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A comparative analysis of phenothiazinium salts for the photosensitisation of murine fibrosarcoma (RIF-1) cells *in vitro*

Ian Walker,[a] Stephen A. Gorman,[b] Russell D. Cox,[b] David I. Vernon,[a] John Griffiths[b] and Stanley B. Brown*[a]

[a] *Centre for Photobiology and Photodynamic Therapy, School of Biochemistry and Molecular Biology, Leeds, LS2 9JT. E-mail: s.b.brown@leeds.ac.uk*

[b] *Centre for Photobiology and Photodynamic Therapy, Department of Colour Chemistry, Leeds, LS2 9JT*

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Photodynamic therapy (PDT) is a treatment combining a photosensitiser, molecular oxygen and visible light of characteristic wavelength to produce cytotoxic reactive oxygen species (ROS). Within our centre, a series of phenothiazinium salts were synthesised and initial characterisation studies performed to determine any potential use for PDT. All photosensitisers within the series were shown to have useful spectral properties for PDT, with absorbance λ_{max} above 667 nm. The Log *P* values of the compounds were shown to range from −0.9 to > +2.0. Furthermore, Log *P* values were shown to be important in determining the site of subcellular localisation and as such the site of photooxidative damage. Derivatives with a Log *P* value of greater than +1.0 were shown to initially localise to the lysosomes then relocalise throughout the cytoplasm following illumination, whereas compounds with intermediate Log *P* values (−0.7 to +1.0) all remained lysosomal. Only methylene blue (Log *P* −0.9) was shown to redistribute to the nucleus upon illumination. Following treatment of RIF-1 cells with each phenothiazinium salt for 1 h and subsequent exposure to 665 nm laser light at a fluence rate of 10 mW cm^{-2} (18 J cm^{-2}), it was determined that the most potent photosensitiser was 260-fold more potent than methylene blue. Furthermore, the PDT efficacy of the photosensitisers was shown to be related to the level of mitochondrial damage induced directly following illumination.

Introduction

Photodynamic therapy (PDT) utilises a photosensitiser, molecular oxygen and visible light to cause selective damage to malignant tissue whilst minimising damage to healthy surrounding tissue. The first drug to gain a licence for PDT was Photofrin® in 1993, which is now approved in many countries across the world to treat a variety of tumour types. Despite its clinical success, Photofrin® is associated with a number of drawbacks, including prolonged skin photosensitivity and a poor absorption above 600 nm. This limits the photosensitiser activation in deep tumours due to the poor penetration of light at shorter wavelengths. Such problems have lead to the search for second-generation photosensitisers with improved properties. One class of photosensitisers under investigation is that of the phenothiazinium salts, with the best-known example being methylene blue.

It is well established that methylene blue is capable of inducing a phototoxic effect *in vitro.* Indeed, methylene blue-PDT (MB-PDT) is known to induced microtubule damage in HeLa cells,[1] and can also inactivate excised rat bladder tumour cells.[2] Other cell lines which have demonstrated sensitivity to MB-PDT include; sarcoma 180, mouse Gardner lymphoma, Ehrlich ascites, mammary adenocarcinoma,[3] human T-cell and B-cell lymphomas,[4] mouse bladder carcinoma,[5] the human melanoma cell line SK-mel-28[6] and human bladder carcinoma cells.[7] Interestingly, a number of multi-drug resistant (MDR) tumour cells have also showed sensitivity to MB-PDT including MA104 (a constitutively MDR cell line derived from the rhesus monkey) and K562-Lucena 1, an MDR cell line that over-expresses the membrane glycoprotein Pgp which confers resistance.[8] Furthermore Wainwright *et al.*[9] demonstrated the sensitivity of the EMT6-R multi-drug resistant cell line to MB-PDT.

The clinical application of MB-PDT has however, been limited[10,11] and is associated with a number of problems, including the lack of *in vivo* efficacy following intravesical or intravenous administration. It was hypothesised that this was due to the hydrophilic nature of the photosensitiser, limiting tumour localisation.[10] Also, upon exposure to light, methylene blue is seen to redistribute from the lysosomes to the cytoplasm and then the nucleus.[12] This provides a potential mutagenicity problem, as it is well established that methylene blue can intercalate DNA and can also photo-induce DNA damage.[13] Furthermore, although methylene blue has an excellent safety record in patients for numerous non-PDT application over a number of decades[14] it shows a relatively small differential between light and dark toxicity *in vitro*.[15] These problems have provided the rationale for the development of new phenothiazinium salt derivatives, with physicochemical properties differing from those of methylene blue.

The literature describes a number of phenothiazinium salt derivatives,[3,6,7,16–18] including a series of symmetrical methylene blue derivatives which were characterised by Mellish *et al.*[15] The series possessed increasing *N*,*N*-dialkylamino chain length (from C1 to C6) at the 3,7-positions of the phenothiazinium chromophore (Fig. 1). The derivatisation was shown to have little effect on the intrinsic photosensitising ability of the compounds but did demonstrate a relationship between lipophilicity and subcellular relocalisation patterns following illumination. These derivatives demonstrated an increased cellular phototoxicity over methylene blue with the propylene blue (with an alkyl chain length of three) compound being the most effective photosensitiser. All derivatives also demonstrated an increased cellular dark toxicity over methylene blue, however the therapeutic ratio between photo-toxicity and dark toxicity was improved in all cases compared to methylene blue.

In order to further study the effect of altering the lipophilicity of photosensitisers, we have produced a further two series of phenothiazinium salts within our centre. The photosensitisers

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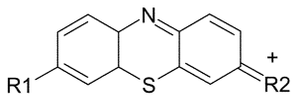


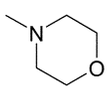
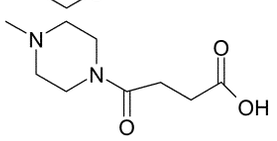
R1	R2	Nomenclature	
$N(CH_3)_2$	**$N(CH_3)_2$**	**Methylene blue**	Ref. 15
$N(C_2H_5)_2$	$N(C_2H_5)_2$	Ethylene blue	Ref. 15
$N(C_3H_7)_2$	**$N(C_3H_7)_2$**	**Propylene blue**	Ref. 15
$N(C_4H_9)_2$	$N(C_4H_9)_2$	Butylene blue	Ref. 15
$N(C_5H_{11})_2$	$N(C_5H_{11})_2$	Pentylene blue	Ref. 15
$N(C_6H_{13})_2$	$N(C_6H_{13})_2$	Hexylene blue	Ref. 15
$N(C_3H_7)_2$	**$N(CH_3)_2$**	**Dimethyl propylene blue derivative (Dimethyl PBD)**	
$N(C_3H_7)_2$	**$N(C_2H_5)_2$**	**Diethyl propylene blue derivative (Diethyl PBD)**	
$N(C_3H_7)_2$	**$N(C_4H_9)_2$**	**Dibutyl propylene blue derivative (Dibutyl PBD)**	
$N(C_3H_7)_2$	**$N(C_5H_{11})_2$**	**Dipentyl propylene blue derivative (Dipentyl PBD)**	
$N(C_3H_7)_2$	**$N(C_6H_{13})_2$**	**Dihexyl propylene blue derivative (Dihexyl PBD)**	
$N(C_3H_7)_2$	**$N(C_2H_4OH)_2$**	**Ethanolamine propylene blue derivative (Ethanolamine PBD)**	
$N(C_3H_7)_2$		**Morpholine propylene blue derivative (Morpholine PBD)**	
$N(C_3H_7)_2$		**Carboxy propylene blue derivative (Carboxy PBD)**	

Fig. 1 Structures of the phenothiazinium salt derivatives. Compounds highlighted in bold text are studied within this paper.

are all propylene blue derivatives (PBD) which possess a structurally different amino moiety on the 7 position of the symmetrical photosensitiser propylene blue. In the first series (designated the 'R2 = alkyl series') (Fig. 1), the newly introduced group is another *N*,*N*-di-*n*-alkylamino moiety in which the alkyl chain increases from C_1 to C_6. The parent compound propylene blue is included in this study since it is obviously a unique, symmetrical member of this particular series. In the second series (designated the 'R2 = non-alkyl' series) the newly introduced group is an oxygen-bearing amino moiety, such as diethanolamino, morpholino or a carboxylic acid attached *via* a piperizine linker (Fig. 1). Methylene blue was also included in the study in order to facilitate direct comparisons with existing literature.

This study further investigates structure function relationships within phenothiazinium salt derivatives. Data were analysed to determine any correlation between phototoxicity, physicochemical properties, intrinsic ability to photo-oxidise biological substrates, cellular uptake and cellular sites of photo-oxidative damage.

Materials and methods

Photosensitisers

Preparation of photosensitisers was performed using an adapted method described by Mellish *et al.*[15] Isolated 7-(*N*,*N*-dipropylamino)-phenothiazin-5-ium triiodide hydrate was subsequently treated with: excess morpholine [to yield 3-morpholino-7-(*N*,*N*-dipropylamino)-phenothiazin-5-ium iodide], diethanolamine [to yield 3-(*N*,*N*-diethanolamino)-7-(*N*,*N*-dipropylamino)-phenothiazin-5-ium iodide], dimethylamine [to yield 3-(*N*,*N*-dimethylamino)-7-(*N*,*N*-dipropylamino)-phenothiazin-5-ium iodide], diethylamine [to yield 3-(*N*,*N*-diethylamino)-7-(*N*,*N*-dipropylamino)-phenothiazin-5-ium iodide], dibutylamine [to yield 3-(*N*,*N*-dibutylamino)-7-(*N*,*N*-dipropylamino)-phenothiazin-5-ium iodide], dipentylamine [to yield 3-(*N*,*N*-dipentylamino)-7-(*N*,*N*-dipropylamino)-phenothiazin-5-ium iodide] or dihexylamine [to yield 3-(*N*,*N*-dihexylamino)-7-(*N*,*N*-dipropylamino)-phenothiazin-5-ium iodide]. Preparation of the 3,7-bis(*N*,*N*-dipropylamino)-phenothiazin-5-ium iodide was performed as described by Mellish *et al.*[15] 3-[4-(3-carboxy-propionyl)-piperazin-1-yl]-7-dipropylamino-phenothiazin-5-ium iodide was synthesised by isolating 3-(*N*,*N*-dipropylamino)-7-piperazin-1-yl phenothiazin-5-ium triiodide (0.26g, 0.50 mmole) in dichloromethane (100 cm^3) and adding succinic anhydride (0.06g, 0.61 mmole). The reaction mixture was refluxed for 21 h and excess solvent was removed by rotary evaporation and the residue triturated with ethyl acetate. The crude solid was then taken up in the minimum volume of methanol.

All compounds were precipitated using excess diethyl ether. Mass spectrometry indicated that the desired species had been produced in every case. Methylene blue (chloride salt) was purchased from BDH (Poole, UK).

Phenothiazinium salt derivatives were dissolved at either 5 or 10 mM in dimethylsulfoxide (DMSO) and stored at 4 °C. Photosensitisers used in cell experiments had a final concentration of <2.5% DMSO, a concentration which demonstrated no discernable effect on cell growth.

Absorbance and fluorescence measurements

Absorbance spectra were measured using a Perkin Elmer Lambda 40 UV/Vis spectrometer. Unless otherwise stated, the phenothiazinium salts were diluted to 1 μM in deionised water and absorbance data collected. Fluorescence measurements for the phenothiazinium salts (1 μM in deionised water at approximately pH 7) were obtained using a Kontron SFM-25 spectrofluorimeter.

Log octanol: buffer partition coefficients

Potassium phosphate buffer (0.1 M pH 7.0) and an equal volume of 1-octanol were agitated to allow saturation, then left to separate overnight. Stock solutions of photosensitisers (5 mM) were prepared in dimethyl sulfoxide. A pre-determined amount of sensitiser was then added to 1425 μl of buffer saturated 1-octanol and 1425 μl 1-octanol saturated buffer. Samples were thoroughly mixed by vortexing, then centrifuged using an MSE bench top centrifuge (2000 *g*, 5 min) to allow separation of the two phases. The distribution of photosensitiser between the two phases was determined by absorbance spectroscopy and

converted to concentrations by comparison to standards prepared in the same buffer or solvent. The Log partition coefficient was calculated as $\log_{10}$ (concentration in octanol/concentration in buffer).

Singlet oxygen generation

A reaction mixture containing 5 ml of 1,3-diphenylisobenzofuran with absorbance of <2 absorbance units at 411 nm (approximately 20 mg L^{-1}) and 5 ml of photosensitiser in methanol (with a final concentration of 7.6–8.1 μM) was illuminated at 4 °C. The light (provided by a 500 W quartz halogen projector) was filtered through a specifically designed tri-compartment cell in which the compartments contained aqueous copper sulfate solution, aqueous Congo Red solution and an infra-red absorbing dye solution in dichloromethane. The concentration of each solution was adjusted to provide filtered light with a transmission window possessing 30% transmittance between 600–700 nm. The generation of singlet oxygen results in the breakdown of 1,3-diphenylisobenzofuran, and was monitored by measuring the decreasing absorbance at 411 nm. Values were normalised by comparison to values obtained with equimolar methylene blue in each case.

Photo-oxidation of tryptophan

A reaction mixture containing 100 μM tryptophan (a non-specific substrate) and 10 μM sensitiser in 60% methanol, 40% 0.1 mM potassium phosphate buffer (pH 7.4) in a total volume of 6 ml was added to a 60 mm petri dish and illuminated at 10 mW cm^{-2} using a Ceram Optec diode laser (665 nm) with variable power output. The light was emitted through a cone shaped fibre optic tip. At 5 min intervals during the irradiation, 100 μl of reaction mixture was removed. This was added to 2.9 ml buffer in a quartz cuvette. Tryptophan fluorescence was measured using a Kontron SFM-25 spectrofluorimeter (λ_{ex} = 280 nm, λ_{em} = 350 nm) and the final concentration was determined by comparison with known standards.

Cell culture. Murine radiation induced fibrosarcoma (RIF-1) cells were cultured in RPMI 1640 culture medium (pH 7.4) supplemented with 10% (v/v) foetal calf serum (FCS), 1% (v/v) glutamine and 0.1% (v/v) gentamicin, at 37 °C in a 5% CO_2 humidified atmosphere. Cells were used at a passage number between 20 and 40 to minimise the risk of genetic drift.

Phototoxicity and dark toxicity

All experiments were performed in a darkened cell culture room with minimal ambient light. Cells were seeded onto 96-well plates at a density of approximately 6.5×10^3 cells $well^{-1}$, and left to attach overnight. The medium was replaced with photosensitiser diluted in culture medium and incubated for 1 h. Following incubation, cells were washed three times with PBS and re-fed with fresh culture medium. Cells were illuminated with 18 J cm^{-2} (10 mW cm^{-2}) of monochromatic light using a Ceram Optec diode laser (665 nm) with variable power output. The light was delivered through a micro-lens tipped optical fibre. Mitochondrial activity (which was used as an indication of cell viability) was measured at 24 h post-treatment using the MTT assay.[19]

Cellular accumulation of photosensitisers

Cells were plated onto 35 mm petri dishes at a density of 1×10^5 cells per dish and allowed to attach and grow overnight. The medium was replaced with medium containing photosensitiser at the PDT LD_{50} concentration and incubated for 1 h at 37 °C. Following incubation, the cells were washed three times with cold PBS (4 °C). 1 ml of 2% (w/v) SDS in water, was then placed in each dish and left for 1 h to fully dissolve the cell monolayer.

Table 1 Phototoxicity, dark toxicity and therapeutic ratio for phenothiazinium salts following a 1 h incubation in RIF-1 cells

Photosensitiser	PDT LD_{50}/μM	Dark LD_{50}/μM	Ratio
Methylene blue	13.1 (±4.8)	122.1 (±21.3)	9
Dimethyl PBD	0.22 (±0.27)	26.2 (±9.1)	119
Diethyl PBD	0.11 (±0.02)	17.53 (±10.49)	159
Propylene blue	0.047 (±0.012)	3.18 (±0.41)	68
Dibutyl PBD	0.09 (±0.02)	4.83 (±0.57)	54
Dipentyl PBD	0.12 (±0.01)	4.76 (±0.81)	40
Dihexyl PBD	0.19 (±0.06)	8.95 (±1.7)	47
Morpholine PBD	13.3 (±3.4)	>250	>19
Ethanolamine PBD	5.1 (±2.3)	>250	>48
Carboxy PBD	22.1 (±2.13)	>250	>11

Cellular photosensitiser levels were determined spectrofluorometrically using the pre-determined optimum excitation and emission wavelengths appropriate for each drug. Quantification was determined by comparison to known standards made up in a solution containing a cell monolayer dissolved in 2% (w/v) SDS. All fluorescence values were obtained using a Kontron SFM-25 spectrofluorimeter.

Protein concentration was determined using the BIO-RAD DC Protein Assay.[20] Final values were expressed as nmoles of photosensitiser per mg of cellular protein.

Subcellular localisation (fluorescence) of photosensitisers

Cells (1×10^4) were seeded into 35 mm petri dishes containing glass cover slips (Mat Tek Corporation, MA, USA) and allowed to attach and grow overnight prior to incubation with photosensitiser for 1 h. Medium was aspirated and cells washed 3 times in PBS before re-incubating with fresh culture medium. Phase images were collected using a 250 ms exposure. Fluorescence images were obtained using a Zeiss Axiovert 135 inverted microscope attached to a Hamamatsu dual mode cooled CCD camera and a 680 nm long pass filter. The light source was a Kinetic Imaging Monokromator providing excitation at 660 nm (27 mW cm^{-2}) continuously for a period of 20 min. Images were collected every minute for analysis.

Mitochondrial function assay

MTT was performed as described above directly after PDT (rather than 24 h post-treatment). PDT treatment was 0.5 μM photosensitiser, 1 h incubation, 18 J cm^{-2} at 10 mW cm^{-2} 665 nm laser light. A protein assay (SRB[21]) was also performed to determine total cell numbers rather than mitochondrial activity.

Statistical analysis

Data was analysed using the Student *t*-test. Data points were analysed as two samples with unequal variance and a two tailed distribution.

Results

The data for the compounds are divided in to two sub-groups to help determine any general trends. The first group contains all the compounds which have alkyl groups on the R2 moiety (called R2 = alkyl). The second group contains all the compounds which have the non-alkyl, oxygen-bearing derivatives (morpholine, ethanolamine and carboxylic acid) on the R2 moiety (called R2 = non-alkyl).

Phototoxicity and dark toxicity

The phototoxicity and dark toxicity of the phenothiazinium salt derivatives following a 1 h incubation is shown in Table 1.

Within the R2 = alkyl series, all compounds were statistically more phototoxic than methylene blue ($P < 0.01$ in each case), with propylene blue being the most phototoxic.

Table 2 This table shows Log partition coefficient values, singlet oxygen generating capabilities (relative values expressed as a % of the methylene blue value), the ability to photo-oxidise tryptophan (relative values expressed as a % of the methylene blue value) and cellular accumulation values for the phenothiazinium salts in RIF-1 cells following a 1 h incubation at the respective PDT LD_{50} concentration (expressed as nmol photosensitiser mg^{-1} cellular protein)

Photosensitiser	Log *P*	Relative singlet oxygen generation	Tryptophan oxidation	Cellular accumulation
Methylene blue	−0.9	100	100	2.38 (±0.27)
Dimethyl PBD	−0.7	82	75	0.48 (±0.07)
Diethyl PBD	+1.0	88	63	0.31 (±0.04)
Propylene blue	+1.2	91	56	0.09 (±0.01)
Dibutyl PBD	+1.7	90	79	0.33 (±0.15)
Dipentyl PBD	+2.0	75	57	0.42 (±0.06)
Dihexyl PBD	>2.0	87	53	0.71 (±0.10)
Morpholine PBD	−0.3	38	89	3.18 (±0.27)
Ethanolamine PBD	0.0	14	33	0.77 (±0.27)
Carboxy PBD	+0.3	31	44	2.03 (±0.51)

The R2 = non-alkyl derivatives generally showed less photodynamic efficacy than the R2 = alkyl series, with PDT LD_{50} values being 106, 282 and 447-fold greater than propylene blue for ethanolamine PBD, morpholine PBD and carboxy PBD, respectively.

All compounds demonstrated an improved phototoxic to dark toxic ratio compared to methylene blue indicating a potential therapeutic advantage.

Cellular accumulation

Cellular accumulation (expressed as nmoles photosensitiser mg^{-1} protein ± SEM, $n = 3$) of phenothiazinium salts following a 1 h incubation at equitoxic concentrations (PDT LD_{50}) is shown in Table 2. The R2 = alkyl series, demonstrated an inverse correlation ($R^2 = 0.946$) between cellular accumulation and PDT LD_{50} values, with the most efficient photosensitiser (propylene blue) requiring the lowest levels of cellular accumulation (0.09 nmol mg^{-1} of cellular protein) to induce a PDT LD_{50} effect. In contrast to this, the least effective photosensitiser in the series (methylene blue) required the highest levels of cellular accumulation (2.38 nmol mg^{-1} of cellular protein) to achieve a PDT LD_{50} effect in RIF-1 cells. This value was significantly higher than all other R2 = alkyl derivatives tested ($P < 0.01$ in all cases).

The R2 = non-alkyl series demonstrated no correlation ($R^2 = 0.26$) between PDT LD_{50} and levels of cellular accumulation following a 1 h incubation at the PDT LD_{50} concentration. RIF-1 cells accumulated 3.18 nmoles mg^{-1} (±0.27) of morpholine PBD derivative, 0.77 nmoles mg^{-1} (±0.27) of ethanolamine PBD or 2.03 nmoles mg^{-1} (±0.51) of carboxy PBD.

Intrinsic photodynamic properties

The derivatives were tested for their ability to generate singlet oxygen by monitoring the photodynamic breakdown of 1,3-diphenylisobenzofuran and comparison to methylene blue (Table 2). It was shown that all derivatives in the R2 = alkyl series possessed comparable singlet oxygen generating ability, with efficacy following the order methylene blue > propylene blue > dibutyl PBD > diethyl PBD > dihexyl PBD > dimethyl PBD > dipentyl PBD. This indicated that increasing the alkylation had no major impact on the ability of the compounds to generate singlet oxygen. The R2 = non-alkyl series was however, shown to be far less effective at generating singlet oxygen and followed the order morpholine PBD > carboxy PBD > ethanolamine PBD.

An alternative method used to determine the intrinsic photodynamic ability of a photosensitiser is to monitor the photodegradation of the biological substrate tryptophan (Table 2). Following exposure to 10 μM photosensitiser and 665 nm laser light, the breakdown of tryptophan was measured [values are based on an average rate over the initial 10 min light exposure (the rate was linear over this time) and are expressed as a relative value to that obtained with methylene blue ($n = 3$)]. Within the R2 = alkyl series, efficiency at inducing tryptophan photooxidation followed the order methylene blue > dibutyl PBD > dimethyl PBD > diethyl PBD > dipentyl PBD > propylene blue > dihexyl PBD. These data demonstrated no obvious correlation between length of alkyl chain and ability to photo-oxidise tryptophan.

In the R2 = non-alkyl series, morpholine PBD was the best photo-oxidiser of tryptophan with a relative value of 89. This value was significantly higher than the values for carboxy PBD ($P < 0.01$) and ethanolamine PBD ($P < 0.01$).

Determination of Log *P*

The Log octanol : buffer partition coefficients of the R2 = alkyl series are shown in Table 2. As expected, the increasing number of alkyl carbons was seen to correlate ($R^2 = 0.88$) with Log *P*. The values ranged from −0.9 for methylene blue to greater than + 2 for dihexyl PBD.

The R2 = non-alkyl derivatives (Table 2) all demonstrated intermediate properties, with Log *P* values of + 0.3, 0.0 and −0.3 for carboxy PBD, ethanolamine PBD and morpholine PBD, respectively.

Absorbance and fluorescence properties

The absorption properties of the series are shown in Table 3. All compounds within the series demonstrated an absorbance peak that would be of therapeutic use (667 to 680 nm). The R2 = alkyl series demonstrated an increase in absorption λ_{max} associated with the increasing alkylation in agreement with Mellish *et al.*[15] The extinction coefficient (ε) values for the R2 = alkyl derivatives in water were all similar with the exception of the dihexyl PBD, which demonstrated a notable decrease. This was associated with a much broader spectral peak and was probably indicative of the compound being aggregated. This was further supported by the fact that the broad spectral peak was not observed when dihexyl PBD was placed in methanol (data not shown).

Within the R2 = non-alkyl series, the large decrease in (ε) values demonstrated by carboxy PBD and ethanolamine PBD was again associated with a very broad spectral peak. This effect was not significantly reduced by diluting the photosensitisers in methanol, however the broad peak was absent upon dilution in pyridine (data not shown) possibly suggesting that the interactions associated with aggregation of these compounds were particularly strong.

Subcellular localisation

Images of RIF-1 cells incubated with phenothiazinium salts are shown in Fig. 2.

The initial fluorescence for each compound showed one of two general patterns, either punctate (dimethyl PBD, morpholine PBD, ethanolamine PBD, carboxy PBD and methylene

Table 3 Absorption and fluorescence properties of the phenothiazinium salts in water

Photosensitiser	Absorption (1 μM in H_2O)			Fluorescence (1 μM in H_2O)	
	λ_{max}/nm	ε/M^{-1} cm^{-1}	Band-width at half peak height/nm	ex λ_{max}/nm	em λ_{max}/nm
Methylene blue	667	42000	70	660	677
Dimethyl PBD	671	66000	57	666	684
Diethyl PBD	671	65000	56	669	687
Propylene blue	677	56000	51	674	689
Dibutyl PBD	678	59000	52	674	689
Dipentyl PBD	680	53000	71	676	690
Dihexyl PBD	680	32000	92	675	690
Morpholine PBD	667	77000	79	666	684
Ethanolamine PBD	668	24000	67	666	684
Carboxy PBD	668	35000	118	663	683

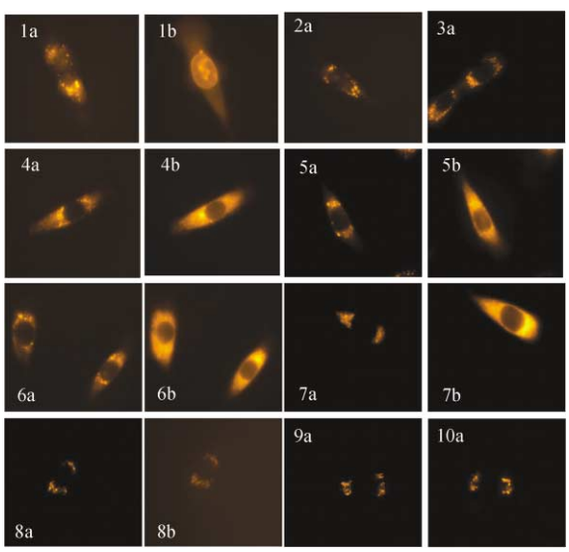


Fig. 2 Images of RIF-1 cells following a 1 h incubation with methylene blue (1), dimethyl PBD (2), diethyl PBD (3), propylene blue (4), dibutyl PBD (5), dipentyl PBD (6), dihexyl PBD (7), ethanolamine PBD (8), carboxy PBD (9) or morpholine PBD (10). Images are fluorescence images at $t=0$ h (a) and fluorescence images after relocalisation where observed (b).

blue), or diffuse with punctate regions (diethyl PBD, propylene blue, dibutyl PBD, dipentyl PBD and dihexyl PBD). The punctate distribution was observed in the perinuclear region of the cytoplasm and was comparable to the fluorescence pattern observed following RIF-1 incubation with the lysosomal dye, acridine orange (data not shown). All compounds appeared to remain extra-nuclear for the initial subcellular localisation.

Upon exposure to monochromatic light (660 nm), dimethyl PBD was associated with a photobleaching effect (Fig. 3). The fluorescence intensity dropped rapidly in the first 3–4 min of the experiment, with the final intensity value being only 9% of the start value. There was no discernible redistribution of the compound with discrete regions still present at the end of the exposure (as determined by image analysis software).

Diethyl PBD showed an initial fluorescence pattern similar to that observed with dimethyl PBD, there was however, also some diffuse fluorescence apparent. Upon exposure to light, the photobleaching effect was similar to dimethyl PBD with fluorescence intensity reducing by 92% over the experiment. Redistribution was not apparent.

Following illumination, propylene blue showed an increase in fluorescence intensity which peaked at 2 min with a value 400% greater than the initial value. This increase in fluorescence intensity (possibly due to a light induce disaggregation) was associated with an apparent redistribution. All punctate regions disappeared and the fluorescence became more diffuse with a mottled appearance.

This redistribution indicated that photosensitiser was present throughout the cell probably accumulating in membranes (due to the lipophilic nature) and mitochondria (due to the charged nature of the compounds and their probable attraction to the membrane potential of the mitochondrial membrane). Following the initial redistribution, the fluorescence intensity was seen to steadily decrease to a final value equal to 21% of the start intensity.

The dibutyl PBD, dipentyl PBD and dihexyl PBD, all demonstrated a very similar pattern of redistribution to that described for propylene blue. Upon exposure to light, the fluorescence intensity also followed a similar trend. The peak intensity occurred at 2 min (250% of the initial value) for dibutyl PBD, 3 min (430% greater than the initial value) for the dipentyl PBD and 7 min (297% greater than the initial value) for dihexyl PBD. In all cases, the increase in fluorescence

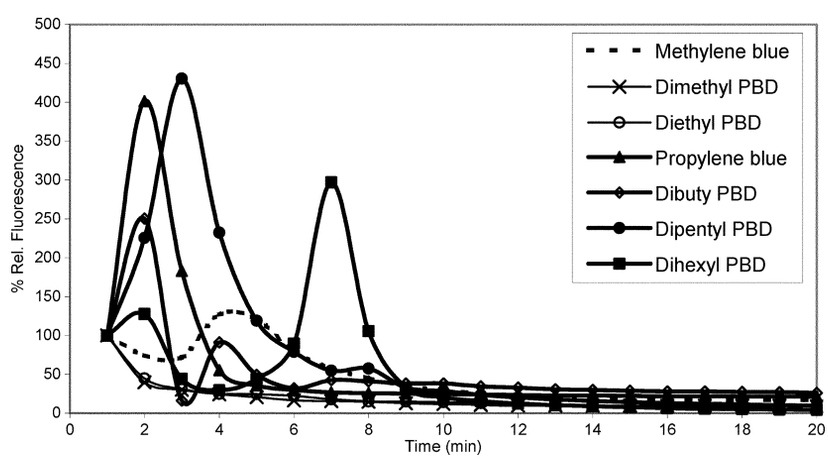


Fig. 3 An example of the cellular fluorescence intensity observed within the cytoplasm during illumination of RIF-1 cells incubated with R2 = alkyl phenothiazinium salts for 1 h (data from one experiment but is representative of the trends observed).

intensity was temporary and was followed by a steady decrease associated with photobleaching.

Within the non-alkyl series, the derivatives all demonstrated similar fluorescence patterns to dimethyl PBD with no redistribution apparent for any of the compounds following exposure to light. The fluorescence intensity (Fig. 4) showed no increase for any of the compounds and was seen to progressively decrease over time, indicating a photobleaching effect. The final fluorescence intensity values were 1, 11 and 26% of the initial values for ethanolamine PBD, morpholine PBD and carboxy PBD derivatives, respectively.

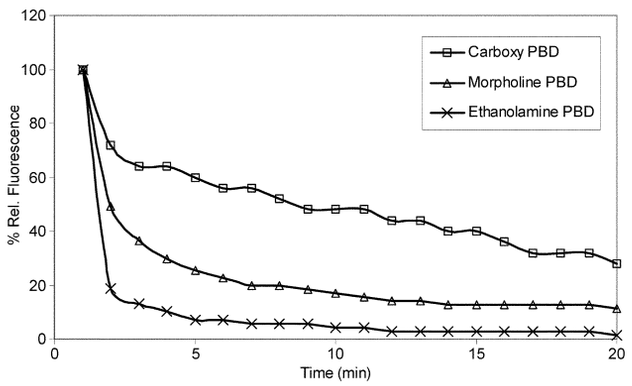


Fig. 4 An example of the cellular fluorescence intensity observed within the cytoplasm during illumination of RIF-1 cells incubated with R2 = non-alkyl phenothiazinium salts for 1 h (data from one experiment but is representative of the trends observed).

Mitochondrial inhibition following PDT

In order to determine any direct mitochondrial photosensitisation by this series, the MTT assay was performed directly following PDT with 0.5 μM photosensitiser and 18 J cm^{-2} of 665 nm laser light. The SRB assay was performed in conjunction with this in order to demonstrate that any decrease in MTT reduction was due to a decrease in mitochondrial function and not simply a reduction in cell number. SRB values were all within 15% of control values indicating that cell numbers remain relatively constant at this early time point (Fig. 5).

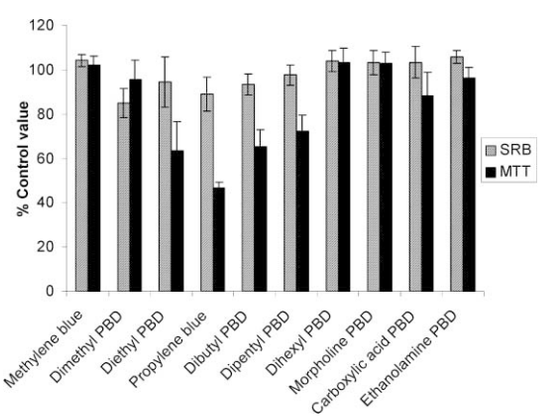


Fig. 5 Results from the mitochondrial function assay in conjunction with the SRB protein assay performed directly following PDT.

The degree of mitochondrial inhibition [expressed as % decrease compared to control values (±SEM $n = 3$)] varied throughout the alkyl series with the order being propylene blue, diethyl PBD, dibutyl PBD, dipentyl PBD. Statistical analysis revealed that only propylene blue, dibutyl PBD and dipentyl PBD demonstrated a statistically significant inhibitory effect of mitochondrial function ($P < 0.05$) directly following PDT.

All derivatives in the R2 = non-alkyl group showed no significant inhibition of mitochondrial function directly following PDT.

Discussion

Within this study we have investigated a series of phenothiazinium salt derivatives with increasing lipophilicity modulated by substitution at the amino position.

Within the R2 = alkyl series, it was shown that increasing the alkyl chain length of the substituent group did not appear to dramatically reduce ability to generate singlet oxygen in methanol. There was however a minor decrease in the ability to photo-oxidise tryptophan in an aqueous environment, possibly reflecting the state of aggregation of the compounds. This minor decrease could not however account for the large different in photodynamic efficacy seen *in vitro*.

The R2 = non-alkyl series were found to have significantly lower capacity to generate singlet oxygen, furthermore the ability to photo-oxidise tryptophan was also dramatically impaired (with the exception of morpholine PBD).

Within the R2 = alkyl series, the general trend regarding phototoxicity, was that increasing alkylation from methylene blue to propylene blue improved the *in vitro* phototoxicity but increasing alkylation between propylene blue and dihexyl PBD reduced phototoxicity, thus the propylene blue compound was the most phototoxic photosensitiser.

The PDT efficacy of the R2 = non-alkyl series was generally lower *in vitro* than the R2 = alkyl derivatives, possibly due to their significantly decreased ability to generate singlet oxygen.

We have demonstrated that the improved phototoxicity of these phenothiazinium salts was not due to an increase in cellular accumulation, indeed the most effective photosensitiser (propylene blue) required the lowest levels of cellular accumulation in order to achieve a PDT LD_{50} effect. These data suggest that the subcellular site of photodamage may be more important than levels of cellular accumulation when determining efficacy.

It has been well established that phenothiazinium salts generally accumulate in the lysosomes following diffusion across the plasma membrane.[22] The acidic nature of the lysosome may then induce the protonation of the phenothiazinium salt chromophore limiting diffusion back across the lysosomal membrane.

The initial fluorescence distribution demonstrated by both the R2 = alkyl and R2 = non-alkyl photosensitisers was generally consistent with these findings.

Upon illumination, a number of the derivatives were shown to relocalise. Methylene blue was seen to dramatically relocalise from the lysosomes to the nucleus in agreement with the literature.[12] The subcellular localisation of the propylene blue, dibutyl PBD, dipentyl PBD and dihexyl PBD was seen to relocalise from a predominantly lysosomal pattern to a pattern of fluorescence distribution similar to that observed following cellular staining with $DiOC_6$ (a membrane stain), however the diffuse ubiquitous fluorescence was interspersed with mottled regions of increased intensity that showed some similarity to the fluorescence distribution pattern seen following incubation with rhodamine 123, indicative of mitochondrial localisation.

Dimethyl PBD, diethyl PBD, ethanolamine PBD, carboxy PBD and morpholine PBD all appeared not to relocalise from the lysosomes following illumination.

Due to the inconclusive nature of the fluorescence images, further experiments were performed in order to determine the degree of mitochondrial damage directly induced following PDT. The R2 = non-alkyl series demonstrated no significant inhibition in mitochondrial function suggesting that the photosensitisers were not located within the mitochondria. Within the R2 = alkyl series, propylene blue, dibutyl PBD and dipentyl PBD caused significant mitochondrial inhibition directly following illumination, suggesting that these compounds must be present within the mitochondria.

Another important result was that all the compounds except for the most hydrophilic, methylene blue (Log P −0.9),

remained extra-nuclear thus limiting the potential for mutagenicity.

We have shown that subtle differences in the structure of photosensitisers can have a dramatic effect upon their *in vitro* photodynamic efficacy. It has been shown that the most vital factor in determining the *in vitro* efficacy is not the intrinsic photosensitising ability of the compound, or indeed the uptake characteristics of the photosensitiser. In fact, it appears that the site of subcellular localisation is the most important factor in determining *in vitro* photodynamic efficacy. Indeed, propylene blue demonstrated the largest light induced inhibitory effect upon mitochondrial function, perhaps explaining why this compound possessed the greatest phototoxicity within the series. We have further demonstrated the link between lipophilicity of the photosensitisers and their subcellular localisation, with the optimum property for mitochondrial localisation within this series being a Log *P* value of +1.2. In order to further investigate such trends, it would be interesting to study a cationic series of phenothiazinium salts with differing structures but the same Log *P* value of +1.2. These types of studies are vital to enable effective drug design for the future.

Abbreviations

MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; PBD, propylene blue derivative.

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References

1 J. C. Stockert, A. Juarranz, A. Villanueva and M. Canete, Photodynamic damage to HeLa cell microtubules induced by thiazine dyes, *Cancer Chemother. Pharmacol.*, 1996, **39**, 167–169.
2 W. B. Gill, A. Taja, D. M. Chadbourne, M. Roma and C. W. Vermeulen, Inactivation of bladder tumor cells and enzymes by methylene blue plus light, *J. Urol.*, 1987, **138**, 1318–1320.
3 J. S. Bellin, S. C. Mohos and G. Oster, Dye-sensitized photoinactivation of tumour cells *in vitro*, *Cancer Res.*, 1961, **21**, 1365–1371.
4 W. H. Boehncke, A. Ruck, J. Naumann, W. Sterry and R. Kaufmann, Comparison of sensitivity towards photodynamic therapy of cutaneous resident and infiltrating cell types in vitro, *Lasers Surg. Med.*, 1996, **19**, 451–457.
5 D. S. Yu, S. Y. Chang and C. P. Ma, Photoinactivation of bladder tumor cells by methylene blue: study of a variety of tumor and normal cells, *J. Urol.*, 1990, **144**, 164–168.
6 L. Rice, M. Wainwright and D. A. Phoemix, Phenothiazine photosensitizers. III. Activity of methylene blue derivatives against pigmented melanoma cell lines, *J. Chemother.*, 2000, **12**, 94–104.
7 G. J. Fowler, R. C. Rees and R. Devonshire, The photokilling of bladder carcinoma cells in vitro by phenothiazine dyes, *Photochem. Photobiol.*, 1990, **52**, 489–494.
8 G. S. Trindade, S. L. Farias, V. M. Rumjanek and M. A. Capella, Methylene blue reverts multidrug resistance: sensitivity of multidrug resistant cells to this dye and its photodynamic action, *Cancer Lett.*, 2000, **151**, 161–167.
9 M. Wainwright, D. A. Phoenix, S. M. Burrow and J. Waring, Cytotoxicity and adjuvant activity of cationic photosensitizers in a multidrug resistant cell line, *J. Chemother.*, 1999, **11**, 61–68.
10 J. L. Williams, J. Stamp, R. Devonshire and G. J. Fowler, Methylene blue and the photodynamic therapy of superficial bladder cancer, *J. Photochem. Photobiol. B*, 1989, **4**, 229–232.
11 K. Orth, A. Ruck, A. Stanescu and H. G. Beger, Intraluminal treatment of inoperable oesophageal tumours by intralesional photodynamic therapy with methylene blue, *Lancet*, 1995, **345**, 519–520.
12 A. Ruck, T. Kollner, A. Dietrich, W. Strauss and H. Schneckenburger, Fluorescence formation during photodynamic therapy in the nucleus of cells incubated with cationic and anionic water-soluble photosensitizers, *J. Photochem. Photobiol. B*, 1992, **12**, 403–412.
13 E. M. Tuite and J. M. Kelly, Photochemical interactions of methylene blue and analogues with DNA and other biological substrates, *J. Photochem. Photobiol. B*, 1993, **21**, 103–124.
14 M. Wainwright and K. B. Crossley, Methylene Blue–a therapeutic dye for all seasons?, *J. Chemother.*, 2002, **14**, 431–443.
15 K. J. Mellish, R. D. Cox, D. I. Vernon, J. Griffiths and S. B. Brown, In vitro photodynamic activity of a series of methylene blue analogues, *Photochem. Photobiol.*, 2002, **75**, 392–397.
16 A. E. Frimberger, A. S. Moore, L. Cincotta, S. M. Cotter and J. W. Foley, Photodynamic therapy of naturally occurring tumors in animals using a novel benzophenothiazine photosensitizer, *Clin. Cancer Res.*, 1998, **4**, 2207–2218.
17 D. J. Ball, Y. Luo, D. Kessel, J. Griffiths, S. B. Brown and D. I. Vernon, The induction of apoptosis by a positively charged methylene blue derivative, *J. Photochem. Photobiol. B*, 1998, **42**, 159–163.
18 J. F. Tremblay, S. Dussault, G. Viau, F. Gad, M. Boushira and R. Bissonnette, Photodynamic therapy with toluidine blue in Jurkat cells: Cytotoxicity, subcellular localisation and apoptosis induction, *Photochem. Photobiol. Sci.*, 2002, **1**, 852–856.
19 T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods*, 1983, **65**, 55–63.
20 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. Randall, Protein measurement with the folin phenol reagent, *J. Biol. Chem.*, 1951, **193**, 265–275.
21 P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney and M. R. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, *J. Natl. Cancer Inst.*, 1990, **82**, 1107–1112.
22 Z. Diwu and J. W. Lown, Phototherapeutic potential of alternative photosensitizers to porphyrins, *Pharmacol. Ther.*, 1994, **63**, 1–35.