic PSs have been reported to bind efficiently to both

Table 1. PS recovery (nM) from lysed cell suspensions after one to three PBS washing steps[a,b]

PS	Number of washing steps prior to lysis		
	1	2	3
TMPyP (**1**)	168.8 ± 30.7	91.1 ± 31.1	40.4 ± 17.6
TNMAP (**2**)	413.8 ± 148.5	102.5 ± 18.9	140.3 ± 83.9
MB (**3**)	84.7 ± 21.4	45.1 ± 10.3	17.2 ± 7.6
TSPP (**4**)	34.1 ± 9.1	0.2 ± 0.1	ND

[a]Incubation of 1×10^8 cells/mL with 7.5 μM PS for 5 min.
[b]Estimated by use of a calibration curve of the PS in PBS containing soluble lysed cell material; ND, none detected.

Gram-positive as well as Gram-negative bacteria,[6] whereas anionic PSs are known to bind and photoinactivate only the former.[7] Although *M. smegmatis* is categorized as Gram-positive, our data unequivocally shows that it behaves in a similar fashion to a Gram-negative bacterium: cationic PSs were effective in mediating the PDI of *M. smegmatis*, anionic ones were clearly unable to do so. Although the extent of PS localization, aggregation, penetration across the mycobacterial membrane and retention (as well as the kinetics of such processes) are beyond the scope of this current study, our PS binding study strongly suggests that the localization and/or binding affinity of the PS to the mycobacterial cell is crucial for the efficacy of the PDI process, and suggests that rapid fluorescence binding assays may be used as an initial screening tool for future PS development.

Recently, O'Riordan *et al.*[5,8] investigated the *in vivo* susceptibility of localized *M. bovis* BCG-induced granulomatous infections in murine models towards PDI. Their results showed promise, with a 1–2 log unit reduction in viable cells. However, the PS employed in that study, verteporfin (an anionic porphyrin) and two phenothiazine derivatives (weakly cationic and similar to MB, **3**), would be predicted by our present study as being non-ideal (in hindsight) candidates for mediating PDI inactivation of mycobacteria due to their poor ability to associate with the negatively charged cell surface. Thus, we conclude that future *in vivo* studies on utilizing PDI to clear mycobacterial infections should focus on the use of highly cationic PSs, with the caveat that other factors such as compound lipophilicity, aggregation or localization also need to be considered.

The results reported herein detail the first fundamental *in vitro* survey of mycobacterial PDI using a range of PS types, concentrations and light doses, and provides the necessary foundation work to help direct future *in vivo* studies. We have observed the greatest extent of mycobacterial cell inactivation (up to 6 log units) reported to date, while maintaining excellent photodynamic conditions of short illumination times and extremely low (nM) PS concentrations. Our data also indicate that highly charged cationic PSs should be employed against Gram-positive mycobacteria, as association with the mycobacterial cell appears to be the primary factor in cell inactivation, albeit moderate to good photophysical properties play a contributing role. With the ongoing development of fibreoptic-based PDT to treat lung tumours and small-cell carcinomas,[9] PDI of adult pulmonary TB might potentially be an attractive alternative treatment option, particularly for MDR-TB where cost-efficient antibiotics are ineffective, or when a need for rapidly reducing bacterial load is required.

Acknowledgements

Part of the data discussed in this article was presented at the 236th ACS National Meeting (abstract INOR-103; Philadelphia, PA; August 2008), and at the Ninth European Biological Inorganic Chemistry Conference (abstract O14; Wroclaw, Poland; September 2008). We thank Professor Jon Lindsey (North Carolina State University) for providing access to a LumaCare PDT light (model LC122). *M. smegmatis* mc^2155 cells were kindly given by Professor William Jacobs (Albert Einstein College of Medicine).

Funding

This research was supported by the North Carolina State University (Department of Chemistry).

Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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