Uptake and cell-killing activities of a series of Victoria blue derivatives in a mouse mammary tumour cell line

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Abstract

The triarylmethane dye Victoria blue BO (VBBO) is a known photosensitizer which has been shown to induce a cytotoxic response *in vitro*. Several novel Victoria blue derivatives, with varying physicochemical properties, have been compared to VBBO, with respect both to dark toxicity and phototoxicity, on a mouse mammary tumour cell line, EMT6. Photosensitizer uptake was observed using confocal fluorescence microscopy. The chemical differences, particularly in the naphthyl substitution of the derivatives were shown to alter the light:dark toxicity differential and the uptake of the photosensitizers.

Introduction

The field of photodynamic therapy (PDT) is now well-established and is gaining increasing acceptance in the treatment of neoplastic disorders (Brown and Truscott, 1993). PDT exerts its cytotoxic effects through a combination of visible light, photosensitizing drug and molecular oxygen (Henderson and Dougherty, 1992). Problems associated with generation porphyrin photosensitizers, such as poor light adsorption in the 'therapeutic window' (600–750 nm) and prolonged skin photosensitization have led to the search for new and improved drugs (Wainwright, 1996).

Much recent progress in drug development has been based on porphyrin derivatives and their analogues, e.g. chlorins and phthalocyanines (Wieman and Fingar, 1992; Monfort et al., 1992; Boyle et al., 1993). However, photosensitizers based on commercial dyes are also of interest because of their familiar and well-established chemistry and synthetic routes. Several examples of commercial photosensitizers have been examined for photocytotoxicity:

acridines, benzophenoxazines, phenothiazines and xanthenes (Iwamoto et al., 1993; Cincotta et al., 1994; Wainwright et al., 1997; Melloni et al., 1988). In most cases, although photocytotoxicity has been demonstrated, there has been concomitant dark toxicity. Few workers have thus far attempted to eradicate the latter by the synthesis of specifically designed photosensitizers based on commercial dyes.

One class of compounds which has received scant attention in this area is the cationic triarylmethane series (Patel et al., 1992). Dyes such as crystal violet have been known for over a century and have indeed been widely used in topical antimicrobial medicine (Browning, 1964). It has been suggested that the flexibility of the ring structure of triarylmethanes causes a fast relaxation of the singlet excited state, and thus a low degree of photosensitizing activity (Wadwa et al., 1988). When immobilised, however, the photosensitizing activity of these compounds is greatly increased, possibly *via* binding of the structure to biomolecules conferring greater structural rigidity (Oster, 1955). The Victoria blue series consists of triarylmethane dyes related to crystal violet, where one of the

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phenyl groups is replaced by naphthyl. Victoria blue BO (VBBO) and Victoria blue R (VBR) (Figure 1) have been reported to exhibit photocytotoxic effects in several mammalian cell lines, though some dark toxicity was observed (Wadwa, Smith and Oseroff, 1988). The phenylamino analogue, Victoria blue B, (VBB, Figure 1) has also shown antitumour activity in animal systems (Riley, 1948).

The mode of action of VBBO has recently been reported to be Type I photosensitization i.e. the intracellular production of superoxide rather than singlet oxygen (Viola et al., 1996). In cultured tumour cell lines, the site of action of VBBO is thought to be in mitochondria where the photosensitizer inhibits Respiratory Complex I (Morgan et al., 1998).

We have synthesised a series of compounds based on the skeleton of VBBO, in order to investigate the effect of physicochemical change upon photodynamic activity. In this paper we report on investigations of the photocytotoxicity and dark toxicity of three novel compounds having different naphthyl substitution on the murine mammary tumour cell line, EMT6. Since the main differences in structure occur at the 4-position of the naphthyl moiety, the different substituted amino groups have been used for compound indication (Figure 1). Thus the Victoria blue analogue having a pyrrolidino group in the 4-naphthyl position is denoted as PVB; the 4-dimethylamino analogue is MVB and the 4-morpholino analogue MOVb.

Materials and methods

Chemicals

VBBO, VBR, VBB and 1-octanol were purchased from Aldrich Chemicals (Poole, Dorset, UK) and were used without further purification. The synthesis of new photosensitizers followed an established procedure has been reported elsewhere (Guinot, Hepworth and Wainwright, 1998). Elemental analysis for the new photosensitizers were correct and purity was confirmed by high performance liquid chromatography and high field ^1H nuclear magnetic resonance spectroscopy (^1H NMR) (Brüker WM250 spectrometer). ^1H NMR was also used to investigate intramolecular interactions of the substituted naphthalenes.

Absorption spectra in methanol, partition coefficients in a pH 7.3 phosphate buffered saline/1-octanol system (Pooler and Valentino, 1979) and the determination of the pK_a values of the photosensitizers

(Goldacre and Phillips, 1949) were measured spectrophotometrically using a Hewlett Packard 8452A diode array spectrophotometer.

Cell culture

The murine mammary tumour cell line (EMT6) was obtained from Zeneca Pharmaceuticals, Macclesfield, Cheshire. mouse mammary tumour cell line, EMT6. The parental cell line (EMT6-S) is sensitive to treatment with doxorubicin, but the sub-line (EMT6-R) is resistant to doxorubicin, over-expresses P-glycoprotein in the cell membrane, and shows cross-resistance to other non-related chemotherapeutic agents (Cox, 1993). Cultures were routinely maintained at 37 °C, 5% CO_2 : 95% air in RPMI 1640 culture medium (Life Technologies, Paisley, UK), supplemented with 10% (v/v) foetal calf serum (M.B. Meldrum Ltd., Bourne End, UK), 200 mM glutamine (Sigma, Poole, UK) and streptomycin ($10\,000\,\mu\text{g}\,\text{ml}^{-1}$)/penicillin ($10\,000\,\text{units}\,\text{ml}^{-1}$) (Sigma). Trypsin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide) and DMSO (dimethyl sulfoxide) were also obtained from Sigma.

Characterisation of drug uptake

Absorbance spectra

Visible spectra were measured using a Hewlett Packard 8452A diode array spectrophotometer and a calibration curve prepared for each to check adherence to the Beer-Lambert law (0–5 mM in methanol). The calibration curves were then used for the determination of the concentration of the dyes in the uptake experiments.

Drug uptake

10 ml aliquots of EMT6-S cells were seeded into 75 cm^2 flasks at a cell density of 8×10^4 cells ml^{-1} , in RPMI 1640 medium, supplemented with 10% (v/v) foetal calf serum, 200 mM L-glutamine and penicillin/streptomycin, as previously described. The cells were then grown to confluence. The medium was aspirated and replaced with varying concentrations of each dye (0–0.625 μM) and the cells incubated for a further 0.5, 1, 2, or 3 h under the same conditions as previously described. Following incubation, the medium was aspirated from each flask, cells were

washed with fresh medium and the cell monolayer removed by the addition of 1 ml 0.25% (w/v) trypsin and 0.5% (w/v) EDTA (in Dulbecco's PBS). The cells were resuspended in 10 ml RPMI to neutralise the action of the trypsin and counted using an improved Neubauer haemocytometer. The cell suspension was centrifuged at 160 g for 5 min and the supernatant removed. The cell pellet was then rinsed twice by resuspension in PBS followed by centrifugation at 160 g for 5 min. The supernatant was aspirated and the photosensitizer was extracted by suspension of the cells in 1 ml methanol for 30 min. The cell suspension was then centrifuged at 160 g for 30 min. The supernatant was removed and the absorbance determined spectrophotometrically. The experiments were performed three times, in duplicate. Specific uptake for each dye was established using a calibration curve and was expressed in picomoles per 10^6 cells.

Localisation studies using confocal microscopy

2 ml aliquots of EMT6 cells were seeded at a cell density of 1×10^4 cells ml^{-1} into 35 mm petri dishes (Falcon, Fahrenheit Laboratories, Rotherham, U.K.) in RPMI 1640 medium, supplemented with 10% (v/v) foetal calf serum, 200 mM L-glutamine and penicillin/streptomycin, as previously described. A sterile quartz coverslip (suprasil, 0.5 mm diameter $\times$ 0.2 mm thick, Heraeus Silica and Metals Ltd., Byfleet, U.K.) was placed into each petri dish and the EMT-6 cells were allowed to attach for three days whilst incubating. The medium was aspirated and replaced with medium containing a 5 μM concentration of VBBO, PVB, MVB, or MOVb, and incubated for three hours, under conditions previously described. Following 3-h incubation, the cells were examined with a scanning laser confocal fluorescence microscope using a helium-neon laser at 633 nm. Untreated cells were also examined for autofluorescence, but none was found.

Phototoxicity: dark toxicity experiments

Light from a circular bank of 24 fluorescent tubes (Phillips/Thorn, 8 W) arranged symmetrically at a distance of 8 cm with maximum emission in the 600–700 nm region and a light fluence of 4 mW cm^{-2} , as measured using a Skye light meter (Skye Instruments Ltd), was used to illuminate the cells which had been exposed to the various dyes. The temperature of the system was monitored constantly during irradiation but no heating effect was observed.

	R	R ¹	λ_{max} (nm) ^a	$\log \epsilon_{\text{max}}$ ^b	$\log P$ ^c	pK_{a} ^d
VBBO	NHEt	NEt ₂	612	4.77	+3.5	11.0
MVB	NMe ₂	NEt ₂	625	4.95	+2.3	8.5
PVB		NMe ₂	632	4.90	+1.8	8.4
MOVb		NMe ₂	622	4.99	+0.9	8.1
VBB	NHPh	NMe ₂	614	4.79	+2.8	7.7
VBR	NHEt	NMe ₂	612	4.82	+1.5	9.5

Figure 1. Victoria blue derivatives. ^a Wavelength of maximum absorption and ^b logarithm of the extinction coefficient measured in 10% aqueous acetic acid; ^c logarithm of the partition coefficient between water and 1-octanol and ^d logarithm of the equilibrium constant measured spectrophotometrically in aqueous buffer.

96 well microtitre plates were seeded with 1000 cells per well (in 200 μl RPMI 1640) and incubated for 2 days. Varying concentrations of each dye (0–5 μM) were added and the cells incubated, as previously, for 3 h. The medium containing the drug was then aspirated and the cells rinsed with 200 μl RPMI 1640, before replacing with a further 200 μl RPMI 1640. Each plate was illuminated for 30 min (light dose = 7.2 J cm^{-2}) or kept dark. Following this treatment, the cells were grown on again for a further 3 days. To evaluate cell viability and thus calculate percentage toxicity, the MTT assay was employed (Carmichael et al., 1987). 25 μl MTT (5 mg ml^{-1}) was added to each well and this was incubated for 5 h. The majority of the medium and MTT were aspirated, taking care not to disturb the formazan crystals, leaving approximately 30 μl in each well. 200 μl DMSO were then added to each well to solubilise the crystals. The plates were shaken for 10 min and the absorbance read on a plate reader (Anthos HT111, measuring filter 540 nm; reference filter 620 nm).

Effect of variable illumination

96 well microtitre plates were set up as previously described. The cells were exposed to the various drug concentrations as before. However, in this series of

Figure 2. Uptake curves of Victoria blue derivatives by EMT-6 cells: VBBO (●); PVB (■); MVB (▲); MOVB (★). Cells (1.5×10^4 /35 mm petri dish) were allowed to attach for 72 h. Drugs (2 ml at $0.625 \mu\text{M}$ in RPMI 1640) were added to the cells and cellular concentration were determined at different time intervals after incubation at 37°C , 5% CO_2 , 95% air. Absorbance of the drug in medium was measured spectrophotometrically before and after incubation and specific uptake by the cells was determined. Each experiment was performed twice using the means from 3 petri dishes in each case (except MOVB where $n = 3$). Error bars represent SEMs.

Figure 3. Effects of drug concentration on the uptake of triaryl-methanes by EMT-6 cells: VBBO (●); PVB (■); MVB (▲); MOVB (★). Experiments were carried out as described in Figure 2 but with varying drug concentrations and one h incubation. Mean points of two experiments are shown (except MOVB where $n = 3$).

experiments, the plates were illuminated for 30 min, 1 h, 2 h or kept in the dark. The photocytotoxicity : dark toxicity ratio effected by each drug was then established using the MTT assay, as previously described.

Results

Absorbance spectra

Each of the dyes absorbed maximally in the red wavelength region (Figure 1). Log P values and pK_a values for the Victoria blue photosensitizers dealt with in this work appear in Figure 1.

Drug Uptake

At a concentration of $0.625 \mu\text{M}$ the initial uptake of VBBO, PVB and MVB over one hour was found to be very similar (Figure 2), with that the VBBO and MVB equilibrating after approximately 2 h. The uptake of PVB between 1–2 h appeared to be slightly lower than VBBO and MVB, but increased thereafter, reaching a similar level to that of VBBO and PVB after 3 h ($\approx 0.025 \text{ pM}/10^6 \text{ cells}$). MOVB showed a much lower rate of uptake than the other three photosensitizers, increasing slowly over three hours (Figure 2). Control experiments showed there to be no artifactual binding of the dye to the plastic petri dishes.

VBBO, PVB, MVB and MOVB all showed a good correlation between drug concentration and cellular uptake after 3-h incubation (Figure 3). The uptake/dose trend was similar for all four drugs tested, and followed the order: VBBO > PVB > MVB > MOVB. This corresponded to the lipophilicity of the agents, with VBBO being the most lipophilic. At very low concentrations ($< 0.156 \mu\text{M}$), the uptake of MVB appeared to be lower than that of the other three drugs.

Mean cytotoxicity following 3-h incubation

VBBO gave the greatest differential between light and dark cytotoxicity at all concentrations, with the optimum differential seen between $0.156 \mu\text{M}$ and $0.312 \mu\text{M}$ (Figure 4). The results for the Victoria blue derivatives indicated that they all gave a lower photocytotoxic response than VBBO, thus decreasing the light dark differential (Figure 4). The IC_{50} values for the light and dark toxicities of each compound are shown in Table 1. Since the light/dark differential varies with concentration, comparison of photosensitizer efficacy has been made by calculating the ratios of the IC_{50} values obtained in the light and dark.

Effect of variable illumination

At a dose of $0.625 \mu\text{M}$ and a light dose of 7.2 J cm^{-2} , VBBO was almost 100% phototoxic and

Figure 4. Percentage cytotoxicity of EMT-6 cells elicited by Victoria blue derivatives at various concentrations: cells kept in darkness (■); cells illuminated at 7.2 J cm^{-2} (●). Cells (1000/well in 96 well plates) were allowed to attach for 48 h. Drugs (200 μl at 0.625 μM in RPMI 1640) were added to the cells and incubated for 3 h. The cells were rinsed with 200 μl RPMI, the medium replaced and exposed to light (7.2 J cm^{-2}) prior to growing on for 3 days at 37°C , 5% CO_2 , 95% air. Error bars represent SEMs.

hence showed no improvement with increased light dose. By contrast, cytotoxicity induced by PVB increased with increased illumination, although this was not a linear relationship (Figure 5). The greatest change in the photocytotoxic response produced by PVB was seen when the light dose was increased to 14.4 J cm^{-2} , with a further, smaller increase seen at 28.8 J cm^{-2} . PVB was less effective overall compared to VBBO at equimolar doses. Similarly, the cytotoxicity induced by MVB increased when the light dose was increased to 14.4 J cm^{-2} , but illumination at 28.8 J cm^{-2} gave no further increase. MVB was also less effective overall compared to VBBO. MOVB was not appreciably phototoxic at the concentrations used, and increased light dose did not enhance this.

Table 1. IC_{50} values/light enhancement for Victoria blue derivatives. The light enhancement factor (LEF) is the ratio of light : dark IC_{50} values. The light : dark toxicity ratio is not constant, but varies with differing concentrations and for this reason has been standardised using the IC_{50} values

Drug	$\text{IC}_{50} (\text{μM})/\text{Dark}$	$\text{IC}_{50} (\text{μM})/\text{Light}$	LEF
VBBO	1.19	0.12	9.9
PVB	3.37	1.26	2.7
MVB	2.35	1.24	1.9
MOVB	2.22	1.79	1.2

Figure 5. Effects of variable light dose on percentage cytotoxicity produced by Victoria blue derivatives at a concentration of 0.625 μ M: VBBO (●); PVB (■); MVB (▲); MOVb (★). Experiments were carried out as described in Figure 4, but were exposed to different light doses. Points are means of at least 4 experiments and error bars represent SEMs.

Localisation studies confocal microscopy

Figure 7 (a and b) shows two different cells treated with 5 μ M VBBO. The dye appeared to be distributed widely throughout the cytoplasm in a punctate pattern, which would be consistent with its reported distribution in mitochondria (Modica-Napolitano et al., 1990; Fiedorowicz et al., 1993). There appeared to be very little fluorescence in the nucleus. Figure 7 (c and d), (e and f) and (g and h) show different cells incubated for three hours with PVB, MVB and MOVb, respectively, all at a concentration of 5 μ M. They all showed a similar intracellular localisation to VBBO in that the dyes were distributed widely throughout the cytoplasm, however, Figure 7 (c) (PVB) and 7(g) (MOVb) suggested some nuclear infiltration.

Discussion

The compounds under discussion can exist in several forms in aqueous media (Figure 7). Triarylmethane dyes are well known to react with to give non-planar, neutral carbinol compounds (Figure 7b). This situation is further complicated for the commercial Victoria blue series (VBBO, VBB and VBR) in that it is also possible for the secondary amino group attached to the naphthyl residue to become deprotonated, again giving a neutral compound – the Homolka Base (Figure 7c). Only the ionised (blue) species (Figure 7a) is photoactive but the neutral forms are expected to exert some influence on pharmacological availability.

The highly lipophilic VBBO ($\log P = +3.5$) exhibited a high level of uptake which is comparable to

Figure 6. Intracellular distribution of Victoria blue derivatives in EMT-6 cells at a concentration of 5 mM, following 3-h incubation shown by confocal microscopy using a helium-neon laser at 633 nm. (a) and (b) VBBO; (c) and (d) PVB; (e) and (f) MVB; (g) and (h) MOVb.

Figure 7. Interaction of Victoria blue BO with water.

Figure 8. The *peri*-interaction in 1-substituted naphthalenes. Repulsion between the amino group and the naphthyl hydrogen shown causes twisting of the amino group out of the plane with loss of conjugation between the lone pair of electrons on nitrogen and the naphthyl chromophore. *N*-Ethyl-1-aminonaphthalene can overcome this repulsion by having the amino hydrogen in close proximity to the naphthyl hydrogen.

that of MVB and PVB ($\log P = +2.3$ and $+1.8$ respectively). MOVB was poorly retained by the cells even after 3 h, possibly due to its much lower lipophilicity ($\log P = +0.9$). This may imply that efficient uptake in the Victoria blue series requires analogues having high, positive $\log P$ values (such as VBB and VBR). Indeed in the current series, the uptake/dose profiles correlate well with trends in $\log P$. The uptake of MVB and PVB does not seem to be the primary factor in their lower cytotoxicities, although low uptake and retention of MOVB may well explain its low efficacy.

Victoria blue BO shows significant dark toxicity. This indicates interaction with a sensitive target site and indeed, VBBO is known to localise in mitochondria (Modica-Napolitano et al., 1990; Fiedorowicz et al., 1993). Confocal microscopy studies showed that VBBO appeared to be distributed throughout the cytoplasm, considerable perinuclear concentration

(Figure 6) supporting the mitochondrial hypothesis. The distribution of the other photosensitizers was also diffuse with some apparent localisation in the mitochondria. In addition, PVB exhibited some nuclear localisation. It would thus appear that a lack of organelle specificity is not responsible for the lower toxicities of the Victoria blue derivatives compared to that of VBBO.

Triarylmethyl cations such as VBBO are non-planar, minimising steric repulsions within the molecule by adopting a propeller-like shape. This lack of coplanarity of the aromatic rings accounts for the absence of singlet oxygen production in chemical tests, although the intermediacy of superoxide has recently been established (Viola et al., 1996). It has been stated that the photosensitizing activity of triarylmethanes is due to enforced molecular coplanarity by interaction with biomolecules (Oster, 1955). To explain the different phototoxicities observed it is therefore necessary to look more closely at the molecular structures involved.

Both VBBO, PVB and MVB exhibit considerable phototoxicity in the EMT6 tumour cell line, whereas MOVB is ineffective in this respect (Table 1 and Figure 4). The high light enhancement factor (LEF, Table 1) for VBBO is due to its efficacy as a photosensitizer *per se*, whereas the other derivatives exhibit lower enhancements. At the IC_{50} for MOVB there is little increase in activity on illumination, emphasising the poor photosensitizing properties of this compound.

While the relative uptakes of the Victoria blue photosensitizers will be governed to a certain extent by their structural make-up, it is a combination of uptake and photosensitizing efficacy which determines cytotoxicity. For efficient photosensitizing activity it is necessary for there to be efficient interaction between the lone pair of electrons on the amino nitrogens and the remainder of the molecule (Figure 8). For both MVB and MOVB there is a lack of coplanarity between the amine group attached to the naphthyl residue because of repulsion between the hydrogens of the amino group and the naphthyl ring (Figure 8). This lack of coplanarity is well known to decrease photosensitizing ability (Cincotta and Foley, 1988). The twisting effects caused by the larger groups can be seen in the 1H NMR data (Table 2) and also in the higher basicity of MOVB than PVB (Figure 1 – no comparison may be made with MVB since this has different aminophenyl functionality). In nuclear magnetic resonance spectroscopy, the 'chemical shift' of a hydrogen atom in a molecule has an inverse relationship to the amount of electron density surrounding it.

Table 2. ^1H Nuclear magnetic resonance shifts for H-2 in the 1-substituted naphthalene system

Group at naphthyl C-1	NHEt		NMe ₂
Chemical shift of naphthyl H-2 (ppm)	6.50	7.02	7.10	7.10

Thus the hydrogen attached to carbon-2 of the naphthalene system – used as it is easily identifiable – (Figure 8) will suffer a decrease in electron density of the group attached to carbon-1 twists out of parallel with the naphthalene ring because this inhibits the interaction between the nitrogen lone pair and the π -system. This twisting out of the plane is caused by steric repulsion between the hydrogen on carbon-8 and the substituent on carbon-1 (the *peri*-interaction, Figure 8). From Table 2 the order of increase in chemical shift of the hydrogen at carbon-2 is NHEt < pyrrolidino < NMe₂ = morpholino.

PVB will suffer to a lesser extent from this repulsion because of the small size of the pyrrolidiny ring. Thus a degree of coplanarity will exist with a concomitant increase in photosensitizing ability compared to MVB and MOVb. VBBO shows the highest photosensitizing ability of the series. This may be because the *N*-ethyl group is able to adopt a configuration which minimises the repulsion mentioned above i.e. a high degree of coplanarity exists between the amine and naphthyl moieties in this case (Figure 8).

In the present study, the presence of a secondary amino group in 4-position of the naphthalene ring of the Victoria blue photosensitizers appears to be of significance as regards photosensitizing ability *in vitro*. The importance of a secondary amino group in a series of cationic photosensitizers has been reported previously for Nile blue and its congeners, but Nile blue is a planar molecule, whereas there is a lack of coplanarity between the naphthyl moiety and the remainder of the molecule in the Victoria blue series. The presence of a tertiary amino group, unless small, in this position greatly inhibits efficient photosensitizing activity due to a lack of coplanarity between the amino group itself and the naphthyl residue. The present work appears to indicate that, in common with other types of photosensitizer, the Victoria blue derivatives have specific cellular sites of action, and that their phototoxic

behaviour is governed by photosensitizing efficiency in combination with sufficient lipophilicity to ensure high levels of intracellular uptake.

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