Dyes and Pigments 82 (2009) 387–391

Contents lists available at ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

Phenothiazinium photosensitisers, Part VI: Photobactericidal asymmetric derivatives

Mark Wainwright [a,*], Katie Meegan [a], Ciara Loughran [a], Richard M. Giddens [b]

[a] School of Pharmacy & Biomolecular Science, Liverpool John Moores University, Liverpool L3 3AF, United Kingdom
[b] Centre for Materials Science, UCLan, Preston PR1 2HE, United Kingdom

ARTICLE INFO

Article history:
Received 11 February 2009
Received in revised form
24 February 2009
Accepted 25 February 2009
Available online 9 March 2009

Keywords:
Asymmetric
Methylene blue
Phenothiazinium derivatives
Photosensitiser
Photoantimicrobial

ABSTRACT

The synthesis of derivatives of Methylene Blue (C.I. Basic Blue 9) has generally employed symmetrical and non-symmetrical dialkylamine functionality in the auxochromic C-3 and C-7 positions of the chromophore. In the present work asymmetric derivatives were synthesised having dialkylamino groups at position 3 and either arylamino or aralkylamino groups at position 7, of the phenothiazinium ring. Physicochemical testing of the derivatives showed that the λ_{max} and ε_{max} values of the asymmetrical derivatives having arylamine substitution were very close to those of the symmetrical bis(dialkylamino) analogues but that the singlet oxygen yields were minimal, in line with previously published work concerning symmetrical bis(arylamino) derivatives. Synthesised asymmetric analogues having benzylamino or cyclohexylamino, rather than arylamino-substitution exhibited restored singlet oxygen generation. As expected, in screening tests against Gram-positive and Gram-negative bacteria, the aralkylamino and cyclohexylamino derivatives were highly active on illumination, presumably via singlet oxygen damage. However, the asymmetric arylamino derivatives were similarly photobactericidal, possibly due to molecular rigidification of these derivatives in the cellular milieu. Considerably increased activity was observed in each class relative to that of the standard methylene blue. In addition, the more lipophilic derivatives exhibited greater activity against *Escherichia coli*. This may be due to increased interaction with the lipid-rich outer membrane of this Gram-negative organism.

1. Introduction

Phenothiazinium derivatives, particularly *Methylene Blue* (C.I. Basic Blue 9) and *Toluidine Blue O* (C.I. Basic Blue 17) (Fig. 1), have been widely used in recent photoantimicrobial research [1–3]. C.I. Basic Blue 9 is itself used in the photodecontamination of blood plasma [4] while toluidine blue has been proposed for use in oral photodisinfection [5]. This reflects the long use and clinical safety of these compounds. However, as with other areas of antimicrobial research, there is a requirement for novel structures with improved activity/toxicity profiles. Novel derivatives based on both symmetrical and asymmetrical dialkylamine substitution have been published [6,7] although there has been no systematic comparison of the relative photobactericidal effects of resulting series. Indeed, there has been little imaginative design beyond the inclusion of simple alkyl or hydroxyalkyl moieties at the auxochromic sites.

The inclusion of pendant aromatic groups in phenothiazinium-based molecules is appealing with respect to the present drug design/discovery programme, due to the greater potential for functionalisation allowed by the aromatic moiety compared to the alkyl groups employed in most of the previous work in auxochromic variation. For example, symmetrical candidates synthesised earlier in the programme contained aryl pendants with a variety of atoms/groups attached, thus allowing heavy atom or reactive group inclusion [8]. However, none of these compounds produced measurable levels of singlet oxygen during *in vitro* chemical testing. As expected, the inclusion of two aryl groups per molecule also led to significant increases in lipophilicity and, indeed, to aqueous insolubility.

Previous work involving the related benzo[*a*]phenoxazinium series – analogues of *Nile Blue* (C.I. Basic Blue 12) – demonstrated that the inclusion of *N*-aryl moieties, e.g. phenyl or 4-tolyl, furnished compounds of greater antibacterial activity (Fig. 2) [9].

While the extension of the investigation with benzo[*a*]phenothiazinium derivatives produced weaker antibacterial candidates, the primary aim of the work was to produce conventional candidate drugs for the treatment of tuberculosis and thus the use of photoactivation was not investigated at the time.

* Corresponding author. Tel.: +44 0151 231 2039; fax: +44 0151 231 2170.
E-mail address: mark_wainwright@hotmail.com (M. Wainwright).

0143-7208/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dyepig.2009.02.011

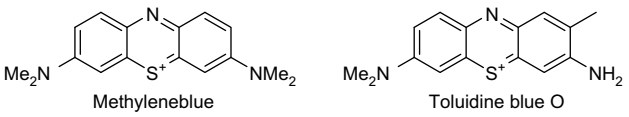


Fig. 1. Lead compounds for phenothiazinium drug development.

The current work utilises phenothiazinium synthesis via an asymmetric approach in an attempt to balance the increased drug design opportunity associated with pendant aromatic substitution with the singlet oxygen production necessary for Type II photodynamic action. In order to examine the practical use of the resulting drug candidates, photoactivity is also presented in terms of photoantibacterial efficacy against standard Gram-positive and Gram-negative organisms. The long-term aim of the work is to provide clinically useful photoantimicrobial agents for use in topical antisepsis and other such applications in healthcare infection control.

2. Experimental

10*H*-Phenothiazine, iodine, dialkylamines, arylamines and solvents were purchased from Sigma–Aldrich, UK, and used without further purification. Photophysical characterisation of the products was carried out using a Hewlett Packard 8452A diode array spectrophotometer. This was also used for the determination of lipophilicity (below). Accurate molecular ion masses were obtained using a Micromass LCT TOF mass spectrometer.

2.1. Synthesis

2.1.1. Phenothiazin-5-ium tetraiodide

10*H*-Phenothiazine (2 g, 10 mmol) was dissolved in 50 ml of dichloromethane at room temperature. A solution of iodine (8 g, mmol) in dichloromethane (150 ml) was added, and the whole stirred for 3 h 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%.

2.1.2. 3-Dialkylaminophenothiazinium triodides

Phenothiazinium tetraiodide (2.15 g, 3 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 min. The reaction was allowed to stir at room temperature for a further 3 h, monitored by thin-layer chromatography on silica gel (3% aqueous NH_4OAc/CH_3OH 1:17). The solution was allowed to stand overnight and the resulting black solid filtered off and washed with cold methanol. Solid products were recrystallised from methanol.

2.1.3. 3,7-Bis(dialkylamino)phenothiazinium iodides

Solid phenothiazin-5-ium tetraiodide (1.00 g, 1.4 mmol) was added in one amount to a solution of the requisite dialkylamine (14 mmol) in dichloromethane (100 ml). The resulting solution was allowed to stir at room temperature for 3 h, then extracted three times with 5% w/v hydroiodic acid (100 ml) and three times with water (100 ml). Post-extraction, the organic layer was dried over sodium sulphate, concentrated to a low volume and precipitated with dry diethyl ether. Reprecipitation was carried out until spectrophotometric analysis gave a peak ratio (λ_{660}:λ_{290}) of >2.2. Compounds impure by thin-layer chromatography at this stage were chromatographed on silica gel (Fisher Scientific, UK) using gradient elution in dichloromethane/methanol.

2.1.4. 3-Dialkylamino-7-arylaminophenothiazinium iodides

To a suspension of 3-dialkylaminophenothiazinium triiodide (0.75 mmol) in methanol (10 ml) was added dropwise the requisite arylamine (1.8 mmol) in 10 ml methanol and the reaction monitored by TLC, as above. Reaction times were in the region of 1.5–2 h. Products were isolated by evaporation of the methanol, redissolution in dichloromethane, extraction with 5% v/v hydroiodic acid then water, drying of the organic layer over anhydrous sodium sulphate, evaporation to a small volume and repeat precipitations in dry diethyl ether. Compounds impure by thin-layer chromatography at this stage were chromatographed on silica gel (Fisher Scientific, UK) using gradient elution in dichloromethane/methanol.

Synthetic yields and analytical data for the derivatives are given in Table 1.

2.2. Singlet oxygen testing

Singlet oxygen production by the photosensitisers was assayed as in previous work [10], except that the decolourisation of 2,3,4,5-tetraphenylcyclopentadienone (TPCPD) in dichloromethane was employed rather than that of 1,3-diphenylbenzisofuran in methanol. Thus the decrease in absorption at 500 nm was monitored spectrophotometrically with time, using methylene blue as a standard photosensitiser. 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 ($t_{1/2}$MBD) thus gives a measure of its photosensitising efficiency. Thus, if the time for the DPIBF absorption to decrease by 50% due to Methylene Blue (MB) photosensitisation is $t_{1/2}$MB, relative singlet oxygen yields for the derivatives are given by:

$$\textit{Relative } {}^1O_2 \textit{ yield} = \frac{t_{1/2}MB}{t_{1/2}MBD}$$

i.e. the lower the $t_{1/2}$ value for the derivative, the greater its $^1O_2$ yield.

2.3. Lipophilicity (*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 [11] based on the relationship:

$$\log P = \log\left\{\frac{\left(A - A^1\right)}{A^1} \cdot \frac{V_w}{V_o}\right\}$$

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.

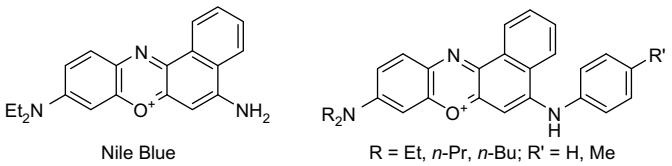
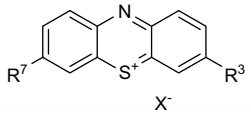


Fig. 2. Nile blue and *in vitro* antitubercular derivatives.

Table 1
Analytical data for the derivatives.



	R^3	R^7	X^-	m/z[a]		% Yield	λ_{max}[b] (nm)	log ε_{max}[b]
				Calc.	Found			
	NMe_2	NMe_2	Cl^-	–	–	–	656	4.88
1a	NEt_2	H	I_3^-	269.11	269.11	43	580	4.29
2a	NEt_2	NEt_2	I^-	340.18	340.18	23	667	4.87
3a	NEt_2	NHC_6H_5	I^-	360.15	360.15	29	656	4.77
3c	NEt_2	NH-4-C_6H_4Me	I^-	374.17	374.17	36	656	4.29
1b	NPr^n_2	H	I_3^-	297.14	297.14	40	580	4.21
2b	NPr^n_2	NPr^n_2	I^-	396.25	396.24	28	672	4.75
3b	NPr^n_2	NHC_6H_5	I^-	388.18	388.18	32	656	4.82
3d	NPr^n_2	NH-4-C_6H_4Me	I^-	402.20	402.20	33	656	4.65
4a	NEt_2	$NHCH_2Ph$	I^-	374.17	374.18	30	644	4.62
4b	NPr_2	$NHCH_2Ph$	I^-	402.20	402.22	32	646	4.65
4c	NEt_2	$NHCH_2$-4-C_6H_4Me	I^-	388.18	388.18	37	648	4.63
4d	NPr_2	$NHCH_2$-4-C_6H_4Me	I^-	416.22	416.22	35	654	4.67
5a	NEt_2	NHC_6H_{11}	I^-	366.54	366.55	21	650	4.72
5b	NPr_2	NHC_6H_{11}	I^-	394.22	394.23	19	652	4.78
6a	NHC_6H_5	NHC_6H_5	I^-	380.12	380.11	–	660	4.57
6b	NH-4-C_6H_4Me	NH-4-C_6H_4Me	I^-	408.15	408.15	–	662	4.73

[a] By ICP-MS.
[b] Measured in MeOH.

2.4. Antibacterial screening

The photobactericidal efficacies of the derivatives in addition to those 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 h at 37 °C in microtitre trays with various concentrations of photosensitiser ranging from 100 to 3 μM, with zero photosensitiser concentrations in each case for control purposes. The trays were then either illuminated for 20 min using an array of 126 light-emitting diodes (660 nm) giving a light dose of 6 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 h at 37 °C. The minimum bactericidal concentrations were then determined as the lowest concentration for each photosensitiser giving no bacterial growth. Each test was repeated to ensure an absolute value for the cited MBC with $n = 6$. Due to the absolute nature of the assay, i.e. complete absence of growth, rather than fractional kill, no statistical treatment of the resulting data was applied.

3. Results and discussion

Synthesis of the 3-dialkylaminophenothiazinium iodides was straightforward, being an established process [7], and the addition of the arylamino groups proceeded as with previous work [8], yielding the target compounds in reasonable yields and without complex separation protocols. Structure development for the current work is shown in Fig. 3, and the overall reaction scheme in Fig. 4.

As with earlier symmetrical bis(arylamino) compounds (e.g. **6a** and **6b**), the λ_{max} values for the asymmetrical dialkylamino-/arylamino derivatives were close to those of C.I. Basic Blue 9 (ca. 660 nm), underlining the significant electronic effect of the arylamino auxochrome on light absorption (Table 1). Obviously, such chromophore extension is not seen, for example, where one dimethylamino group in C.I. Basic Blue 9 is replaced by methylamino (i.e. MB → Azure B, C.I. 52010), and a hypsochromic shift of approximately 20 nm is observed relative to the lead compound. Although it had been suggested that compounds halfway between the bis(dialkylamino)- and bis(arylamino)phenothiaziniums would maintain singlet oxygen yields alongside increased potential for peripheral drug design – utilising substitution patterns in the pendant aryl group – the presence of a single arylamino moiety was found to be sufficient to decrease the singlet oxygen yield to insignificance in the spectrophotometric assay (compounds **3a**–**d**, Table 1). Possibly the aryl groups employed, being directly attached to an auxochromic nitrogen, allowed increased deactivation of the excited chromophore via a twisted intramolecular charge transfer (TICT) mechanism, as has been suggested for similar systems, such as those based on the benzo[*a*]phenothiazinium [12] and triarylmethane chromophores [13].

Beside the arylamine derivatives, the symmetrical phenothiazinium salts, i.e. the tetraethyl and tetrapropyl derivatives of methylene blue (**2a** and **2b** respectively), behaved in typical photosensitiser fashion, although with lower singlet oxygen yields than the parent compound, in agreement with data reported by Mellish et al. [6]. Also as expected, the increase in alkyl group size led to increased lipophilicity (Table 2). Unsurprisingly, the spacing of the chromophore and aryl moieties using a methylene (benzylic) group restored the ability to produce singlet oxygen *in vitro* in compounds **4a**–**4d**. Similarly, compounds **5a** and **5b**, being direct analogues of **3a** and **3b**, with the pendant *N*-phenyl moieties replaced by *N*-cyclohexyl, also produced singlet oxygen in the spectrophotometric assay (Table 1).

Bacterial screening of the series against *S. aureus* and *E. coli*, as standard representatives of the Gram-positive and Gram-negative bacterial classes respectively, demonstrated that the apparent loss of photosensitising activity in the *N*-aryl derivatives pertained only *in vitro*, since differential light/dark bactericidal effects were exhibited in practice (Table 2). Thus it may be that the proposed TICT relaxation mechanism in solution was not possible in cellular media, and this can be explained on the grounds of biomolecular

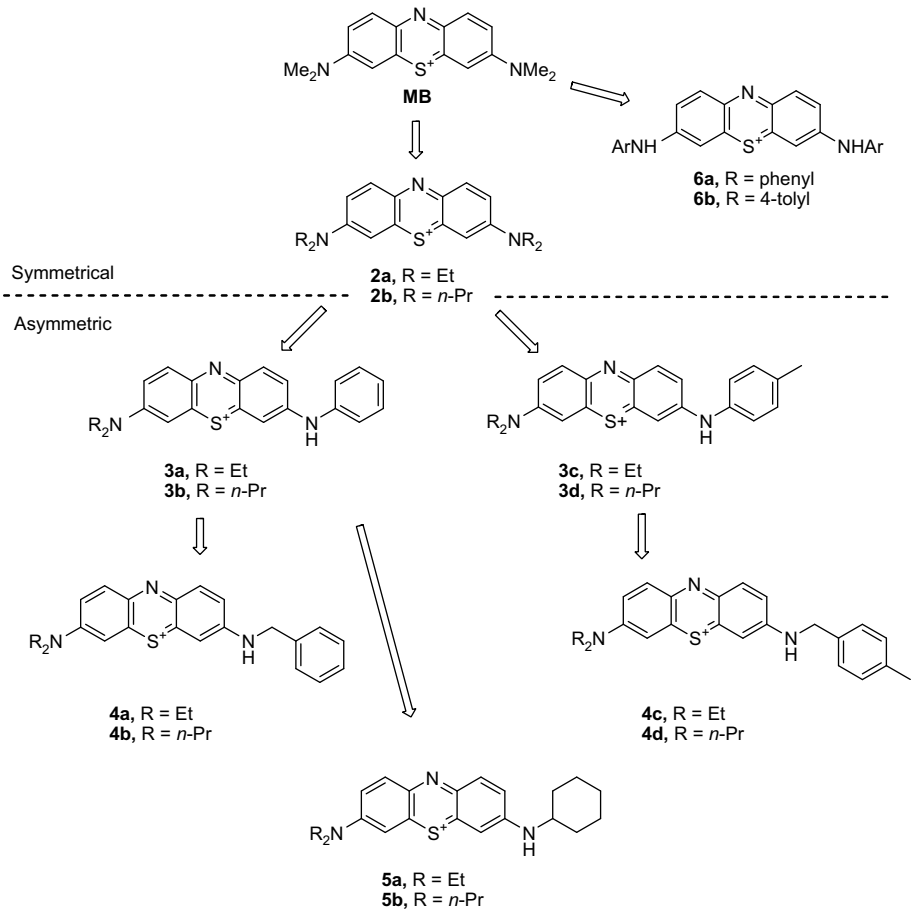


Fig. 3. Phenothiazinium structure development.

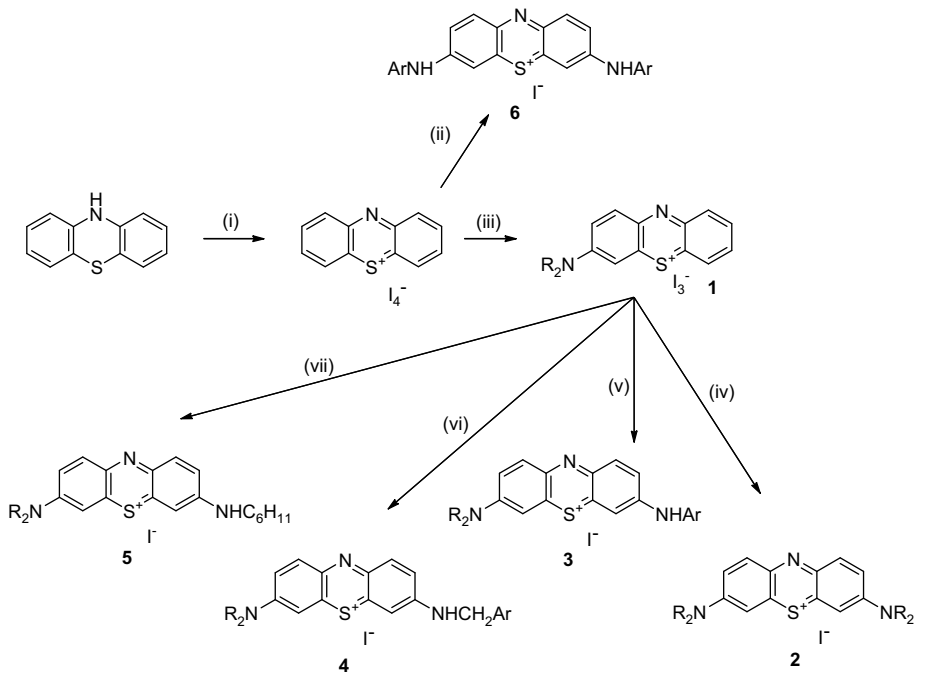


Fig. 4. Stepwise synthesis of symmetrical and asymmetrical phenothiazinium derivatives. (i) I_2, $CHCl_3$, r.t.; (ii) $ArNH_2$, $CHCl_3$, r.t. (iii) NHR_2 (0.5 equiv), MeOH, r.t.; (iv) NHR_2, MeOH, r.t.; (v) $ArNH_2$, MeOH, r.t.; (vi) $ArCH_2NH_2$, MeOH, r.t.; (vii) cyclohexylamine, MeOH, r.t.

Table 2
Antibacterial data for the derivatives.

Compound	MBC[a] (μM)				Rel. $^1O_2$[b]	log *P*
	S. aureus		*E. coli*			
	Light	Dark	Light	Dark		
MB	25	100	25	>100	1.00	−0.1
2a	6.25	100	6.25	100	0.55	+0.8
3a	12.5	>100	6.25	25	[c]	+1.6
3c	25	100	25	>100	[c]	+1.7
2b	≤3.13	50	6.25	>100	0.59	+1.1
3b	12.5	25	≤3.13	100	[c]	+1.9
3d	12.5	>100	≤3.13	>100	[c]	>2.0
4a	6.25	>100	6.25	>100	0.69	>2.0
4b	12.5	100	≤3.13	100	0.47	>2.0
4c	6.25	50	6.25	100	0.57	>2.0
4d	12.5	>100	≤3.13	>100	0.38	>2.0
5a	12.5	>100	≤3.13	>100	0.72	+1.8
5b	12.5	100	6.25	50	0.84	+1.8
6a	12.5	50	12.5	50	[c]	ND
6b	12.5	12.5	50	100	[c]	ND

[a] Minimum bactericidal concentration.
[b] Yield of singlet oxygen relative to that of MB.
[c] $^1O_2$ measurement ≤2% of that of MB; ND – not determined.

binding of the phenothiazinium derivatives, leading to molecular rigidification. Similar effects have been reported for triarylmethane compounds such as crystal violet [14]. Since the *N*-aralkyl derivatives (**4a**–**d**) used in the present study demonstrated singlet oxygen production *in vitro* and photoactivity in the cellular challenge, it may be assumed that the TICT mechanism is not a significant relaxation factor in these compounds. The utility of aromatic ring inclusion in the auxochromic group, in terms of increasing the range of drug design, is thus justified, whether the aryl moiety is attached to the auxochromic nitrogen directly or via a spacer. However, the previously reported 3,7-bis(arylamino) derivatives (**6a**, **6b**) exhibited only slightly increased activity on illumination (Table 2).

In comparison to the lead compound, methylene blue, almost all of the new derivatives were better photobactericidal agents against both organisms (Table 2). As the singlet oxygen yields for the derivatives were either poorer than the lead compound or non-measurable (Table 2), this probably indicates improved bacterial uptake for the derivatives, or more critical targeting. In addition, the Gram-negative bacterium *E. coli* was generally more susceptible to the derivatives than was *S. aureus*. This may reflect the greater hydrophobicity of the derivatives (positive log *P* values, Table 2) – resulting from increased hydrocarbon content – since it is well known that Gram-negative organisms have a lipid-rich outer membrane.

Considering the dark toxicity data, the new derivatives exhibited similar effects to those of methylene blue, most examples having low toxicity, with no discernible structural correlation (Table 2). This is in line with the landmark study by Crossley, Clapp and co-workers wherein a large number (ca. 120) of benzo[*a*]phenoxazinium salts were synthesised and assayed in mice for activity against *Mycobacterium bovis*, a model for the tuberculosis bacterium, *Mycobacterium tuberculosis* [9]. Approximately one quarter of the derivatives tested exhibited higher activity against *M. bovis* than the standard therapeutic, streptomycin. However, it was noticeable that there was no significant difference in activity between derivatives with similarly substituted aromatic rings whether the ring was directly attached to the auxochromic amino nitrogen or separated from it by a methylene spacer. In addition, it was reported that identically-substituted benzo[*a*]phenothiazinium derivatives were much less active against *M. bovis* than the corresponding benzo[*a*]phenoxazinium analogues [15]. Again, it should be emphasised that the Crossley study was aimed at conventional therapeutic development, and not photobactericides.

4. Conclusion

The current study thus proves, again, that it is possible to synthesise relatively simple derivatives of the lead compound methylene blue which are more active as photobactericidal agents and which exhibit low inherent (dark) toxicities. In the present case, the inclusion of pendant aromatic moieties also increases the potential for novel photosensitiser design, via non-chromophoric functionalisation. Such functionalisation may be carried out in order to tailor the physicochemical profile of the resulting molecule whilst conserving photosensitising capabilities.

The positive results from the current work are being followed up by similar studies on the photoproperties of candidates having inclusion of various groups within the pendant aromatic moieties.

References

[1] Wainwright M. The development of phenothiazinium photosensitisers. Photodiagnosis and Photodynamic Therapy 2005;2:263–72.
[2] Wagner SJ, Skripchenko A, Robinette D, Foley JW, Cincotta L. Factors affecting virus photoinactivation by a series of phenothiazine dyes. Photochemistry and Photobiology 1998;67:343–9.
[3] Zeina B, Greenman J, Purcell WM, Das B. Killing of cutaneous microbial species by photodynamic therapy. British Journal of Dermatology 2001;144:274–8.
[4] Lambrecht B, Mohr H, Knuever-Hopf J, Schmitt H. Photoinactivation of viruses in human fresh plasma by phenothiazine dyes in combination with visible light. Vox Sanguinis 1991;60:207–13.
[5] Komerik N, Nakanishi H, MacRobert AJ, Henderson B, Speight P, Wilson M. In vivo killing of *Porphyromonas gingivalis* by toluidine blue-mediated photosensitization in an animal model. Antimicrobial Agents and Chemotherapy 2003;47:932–40.
[6] Mellish KJ, Cox RD, Vernon DI, Griffiths J, Brown SB. *In vitro* photodynamic activity of a series of methylene blue analogues. Photochemistry and Photobiology 2002;75:392–7.
[7] Strekowski L, Hou DF, Wydra RL, Schinazi RF. A synthetic route to 3-(dialkylamino)phenothiazin-5-ium salts and 3,7-disubstituted derivatives containing two different amino groups. Journal of Heterocyclic Chemistry 1993;30:1693–6.
[8] Wainwright M, Grice NJ, Pye LEC. Phenothiazine photosensitisers. Part II. 3,7-Bis(arylamino)phenothiazines. Dyes and Pigments 1999;42:45–51.
[9] Crossley ML, Turner RJ, Hofmann CM, Dreisbach PF, Parker RP. Chemotherapeutic dyes. II. 5-Arylamino-9-dialkylaminobenzo[*a*]phenoxazines. Journal of the American Chemical Society 1952;74:578–84.
[10] Wainwright M. Phenothiazinium photosensitisers V. Photobactericidal activities of chromophore-methylated phenothiazinium salts. Dyes and Pigments 2006;73:7–12.
[11] Pooler J, Valenzo DP. Physicochemical determinants of the sensitizing effectiveness for photooxidation of nerve membranes by fluorescein derivatives. Photochemistry and Photobiology 1979;30:491–8.
[12] Cincotta L, Foley JW, Cincotta AH. Novel phenothiazinium photosensitizers for photodynamic therapy. SPIE Advance in Photochemotherapy 1988;997:145–53.
[13] Nair V, Thomas S, Mathew SC, Abhilash KG. Recent advances in the chemistry of triaryl- and triheteroarylmethanes. Tetrahedron 2006;62:6731–47.
[14] Dobson J, Wilson M. Sensitization of oral bacteria in biofilms to killing by light from a low-power laser. Archives of Oral Biology 1992;37:883–7.
[15] Clapp RC, English JP, Fellows CE, Forsythe J, Grotz RE, Shepherd RG. Chemotherapeutic dyes. V. Benzo[*a*]phenothiazines and benzo[*a*]phenazines. Journal of the American Chemical Society 1952;74:1994–6.