Phenothiazinium photosensitisers XI. Improved toluidine blue photoantimicrobials.

Mark Wainwright, Ciara O'Kane, Sophie Rawthore

School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University, Byrom Street Liverpool L3 3AF, United Kingdom

Author for correspondence, Email: mark_wainwright@hotmail.com

Abstract

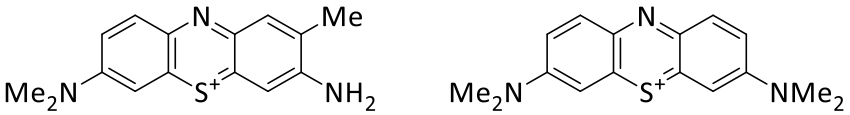
The phenothiazinium derivative toluidine blue O (TBO) is widely employed as a photoantimicrobial agent in clinical trialling, particularly in dentistry. However, its activity against a range of pathogenic microbial species is not significantly different to that of the standard photoantimicrobial methylene blue. In the current study, derivatives of TBO with varying hydrocarbon substitution in chromophore position 2 were synthesised via the established anilinethiosulphonic route, using the mild oxidant silver(II) carbonate to allow substituent preservation.

The resulting series of analogues demonstrated the expected increases in visible absorption wavelength and lipophilicity with increasing hydrocarbon content, as well as decreased aggregation for derivatives with bulkier substituents, and all produced singlet oxygen on illumination *in vitro*. Screening against a range of bacterial and fungal pathogens relevant to infection control showed remarkable increases in activity relative to the parent compound, particularly against the clinically important Gram-negative bacterium *Pseudomonas aeruginosa.* In addition, in order to demonstrate clinical relevance, the photoactivities of the new derivatives against microbial targets were compared to conventional antibacterial and antifungal drugs, as well as biocides commonly used for local disinfection. Activity here was also generally greater than that of the conventional agents used for comparison, considerably so relative to the local disinfectant agents.

Keywords: antibacterial; antifungal; disinfectant; phenothiazinium; photoantimicrobial; toluidine blue derivatives.

1. Introduction

Toluidine blue O (tolonium chloride, TBO, Figure 1) is a phenothiazinium derivative related to methylene blue (MB, Figure 1). It has been used widely in the biological arena as a stain in the pathology of both cancer and microbial disease [1,2]. More recently TBO has been widely examined as a photoantimicrobial agent aimed at oral disinfection [3,4], and is a lead compound in azine photosensitiser research and development [5].



TBO MB

Figure 1. Accepted structures for toluidine blue O and methylene blue cations.

Where comparative testing of the photoantimicrobial efficacies of MB and TBO have been carried out, little overall difference has been reported [6]. However, as the principal lead compound for photoantimicrobial research, MB has a significantly fuller pedigree in terms of the range of bacterial, fungal, viral and protozoal challenges reported [7]. Logically, both are established to interact with anionic groups at the external surfaces of the microbial target, e.g. with lipoteichoic acids [8], and to displace stabilising metal cations in membrane structures [9].

The use of TBO in biological staining often utilises the property of metachromasia. This is exhibited as a change in colour of the stained tissue due to the significant aggregation of the highly planar, substituted phenothiazinium cation [10]. As the chromophore concerned is established as a planar moiety, this infers that neither the auxochromic dimethylamino- or amino- groups, nor the methyl at position 2 of the ring system cause any deviation from this. Aggregates can be observed in the visible spectrum of aqueous TBO – and similarly for other phenothiazinium cations - as a broad shoulder to lower wavelengths extending from the λ_{max} [6].

As part of the ongoing programme of photosensitiser discovery, improved derivatives of toluidine blue were sought – particularly for photoantimicrobial analoguing - by variation of the group at C-2 of the chromophore. Previous work has demonstrated that the use of increased chromophore methylation leads to greater photoantimicrobial activity in derivatives of both methylene blue and azure A [11,12].

2. Materials & Methods

2.1 Synthesis

Substituted anilines, *p*-phenylenediamine derivatives, silver carbonate (5% w/w on celite) 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 singlet oxygen yield and of lipophilicity. Accurate molecular ion masses (m/z) for the derivatives were obtained using a Micromass LCT TOF mass spectrometer.

2-Amino-5-dimethylaminophenylthiosulphonic acid

N,N-Dimethyl-*p*-phenylenediamine sulphate (130 mmol) was added to a mechanically stirred solution of aluminium sulfate octadecahydrate/water (43.6 g, 65 mmol/100 ml). To this was added a solution of sodium thiosulfate in water (22.0 g, 139 mmol/80 ml) followed by zinc chloride in water (8.8 g, 63 mmol/12 ml). The reaction solution was cooled to 0 °C and aqueous potassium dichromate (5.0 g, 17 mmol in 20 ml water) was added dropwise over a 30 minute period. Following this addition, the mixture was allowed to stir for 2 hours. During the last 30 minutes the temperature was allowed to rise to 10 °C causing the formation of a viscous precipitate. This was isolated by filtration and washed with water followed by acetone. Yield=15.87 g (49%), m.p. 190 °C (dec.)

2-Alkyl-3-amino-7-dimethylaminophenothiazinium derivatives
2-Amino-5-dimethylaminophenylthiosulphonic acid (4 mmol) and 2-alkylaniline (5 mmol) were refluxed in 120 ml methanol and silver carbonate on celite (5 g, 50% w/w) was added slowly over 0.5 h. The reaction mixture was refluxed for a further hour, filtered through a celite pad and the filtrates evaporated. The residue was extracted with dichloromethane and purified by column chromatography on silica.

3-Amino-7-dimethylamino-2-methylphenothiazinium hydrogensulphate (toluidine blue O, 1a)
Prepared as above from 2-amino-5-dimethylaminophenylthiosulphonic acid and 2-methylaniline. Blue-black powder, yield = 460 mg, 31%; m/s $C_{15}H_{16}N_3S$ requires 270.11, found 270.11; λ_{max} (MeOH) 629 nm.

3-Amino-7-(dimethylamino)-2-ethylphenothiazinium hydrogensulphate (1b)

From 2-amino-5-dimethylaminobenzenethiosulphonic acid and 2-ethylaniline as violet-blue crystals, yield = 273 mg, 18%; m/z, $C_{16}H_{18}N_3S$ requires 284.40, found 284.42; λ_{max} (MeOH) 634 nm.

3-Amino-7-(dimethylamino)-2-*n*-propylphenothiazinium hydrogensulphate (1c)

From 2-amino-5-dimethylaminobenzenethiosulphonic acid and 2-*n*-propylaniline as blue-black powder, yield = 302 mg, 19%; m/z, $C_{17}H_{20}N_3S$ requires 298.43, found 298.40; λ_{max} (MeOH) 636 nm.

3-Amino-2-*tert*-butyl-7-(dimethylamino)phenothiazinium hydrogensulphate (1d)

From 2-amino-5-dimethylaminobenzenethiosulphonic acid and 2-*tert*-butylaniline as black powder, yield = 487 mg, 30%; m/z, $C_{18}H_{22}N_3S$ requires 312.45, found 312.41; λ_{max} (MeOH) 637 nm.

3-Amino-7-(dimethylamino)-2-phenylphenothiazinium hydrogensulphate (1e)

From 2-amino-5-dimethylaminobenzenethiosulphonic acid and 2-aminobiphenyl as dark blue powder, yield = 455 mg, 26%; m/z, $C_{20}H_{18}N_3S$ requires 332.44, found 332.41; λ_{max} (MeOH) 639 nm.

2.2 Singlet oxygen testing

Singlet oxygen production by the photosensitisers was assayed as in previous work [13], using the decolourisation of 2,3,4,5-tetraphenylcyclopentadienone (TPCPD) in dichloromethane. Thus the decrease in absorption of TPCPD 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 toluidine blue derivatives under identical conditions ($t_{½}$TBD) thus gives a measure of its photosensitising efficiency. Thus, if the time for the DPIBF absorption to decrease by 50% due to TBO photosensitisation is $t_{½}$TBO, relative singlet oxygen yields for the derivatives are given by:

$$\text{Relative } {}^1O_2 \text{ yield } = \frac{t_{1/2}\text{TBO}}{t_{1/2}\text{TBD}}$$

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 [14] based on the relationship:

$$\text{Log}P = \text{Log}\left\{\frac{(A - A^1)}{A^1} \cdot \frac{V_W}{V_O}\right\}$$

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.

2.4 Antimicrobial Screening/Comparison

The photobactericidal efficacies of the derivatives in addition to that of the known photosensitiser methylene blue were measured against both Gram positive *Staphylococcus aureus* (NCTC 6571) and *Enterococcus faecalis* (NCIMB 13280) and Gram negative *Escherichia coli* (NCTC 10418), *Proteus mirabilis* (NCIMB 5887) and a clinical strain of *Pseudomonas aeruginosa* (*P. aeruginosa*) bacteria (courtesy of the Clatterbridge Hospital, Bebington, UK) . 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

doubling dilutions from 100 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.

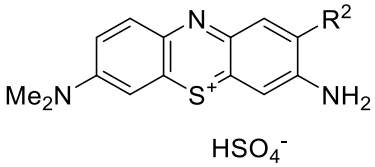
The standard antibacterial agent levofloxacin and several disinfectants (chlorhexidine digluconate, cetyltrimethylammonium bromide, benzalkonium chloride and Triclosan, purchased from Sigma-Aldrich, Gillingham UK) were screened for activity in the same way as above.

Antifungal screening was carried out similarly against the yeast *Candida albicans* (NCPF 8179), using Sabouraud broth and agar. The standard antifungal agent fluconazole (Sigma-Aldrich, Gillingham, UK) was included in this test for comparison.

3. Results & Discussion

3.1 Photosensitisers

Toluidine blue and its analogues were synthesised in straightforward manner, in low-moderate yields (around 30%), and normally requiring purification by column chromatography.
Given the importance of photosensitiser action wavelength in avoiding endogenous light absorption – typically by blood – the derivatives exhibited the expected increases in λ_{max} with electron release of the groups added at C-2 of the phenothiazinium chromophore (Table 1).



R^2	λ_{max} (nm, MeOH)	$^1O_2$ yield*	LogP
Me	629	1.00	-0.20†
Et	634	1.49	-0.46
n-Pr	636	1.24	+0.29
t-Bu	637	1.79	+0.84
Ph	639	1.87	+0.63

Table 1. Relevant properties for the toluidine blue derivatives (* relative to toluidine blue; †Lit. -0.21[15])

Similarly, the LogP values of the derivatives increased, as expected, with hydrocarbon content (Table 1), with most resulting compounds being in the range normally considered to be amphiphilic, and all retaining water solubility.

All derivatives were shown to produce singlet oxygen in reasonable yields in the *in vitro* test (Table 1), although this may not reflect activity against target cells, since this is dependent on the eventual cellular environment / biomolecular binding of the photosensitiser.

Inspection of the visible spectra of the alkyl toluidine blue derivatives revealed a lessening of the lower-wavelength shoulder to the main peak absorption in the red region with increasing alkyl group size at C-2 (TBO and the *t*-butyl derivative shown in Figure 2). As noted, the λ_{max} was shifted with increasing hydrocarbon group size, via hyperconjugation, as expected. The decreased shoulder with increased alkyl size indicates a drop in aggregational behaviour, logically due to the difficulty in packing caused by the extra asymmetrical volume incurred. This volume change is demonstrated in the energy-minimised, space-filling models of TBO and its 2-*tert*-butyl derivative in Figure 3. However, the 2-phenyl derivative demonstrated intermediate aggregation, with the shoulder more emphasised (Figure 2). While the phenyl moiety is relatively large, even compared to *tert*-butyl, it is assumed that it adopts a coplanar orientation with the phenothiazinium chromophore at higher concentrations, allowing aggregation to occur.

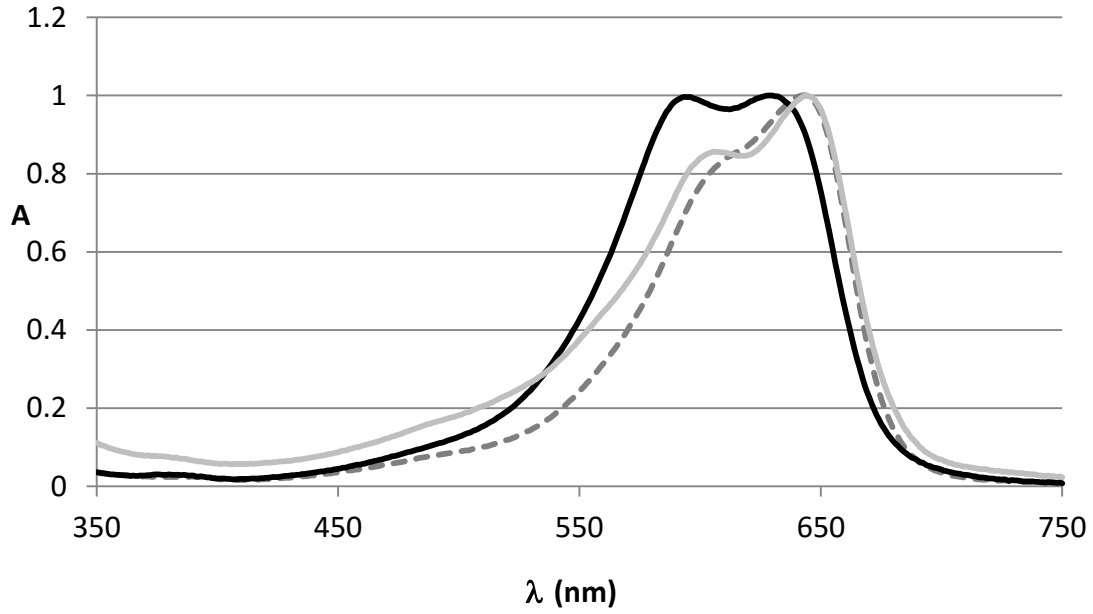


Figure 2. Aqueous visible absorption by TBO (black), *t*-BuTB (grey dashes) and PhTB (mid-grey), showing variation in the aggregational, lower wavelength, shoulder (photoantimicrobial concentration approx. 15 μM).

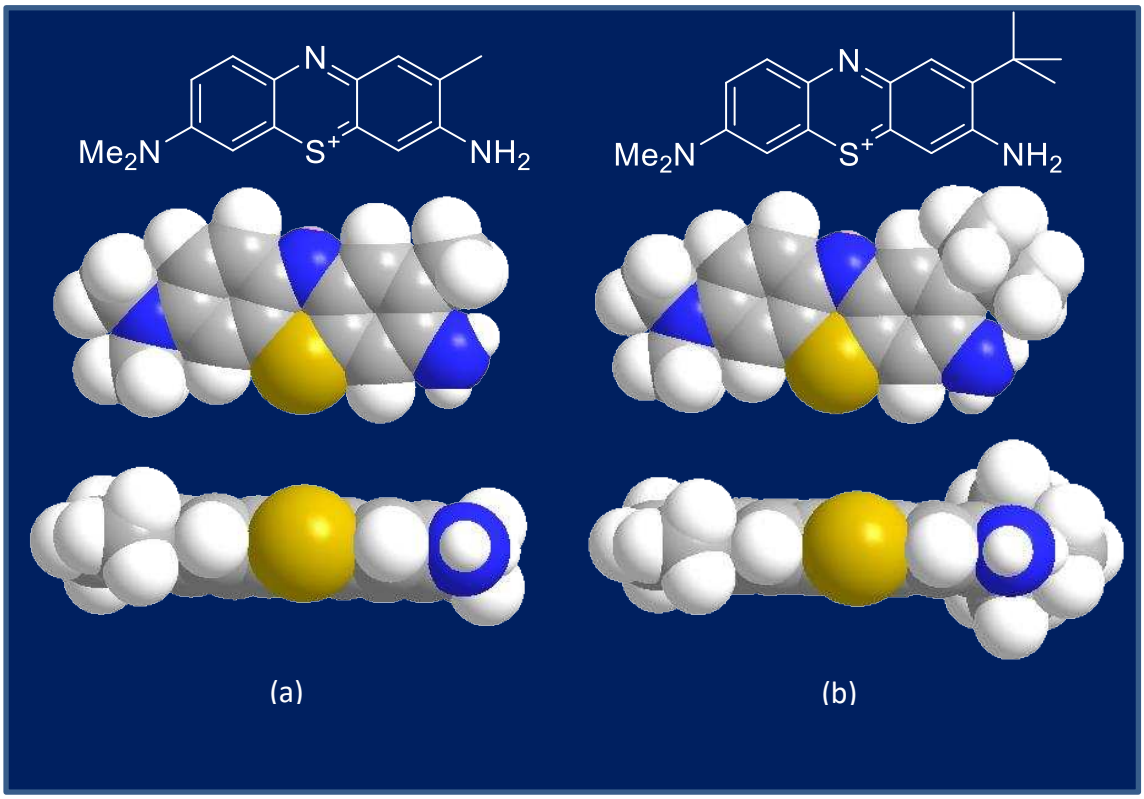


Figure 3. Increased asymmetric molecular volume due to 2-*tert*-butyl group substitution (b) compared to TBO (a) (energy minimised structures using ChemDraw 3D software).

3.2 Antimicrobial efficacy

Photoantimicrobial activity among the derivatives was first screened against the standard Gram-positive/Gram-negative bacteria *S. aureus* and *E. coli*, respectively, and the yeast *Candida albicans*. A subset were further screened against a range of clinically relevant pathogens, the 2-phenyl analogue being excluded due to solubility problems. The derivatives generally exhibited significant improvements on the lead compound, toluidine blue, but also on the principally-used photoantimicrobial compound, methylene blue (Table 2). In addition, the activity profile was not decreased against Gram-negative species, as has been widely reported in the literature for both lead compounds [16]. Such broad-spectrum potential is important given the polymicrobial nature of many clinical infections and would aid in local 'photoempirical' administration for wounds at the clinical triage stage, this being a logical application of photoantimicrobials, allowing conservation of conventional systemic drugs. The efficacy of TBO against bacteria in biofilms [17] and in trialling against suitable conditions/presentations *in vivo*, such as onychomycosis [18] and osteomyelitis [19] is also encouraging, both for clinical involvement outside dentistry and in the search for improved photoantimicrobial agents.

R^2	MBC (μM)						
	S. aureus (Gram +)	*E. coli* (-)	*Enterococcus faecalis* (+)	*Proteus mirabilis* (-)	*P. acnes* (+)	*P. aeruginosa* (-)	*Candida albicans*
Me	7.36	7.36	7.36	14.71	0.78	7.36	12.5
Et	3.13	1.47	1.35	5.50	-	3.13	3.13
n-Pr	3.13	1.32	0.33	5.70	0.39	0.78	0.78
t-Bu	3.13	3.13	0.33	3.13	0.78	0.39	3.13
Ph	3.13	3.13	-	-	-	-	0.78
MB	25	25	100	0.20	50	25	3.13
Levo	6.26	3.13	100	3.13	-	25	-
Fluc	-	-	-	-	-	-	25
BPO	-	-	-	-	56	-	-

Table 2. Photoantimicrobial activity for TBO analogues (MBC – minimum bactericidal concentration; MB – methylene blue; Levo – levofloxacin, standard antibacterial; Fluc – fluconazole, standard antifungal; BPO – benzoyl peroxide, standard topical acne treatment).

Given that interaction between the target organism and the photoantimicrobial agent is essential to successful cell kill, the improvements in activity among the derivatives may be due both to the increased lipophilicity and decreased propensity for aggregation noted above (Table 1 / Figure 2).

Comparators were included in the study to provide some indication of clinical relevance and potential and, here again, the new derivatives generally exhibited similar or increased activities on illumination - for example, in comparison to the fluoroquinolone derivative levofloxacin against *Enterococcus faecalis* and *Pseudomonas aeruginosa*, or the imidazole antifungal fluconazole against *Candida albicans* (Table 2). Similarly, those derivatives screened against *Propionibacterium acnes* (*P. acnes*) were much more effective (MBC < 1 μM) than the standard acne medication benzoyl peroxide (MBC = 56 μM).

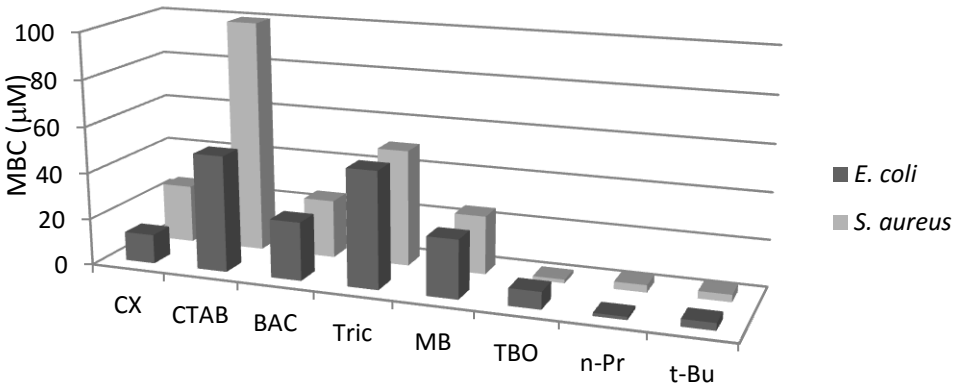


Figure 4. Photobactericidal efficacies vs. standard bacteria of a range of photosensitisers measured against standard disinfectants (abbreviations as above, except: CX – chlorhexidine digluconate; CTAB – cetyltrimethylammonium bromide; BAC – benzalkonium chloride; Tric – triclosan).

Given the potential utility of these agents in local disinfection, testing was also carried out against standard topical disinfectants (Figure 4), once again demonstrating the high comparative efficacies of the derivatives. Since three of the topical agents tested were of the quaternary cationic class (chlorhexidine digluconate, cetyl trimethylammonium bromide and benzalkonium chloride), it is reasonable to suppose that similar areas of the target cells – regions containing anionic groups in the cell wall/outer membrane for Gram-positive and Gram-negative bacteria respectively - were attacked both by these and the photoantimicrobials, but that reactive oxygen generation by the latter ensured rapid chemical oxidation of such regions, rather than merely physical interruption in the case of the disinfectant agents.

4. Conclusions

The established phenothiazinium dyes, having suitable light absorption and photosensitising profiles, have been investigated throughout much of the renaissance of research into the photodynamic therapy of cancer and photoantimicrobial action [20]. Lead compounds such as toluidine blue are structurally simple with relatively straightforward syntheses, allowing facile analogue production.

MB has been reported to produce more singlet oxygen than TBO in various formulations *in vitro* [21], thus the improved performance of the new derivatives in the current work is encouraging in this respect, and is, indeed, reflected in the increased photoantimicrobial efficacies reported here.

The toxicology of new drug candidates is, of course, paramount in establishing a route forward towards clinical trialling. One of the better candidates from the current work is currently beginning initial small scale testing in this respect and this work will be reported at a later date.

References

1. P. Paudyal, F.D. Flohr, C.D. Llewellyn, A systematic review of patient acceptance of screening for oral cancer outside of dental care settings**,** Oral Oncology 50 (2014) 956-962.
2. M.A. Jaber, Dental practitioner's knowledge, opinions and methods of management of oral premalignancy and malignancy, Saudi Dent. J. 23 (2011) 29-36.
3. J.Y. Nagata, N. Hioka, E. Kimura, V.R. Batistela, R.S.S. Terada, A.X. Graciano, M.L. Baesso, M.F. Hayacibara, Antibacterial photodynamic therapy for dental caries: Evaluation of the photosensitizers used and light source properties, Photodiag. Photodyn. Ther. 9 (2012) 122-131.
4. 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. Opin. Pharmacol. 13 (2013) 731-762.
5. M. Wainwright, Tricyclic cationic chromophores as models for new photoantimicrobials, J. Braz. Chem. Soc. 26 (2015) 2390-2404.
6. M.N. Usacheva, M.C. Teichert, M.A. Biel, The role of the methylene blue and toluidine blue monomers and dimers in the photoinactivation of bacteria, J. Photochem. Photobiol. B Biol. 71 (2003) 87-98.
7. M. Wainwright, Phenothiazinium salts as antimicrobial photosensitising agents, in *Photodynamic Inactivation of Microbial Pathogens*, Eds G. Jori, M.R. Hamblin, RSC, (2011) 19-43.
8. M.K. Pal, T.C. Ghosh TC, Induction of metachromasia and circular dichroism in the dye 1,9-dimethyl methylene blue by *S. aureus* wall teichoic acid, Indian J. Biochem. Biophys. 27 (1990) 176-178.
9. T. Dai, Y.Y. Huang, M.R. Hamblin, Photodynamic therapy for localized infections—State of the art, Photodiag. Photodyn. Ther. 6 (2009) 170-188.
10. G. Sridharan, A.A. Shankar, Toluidine blue: a review of its chemistry and clinical utility, J. Oral Maxillofac. Pathol. 16 (2012) 251-255.
11. 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.
12. M. Wainwright, Phenothiazinium photosensitisers V. Photobactericidal activities of chromophore-methylated phenothiazinium salts, Dyes Pigments 73 (2006) 7-12.
13. M. Wainwright, J. Antczak, M. Baca, C. Loughran, K. Meegan, Phenothiazinium photoantimicrobials with basic side chains, J. Photochem. Photobiol. B Biol. 150 (2015) 38-43.
14. J. Pooler, D.P. Valenzo, Physicochemical determinants of the sensitizing effectiveness for photooxidation of nerve membranes by fluorescein derivatives, Photochem. Photobiol. 30 (1979) 491-498.
15. M. Wainwright, The development of phenothiazinium photosensitisers, Photodiag. Photodyn. Ther. 2 (2005) 263-272.
16. M.N. Usacheva, M.C. Teichert, M.A. Biel, Comparison of the methylene blue and toluidine blue photobactericidal efficacy against gram-positive and gram-negative microorganisms, Lasers Surg. Med. 29 (2001) 165-173.
17. L.P. Rosa, F.C. da Silva, S.A. Nader, G.A. Meira, M.S. Viana, Antimicrobial photodynamic inactivation of *Staphylococcus aureus* biofilms in bone specimens using methylene blue,

toluidine blue ortho and malachite green: An *in vitro* study**,** Arch. Oral Biol. 60 (2015) 675-680.
18. J.P. Tardivo, M. Wainwright, M. Baptista, Small scale trial of photodynamic treatment of onychomycosis in São Paulo, J. Photochem. Photobiol. B Biol. 150 (2015) 66-68.
19. J. Alves dos Reis, J. Nunes dos Santos, B.S. Barreto, P. Nascimento de Assis, P.F. Almeida, A.L.B. Pinheiro, Photodynamic Antimicrobial Chemotherapy (PACT) in osteomyelitis induced by *Staphylococcus aureus*: Microbiological and histological study, J. Photochem. Photobiol. B Biol. 149 (2015) 235-242.
20. T. Maisch, Strategies to optimize photosensitizers for photodynamic inactivation of bacteria, J. Photochem. Photobiol. B Biol. 150 (2015) 2-10.
21. E.P. Prochnow, M.R. Martins, C.B. Campagnolo, R.C. Vianna, M.A. Villetti, K.Z. Kantorski, Antimicrobial photodynamic effect of phenothiazinic photosensitizers in formulations with ethanol on *Pseudomonas aeruginosa* biofilms, Photodiag. Photodyn. Ther, 2016, in Press.