Pure Appl. Chem., Vol. 83, No. 4, pp. 733–748, 2011.
doi:10.1351/PAC-CON-11-01-05

Toward targeted photodynamic therapy*

David Phillips

Department of Chemistry, Imperial College London, Exhibition Road, London SW7 AZ, UK

Abstract: The sensitizers in common use for photodynamic therapy (PDT) are summarized, and approaches to the improvement of these outlined. Selectivity in the targeting of sensitizers to tumor cells and tissue is highly desirable, as is water solubility and prevention of aggregation. Some new free sensitizers are described, based upon the *pyropheophorbide a* (PP*a*) structure, and their photophysical properties, distribution in cells via confocal fluorescence microscopy, and cell kill properties described. A novel approach to targeting is to covalently attach such sensitizers to monoclonal antibody fragments, and recent work on the attachment of *pyropheophorbide a* to such monoclonal antibody fragments is reviewed, with a demonstration of the increased efficiency of cell kill, and the treatment of a solid human tumor in a mouse model described. Finally, an alternative method of achieving selectivity based upon two-photon excitation (TPE) using porphyrin dimer sensitizers is reviewed, and the use of these to kill tumor cells is compared with the use of a commercially available PDT sensitizer (Visudyne). TPE of a porphyrin dimer sensitizer is shown to be capable of sealing blood vessels in a mouse model.

Keywords: aggregation; monoclonal antibody fragments; photodynamic therapy; pyropheophorbide *a*; two-photon excitation.

INTRODUCTION

This paper is dedicated to the memory of George Porter, The Lord Porter of Luddenham (Fig. 1), Nobel Laureate, on the occasion of my award of the Porter Medal. It is an honor to be included in the roll of eminent scientists who have thus far been awarded the medal, beginning of course with George Porter himself (1986).

Porter was a superb scientist who made major contributions in the development of instruments, notably flash photolysis, for which he was awarded the Nobel Prize for Chemistry in 1967, later for laser flash photolysis; in photosynthesis and artificial photosynthesis, and in the promotion of science through public engagement and via political influence.

While one could not hope to be in the same league, my career has some parallels. In instrument development, we made a significant contribution to time-correlated single-photon counting (TCSPC) [1], being among the first to recognize that mode-locked, cavity-dumped, or pulse-picked lasers were an ideal source for these studies. A later application of TCSCP was in fluorescence lifetime imaging microscopy, where gated techniques were also used [2,3]. Diffuse reflectance flash photolysis has been modified for use in widely scattering biological systems such as cells [4,5], and major advances were made in the application of time-resolved vibrational techniques, from nanosecond through to sub-

*Paper based on the acceptance lecture for a 2010 Porter Medal, presented at the XXIIIrd IUPAC Symposium on Photochemistry, Ferrara, Italy, 11–16 July 2010. Other presentations are published in this issue, pp. 733–930.

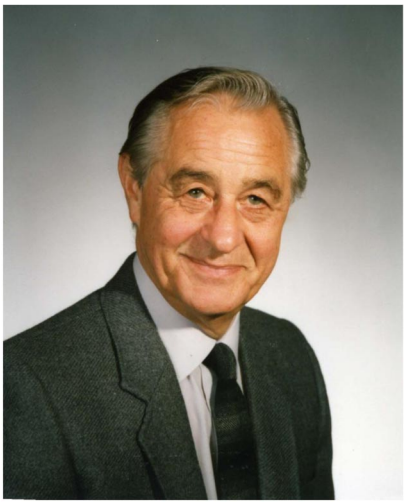

Fig. 1 George Porter, Nobel Laureate.

picosecond, both time-resolved resonance Raman and infrared spectroscopies being studied [6–8]. We report here, however, work in which we have been engaged for the past 20 years or so, that of photodynamic therapy (PDT), concentrating on most recent developments.

PHOTODYNAMIC THERAPY

PDT, the use of a light-absorbing sensitizer; light, nowadays usually laser light; in the presence of molecular oxygen, was first used by von Tappenheimer in 1903 [9]; but modern use really took off in the 1970s when Dougherty used fluorescein as a sensitizer to treat in vivo mammary implants [10]. Despite growing use since then, there is still a need for better take-up of this exciting therapy by the medical profession.

PDT is the minimally invasive procedure used in treating a range of cancerous diseases [11], infections [12], and recently, in ophthalmology to treat the wet form of age-related macular degeneration (AMD) [13]. The photodynamic action relies on the simultaneous interaction between a nontoxic photosensitizer molecule, visible light, and molecular oxygen, offering in its most commonly used form, dual selectivity through preferential uptake of the photosensitizer by diseased cells and the selective application of light. Following activation with visible light of the appropriate wavelength, the photosensitizer generates reactive oxygen species (ROS), primarily the reactive singlet state of molecular oxygen, "singlet oxygen", $O_2(a^1\Delta_g)$, through energy transfer from the long-lived triplet state of the sensitizer to ground-state triplet oxygen, $O_2(X^3\Sigma_g^-)$ via an electron exchange mechanism (Fig. 2).

Other photochemical products of energy and/or electron transfer can include radicals, e.g., the superoxide anion $O_2^{-\bullet}$ and the hydroxyl radical $OH^\bullet$. Production of these short-lived species within biological tissues leads to localized cell death via irreversible damage to cellular components such as proteins, lipids, and DNA [14]. The lifetime of singlet oxygen in an aqueous environment is 3.5 μs [15], which is expected to shorten further in a cellular environment due to quenching of the singlet oxygen by substrate species. (If this were not so, the singlet oxygen would be chemically inert [16–18].) Spectroscopic data from cells [17–19] clearly indicate that singlet oxygen is unable to diffuse beyond the intracellular domain where it was produced, in part due to a high viscosity of the intracellular environment. Thus, the primary site of ROS generation determines the first point of damage to the cell. Consequently, the subcellular localization as well as the selective accumulation of photosensitizers in diseased cells are important factors in determining PDT efficacy.

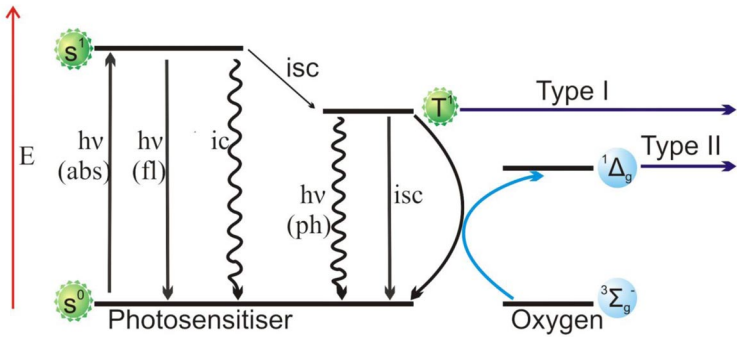

Fig. 2 Jablonski diagram showing the production of singlet oxygen via energy transfer to ground-state oxygen from the excited triplet state of a photosensitizer (from ref. [36] with permission).

It should be noted that although singlet oxygen is widely accepted as the dominant mechanism in PDT, electron-transfer processes can, in principle, play a role. Thus, difficult diffuse reflectance studies on the effect of di-sulfonated aluminum phthalocyanine photosensitizers on various bacteria (*S. mutans*, *P. gingivalis*, and *E. coli*, and the fungus *Candida albicans*) revealed the spectral signature of the radical anion of the sensitizers, clearly implicating electron transfer [4,5]. Recent work by Arnaut et al. [20] has also indicated that fluorinated sulfonamide tetraphenyl bacteriochlorin sensitizers have cell kills in excess of those expected by the singlet oxygen route alone, again implying that the electron-transfer mechanism, ultimately producing superoxide, hydroxyl or peroxide, may be of importance.

Tumor destruction post-PDT treatment takes place in two ways, by initiation of *necrosis* or *apoptosos* (programmed cell death). Necrosis is caused by sudden cell damage, which leads to the rupture of or damage to the plasma membrane, cell lysis, and tissue inflamation. The apoptotic process is initiated when a cell given the appropriate signals begins to shrink. Eventually, the cell is taken up by phagocytes. A tumor may also be destroyed by an indirect process resulting from damage to vasculature associated with the tumor, which is thus starved of nutrients and oxygen, leading to tumor infarction. Tumor hypoxia is both welcomed in PDT, since it leads to ischemia-related cell death, but has an adverse effect, at the same time, since oxygen is required for the intial PDT process. Most treatments overcome this problem by using fractionated (multiple) light doses with a time interval in between, such that hypoxic tissue is re-oxygenated before the next dose of light.

Photosensitizers

The key component of PDT is the photosensitizer, which has to possess a number of crucial properties, including strong absorption in the red (600–800 nm) allowing photo-activation deep in tissue, some selectivity in the uptake, or retention by malignant cells, the ability to efficiently generate singlet oxygen or other ROS, and minimal dark toxicity.

Photofrin®, a first-generation photosensitizer, was the first to be approved for clinical use, and has been used to treat advanced and early-stage lung cancers, gastric cancers, esophagal adenocarcinoma, and cervical and bladder cancers [21,22]. Photofrin has a number of limitations including prolonged skin photosensitivity and poor absorption in the red, and more recent efforts have concentrated on so-called second-generation photosensitizers with substantially improved properties over Photofrin. A majority of these second-generation photosensitizers are based on modified tetrapyrrolic macrocyles (porphyrinoids) with excellent absorption profiles at longer wavelengths and include both naturally derived and synthetic molecules, such as Photosens (a trisulfonated aluminum phthalocyanine) [23]; Foscan (5,10,15-tetrakis-[*m*-hydroxyphenyl]chlorin) [24,25]; Visudyne (benzoporphrin derivative,

BPD), largely used to treat wet-form AMD [26]; Levulan (5-aminolaevulinic acid) [27], which undergoes biosynthetic transformation to protoporphyrin IX, and other chlorins, bacteriochlorins, benzoporphyrin derivatives, phthalocyanines [28], and naphthalocyanines.

There are in excess of 1450 molecules identified in the literature as being potentially of use in PDT. Very few of these will be used for this purpose, due to the high cost of introduction into the clinic associated with Phase I, II, and III clinical trials. Only those sensitizers with *exceptional* properties will negotiate these financial hurdles. Since the currently approved sensitizers have quantum yields of ROS formation generally of 0.5 or above, the maximum benefit that can be reached with new sensitizers based upon photochemical performance alone is a factor of two, unless a photochemical mechanism involving chain oxidation can be achieved. It is thus the belief of this author that the choice of new photosensitizers will be based upon their *aggregation* and *biological* properties rather than photochemical, and that key to this is the targeting of water-soluble, non-aggregating sensitizers to the tissue to be destroyed.

Aggregation

Aggregation is a problem with many if not most sensitizers based upon porphyrin and porphyrin-like compounds. Dimers of most molecules are photochemically inert (but see later section on two-photon excitation, TPE), due to self-quenching of the singlet excited state of these, thus preventing formation of the photochemically active triplet state. A typical case in point is disulfonated aluminum phthalocyanines and the corresponding zinc compounds, where the fluorescence and triplet-state yields of these compounds, and the yield of singlet oxygen production are strongly pH-dependent, due to the formation of inert aggregates at low pH [29–31]. Unfortunately, the pK_a value for these compounds means that at physiological pH, aggregation still occurs, rendering the sensitizers less than optimal, though still used. Tri-sulfonated aluminum phthalocyanines are less aggregated, being more water-soluble, and are used in PDT as "Photosens". The presence of inert dimers of aluminum phthalocyanines, which are capable of quenching dynamically the excited singlet state of the monomeric, active sensitizer is a process that has to be invoked in order to explain the photophysics of these compounds in cells, in lipids, and in concentrated aqueous solution [32–34].

For all sensitizers, provision of a water solubilizing group reduces the tendency to aggregation, and successful sensitizers will undoubtedly have this feature, though liposolubility is also desirable. In recent work, we have synthesized some water-soluble sensitizers based upon the pyropheophorbide *a* (PP*a*) structure, compounds **1** and **2** (Fig. 3) [35,36].

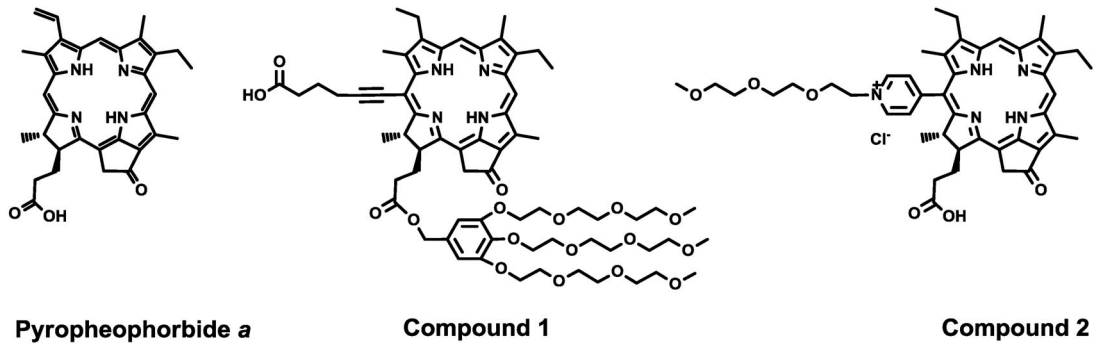

Fig. 3 Structures of new pheophorbide-based photosensitizers [35]. Reproduced by permission of the Royal Society of Chemistry (RSC) for the European Society for Photobiology (ESP), the European Photochemistry Association (EPA), and RSC.

New sensitizers

It is evident from both absorption and fluorescence spectra that in an aqueous environment all three photosensitizers show significant aggregation. Dyes **1** and **2** display increased fluorescence intensity compared to PP*a* in water, and this together with solubility data indicates that aggregation is significantly reduced for these compounds, increasing their bioavailability. 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 [37,38].

Photophysical properties of PP*a*, **1** and **2**, are summarized in Table 1. Both **1** and **2** have higher singlet oxygen quantum yields than PP*a*, Table 1. We have also determined that for the three photosensitizers 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 PP*a* (0.73 compared to 0.5) leads to a marked decrease in fluorescence efficiency. Fluorescence is a useful property of a PDT photosensitizer, which aids in photodiagnosis and tumor localization, however, $\phi_f > 0.1$ is deemed sufficient for these purposes. Thus, on the basis of the photophysical properties, **2** is the best photosensitizer in the current series.

Table 1 Photophysical parameters of the photosensitizers under study[a].

Compound	PP*a*	**1**	**2**
λ_{max}(em)/nm	675, 722	677, 731	690, 755 (shoulder)
ϕ_f	0.30	0.26	0.15
ϕ_Δ	0.50	0.56	0.73
τ_Δ/μs	30	30	30

[a]λ_{max}(em) is the peak fluorescence wavelength; ϕ_f is the fluorescence quantum yield determined vs. PP*a* in toluene ($\phi_f = 0.3$) 5 % error; ϕ_Δ is the singlet oxygen quantum yield determined vs. PP*a* in toluene ($\phi_\Delta = 0.5$), 10 % error; τ_Δ is the singlet oxygen lifetime. From ref. [35].

Phototoxicity

There is no single standard for measuring phototoxicity of a photosensitizer, due to the large number of variables that can determine the PDT efficiency in a given system. These factors are (i) photosensitizer concentration, (ii) incubation time, (iii) irradiation time and intensity, (iv) the cell type used, and other factors. Two cancer cell types commonly used in our laboratory, SKOV3 and KB, were used to test the PDT efficiency for PP*a*, **1** and **2**. Irradiation time and fluency as well as the incubation time has been previously optimized for PP*a*, and in this work we have kept those conditions constant while measuring the effect of photosensitizer 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.

The experimental dose–cytotoxicity curves are shown in Fig. 4. Promisingly, all photosensitizers demonstrated insignificant dark cytotoxicity up to concentrations of 100 μM, for both cell lines.

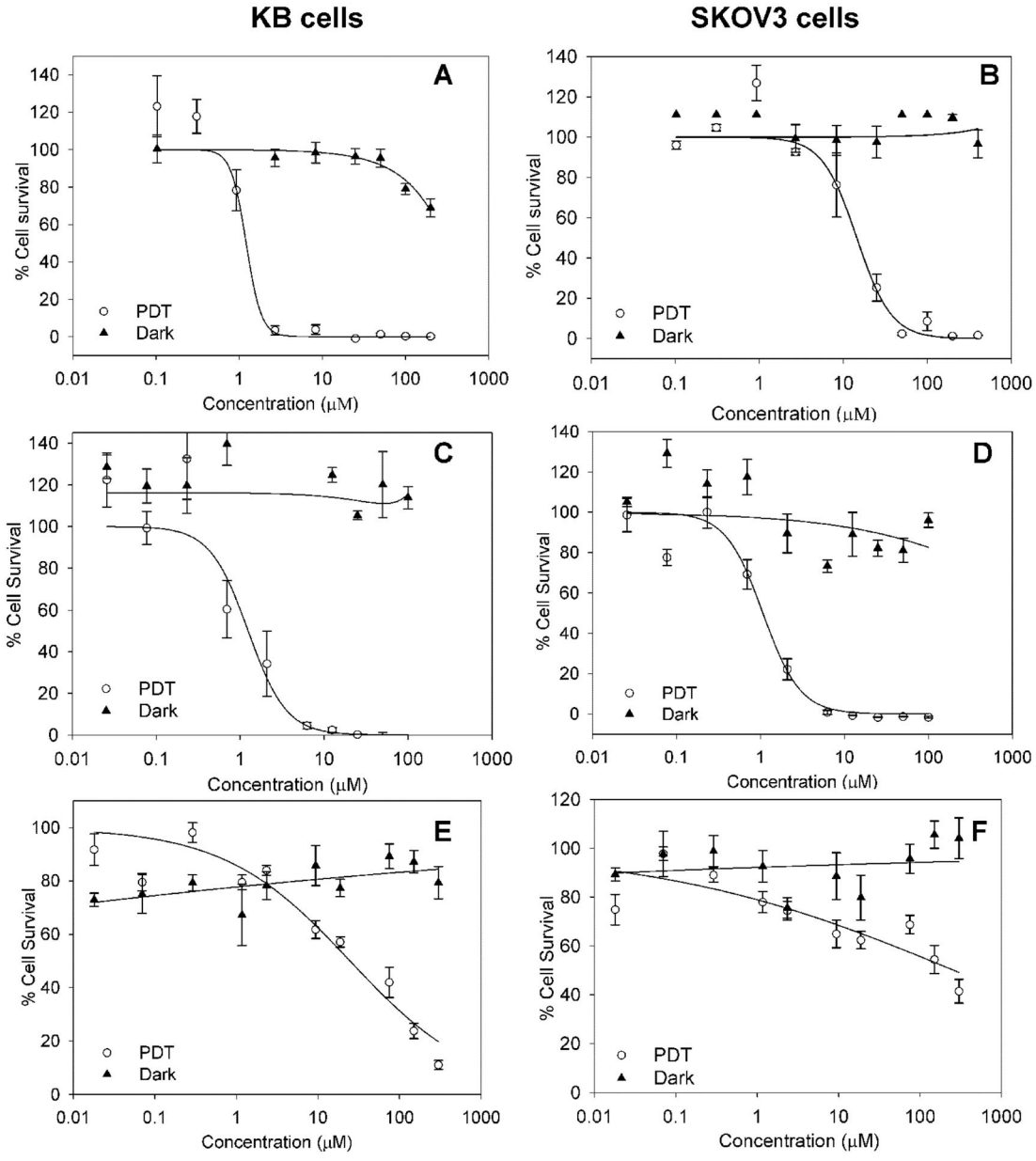

Fig. 4 Cell cytotoxicity of PPa-derived photosensitisers on tumor cells. Dose titration of photosensitisers on KB cells (all left panels) and SKOV3 (all right panels). Panels A, B (PPa), C, D (compound 1) and E, F (compound 2). Reproduced from ref. [35] by permission of RSC for ESP, EPA, and RSC.

As shown in Fig. 4, PP*a*, **1** and **2** differ significantly in phototoxicity, following the order **1** > PP*a* > **2**. The IC_{50}s are summarized in Table 2. The phototoxicity trend **1** > PP*a* > **2** is valid for both cell lines studied, however, an equally low concentration of **1** and PP*a* is required to achieve 50 % cell kill with KB cell line. We have always observed that the KB tumor cell line is more sensitive to PDT damage than the SKOV3. Even with the less potent photosensitizer, PP*a* (compared to **1**), the damage threshold for KB cells is achieved at relatively low concentration. For SKOV3 cells, 13× higher con-

centration is required to achieve achieve 50 % cell kill with PP*a* compared to **2**. For the KB cell line, both PP*a* and **1** are over 20-fold more potent than **2**, Table 2.

Table 2 Concentration of the photosensitizer required to kill 50 % of cells (IC_{50}) in a PDT experiment with either SKOV3 or KB cell lines.

Compound	IC_{50} (μM) on SKOV3 cells	IC_{50} (μM) on KB cells
PP*a*	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

From ref. [35].

Confocal microscopy

This trend in phototoxicity, **1** > PP*a* > **2**, is particularly surprising given the higher solubility and higher singlet oxygen yields for both **1** and **2** and ϕ_Δ (**2**) > ϕ_Δ (**1**). To investigate the reasons behind such a drastic difference in phototoxicity of **1** and **2**, confocal fluorescence microscopy was used to determine the intracellular localization of **1**, **2** and PP*a* in cells.

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

Intracellular localization is clearly a crucial factor in PDT efficiency, since the diffusion distance of ROS is very small and thus intracellular localization translates directly to the photosensitizer's ability to target vulnerable organelles within cells. For example, previously, for photosensitizers that can efficiently kill diseased human cells, intracellular localization in the mitochondria was found to be important [39]. Therefore, we have investigated intracellular localization of **1** and **2** and compared it to PP*a* by monitoring their fluorescence at 700 nm using confocal microscopy in live preincubated cells.

Confocal microscopy has been previously used to demonstrate that PP*a* derivatives localize in various hydrophobic membrane-like domains within cells [40,41]. The fluorescence confocal images of SKOV3 cells preincubated with **1** and **2** are shown in Figs. 5–7. The images of SKOV3 cells incubated with PP*a* are also shown for comparison. It is clear that while the intracellular localization of **1** is very similar to that of PP*a*, the pattern of intracellular fluorescence of **2** is strikingly different (Fig. 4i). Unlike PP*a* and **1**, the photosensitizer **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) are shown in Figs. 5 and 6, respectively. We observed significant co-localization of **1** and PP*a* with Mitotracker® Green FM and no co-localization for **2**, Fig. 5.

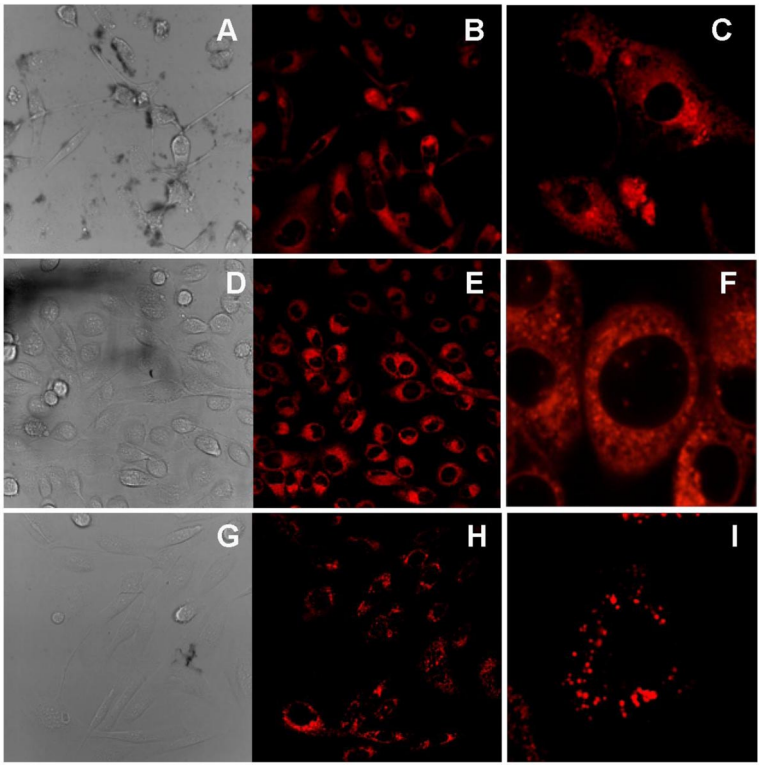

Fig. 5 Confocal fluorescence microscopy of PP*a*-derived photosensitizers on SKOV3 cells. Left panel are white light transmission images, middle panel are photosensitizer fluorescence images, with the right panel showing cells magnified. Panels A–C (PP*a*), D–F (compound **1**) and G–I (compound **2**). Reproduced from ref. [35] by permission of RSC for ESP, EPA, and RSC.

It is likely that **1** is localized in the plethora of intracellular membranes, similar to the parent MePP*a* [42]. In contrast, the pattern of intracellular fluorescence of **2** is very different and does not show a pattern similar to any organelles that are considered crucial in PDT, such as ER, Golgi, or plasma membrane. Significantly, we saw similar co-localization of **1** and **2** in lysosomes (Fig. 6), consistent with increased hydrophilicity but suggesting that lysosomal distribution does not account for the observed difference in PDT potency.

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

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 taken up by SKOV cells with similar speed and to a similar extent. The difference in intracellular distribution is likely to be due to different mechanisms of intracellular uptake of **1** and **2**, explained by markedly different hydrophilic properties

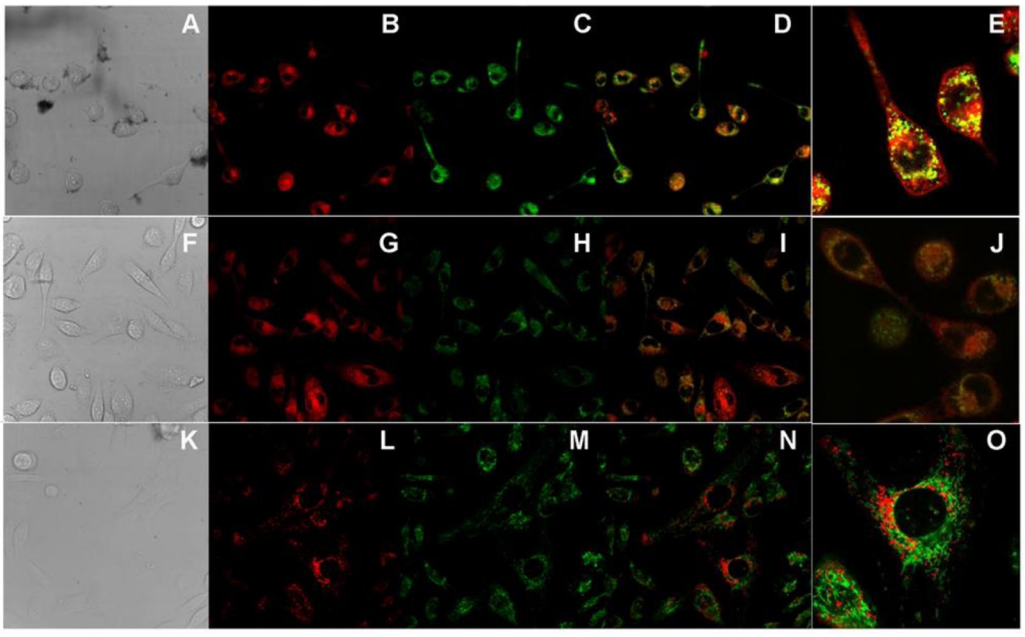

Fig. 6 Confocal fluorescence microscopy of PP*a*-derived photosensitizers on SKOV3 cells, co-stained with Mitotracker® Green FM. Left panel are white light transmission images, second panel are photosensitizer fluorescence images, third panels show the mitotracker co-stain with the right panel showing the overlaid images. The far right panels show the overlaid cell images magnified. Panels A–E (PP*a*), F–J (compound **1**) and K–O (compound **2**). Reproduced from ref. [35] by permission of RSC for ESP, EPA, and RSC.

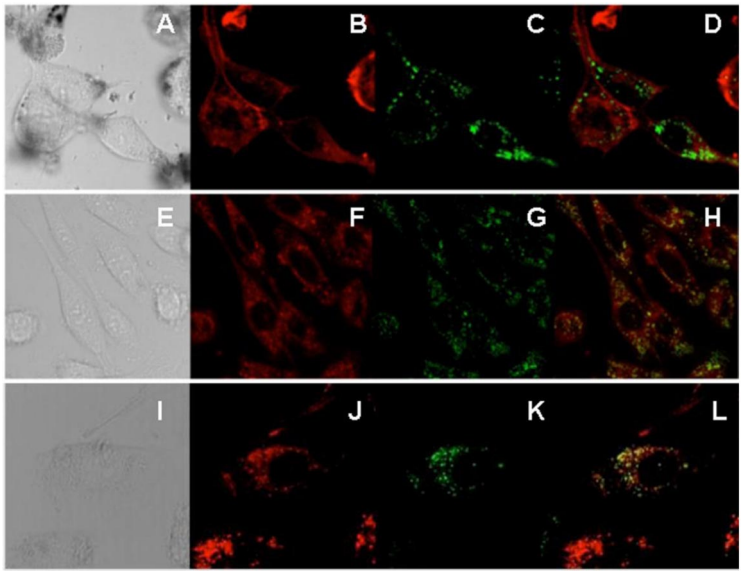

Fig. 7 Confocal fluorescence microscopy of PP*a*-derived photosensitizers on SKOV3 cells, co-stained with LysoTracker® Green. Left panel are white light transmission images, second panel are photosensitizer fluorescence images, third panels show the lysotracker co-stain with the right panel showing the overlaid images. Panels A–D (PP*a*), E–H (compound **1**) and I–L (compound **2**). Reproduced from ref. [35] by permission of RSC for ESP, EPA, and RSC.

TARGETING

Currently used free sensitizers achieve targeting only in the ratio of 2–5:1 tumor to normal tissue; improving this by at least an order or magnitude could reduce dramatically the dosage required for the PDT effect, and reduce considerably side effects such as skin sensitivity.

The principal means to achieve targeting include

i. whole antibodies,
ii. monoclonal antibody fragments,
iii. peptides, sugars, folic acid,
iv. multifunctional nanoparticles, and
v. spatial targeting using TPE.

Whole antibodies have proved to be largely unsuccessful for PDT, due to solubility problems. The use of peptides, folic acids and sugars, and nanoparticles has been widely studied, but can be exemplified by the work of Frochot et al. [43–47]. Our own work has concentrated upon single-chain monoclonal antibody fragments on which typically 8–10 sensitizers per monoclonal can be achieved, without causing the aggregation that plagues free sensitizers (Fig. 8).

The sensitizers are attached to the monoclonal antibody fragments via a peptide linkage to the lysine amino acids in the monoclonal. For some antibodies, lysines in the binding pocket have thus to be genetically altered so as to prevent attachment of a sensitizers in this location, which would eliminate specific binding. The details of the chemistry associated with the attachment of the sensitizer to the monoclonals, the choice of targets, and comparison with existing sensitizers are all outside the scope of this article, but a typical result of this type of targeted PDT is shown in Fig. 9, in which a thrice-repeated light treatment on mice bearing a human carcinoma is shown to be completely successful in the case of the monoclonal antibody-sensitizer conjugate in eradicating the tumor, whereas the free sensitizer merely arrests growth for a time before re-growth occurs. This result has been repeated with a variety of targets for different tumors, and a range of different sensitizers, and in the view of the author, provides the prospect of a real advance in the appeal and efficacy of PDT.

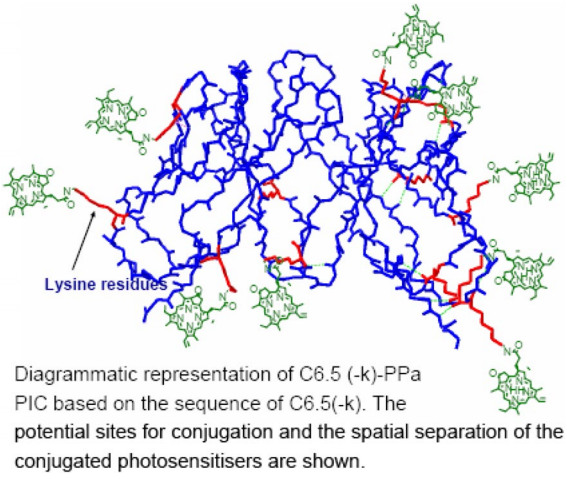

Fig. 8 Monoclonal antibody fragment with covalently linked PP*a* sensitizers. Reproduced from ref. [36] with permission.

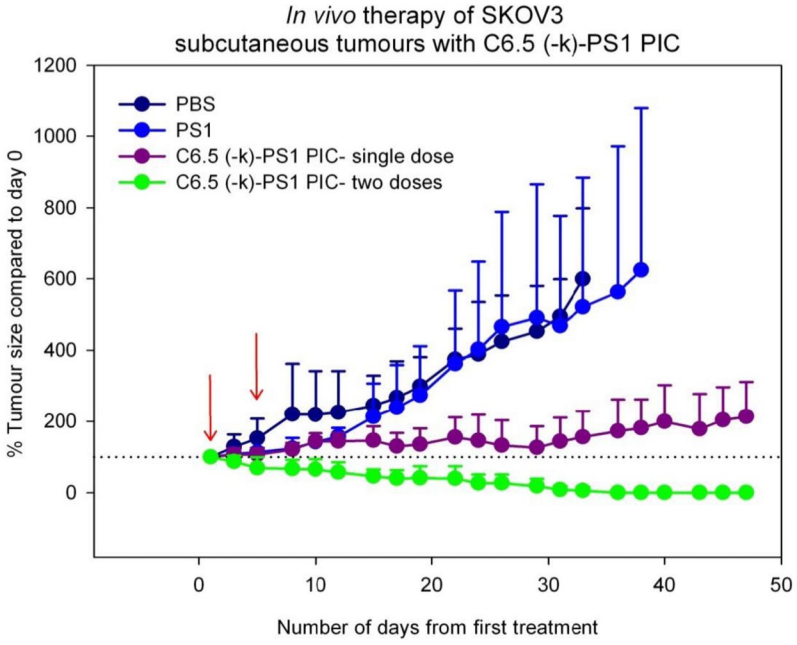

Fig. 9 In vivo PDT therapy using C6.5(-k)-PS1 PIC. Treatment of nude mice bearing SKOV3 tumors using 1 mg ml^{-1} of C6.5(-k)-PS1 PIC. Arrows (—) indicate day of injection followed by illumination on the same day; second arrow does not apply for the single-dose treatment (●). Samples were injected per animal as follows: 1 mg ml^{-1} solution of PIC (● and ●) (200 µg of scFv, 50.6 µg PS1 assuming a 1:6 LR), control samples saline (●) and free PS1 (135 µg) (●). All animals were illuminated 4 h later. Dotted line indicates 0 % tumor size (n = 4 for each dose/sample). Error bars are standard deviation and shown in one direction for clarity (from ref. [36] with permission).

TWO-PHOTON EXCITATION

The second approach to targeting used in the author's laboratories is that of TPE, such that it is only at the focal point of a pulsed laser that there is sufficient intensity for the two-photon process to occur. The effects are thus spatially confined and thus are controlled by the focusing of the laser. The sensitizers developed by Harry Anderson's group in Oxford, which have very high cross-sections for two-photon absorption, are shown in Fig. 10, and the effect on cells illustrated in Fig. 11, from which it can be seen that although the one-photon effect is poorer in comparison with the commercial sensitizers Visudyne, in TPE, the porphyin dimers are much more effective [48,49]. TPE has the advantage of using red or infrared light, which penetrates tissue much more readily that visible light needed for one-photon excitation. The two-photon process may thus have some potential as a means of achieving spatial selectivity in PDT, though it must be admitted that there are practical difficulties associated with focusing lasers within highly scattering media such as human tissue. Nevertheless, the principle has been demonstrated by the two-photon PDT sealing of blood vessels in mice, Fig. 12 [49].

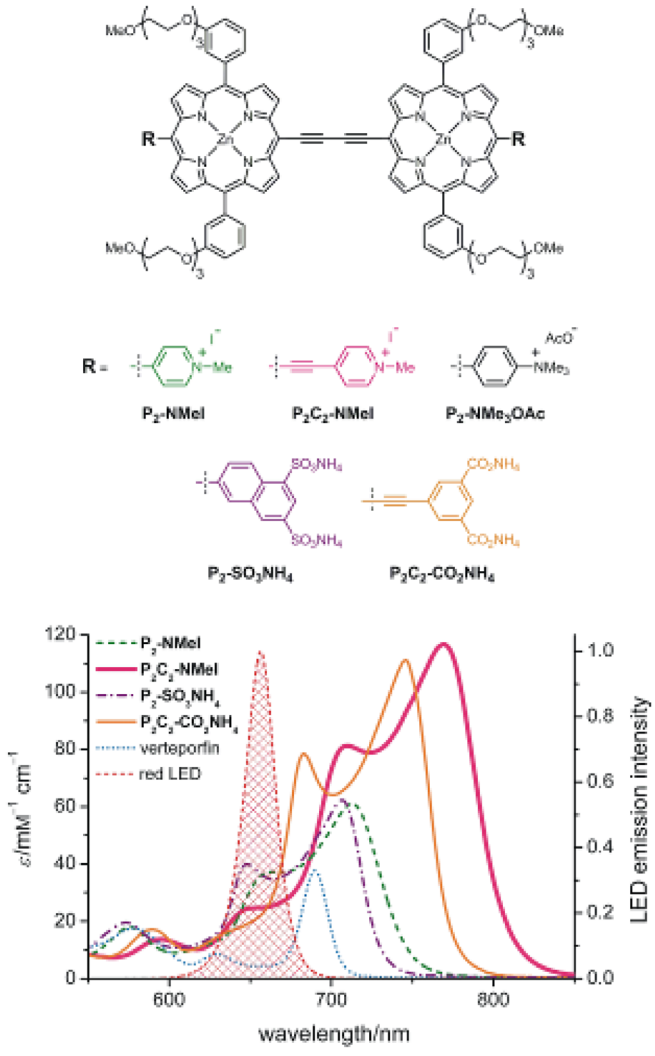

Fig. 10 Structures and absorption spectra of two-photon porphyrin dimer sensitizers (from ref. [37] with permission of RSC).

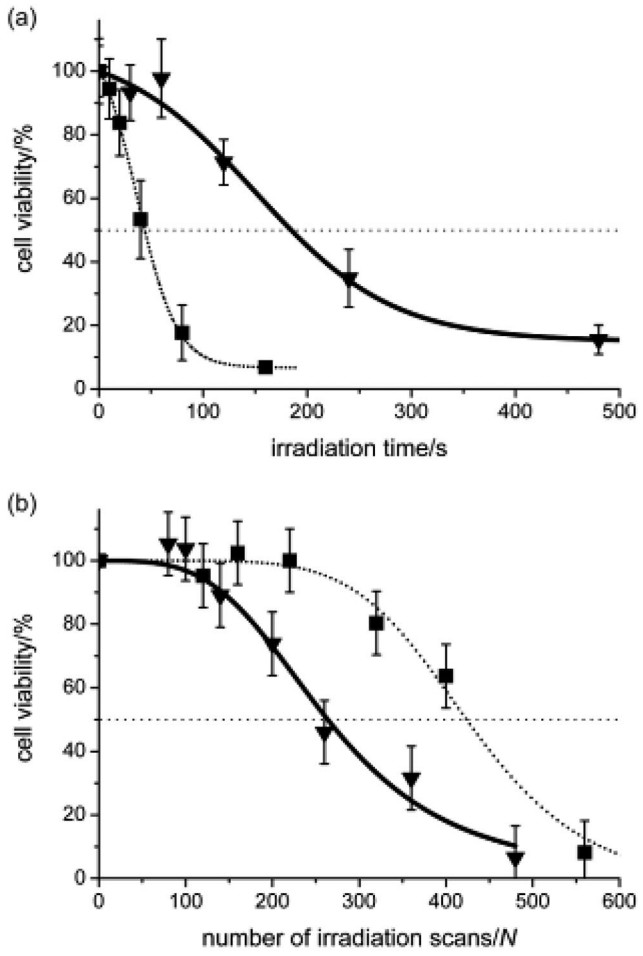

Fig. 11 Viability of cells incubated with porphyrin dimer sensitizers (triangles) in comparison with the commercial photosensitizer Visudyne (squares) in (a) one-photon excitation, (b) TPE (from ref. [48] with permission of RSC).

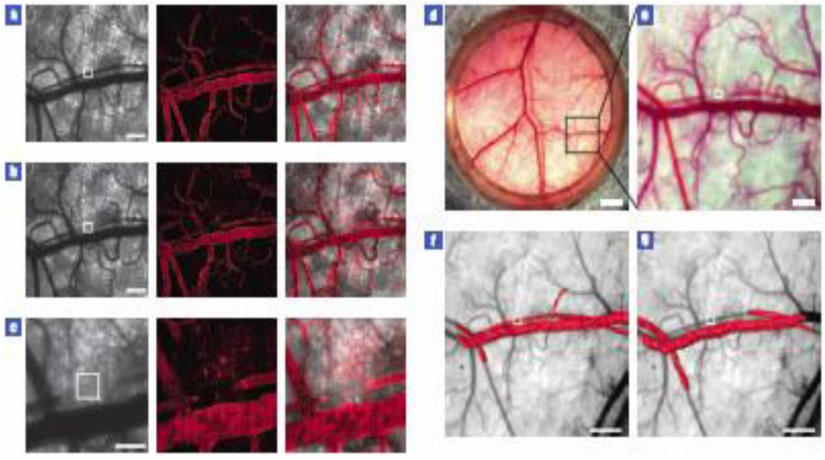

Fig. 12 Two-photon sealing of blood vessel in mouse model (from ref. [49] with permission).

CONCLUSIONS

We have investigated the PDT efficiency of two new second-generation photosensitizers, based on the pheophorbide-*a* structure. Both photosensitizers show singlet oxygen production efficiencies comparable to those of clinical photosensitizers, and higher than PP*a*. Furthermore, even though they demonstrate a lower tendency to aggregate than the parent PP*a*, the PDT efficiencies of the new photosensitizers do not follow the simple trend derived from the photophysical properties, but biodistribution in cells is of critical importance.

We have shown that sensitizers covalently attached to monoclonal antibody fragments are much more efficient at cancer cell kill, and in destruction of tumors in a mouse model.

A new family of PDT photosensitizer porphyrin dimers with high two-photon cross-sections offer a significant improvement in TPE over the commercial photosensitizer Verteporfin. This has been shown to be effective TPE-PDT in vivo in a living mammal. Although the energy density required for two-photon vessel closure with near-infrared light is higher than would be required for one-photon closure with visible light, there is no detectable photothermal damage because the tissue is essentially transparent at the illumination wavelength (920 nm). This study highlights the feasibility of two-photon PDT as a new strategy for targeted occlusion of blood vessels.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge my many colleagues from Imperial College who have collaborated on imaging (Daniel Davis, Paul French, Klaus Suhling, Marina Kuimova and their research groups); in PDT colleagues in PhotoBiotics (Mahendra Deonarain, Gokhan Yahioglu, Lionel Milgrom, and Marina Kuimova and the research students and postdoctorals who contributed); to my Oxford colleague, Harry Anderson and his group, and to my very many students and postdoctorals who contributed to our own efforts in PDT. I acknowledge with gratitude the collaboration I enjoyed with the University College Hospital group in the National Medical Laser Centre (Steve Bown, Colin Hopper, and Sandy MacRobert).

REFERENCES

1. D. V. O'Connor, D. Phillips. *Time-Correlated Single-Photon Counting*, p. 288, Academic Press, London (1984).
2. D. S. Elson, J. Requejo-Isidro, I. Munro, F. Reavell, J. Siegel, K. Suhling, P. J. Tadrous, R. Benninger, P. M. P. Lanigan, J. McGinty, C. Talbot, B. Treanor, S. Webb, A. Sandison, A. Wallace, D. M. Davis, J. Lever, M. A. A. Neil, D. Phillips, G. W. Stamp, P. M. W. French. *Photochem. Photobiol. Sci.* **3**, 795 (2004).
3. K. Suhling, P. M. W. French, D. Phillips. *Photochem. Photobiol. Sci*. **4**, 13 (2005).
4. T. C. Oldham, D. Phillips. *J. Phys. Chem.* **103**, 9333 (1999).
5. J. A. Lacey, D. Phillips. *Phys. Chem. Chem. Phys.* **4**, 232 (2002).
6. M. Towrie, D. C. Grills, J. Dyer, J. A. Weinstein, P. Matousek, R. Barton, P. D. Birley, N. Subramanian, W. M. Kwok, C. Ma, D. Phillips, A. W. Parker, M. W. George. *Appl. Spectrosc.* **57**, 367 (2003).
7. W. M. Kwok, M. W. George, D. C. Grills, C. Ma, P. Matousek, A. W. Parker, D. Phillips, W. T. Toner, M. Towrie. *Angew. Chem., Int. Ed.* **42**, 1826 (2003).
8. P. Matousek, M. Towrie, C. Ma, W. M. Kwok, D. Phillips, W. T. Toner, A. W. Parker. *J. Raman Spectrosc.* **32**, 983 (2001).
9. D. E. J. G. J. Dolmans, D. Fukumura, R. K. Jain. *Nat. Rev. Cancer* **3**, 380 (2003).
10. In *Chemical Aspects of Photodynamic Therapy*, R. Bonnet (Ed.), Gordon and Breach, London (2000).
11. P. J. Lou, L. Jones, C. Hopper. *Technol. Cancer Res. Treat.* **2**, 311 (2003).
12. M. R. Hamblin, T. Hassan. *Photochem. Photobiol. Sci.* **3**, 436 (2004).
13. N. M. Bressler, S. B. Bressler. *Invest. Opthalmol. Vis. Sci.* **41**, 624 (2000).
14. S. Pervaiz. *FASEB J.* **15**, 612 (2001).
15. S. Y. Egorov, V. F. Kamalov, N. I. Koroteev, A. A. Krasnovsky, B. N. Toleutaev, S. V. Zinukov. *Chem. Phys. Lett.* **163**, 421 (1989).
16. E. Skovsen, J. W. Snyder, J. D. C. Lambert, P. R. Ogilby. *J. Phys. Chem. B* **109**, 8570 (2005).
17. M. K. Kuimova, G. Yahioglu, P. R. Ogilby. *J. Am. Chem. Soc.* **131**, 332 (2009).
18. S. Hatz, L. Poulsen, P. R. Ogilby. *Photochem. Photobiol.* **84**, 1284 (2008).
19. J. Moan, K. Berg. *Photochem. Photobiol*. **53**, 549 (1991).
20. E. F. F. Silva, C. Serpa, J. M. Dabrowski, C. P. Montairo, S. J. Formosinho, G. Stochel, K. Urbanska, S. Simoes, M. M. Pereira, L. G. Arnaut. *Chem.—Eur. J.* **16**, 9273 (2010).
21. A. E. O'Connor, W. M. Gallagher, A. T. Byrne. *Photochem. Photobiol.* **85**, 1053 (2009).
22. J. J. Schuitmaker, P. Baas, H. L. L. van Leengoed, F. W. van der Muelen, W. M. Star, N. van Zandwijk. *J. Photochem. Photobiol.* **34**, 3 (1996).
23. V. V. Sokolov, V. I. Chissov, R. I. Yakubovskaya, E. I. Aristarkhova, E. V. Filonenko, T. A. Belous, G. N. Vorozhtsov, N. N. Zharkova, V. V. Smirnov, M. B. Zhitkov. *SPIE Proceedings, Vol. 2625, Photochemotherapy and Photodynamic Therapy and Other Modalities*, B. Ehrenberg, G. Jori, J. Moan (Eds.), pp. 281–287 (1996).
24. W. M. Sharman, C. M. Allen, J. E. van Lier. *Drug Discovery Today* **4**, 507 (1999).
25. H. J. Nyst, I. B. Tan, F. A. Stewart, A. J. M. Balm. *Photodiag. Photodyn. Ther.* **6**, 3 (2009).
26. K. J. Mellish, S. B. Brown. *Exp. Opin. Pharmacother.* **2**, 351 (2001).
27. J. E. Brown, S. B. Brown, D. I. Vernon. *Coloration Technol.* **115**, 249 (1999).
28. D. Phillips. *Prog. React. Kinet.* **22**, 175 (1997).
29. D. Phillips, S. Dhami, R. B. Ostler, Z. Petrasek. *Prog. React. Kinet. Mech.* **28**, 299 (2003).
30. S. Dhami, D. Phillips. *J. Photochem. Photobiol. A* **100**, 77 (1996).
31. R. B. Ostler, A. D. Scully, A. G. Taylor, I. R. Gould, T. A. Smith, A. Waite, D. Phillips. *Photochem. Photobiol.* **71**, 397 (2000).
32. S. Dhami, G. Rumbles, A. J. MacRobert, D. Phillips. *Photochem. Photobiol.* **65**, 85 (1997).

33. Z. Petrasek, R. B. Ostler, I. V. Eigenbrot, D. Phillips. *SPIE Proc.* **3602**, 285 (1999).
34. A. D. Scully, R. B. Ostler, A. J. MacRobert, A. W. Parker, C. de Lara, P. O'Neil, D. Phillips. *Photochem. Photobiol.* **68**, 199 (1998).
35. I. Stamati, M. K. Kuimova, M. Lion, G. Yahioglu, D. Phillips, M. P. Deonarain. *Photochem. Photobiol. Sci*. **9**, 1033 (2010).
36. I. Stamati. *Targeted PDT for cancer using photoimmunoconjugates based on pyropheophorbide-a derivatives*, Ph.D. Thesis, Imperial College London (2010).
37. M. K. Kuimova, H. A. Collins, M. Balaz, E. Dahlstedt, J. A. Levitt, N. Sergent, K. Suhling, M. Drobizhev, N. S. Makarov, A. Rebane, H. L. Anderson, D. Phillips. *Org. Biomol. Chem.* **7**, 889 (2009).
38. M. S. C. Foley, A. Beeby, A. W. Parker, S. M. Bishop, D. Phillips. *J. Photochem. Photobiol. B* **38**, 10 (1997).
39. N. L. Oleinick, R. L. Morris, T. Belichenko. *Photochem. Photobiol. Sci.* **1**, 1 (2002).
40. X. Sun, W. N. Leung. *Photochem. Photobiol*. **75**, 644 (2002).
41. I. J. Macdonald, J. Morgan, D. A. Bellnier, G. M. Paszkiewics, J. E. Whitaker, D. J. Litchfield, T. J. Dougherty. *Photochem. Photobiol.* **70**, 789 (1999).
42. Y. Tan, C. S. Xu, X. S. Xia, H. P. Yu, D. Q. Bai, Y. He, A. W. N. Leung. *Laser Phys. Lett.* **6**, 321 (2009).
43. R. Schneider, F. Schmitt, C. Frochot, Y. Fort, N. Lourette, F. Guillemin, J. F. Muller, M. Barberi-Heyob. *Bioinorg. Med. Chem.* **13**, 2799 (2005).
44. J. Gravier, R. Schneider, C. Frochot, T. Bastogne, F. Schmitt, J. Didelon, F. Guillemin, M. Barberi-Heyob. *J. Med. Chem.* **51**, 3867 (2008).
45. N. Thomas, D. Bechet, P. Becuwe, L. Tirand, R. Vanderesse, C. Frochot, F. Guillemin, M. Barberi-Heyob. *J. Photochem. Photobiol. B* **96**, 101 (2009).
46. D. Bechet, P. Couleaud, C. Frochot, M. L. Viriot, M. Barberi-Heyob. *Trends Biotechnol.* **26**, 612 (2008).
47. P. Couleaud, V. Morosimi, C. Frochot, S. Richeter, L. Raehm, J. O. Durand. *Nanoscale* 20I10 1039/c0nr00096e (2010).
48. E. Dahlstedt, H. A. Collins, M. Balaz, M. K. Kuimova, M. Khurana, B. C. Wilson, D. Phillips, H. L. Anderson. *Org. Biomol. Chem.* **7**, 897 (2008).
49. H. A. Collins, M. Khurana, E. Moriyama, A. Mariammpillai, E. Dahlstedt, M. Balaz, M. K. Kuimova, D. Phillips, B. C. Wilson, H. L. Anderson. *Nat. Photonics* **2**, 420 (2008).

