Phenothiazinium Photoantimicrobials with Basic Side Chains

Mark Wainwright, Joanna Antczak, Martyna Baca, Ciara Loughran and Katie Meegan
School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF, United Kingdom

*Author for correspondence, email: mark_wainwright@hotmail.com

Abstract
Derivatives of the standard cationic photosensitiser, methylene blue, were synthesised, having extra amino (basic) functionality in the auxochromic side-chain. The resulting analogues were profiled for photodynamic activity in vitro, and screened against standard Gram-positive and Gram-negative bacteria for photobactericidal activity. The substitution pattern of the derivatives was such that ionisation of the amino groups *in situ*, via protonation, provided a range of charge distribution and degree of charge across the molecular framework.
While most examples exhibited greater activity than the lead compound, in addition to similar activity to the known, but more powerful, phenothiazinium photoantimicrobial, dimethyl methylene blue, this was also associated with relatively high dark toxicity, inferring that these compounds were targeting crucial structures before illumination.
One derivative having an asymmetrical structure, with separation between a lipophilic and a hydrophilic region exhibited a combination of very high phototoxicity coupled with very low dark effects, against both the standard screen and an additional one containing further, relevant pathogen species, including *Candida albicans*. It is suggested that the great activity of this analogue is due to efficient membrane targeting.

Running header: Basic Phenothiazinium Derivatives

Keywords: basic side chain; dimethyl methylene blue; phenothiazinium; photoantimicrobial; photosensitiser.

1. Introduction

The phenothiazinium salt methylene blue (Figure 1) has considerable antiquity among dyes used in biology and medicine, having featured in the vanguard of colorants for microscopy in the late 19th Century. It was also a lead compound in the work of Koch, Ehrlich and others in their development of the theory of selective toxicity and, subsequently, of modern chemotherapy [1].

Methylene blue was used by Guttmann and Ehrlich in the clinical cure of falciparum malaria in 1891 [2], and this became the basis of antimalarial research later carried out at IG Farben in the immediate aftermath of the First World War.

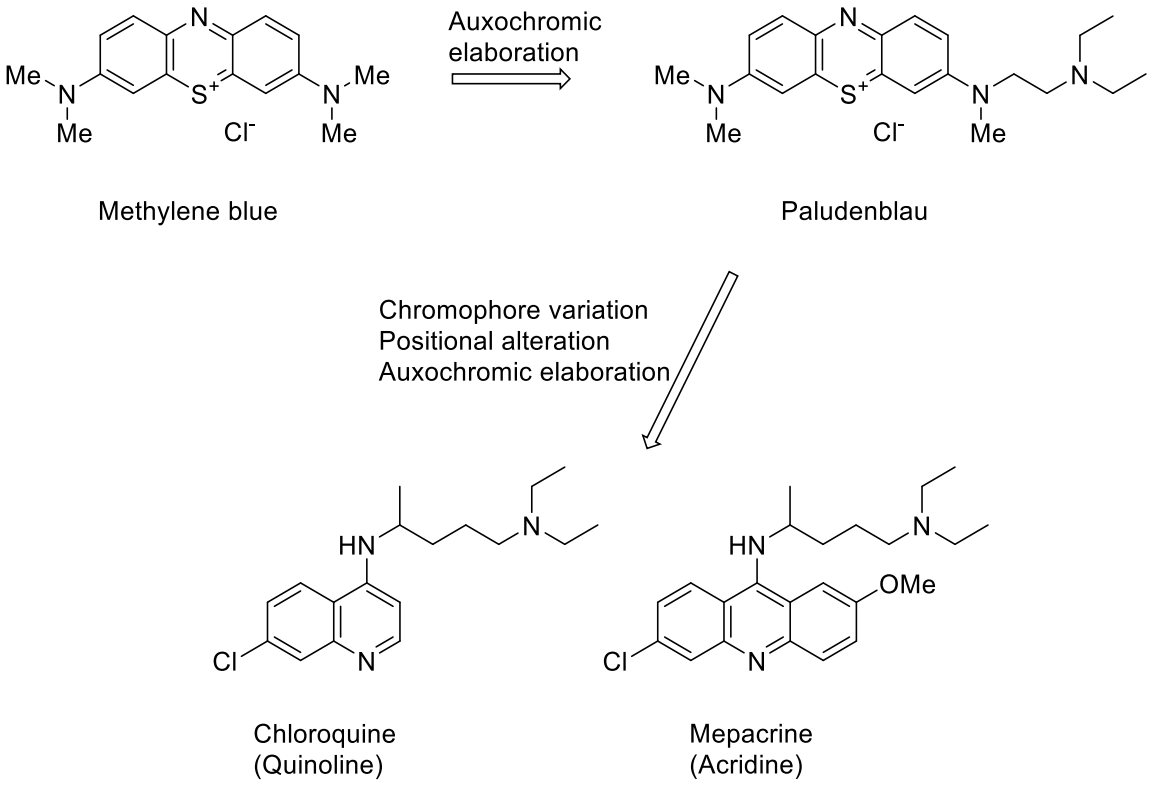


Figure 1. Methylene blue and early antimalarial drugs

Among derivatives of the lead compound was a group of methylene blue analogues having alterations in one of the dimethylamino auxochromes, a constituent methyl group being extended in length and terminated with another amine (Figure 1). The resulting aminoalkylamino chain, and its elaborations, was found to increase antimalarial activity in the canary model developed by Ernst Roehl [3]. Further testing led to the development of *Paludenblau* (Figure 1) although this was eventually discontinued in the face of less staining antimalarials such as the acridine mepacrine and the quinoline chloroquine (Figure 1) [4].

In related work based on the acridine chromophore, it was found that the size of the side chain was influential on the type of activity observed, longer aminoalkylamino moieties being associated with antiplasmodial activity, whereas simpler alkylamino- or amino groups endowed compounds useful against bacteria (e.g. proflavine, aminacrine) [5]. In addition, the combination of a planar chromophore and an aminoalkylamino side chain has been used more recently to target nucleic acid, both in anticancer and antiviral applications [6].

As part of an ongoing photosensitiser discovery programme, novel photoantimicrobial compounds have been sought, based on the phenothiazinium chromophore and, indeed, using methylene blue as a lead structure. While the low human toxicity of this compound is evidenced by its use on a daily basis in healthcare concerns around the world, it remains a relatively weak photoantimicrobial *per se*.

Since the analogues of methylene blue produced by IG Farben, including methylene blue itself, were realised via traditional (i.e. dye industry) synthetic routes from the requisite *p*-phenylenediamine/aniline derivatives, the range of compounds available was relatively small due to the strong oxidising agents employed, such as acidic dichromate, which would also denature less robust groups in the reactants. In addition, the aqueous reaction media employed presented solubility difficulties with e.g. higher alkyl or halogenated derivatives.

In terms of expanding the range of derivatives available for screening as photosensitisers, the replacement of chromium(VI) with weaker oxidants such as silver(I) and the use of methanol as a reaction solvent represent a positive move, particularly in producing ring-substituted derivatives [7]. Similarly, synthesis of derivatives from 10*H*-phenothiazine has expanded the range of auxochromic groups enormously [8-10], especially since this allows facile production of asymmetrically-substituted derivatives.

The current work covers the synthesis of asymmetrical and symmetrical phenothiazinium derivatives having additional amino functionality in one or both auxochromic groups and the screening of these compounds against bacteria and fungi [11].

2. Materials and Methods

10*H*-Phenothiazine, bromine, iodine, all amines and silica gel were obtained from Sigma Aldrich (UK). Solvents were obtained from Fischer UK. Spectrophotometric measurements were carried out using a Hewlett Packard 8452A diode array spectrophotometer. Accurate molecular ion masses for the derivatives were obtained using a Micromass LCT-TOF mass spectrometer.

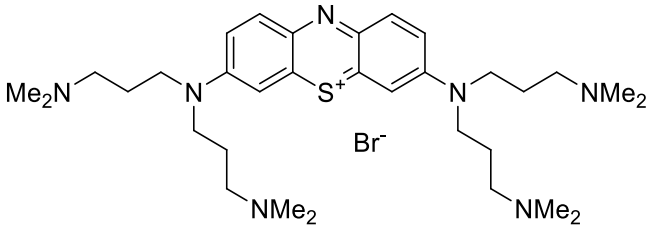
2.1 Symmetrical photosensitisers, general route

3,7-Dibromophenothiazinium bromide

10*H*-Phenothiazine (2 g, 10 mmol) was dissolved in 150 mLof glacial acetic acid at room temperature. Bromine (10 mL, mmol) was added in one amount and the reaction stirred vigorously for one minute. Water (400 mL) was then added in one amount, producing a red-brown solution and a red-black amorphous solid, isolated by filtration at the pump. The solid was washed with water and then with ether until the washings were colourless. The resulting dark red solid was powdered and dried *in vacuo* at room temperature to constant weight, yield = 4.96 g, 84%.

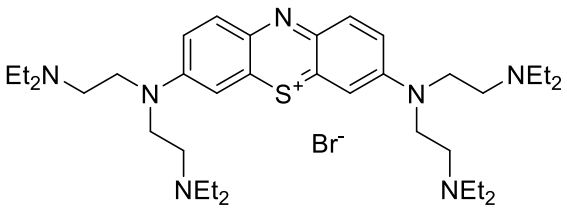
The alkylaminoalkylamino compound (15 mmol) was dissolved in dichloromethane (100 mL) at room temperature, and solid 3,6-dibromophenothiazinium tribromide (1.00g, 1.7 mmol) was added in one amount. The resulting green-blue solution was stirred at room temperature for a further two hours at room temperature. Product isolation was achieved by aqueous washing of the reaction mixture, drying and concentration of the resulting organic solution and precipitation with dry ether. Further purification by column chromatography, (SiO_2 / CH_2Cl_2, CH_3OH), was carried out where necessary.

3,7-Bis(bis(3-(dimethylamino)propyl)amino)phenothiazinium bromide (1a)



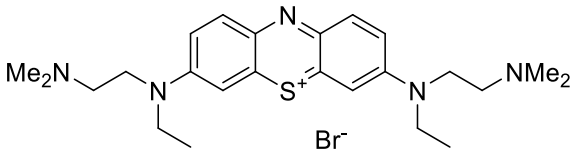
From bis(3-(dimethylaminopropyl)amine, as a blue black solid, yield = 228 mg, 21 %; HRMS (ESI) calcd for $C_{32}H_{54}N_7S$ requires 568.42, found 568.40; λ_{max} (MeOH) 668 nm.

3,7-Bis(bis(3-(diethylamino)propyl)amino)phenothiazinium bromide (1b)



From bis(2-(diethylamino)ethyl)amine, as a blue black solid, yield = 396 mg, 31 %; HRMS (ESI) calcd for $C_{36}H_{62}N_7S$ requires 624.48, found 624.49; λ_{max} (MeOH) 667 nm.

3,7-Bis((2-(dimethylamino)ethyl)(ethyl)amino)phenothiazinium bromide (1c)



From (2-(dimethylamino)ethyl)ethylamine as a blue black solid, yield = 187 mg, 22 %; HRMS (ESI) calcd for $C_{28}H_{44}N_5S$ requires 426.27, found 426.28; λ_{max} (MeOH) 662 nm.

2.2 Asymmetric photosensitisers, general route

Phenothiazinium tetraiodide

10*H*-Phenothiazine (2 g, 10 mmol) was dissolved in 50 mL of dichloromethane at room temperature. A solution of iodine (8 g, 32 mmol) in dichloromethane (150 mL) was added, and the whole stirred for three hours at room temperature. The resulting purple-black solid was filtered at the pump, washed free of iodine with dichloromethane, powdered and dried to constant weight. Yield of black powder = 6.05 g, 86%.

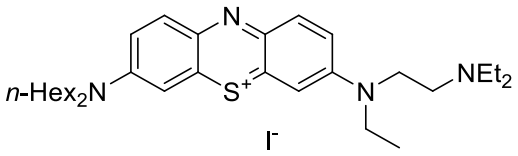
3-Dialkylaminophenothiazinium triiodides

Phenothiazinium tetraiodide (2.15 g, mmol) was dissolved in methanol (20 mL) at room temperature and a solution of dialkylamine (7.6 mmol) in methanol (20 mL) added dropwise over 20 minutes. The reaction was allowed to stir at room temperature for a further three hours, monitored by thin layer chromatography (SiO_2 / 3% aqueous NH_4OAc in CH_3OH). The solution was allowed to stand overnight and the resulting black solid filtered off and washed with cold methanol.

3-Dialkylamino-7-dialkylaminoalkylaminophenothiazinium iodides

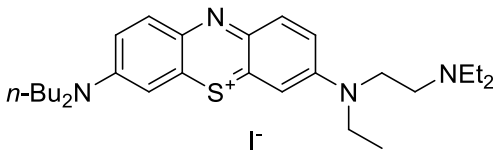
To a suspension of 3-dialkylaminophenothiazinium triiodide (0.75 mmol) in methanol (10 mL) was added dropwise the requisite dialkylaminoalkylamine (1.8 mmol) in 10 mL methanol. The reaction was allowed to stir at room temperature for 3 hours and then to stand overnight. The resulting black solid was filtered at the pump, dried and then recrystallised from the minimum of methanol. Several examples required further purification by column chromatography, (SiO_2 / CH_2Cl_2, CH_3OH).

3-(Di-*n*-hexylamino)-7-((2-(diethylamino)ethyl)(ethyl)amino)phenothiazinium iodide (2a)



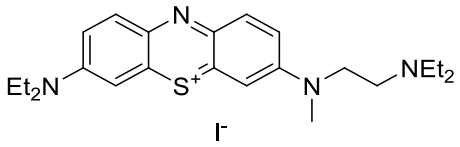
From 3-di-*n*-hexylaminophenothiazinium triiodide and (2-(diethylamino)ethyl)ethylamine as a black solid, yield = 171 mg, 35 %; HRMS (ESI) calcd for $C_{32}H_{51}N_4S$ requires 523.38, found 523.38; λ_{max} (MeOH) 660 nm.

3-(Di-*n*-butylamino)-7-((2-(diethylamino)ethyl)(ethyl)amino)phenothiazinium iodide (2b)



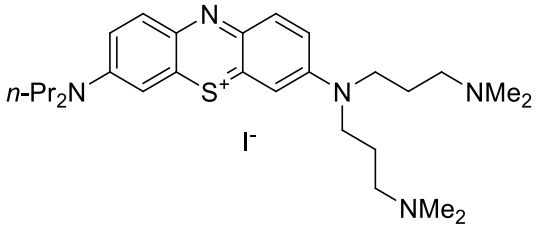
From 3-diethylaminophenothiazinium triiodide and (2-(dimethylamino)ethyl)ethylamine as a black solid, yield = 129 mg, 29 %; HRMS (ESI) calcd for $C_{28}H_{43}N_4S$ requires 467.32, found 467.28; λ_{max} (MeOH) 665 nm.

3-(Diethylamino)-7-((2-(diethylamino)ethyl)(methyl)amino)phenothiazinium iodide (2c)



From 3-diethylaminophenothiazinium triiodide and (2-(diethylamino)ethyl)methylamine as a black solid, yield = 102 mg, 26 %; HRMS (ESI) calcd for $C_{23}H_{33}N_4S$ requires 397.24, found 397.20; λ_{max} (MeOH) 652 nm.

3-(Bis(3-(dimethylamino)propyl)amino)-7-(di-*n*-propylamino)phenothiazinium iodide (2d)



From 3-di-*n*-propylaminophenothiazinium triiodide and bis(3-(dimethylaminopropyl)amine as a black solid, yield = 151 mg, 33 %; HRMS (ESI) calcd for $C_{28}H_{44}N_5S$ requires 482.33, found 482.29; λ_{max} (MeOH) 661 nm.

2.3 Singlet oxygen production

Singlet oxygen production by the photosensitisers was assayed using the decolourisation of 2,3,4,5-tetraphenylcyclopentadienone (TPCPD) in dichloromethane. Thus the decrease in absorption at 500 nm was monitored spectrophotometrically with time as in the method of Cincotta *et al.* [12]. The singlet oxygen yield for the standard photosensitiser, methylene blue ($\Phi_{\Delta MB}$) is given as 0.443 [13]. By assuming that the decrease in absorption of TPCPD at 500 nm is directly proportional to its reaction with singlet oxygen, the time for a 50% decrease in absorption caused by each of the derivatives under identical conditions ($t_{½Der}$) thus gives a measure of its photosensitising efficiency. Thus, the time for the TPCPD absorption to decrease by 50% due to MB photosensitisation ($t_{½MB}$) was taken as 1.0. To calculate the singlet oxygen yield for the derivatives ($\Phi_{\Delta Der}$), the following formula was used:

$$\Phi_{\Delta Der} = \Phi_{\Delta MB} \bullet \frac{t_{1/2MB}}{t_{1/2Der}}$$

2.3 Hydrophilic-lipophilic balance (Log*P*)

The lipophilicities of the photosensitisers were calculated in terms of log *P*, the logarithm of their partition coefficients between phosphate-buffered saline and 1-octanol. The data were calculated using the standard spectrophotometric method [13] based on the relationship:

$$LogP = Log\left\{\frac{(A - A^1)}{A^1} \bullet \frac{V_W}{V_O}\right\}$$

where A and A^1 are the absorption intensities before and after partitioning respectively and V_w and V_o are the respective volumes of the aqueous and 1-octanol phases. Determinations were repeated three times.

2.4 Antibacterial Screening

The photobactericidal efficacies of the derivatives in addition to that of the known photosensitiser methylene blue were measured against a Gram positive and a Gram negative organism, *Staphylococcus aureus* (NCTC 6571) and *Escherichia coli* (NCTC 10418) respectively. Both strains were grown in Mueller-Hinton Broth and then diluted to a concentration of 10^6 colony-forming units/mL. Aliquots of the strains were then incubated for 1 hour at 37 °C in microtitre trays with various concentrations of photosensitiser in a range of from 100 to 3 μM, with zero photosensitiser concentrations in each case for control purposes. The trays were then either illuminated for twenty minutes using an array of light-emitting diodes (660 nm) giving a light dose of 6.2 J cm^{-2} or alternatively foil-covered to provide dark controls. From each well showing an inhibition of growth of the micro-organism, 1 μl was sub-cultured on nutrient agar, using the Miles-Misra method, and incubated for 18 hours at 37 °C. The minimum bactericidal concentrations were then determined as the lowest concentration for each photosensitiser giving no bacterial growth.

The candidate compound performing best in this initial screen (2d) was then tested further against the following clinically relevant organisms: *Enterococcus faecalis* (NCIMB 13280), *Proteus mirabilis* (NCIMB 5887) and a clinical strain of *Klebsiella pneumoniae* (isolated at the Clatterbridge Hospital, UK), using the same culture methodology. Antifungal screening was carried out similarly against the yeast *Candida albicans* (NCPF 8179), using Sabouraud broth and agar.

3. Results and Discussion

3.1 Photosensitisers

A series of phenothiazinium derivatives having dialkylamino-type substitution was produced as expected, the maximum wavelength of absorption in aqueous media being in line with that of previous series of methylene blue analogues, in the region of 660-670 nm [14-16]. This is simply explained, given the reactivity of the secondary amino function present in the diamines and triamines employed in the syntheses, i.e. the resulting products were, effectively,3,7-bis(dialkylamino)phenothiazinium derivatives, similar to methylene blue. Pure compound yields were low (20-30%), again in line with previous work employing halogen oxidation protocols [14-16]. Attempts to extend the series utilising primary alkylamino reactants were unsuccessful using the same protocols (data not included), the reactants being recovered unchanged. Each of the products reported was purified by column chromatography on silica gel.

The compounds examined in the present study may be broadly classified via substitution pattern and degree. Thus the derivatives might be symmetrically or unsymmetrically substituted with respect to both auxochromic moieties and each of these groups might be abasic, mono- or dibasic, depending on the number of amino termini so possessed. While all of the compounds are at least permanently monocationic due to the delocalised chromophoric charge (i.e. as for methylene blue), the basicity of the free tertiary amino moieties in the various side chains means that additional, though localised, cationic sites would be formed by ionisation (*N*-protonation) in aqueous media. Thus compounds 2a-c might be considered as dicationic, compounds 1c and 2d tricationic and compounds 1a and 1b pentacationic (e.g. Figure 2).

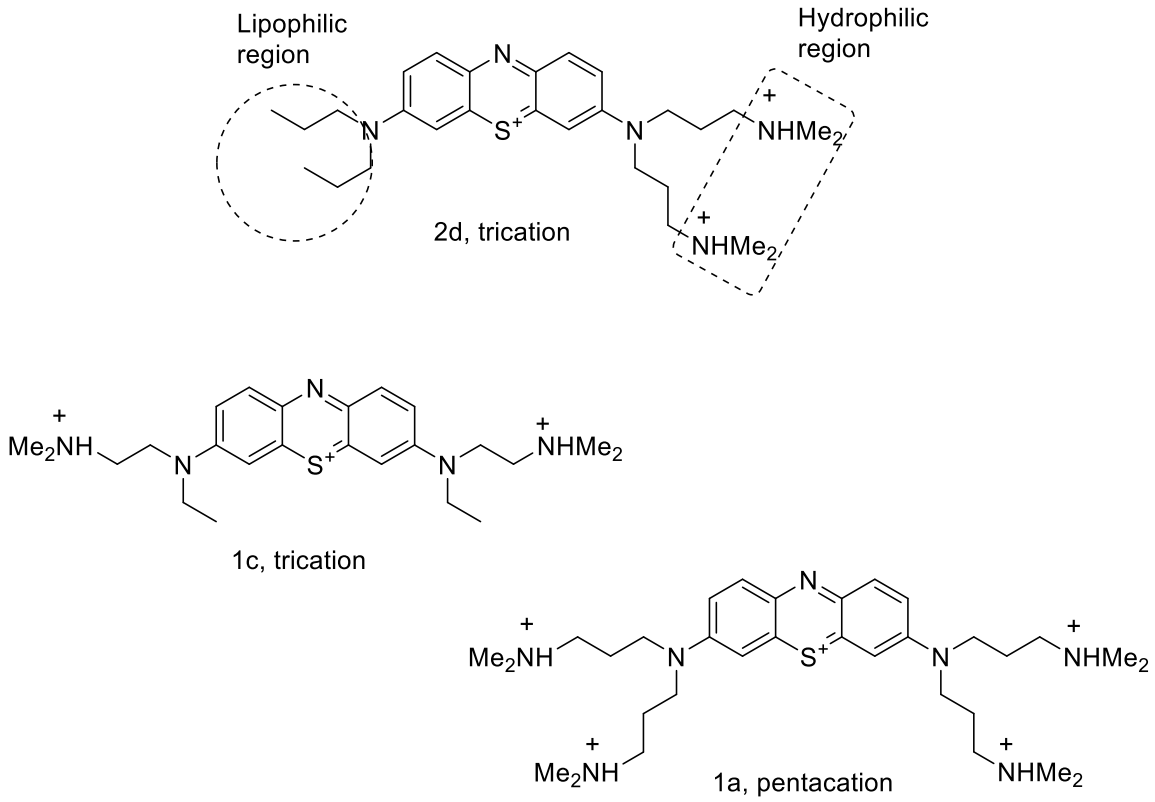


Figure 2. Polycationic forms of the derivatives.

All of the derivatives produced singlet oxygen in the standard spectrophotometric cyclopentadienone oxidation screen (Table 1), although, unsurprisingly, *in vitro* yields did not correlate with data resulting from the antimicrobial screening. Generally the derivatives exhibited increased lipophilicities (i.e. more positive Log P values compared to the parent compound, methylene blue, Table 1), which were generally concomitant with increased hydrocarbon content in the auxochromic function, while the presence of the maximum four ionisable dialkylamino termini (e.g. compound 1b, Log P +0.11) provided the most hydrophilic examples of the current series.

Compound	λ_{max} (nm, MeOH)	Log P	Relative yield of singlet oxygen
MB	656	-0.10	1.0
1a	668	+0.21	0.6
1b	667	+0.11	0.3
1c	662	+0.76	0.2
2a	660	+1.25	0.4
2b	665	+1.09	0.3
2c	652	+0.74	1.4
2d	661	+0.57	0.2
DMMB	648	+1.11	1.2

Table 1. Measured physicochemical and photoproperties for the new photosensitisers and comparators employed in the study

3.1 Antimicrobial efficacy

As noted above, although it is used in several clinical photodynamic protocols, methylene blue is not an ideal photoantimicrobial candidate. While the relatively high MBC values exhibited against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) (Figure 3) are offset by a known lack of human toxicity, its dark/light differential is also low. Most of the compounds tested in the current study were much more efficient than the parent compound in cell killing. The known analogue dimethyl methylene blue was included as an established, more powerful photosensitiser.

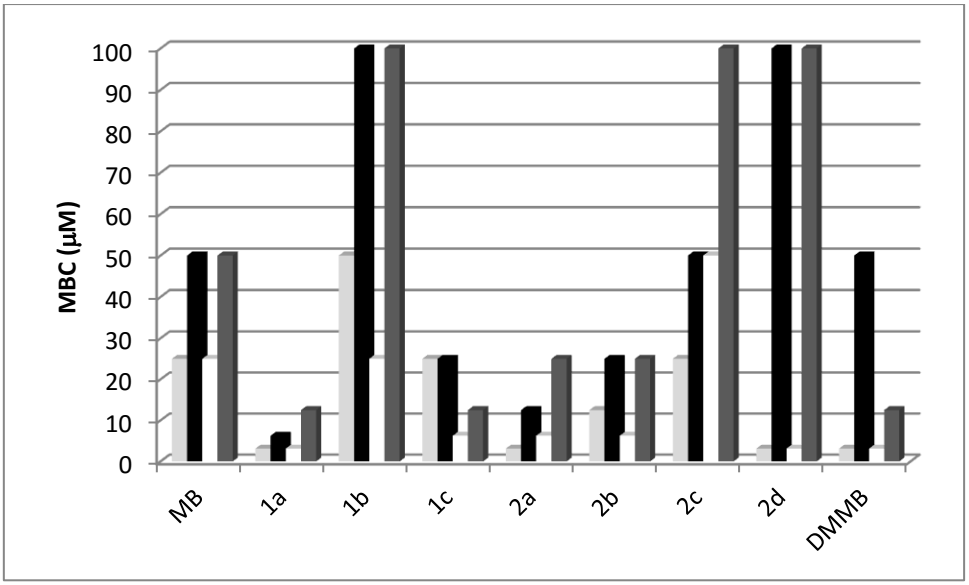


Figure 3. Photobactericidal data for methylene blue, dimethyl methylene blue (DMMB) and the derivatives. MBC = minimum bactericidal concentration giving total bacterial kill. Key: activity against *Staphylococcus aureus*, pale grey (+ light), black (dark); activity against *Escherichia coli*, white (+ light), dark grey (dark).

Among the synthesised series of analogues, noticeably, the established antimalarial derivative, paludenblau (2c), was a weak photobactericidal agent, similar to methylene blue as suggested above, despite being the most efficient producer of singlet oxygen in the spectrophotometric assay (Table 1). This suggests a lack of uptake and/or interaction with the bacterial target. However, asymmetrical compounds of similar pattern but having greater alkyl content were highly effective against both Gram-types used in the initial screen (Figure 3), although with variable dark toxicities, indeed having similar dark:light ratios to those of methylene blue and dimethyl methylene blue.

Conversely, compound 2d exhibited the lowest minimum photobactericidal concentration (≤ 3.125 μM) combined with the highest dark toxicity (≥ 100 μM) in the standard antibacterial screen. Given this activity, it was then tested against three further relevant bacterial strains and the yeast, *Candida albicans*. It should be noticed that two of the bacterial strains, *Klebsiella pneumoniae* and *Enterococcus faecalis*, have particular clinical significance in terms of their common involvement in conventional-resistant infectious disease, such as hospital-acquired pneumonia and urinary tract infections, respectively [17,18]. Compound 2d was highly active against each of these follow-on strains (e.g. MBC vs. *E. faecalis* = 0.19 μM), and again exhibited no measurable dark toxicity (Table 2). It is emphasised that 2d exhibited excellent photoanti*microbial* activity, given the similar high efficacy exhibited against the yeast challenge.

	E. faecalis		*P. mirabilis*		*K. pneumoniae*		*C. albicans*	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
2a	0.19	100	0.39	100	3.13	100	3.13	100

Table 2. Antimicrobial activity for compound 2d. Minimum antimicrobial concentration (in μM) giving total kill.

In terms of the ionisation pattern discussed above, compound 2d, being potentially tricationic (Figure 2), exhibits one which has been shown to be active by several groups [19,20]. Thus the molecule has recognisable lipophilic and hydrophilic regions which may aid in orientation in membranous regions in the microbial cell. The tricationic example 1c has a different (symmetrical) distribution of positive charge, as is the case also for the potentially pentacations formed by compounds 1a and 1b (Figure 2). The lower dark:light toxicity ratio for these derivatives suggests that – if the target is a membrane – they are more generally destructive in terms of membrane structural integrity, whereas compound 2d causes much greater damage, but only on illumination, across the range of microbial targets.

4. Conclusion

A range of new derivatives of methylene blue, having basic functionality in the side chain, have been synthesised and tested. The presence of functional groups other than hydroxyl (HO-) is relatively unusual in phenothiazinium derivatives. While most of the series were, as expected, more active than the parent compound, methylene blue, several of the new analogues were also more active than dimethyl methylene blue, a powerful photosensitiser which has been suggested as a replacement for methylene blue itself in clinical contexts [21], but which should not be employed due to the high mammalian toxicity reported from *in vitro* studies [22]. The large differential dark:light toxicity shown by the asymmetric compound 2d throughout the current study is most encouraging in this respect, but this will obviously require further testing/analogue comparison to allow significant further progress.

References

[1] M. Wainwright, K.B. Crossley, Methylene blue – a therapeutic dye for all seasons? J. Chemother. 14 (2002) 431-443.

[2] P. Ehrlich, P. Guttmann, Ueber die Wirkung des Methylenblau bei Malaria, Berlin. Kiln. Woch. 28 (1891) 953-956.

[3] A. Haberkorn, A. Harder, G. Greif, Milestones of protozoan research at Bayer, Parasitol. Res. 87 (2001) 1060-1062.

[4] W. Schulemann. Synthetic anti-malarial preparations, Proc. Roy. Soc. Med. 25 (1932) 897-905.

[5] A. Albert, in A. Albert, The Acridines (2nd Ed.) Arnold 1966, pp. 434-468.

[6] R. Henschler, E. Seifried, N. Mufti, Development of the S-303 pathogen inactivation technology for red blood cell concentrates, Transfus. Med. Hemother. 38 (2011) 33-42.

[7] M. Wainwright, Phenothiazinium photosensitisers V. Photobactericidal activities of chromophore-methylated phenothiazinium salts, Dyes Pigments 73 (2006) 7-12.

[8] K.J. Mellish, R.D. Cox, D.I. Vernon, J. Griffiths, S.B. Brown, In vitro photodynamic activity of a series of methylene blue analogues, Photochem. Photobiol. 75 (2002) 392-397.

[9] S.A. Gorman, A.L. Bell, J. Griffiths, D. Roberts, S.B. Brown, The synthesis and properties of unsymmetrical 3,7-diaminophenothiazin-5-ium salts: potential photosensitisers for photodynamic therapy, Dyes Pigments 71 (2006) 153-160.

[10] Wainwright M, Meegan K, Loughran C, Giddens RM. Phenothiazinium photosensitisers VI. Photobactericidal asymmetric derivatives. *Dyes and Pigments* 2009; 82: 387-391.

[11] Pharmalucia Ltd, Compounds and methods relating thereto, WO/2010/097626.

[12] L. Cincotta, J.W. Foley, A.H. Cincotta, Novel phenothiazinium photosensitizers for photodynamic therapy, SPIE Adv. Photochemother. 997 (1988) 145-153.

[13] L. Cincotta, J.W. Foley, A.H. Cincotta, Novel red-absorbing benzo[*a*]phenoxazinium and benzo[*a*]phenothiazinium photosensitizers: in vitro evaluation, Photochem. Photobiol. 46 (1987) 751-758.

[14] M. Wainwright, K. Meegan, C. Loughran, R.M. Giddens, Phenothiazinium photosensitisers VI. Photobactericidal asymmetric derivatives, Dyes Pigments 82 (2009) 387-391.

[15] M. Wainwright, S. Brandt, A. Smith, A. Styles, K. Meegan, C. Loughran, Phenothiazinium photosensitisers VII. Novel substituted asymmetric *N*-benzylphenothiaziniums as photoantimicrobial agents, J. Photochem. Photobiol. B Biol. 99 (2010) 74-77.

[16] M. Wainwright, A. Shah, K. Meegan, C. Loughran, A. Smith, N. Valli, Phenothiazinium-fluoroquinolone drug conjugates. Int. J. Antimicrob. Agents 35 (2010) 405-409.

[17] Y.M. Ah, A.J. Kim, J.Y. Lee, Colistin resistance in *Klebsiella pneumoniae,* Int. J. Antimicrob. Agents 44 (2014) 8-15.

[18] A. Zirakzadeh, R. Patel, Vancomycin-resistant enterococci: colonization, infection, detection and treatment, Mayo Clin. Proc. 81 (2006) 529-536.

[19] P. Margaron, R. Langlois, J.E. van Lier, Photodynamic propertie of naphthosulfobenzoporphyrazines, novel asymmetric amphiphilic phthalocyanine derivatives, J Photoche. Photobiol. B Biol. 14 (1992) 187-199.

[20] W. Duan, P.C. Lo, L. Duan, W.P. Fong, D.K.P. Ng, Preparation and in vitro photodynamic activity of amphiphilic zinc(II) phthalocyanines substituted with 2-(dimethylamino)ethylthio moieties and their N-alkylated derivatives, Bioorg. Med. Chem. 18 (2010) 2672-2677.

[21] R. Yin, T. Dai, P. Avci, A.E.S. Jorge, W.C.M.A. de Melo, D. Vecchio, Y.Y. Huang, A. Gupta, M.R. Hamblin, Light-based anti-infectives: ultraviolet C irradiation, photodynamic therapy, blue light and beyond, Curr. Op. Pharmacol. 13 (2013) 731-762.

[22] M. Wainwright, D.A. Phoenix, L. Rice, S.M. Burrow, J.J. Waring, Increased cytotoxicity and photocytotoxicity in the methylene blue series via chromophore methylation, J. Photochem. Photobiol. B: Biol. 40 (1997) 233-239.