

Novel photosensitisers derived from pyropheophorbide-*a*: uptake by cells and photodynamic efficiency *in vitro*†

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Received 1st March 2010, Accepted 27th April 2010

First published as an Advance Article on the web 8th June 2010

DOI: 10.1039/c0pp00038h

Photodynamic Therapy (PDT) is a minimally invasive procedure used for treating a range of neoplastic diseases, which utilises combined action of light and a PDT drug called a photosensitiser. The efficiency of this treatment depends crucially on the properties of the photosensitiser used, namely on its efficient uptake by cells or by the surrounding vasculature, intracellular localisation, minimal dark toxicity and substantial phototoxicity. In this report we compare the spectroscopic properties, cell uptake and *in vitro* phototoxicity of two novel hydrophilic photosensitisers derived from pyropheophorbide-*a* (PPa). Both new photosensitisers have the potential to form bioconjugates with antibody fragments for targeted PDT. We find that the photophysical properties of both new photosensitisers are favourable compared to the parent PPa, including enhanced absorption in the red spectral region and substantial singlet oxygen quantum yields. Both molecules show efficient cellular uptake, but display a different intracellular localisation. Both new photosensitisers exhibit no significant dark-toxicity at concentrations of up to 100 μM . The phototoxicity of the two photosensitisers is strikingly different, with one derivative being 13 times more efficient than the parent PPa and another derivative being 18 times less efficient in SKOV3 ovarian cancer cells. We investigate the reasons behind such drastic differences in phototoxicity using confocal fluorescence microscopy and conclude that intracellular localisation is a crucial factor in the photodynamic efficiency of pheophorbide derivatives. These studies highlight the underlying factors behind creating more potent photosensitisers through synthetic manipulation.

Introduction

Photodynamic Therapy (PDT) is a minimally invasive procedure used in treating a range of cancerous diseases,¹ infections² and, recently, in ophthalmology to treat the wet form of age-related macular degeneration (AMD).³ The photodynamic action relies on the simultaneous interaction between a non-toxic photosensitiser molecule, visible light and molecular oxygen, offering dual selectivity through preferential uptake of the photosensitiser by diseased cells and the selective application of light. Following activation with visible light of the appropriate wavelength, the photosensitiser generates reactive oxygen species (ROS), primarily the reactive singlet state of molecular oxygen, called singlet oxygen, $\text{O}_2(\text{a}^1\Delta_g)$, through energy transfer to the ground state triplet oxygen, $\text{O}_2(\text{X}^3\Sigma_g^-)$. Other photochemical products of energy and/or electron transfer include radicals, *e.g.* the superoxide anion $\text{O}_2^{\cdot-}$ and hydroxyl radical OH^\cdot . Production of these short-lived species within biological tissues leads to localised cell death *via*

irreversible damage to cellular components such as proteins, lipids and DNA.⁴

The lifetime of singlet oxygen in an aqueous environment is 3.5 μs ,⁵ which is expected to shorten further in a cellular environment due to quenching.^{6–8} The diffusion distance and therefore the spatial domain of intracellular activity of $\text{O}_2(\text{a}^1\Delta_g)$ has been estimated as 100 nm or less.^{8,9} The spectroscopic data from cells^{7,8} clearly indicate that singlet oxygen is unable to diffuse beyond the intracellular domain where it has been produced, in part due to a high viscosity of the intracellular environment. The limited diffusion distance of ROS in biological systems means that diffusion only occurs at the intracellular level and furthermore, that the primary site of ROS generation determines the first point of damage to the cell. Consequently, the subcellular localisation as well as the selective accumulation of photosensitisers in diseased cells are important factors in determining PDT efficacy.

A key component of PDT is the photosensitiser which has to possess a number of key properties including absorption in the red (600–800 nm) allowing photoactivation within deeper tissues, selective uptake by malignant cells, the ability to efficiently generate singlet oxygen and minimal dark toxicity. Since the clinical approval of Photofrin[®], a first-generation photosensitiser with a number of limitations such as prolonged skin photosensitivity and poor absorption in the red, efforts have concentrated on so-called second generation photosensitisers with substantially improved properties over Photofrin[®]. A majority of these second

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† This article is published as part of a themed issue in appreciation of the many important contributions made to the field of molecular photophysics by Jan Verhoeven.

Table 1 Photophysical parameters of the photosensitisers under study^a

Compound	PPa	1	2
λ_{max} (em)/nm	675, 722	677, 731	690, 755 (shoulder)
ϕ_f (toluene)	0.30	0.26	0.15
ϕ_{Δ} (toluene)	0.50	0.56	0.73
ϕ_f (water)	4×10^{-3}	0.01	2×10^{-3}

^a λ_{max} (em) is the peak fluorescence wavelength; ϕ_f is the fluorescence quantum yield determined vs. PPa in toluene ($\phi_f = 0.3$),²⁷ 5% error; ϕ_{Δ} is the singlet oxygen quantum yield determined vs. PPa in toluene ($\phi_{\Delta} = 0.5$),²⁸ 10% error; ϕ_f in water was determined in the presence of 2% DMSO.

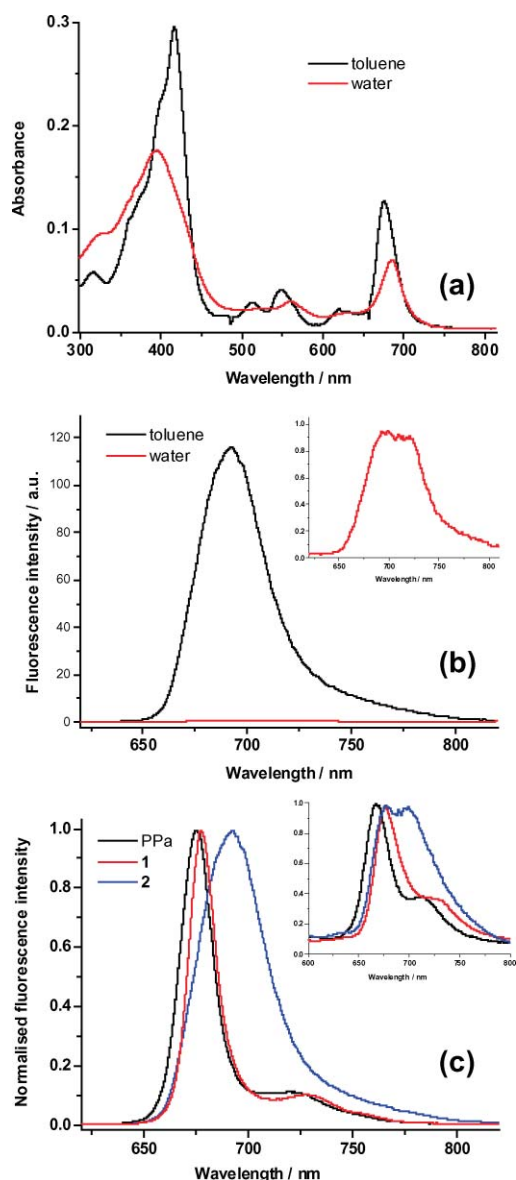


Fig. 1 Absorption (a) and fluorescence (b) spectra of **2** in toluene and in water; (c) normalised fluorescence spectra of **1**, **2** and PPa in toluene (main body) and in water (insert).

of the positive charge on **2**. We expect that increased solubility of photosensitisers **1** and **2** (together with reduced aggregation of **1**) will increase their bioavailability and will aid the delivery of **1** and **2** to the target site in the body and the cell. However, it

is still expected that in the cellular environment these derivatives will be trafficked to the hydrophobic cellular domains and/or will associate with the intracellular proteins which will further assist in their disaggregation.²⁶

Photophysical properties of PPa, **1** and **2** are summarised in Table 1. Singlet oxygen quantum yields were determined by directly monitoring fluorescence of singlet oxygen at 1270 nm following 420 nm excitation of each photosensitiser. We have established that both **1** and **2** have higher singlet oxygen quantum yields than PPa, Table 1. We have also determined that for the three photosensitisers under study the sum of ($\phi_{\Delta} + \phi_f$) is approximately constant and equals *ca.* 0.8. Thus a substantial increase in ϕ_{Δ} for **2** compared to PPa (0.73 compared to 0.5) leads to a marked decrease in fluorescence efficiency. Fluorescence is a useful property of a PDT photosensitiser, which aids in photo-diagnosis and tumor localisation, however $\phi_f > 0.1$ is deemed sufficient for these purposes. Thus on the basis of the photophysical properties, **2** is the best photosensitiser in the current series.

Phototoxicity

There is no single standard for measuring phototoxicity of a photosensitiser, due to the large number of variables which can determine the PDT efficiency in a given system. These factors are (i) photosensitiser concentration, (ii) incubation time, (iii) irradiation time and intensity, (iv) the cell type used and other factors. We have chosen two cell types commonly used in our laboratory: SKOV3 and KB, to test the PDT efficiency for PPa, **1** and **2**. Irradiation time and fluency as well as the incubation time has been previously optimised for PPa¹⁹ and in this work we have kept those conditions constant while measuring the effect of photosensitiser concentration on the overall cell kill, observed 48 h following irradiation. The 48 h period was deemed sufficient to capture the effect of both apoptosis and necrosis on the cell death.

For all photosensitiser concentrations the dark toxicity was determined, *i.e.* cell viability measured without light irradiation. It is important that a photosensitiser is not significantly dark-toxic, *i.e.* it should be harmless to the healthy tissues in the body which have not been exposed to light treatment.

The experimental dose-cytotoxicity curves are shown in Fig. 2. Promisingly, all photosensitisers demonstrated insignificant dark cytotoxicity up to concentrations of 100 μM , for both cell lines. We note that some dark toxicity has been recorded for **2** with KB cells. We attribute this to the fact that KB cells are generally more sensitive to cytotoxins than SKOV3 cells. Furthermore, there are two parameters alleviating the unwelcome dark toxicity. Since we are developing derivatives for use in targeted PDT, in the future we should be able to target cells faster and more efficiently to allow any non-specifically targeted photosensitiser to be washed away *in vitro*. Thus, *in vivo*, dark toxicity should not pose a concern as the working administered concentrations of the photosensitiser as part of a conjugate will be much less due to the benefit of targeting.

As shown in Fig. 2, PPa, **1** and **2** differ significantly in phototoxicity, following the order **1** > PPa > **2**. The IC₅₀s are summarised in Table 2. The phototoxicity trend **1** > PPa > **2** is valid for both cell lines studied, however, an equally low concentration of **1** and PPa is required to achieve 50% cell kill with the KB cell line. We have always observed that the KB tumour

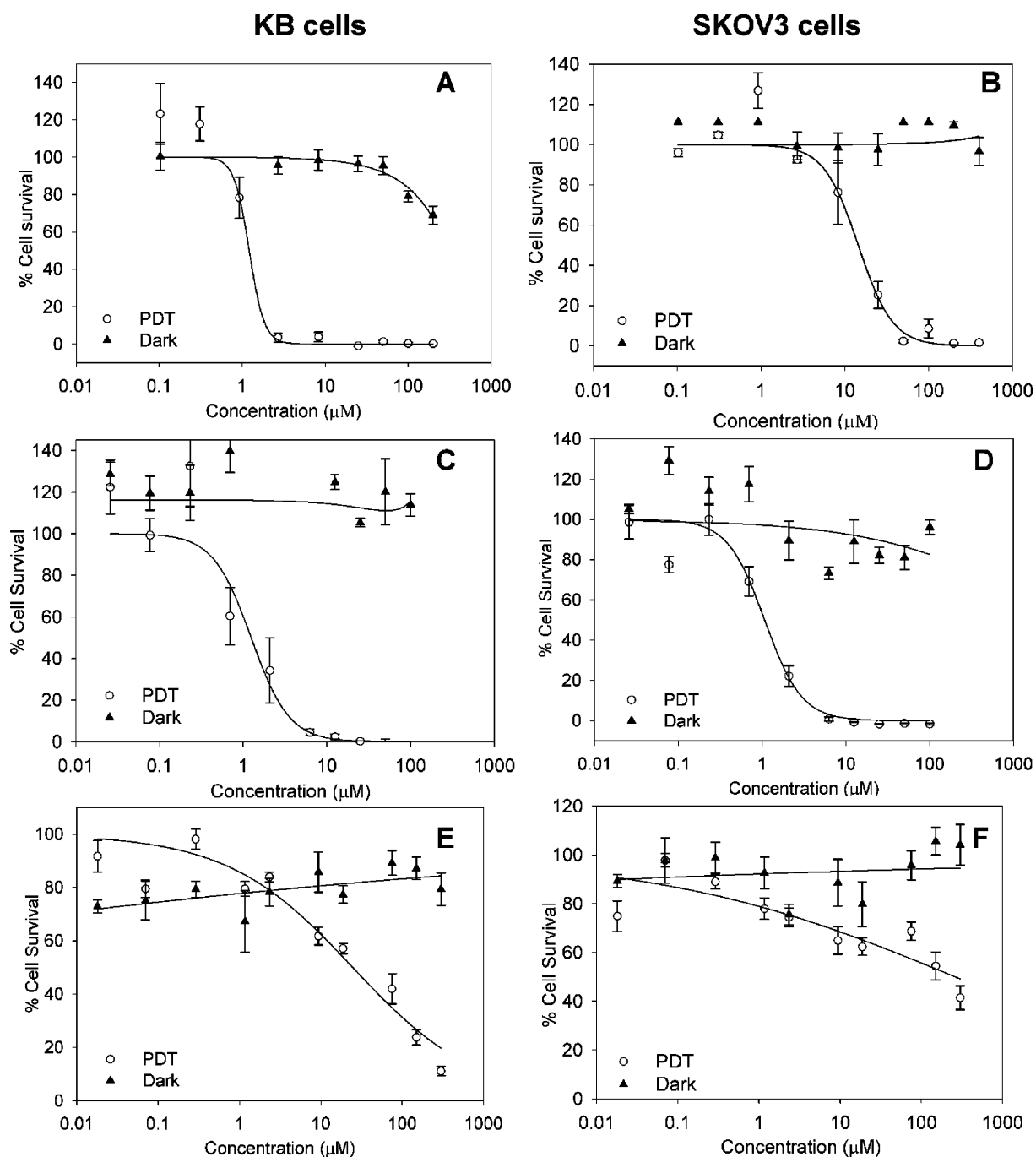


Fig. 2 Cell cytotoxicity of PPa-derived photosensitisers on tumour cells. Dose titration of photosensitisers on KB cells (all left panels) and SKOV3 (all right panels). Panels A, B (PPa), C, D (compound **1**) and E, F (compound **2**).

Table 2 The concentration of the photosensitiser required to kill 50% of cells (IC_{50}) in a PDT experiment with either SKOV3 or KB cell lines^a

Compound	$IC_{50}/\mu\text{M}$ on SKOV3 cells	$IC_{50}/\mu\text{M}$ on KB cells
PPa	14.5 ± 3.2	1.2 ± 0.4
1	1.1 ± 0.2	1.2 ± 0.5
2	259.6 ± 31	24.7 ± 11.7

^a Determined from data shown in Fig. 2.

cell line is more sensitive to PDT damage than the SKOV3. This may be due to a variety of mechanisms which are currently under study. Therefore, even with the less potent photosensitiser, PPa (compared to **1**), the damage threshold for KB cells is achieved at relatively low concentration and we can not differentiate between the PDT efficiency of **1** and PPa in KB cells.

For SKOV3 cells, a 13-fold higher concentration is required to achieve 50% cell kill with PPa compared to **1** and an 18-fold lower concentration is required to achieve 50% cell kill with PPa

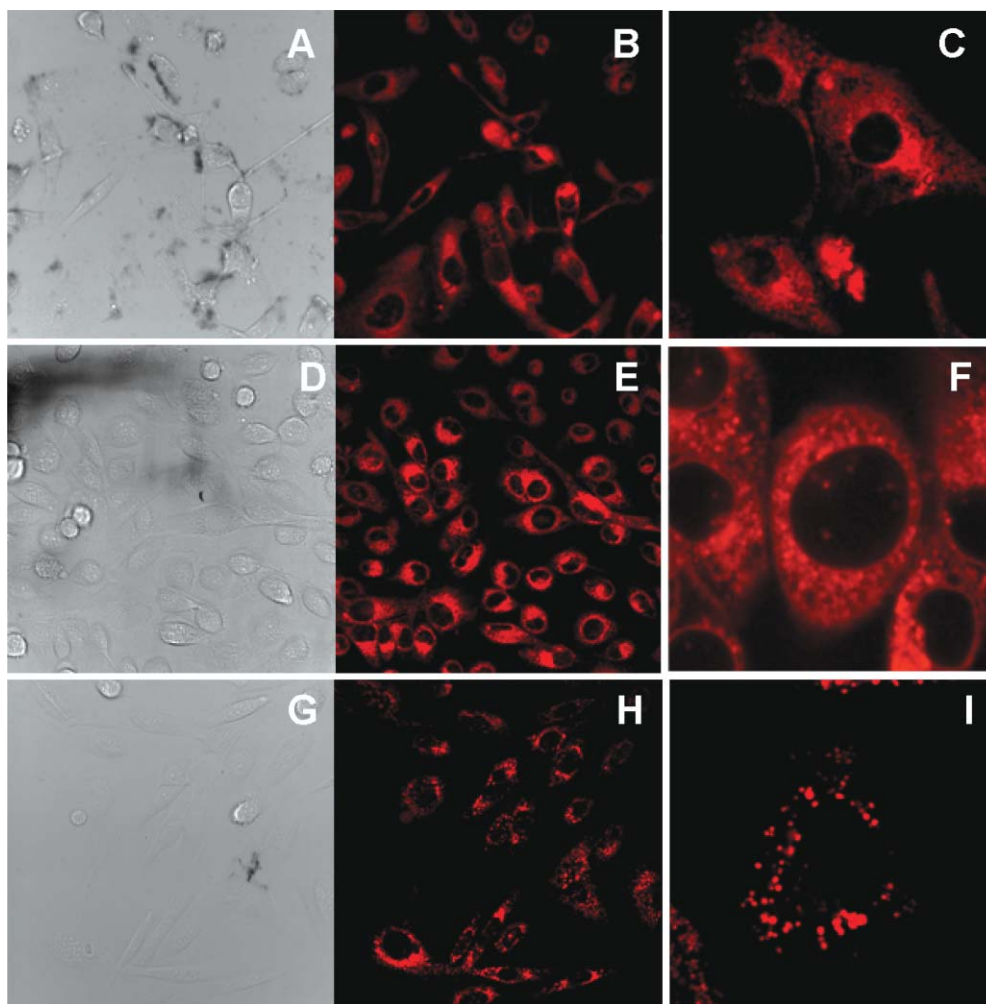


Fig. 3 Confocal fluorescence microscopy of PPa-derived photosensitisers on SKOV3 cells. Left panel are white light transmission images, middle panel are photosensitiser fluorescence images, with the right panel showing cells magnified. Panels A–C (PPa), D–F (compound **1**) and G–I (compound **2**).

compared to **2**. For the KB cell line, both PPa and **1** are over 20-fold more potent than **2**, Table 2.

This trend in phototoxicity, $\mathbf{1} > \text{PPa} > \mathbf{2}$, is particularly surprising given the higher solubility and higher singlet oxygen yields for both **1** and **2** and $\phi_{\Delta}(\mathbf{2}) > \phi_{\Delta}(\mathbf{1})$. To investigate the reasons behind such drastic difference in phototoxicity of **1** and **2**, confocal fluorescence microscopy was used to determine the intracellular localisation of **1**, **2** and PPa in cells.

Confocal microscopy

The phototoxicity of a compound depends on the extinction coefficient at the activation wavelength, photosensitiser concentration, its quantum yield of singlet oxygen production and, finally, the intracellular localisation of the photosensitiser. We cannot explain the poor phototoxicity of **2** based on the first three factors, since they are either identical for all three photosensitisers studied or even more favourable for **2**.

On the other hand, the intracellular localisation could be a crucial factor in PDT efficiency, since the diffusion distance of ROS is very small and thus intracellular localisation translates directly to the photosensitiser's ability to target vulnerable organelles

within cells. For example, previously, for photosensitisers which can efficiently kill diseased human cells, intracellular localisation in the mitochondria was found to be important.²⁹ Therefore we have investigated intracellular localisation of **1** and **2** and compared it to PPa by monitoring their fluorescence at 700 nm using confocal microscopy in live preincubated cells.

Confocal microscopy has been previously used to demonstrate that PPa derivatives localise in various hydrophobic membrane-like domains within cells.^{30,31} The fluorescence confocal images of SKOV3 cells preincubated with **1** and **2** are shown in Fig. 3–5. The images of SKOV3 cells incubated with PPa are also shown for comparison. It is clear that while the intracellular localisation of **1** is very similar to that of PPa, the pattern of intracellular fluorescence of **2** is strikingly different (Fig. 3I). Unlike PPa and **1**, the photosensitiser **2** shows punctate distribution inside the cells, possibly in intracellular vesicles.

Co-staining experiments using the organelle stain Mitotracker® Green FM (mitochondria) and LysoTracker® Green (lysosomes) were performed in order to determine the subcellular regions targeted by **1** and **2**, Fig. 4 and 5 respectively. These probes were chosen since both **1** and **2** show distribution in cells, which is similar to that of mitochondria and lysosomes. Additionally,

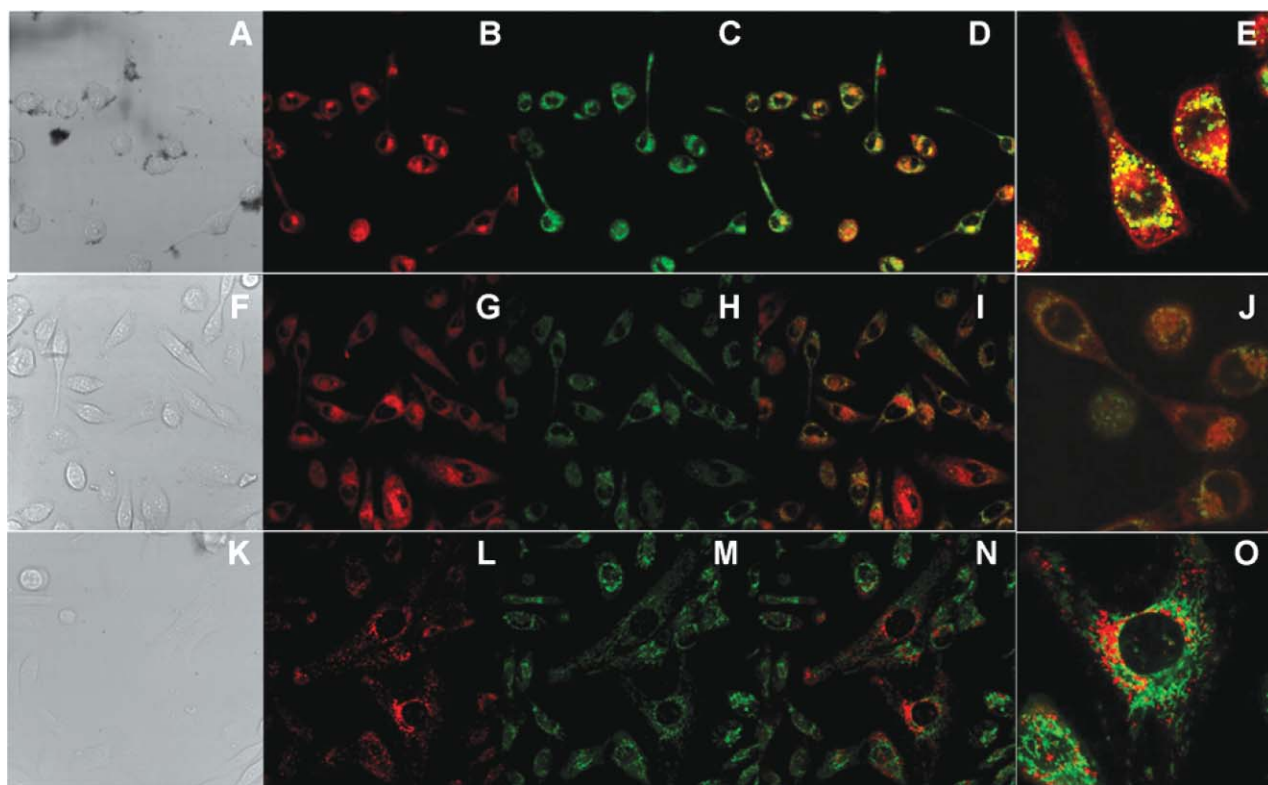


Fig. 4 Confocal fluorescence microscopy of PPa-derived photosensitisers on SKOV3 cells, co-stained with Mitotracker® Green FM. Left panels are white light transmission images, second panels are photosensitiser fluorescence images, third panels show the mitotracker co-stain, with the right panels showing the overlaid images. The far right panels show the overlaid cell images magnified. Panels A–E (PPa), F–J (compound **1**) and K–O (compound **2**).

mitochondria are known to be an important target organelle for efficient PDT²⁹ and the parent photosensitiser PPa methyl ester = MePPa is known to localise in mitochondrial membranes.³² Lysosomal staining was chosen because previous work has shown that more soluble sensitisers tend to localise to the lysosomes.³³

We observed significant colocalisation of **1** and PPa with Mitotracker® Green FM and no co-localisation for **2**, Fig. 4.

It is likely that **1** is localised in the plethora of intracellular membranes, similar to the parent MePPa.³² In contrast, the pattern of intracellular fluorescence of **2** is very different and does not show a pattern similar to any organelles which are considered crucial in PDT, such as ER, Golgi or plasma membrane. Significantly, we saw similar co-localisation of **1** and **2** in lysosomes (Fig. 5), consistent with increased hydrophilicity but suggesting that lysosomal distribution does not account for the observed difference in PDT potency.

Thus, the different localisation of **1** and **2** in cells does appear to be responsible for poor PDT efficiency of **2**, this in spite of the very favourable photophysical properties of **2**.

Another contributing factor to the difference in PDT efficiency could be the relative uptake of the compounds. We have estimated the intracellular concentration by monitoring fluorescence intensity in confocal images of cells incubated with solutions of **1** and **2**. These data indicate that both dyes are being taken up by SKOV cells with similar speed and to a similar extent. The difference in intracellular distribution is likely to be a consequence of different mechanisms of intracellular uptake of **1** and **2**. The uptake mechanism, in turn, can be explained by markedly

different hydrophilic properties of **1** and **2**. In an attempt to better understand the behaviour of the compounds with respect to hydrophobic domains, *e.g.* biological membranes, we estimated octanol–water partition coefficient values using the Pallas 7.3 software. The octanol–water system is a good descriptor of a cell membrane biphasic environment.^{34,35} It is generally accepted that octanol partition coefficients are difficult to obtain and reproduce experimentally,³⁶ hence the Pallas software was used to provide good approximations allowing comparisons to be made. We obtained values for PPa (3.20 ± 0.58), **1** (3.63 ± 1.66) and **2** (0.37 ± 0.9). These values indicate that **2** is considerably more hydrophilic than PPa and **1**, consistent with the presence of a net charge on **2**. The tendency of PPa and **1** to localise in the mitochondrial membranes and other protein- and lipid-rich compartments (as shown by others for PPa^{30–32}) is again consistent with less hydrophilic compounds and high log *P* values such as obtained for both of these molecules. Thus the hydrophilic properties of **1**, **2** and PPa appear to correlate with the apparent intracellular localisation of these compounds.

Conclusions

We have investigated the PDT efficiency of two new second-generation photosensitisers, based on the pheophorbide structure. Both photosensitisers show singlet oxygen production efficiencies comparable to those of clinical photosensitisers, and higher than PPa. Furthermore, even though they demonstrate a lower tendency to aggregate than the parent PPa, the PDT efficiencies

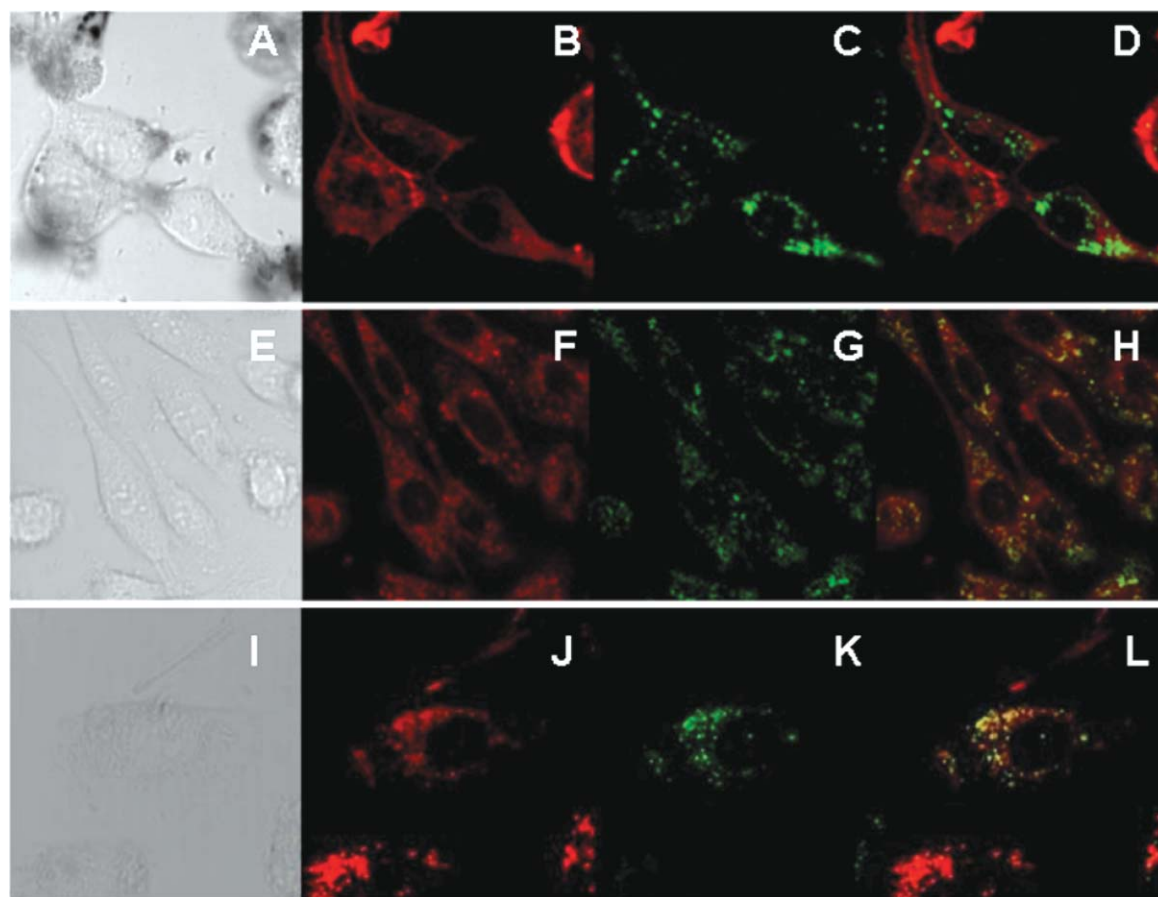


Fig. 5 Confocal fluorescence microscopy of PPa-derived photosensitisers on SKOV3 cells, co-stained with LysoTracker® Green. Left panels are white light transmission images, second panels are photosensitiser fluorescence images, third panels show the lysotracker co-stain with the right panel showing the overlaid images. Panels A–D (PPa), E–H (compound **1**) and I–L (compound **2**).

of the new photosensitisers do not follow the simple trend derived from the photophysical properties.

We along with other studies have demonstrated that a key factor affecting the PDT efficiency of these molecules *in vitro* is their intracellular localisation. The nature of action of PDT where transient species like ROS with extremely short lifetimes and limited diffusion distances are produced on irradiation of a photosensitiser, highlights the fact that the subcellular localisation of a photosensitiser is one of the key properties influencing the efficiency and extent of cell damage as well as cellular response and the mechanism of cell death. Our data on the vastly different PDT effect with **1** and **2** can be rationalised by the fact that **1** localises in the hydrophobic membrane-rich compartments such as mitochondria, while **2** does not. Previously, we and others have shown that receptor targeting of photosensitisers increases specificity and potency.^{15,16,19,20} What remains to be seen is whether the photosensitiser, upon entering the cell *via* a cell-surface receptor, follows the intracellular sorting route of the receptor (*e.g.* endosomal pathway, much like **2** is predicted to follow) or whether it redistributes to more lipophilic compartments (mitochondria, ER and other compartments, like PPa and **1** are predicted to follow). This will have implications for the design of future, more potent and specific PDT agents. Further work is concentrating on the synthesis of so called third-generation PDT

photosensitiser constructs, containing **1** and **2**, whereby the drug could be delivered to both the target organelle and the target organ in the body by specific carriers, *e.g.* single chain antibody fragments.^{16,17}

Experimental

The photosensitisers **1** and **2** were obtained from PhotoBiotics Ltd [UK Patent application reference no. GB 0904825.7]. Ppyropheophorbide-*a* (PPa) was obtained from Frontier Scientific Ltd and used as received. Spectroscopic grade toluene (Aldrich) and triply distilled water were used for all spectroscopic measurements. Air-saturated solutions were used for singlet oxygen yield determination.

Singlet oxygen generation was detected by its phosphorescence at 1270 nm using a North Coast Scientific EO-817P germanium photodiode detector. A frequency-tripled Nd:YAG (Continuum Surelite I-10) pumped dye laser (Lambda Physik, Coumarin 120 laser dye) was used as the excitation source providing 0.01–1.0 mJ per pump pulse at the sample between 420–430 nm, with a pulse duration of around 10 ns. The singlet quantum yields were calculated by a comparative method using PPa as a standard ($\phi_{\Delta} = 0.5$).²⁸ The fluorescence quantum yields were determined relative to PPa ($\phi_f = 0.3$).²⁷

Partition coefficients, log *P*

The octanol-water partition coefficient (*P*), which is referred to as the logarithmic ratio of octanol : water, is used to give a quantitative description of the lipophilicity of a compound in relation to its structure. We have obtained theoretical values using the Pallas 3.7.1.1 software from CompuDrug International Inc.

Cell culture

Two different human-derived tumour cell lines: SKOV3 and KB were obtained from the European Collection of Cell Cultures (ECACC) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum, penicillin and streptomycin antibiotics (1%) and passaged when 70–90% confluent in 75 cm² flasks. The cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere.

Cell preparation for confocal fluorescence imaging

For the imaging experiments, the cells were seeded at 2×10^4 cells per well in 0.2 mL of phenol red free culture medium in untreated 8 well coverglass chambers (Lab-Tek™, Nunc) and allowed to grow for 24 h. In the case of single staining with photosensitisers, the culture media was replaced with prewarmed phenol red free DMEM (0.5% DMSO) containing the photosensitiser and incubated at 37 °C in a humidified 5% CO₂ atmosphere for 20 h. Following incubation, the chambers were washed twice with DMEM and images were collected. In the case of organelle staining using fluorescent probes (Invitrogen) the culture medium was replaced by DMEM or HBSS buffer containing the tracker and subsequently incubated for 15 mins at 4 °C and a further 60 mins at 37 °C before replacing with fresh medium and imaging. Imaging was performed using a confocal laser scanning microscope (Leica TCS SP5), coupled to a CW argon-ion laser (488 nm line) and a diode laser (405 nm). The fluorescence emission of the photosensitisers in the cells was spectrally dispersed using a prism and detected using a photomultiplier tube. A water immersion 63× objective (*NA* = 1.23) was used to image.

In vitro cytotoxicity

Cells were trypsinised and seeded at 2×10^3 cells per well for KB and 3×10^3 cells per well for SKOV3 into 96-well plates and incubated over two nights for KB and overnight for SKOV3 at 37 °C and 5% CO₂. The cells were then washed once in phenol red-free DMEM and appropriately diluted photosensitiser solution (in DMEM, 2% DMSO) was added to the appropriate wells under subdued lighting. DMEM or Triton X-100 (1%) were added to control wells. After 2 h incubation in the dark at 37 °C, 5% CO₂, cells were washed three times with PBS and 100 µl of DMEM was added to each well. Wells were exposed to light from a High Powered-Devices 670 nm laser used at 0.5 W for 10 s. Controls included wells with photosensitiser added and no exposure to light, or DMEM added and exposure to light as well as Triton X-100 (1%) with or without light exposure. Cells that had no photosensitiser added and no exposure to light were included as overall controls. Cells were incubated in the dark at 37 °C, 5% CO₂ for 48 h after which time, a cell titre assay was performed according to the manufacturer's instructions. The Promega Cell

Titre 96® system was used which involves the conversion by live cells of a tetrazolium compound (MTS) into a formazan dye which is measurable by its absorbance at 490 nm.

Acknowledgements

MPD, GY and DP are co-founders and minor shareholders in PhotoBiotics, an Imperial College spin-out company which co-funded this research. This work was additionally funded by a European Union FP6 grant (Immuno-PDT, reference: LSHC-CT-2006-037489) and Cancer Research UK (reference: C18960). MKK is thankful to the EPSRC Life Sciences Interface Programme for a personal fellowship, EP/E038980/1. The confocal fluorescence imaging was performed at the FILM facility, Imperial College London.

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