Photochemical & Photobiological Sciences

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/pps

Photochemical & Photobiological Sciences

PERSPECTIVE

Photodynamic Therapy (PDT) of Cancer: From a Local to a Systemic Treatment

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Janusz M. Dąbrowski*[a] and Luis G. Arnaut*[b, c]

Photodynamic therapy (PDT) requires a medical device, a photosensitizing drug and adequate use of both to trigger biological mechanisms that can rapidly destroy the primary tumour and provide long-lasting protection against metastasis. We present a multidisciplinary view of the issues raised by the development of PDT. We show how the spectroscopy, photophysics, photochemistry and pharmacokinetics of photosensitizers determine mechanism of cell death and clinical protocols. Various examples of combinations with chemotherapies and immunotherapies illustrate the opportunities to potentiate the outcome of PDT. Particular emphasis is given to the mechanisms that can be exploited to establish PDT as a systemic treatment of solid tumours and metastatic disease.

Background

Photodynamic therapy (PDT) is a clinically approved, minimally invasive therapeutic procedure, which is entering the mainstream of the cancer treatments.[1] PDT is a two-steps procedure. First, a drug that absorbs light in the phototherapeutic window (650-850 nm), where tissues are more transparent, is administered to the organism. Next, after a time named the drug-to-light interval (DLI), the target tissue is irradiated. The drug is inactive in the dark, but when electronically excited transfers an electron to molecular oxygen or other electron acceptors leading the formation of superoxide anion and radicals (type I reaction), or transfers its electronic energy to ground-state molecular oxygen generating singlet oxygen ($^1\Delta_g$) (type II reaction).[1-5] The oxidative stress due to the local generation of reactive oxygen species (ROS) triggers three main biological mechanisms that make PDT an effective anti-cancer procedure: vascular shutdown and consequent massive ischemic death of tumour tissue,[6-7] direct killing of tumour cells induced by the oxidative stress inside the cells,[8-9] and PDT-induced acute local and systemic inflammation that eventually stimulates T-cell activation and generate antitumour immune memory and systemic responses.[10]

PDT is a carrefour of physical and life sciences, and its success in oncology depends on contribution from physics, chemistry and pharmacology. Figure 1 illustrates the interplay of the more relevant contributions from these fields. Elegant reviews have recently highlighted various perspectives of PDT, such as the physics of dosimetry,[11] the synthesis of photosensitizing drugs with stronger absorption in the phototheraputic window,[12-13] or biological mechanisms of PDT.[1,14] Our vantage point is that of developing treatment regimens with tailored photosensitizers to intensify the biological mechanisms of PDT and optimize PDT efficacy.

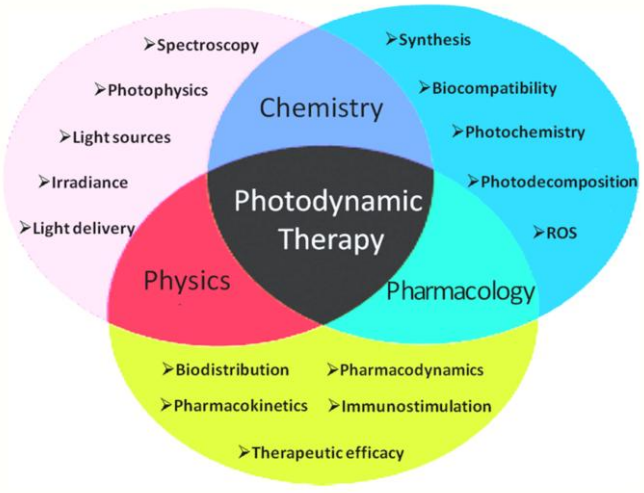

Fig.1 Photodynamic therapy as an interdisciplinary therapeutic approach involving chemistry, physics and pharmacology.

Our work is based on a multidisciplinary approach to PDT. This review begins with a brief presentation of the properties of photosensitizers in clinical use, because they provide the grounds to understand phototoxicity. We highlight properties related to the photophysics, photochemistry and pharmacokinetics of the photosensitizers. The success of PDT is expected to depend on the ability to tailor the treatment regimens to the properties of the photosensitizers and the

[a] Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Kraków, Poland.
[b] Chemistry Department, University of Coimbra, 3004-535 Coimbra, Portugal.
[c] Luzitin SA, Ed. Bluepharma, 3045-016 Coimbra, Portugal.
* Dr. J. M. Dabrowski, phone: +48126632293, fax: +48126340515, e-mail: jdabrows@chemia.uj.edu.pl
* Prof. Luis G. Arnaut, fax: +35123927703, e-mail: lgarnaut@ci.uc.pt
† *Part of the data in this paper were presented during the 16th International Congress on Photobiology held in Cordoba, Argentina, in September (8th - 12th), 2014.*

clinical condition. We reveal how the rationale for a clinical protocol evolved from the investigation of treatment regimes. The acceptance of PDT in oncology will ultimately be tied with its shift from a local to a systemic therapy. Consequently, we will conclude with a discussion of the molecular mechanisms of cellular death and their ability to stimulate immune responses after PDT.

Photosensitizers

Most of synthetic photosensitizers investigated for PDT of cancer are porphyrins,[15-20] chlorins,[19-27] bacteriochlorins[12-13, 28-37] or phthalocyanines.[38-41] The investigation of photosensitizers for PDT that are not based on tetrapyrrole macrocycles has been considerably less extensive, but structural modification of fullerenes,[42] cyanines,[43] hypericins[44] or semiconductors[45] has allowed for manipulation of photochemotherapeutic properties to an interesting degree.[46] However, the photosensitizers in clinical use for the treatment of solid tumours are still porphyrin and phthalocyanine derivatives, and they are the focus of this work. Although precursors of protoporphyrin IX such as the 5-aminolevulinic acid (Levulan®), and its methyl (Metvix®), hexyl (Hexvix®) or benzyl (Benzvix®) ester derivatives have met with considerable success in topical applications of PDT, they will not be discussed here because the actual photosensitizer is protoporphyrin IX. Structures of photosensitizers in clinical use or in active clinical trials in the European Union or in the United States are shown in Figure 2 and their most relevant properties are presented in Table 1.

Table 1. Characteristics of clinically used photosensitizers.

Sensitizer	λ_{max} [nm]	ε_{max} M^{-1} cm^{-1}	Φ_{pd} [a]	Dose mg/kg	Light dose J/cm^2	DLI h	Cancer indications
Porfimer sodium Phorofrin	630	$3 \cdot 10^3$	$5 \cdot 10^{-5}$	2-5	100-200	24-48	lung, oesoph., bladder
Temoporfin Foscan mTHPC	652	$3 \cdot 10^4$	$2 \cdot 10^{-5}$	0.1-0.3	8-12	24-48	Head, neck, lung
Silicon phthalocyanine (**Pc4**)	675	$2 \cdot 10^5$	-	0.5-2	100	24-72	c.neoplasms
Verteporfin Visudyne BPD-M	688	$3.3 \cdot 10^4$	$5 \cdot 10^{-5}$	0.1-2	100-200	0.5-3	skin, pancreas AMD
Talaporfin Laserphyrin	660	$4 \cdot 10^4$	$8 \cdot 10^{-4}$	0.5-3.5	25-100	24-72	liver, pancreas
Padeliporfin Stakel WST11	762	$1.1 \cdot 10^5$	$1 \cdot 10^{-3}$	4	200	0.25	prostate
Redaporfin LUZ11	743	$1.4 \cdot 10^5$	$8 \cdot 10^{-6}$	0.5	50	0.25	head and neck

[a]Aqueous solution, with surfactactants or serum proteins for liphophilic sensitizers[47-48]

Porfimer sodium, a hematoporphyrin derivative, was the first PDT sensitizer approved by the US Food and Drug Administration (FDA). It gave encouraging results in the treatment of certain types of cancer (e.g., lung cancer, oesophagus),[49] but the medical community did not perceive it as a breakthrough. The underachievement of porfimer sodium can be related to some of its properties: (i) it is complex mixture of porphyrin dimers and higher oligomers,[50] (ii) the low molar absorption coefficient at 630 nm decreases the likelihood of excitation and requires relatively high doses (in mg/kg) to obtain therapeutic effects,[51] (iii) the tissue penetration of 630 nm light is low, (iv) it forms aggregates in water with low singlet oxygen quantum yields,[51] (v) selectivity between tumour and normal tissues is poor, (vi) skin phototoxicity in patients lasts for four to six weeks after administration.[52-53] This long photosensitivity period is due to the mean apparent elimination half-life of 21.5 days of porfimer sodium.[54]

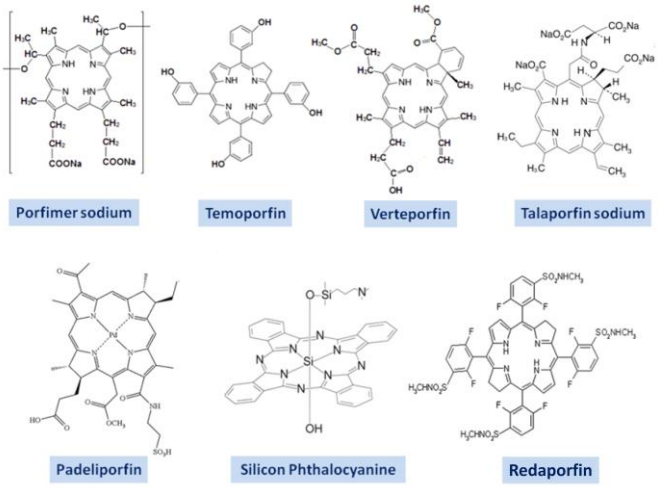

Fig. 2 Chemical structures of selected clinically used photosensitizers.

Second-generation synthetic photosensitizers such temoporfin or silicon phthalocyanine 4 (Pc4) have intense absorption bands at longer wavelengths, enabling treatments to increasing depths, and shorter skin photosensitivity periods. The terminal plasma half-life of temoporfin is 65 h, leading to 15 days daylight quarantine,[55] and that of Pc4 is 28 h.[56] Clinical protocols with temoporfin prescribe drug doses of 0.1-0.15 mg/kg and illumination of tumours with red light (652 nm) with a total dose of 10–20 J/cm^2 after DLI of 4–6 days. This long DLI is based on the times at which maximum drug concentration differentials have been measured between the tumour and the surrounding normal tissues.[57-58] Generally, synthetic porphyrin derivatives clear more slowly from the tumour than from normal tissues, and longer drug-to-light intervals reduce side effects.[59] Successful semi-synthetic photosensitizers of second-generation photosensitizers, such as verteporfin (benzoporphyrin derivative monoacid ring A, BPD-MA) or talaporfin (mono-(L)-aspartylchlorin-e6, NPe6), are based on the chlorin macrocycle and also exhibit intense absorption band in the red. Verteporfin was particularly successful in the treatment of age-related macular degeneration (AMD) under the trade name Visudyne®. Verteporfin has a maximum plasma concentration at the end of infusion and is used in the treatment of AMD with DLI = 15 minutes. Moreover, verteporfin has an elimination half-life of 5 to 6 h, which reduces the period of skin photosensitivity to less than 48 h.[60] Motivated by its success AMD, verteporfin

was investigated in oncology, namely in the treatment of multiple nonmelanoma skin cancers,[61] or pancreatic cancer.[62] Such studies used DLI of 1 to 3 hours, skin photosensitivity after the treatment was not reported, and the general conclusion was that PDT with verteporfin was feasible and safe. The claimed advantages of verteporfin over temoporfin and hematoporphyrin derivatives have not yet led to marketing approval for cancer indications.

More recently, two photosensitizers based on the bacteriochlorin macrocycle reached clinical trials: padeliporfin (WST11) and redaporfin (LUZ11). The purported advantage of this last generation of photosensitizers in their strong absorption in a spectral region (740-780 nm) that combines enough energy for irreversible energy or electron transfer to molecular oxygen with sufficient tissue penetration ability. Padeliporfin has an elimination half-live in the range of minutes,[63] which reflects its lability, common to photosensitizers derived from natural bacteriochlorophylls or bacteriochlorins. The fast elimination minimizes the risk of skin photosensitivity but required a clinical protocol for prostate cancer where a 10 minutes infusion of WST11 is followed by continuous irradiation of the prostate through optical fibres for 20-25 minutes. The drug dose of 4 mg/kg and 200 J/cm light turned out to be the optimal treatment conditions for vascular-PDT (i.e., DLI<15 min, when the drug is in the vascular compartment), resulting in over 80% of patients treated with this protocol having a negative biopsy at 6 months.[64] Interestingly, WST11 generates ROS almost exclusively through type I reactions.

Redaporfin is a synthetic bacteriochlorin specially designed to combine the spectroscopic properties of bacteriochlorins with photostability. As shown in Table 1, the photodecomposition quantum yield of redaporfin in Cremophor EL:Ethanol:NaCl 0.9% (0.2:1:98.8, v:v:v) solution, $\Phi_{pd}=8\times10^{-6}$, is the lowest in that table. This was achieved with the introduction of fluorine atoms in the ortho positions of the phenyl rings of tetraphenylbacteriochlorins, which raises the oxidation potential of redaporfin to 0.80 V vs. SCE.[33] Additionally, these halogen atoms increase the triplet quantum yield by promoting the spin-orbit coupling in the electronically excited orbital, a phenomenon known as the heavy-atom effect.[65] Redaporfin also has sulfonamide substituents in the meta positions of the phenyl rings to modulate amphiphilicity and further stabilize the structure. Its *n*-octanol-water partition coefficients is $P_{OW}=1.9$.[33] The increased stability of redaporfin also has implications in its pharmacokinetics: ca. 80% of redaporfin is cleared from the plasma of minipigs in 12 h but the terminal plasma half-life is long.[37] This enables the use of redaporfin both for vascular-PDT and for cellular-PDT (i.e., DLI>24 h and the drug diffused into the tumour tissue).[66] Skin photosensitivity has not yet been observed in the on-going clinical trial, although the therapeutic benefit is already evident. Redaporfin was shown to interact with molecular oxygen both through type I and type II reactions.[33]

It is very difficult to observe and quantify singlet oxygen emission from single cells or from tissues.[67] Thus, singlet oxygen quantum yields (Φ_Δ) are usually reported in organic solvents. However, such values of Φ_Δ can be misleading because the concentration of oxygen drops from $[O_2]\approx2$ mM in organic solvents to ca. 10 μM in tissues at the onset of hypoxia. Moreover, the diffusion rate constant decreases from $k_q\approx2\times10^9$ $M^{-1}s^{-1}$ in solution to 4×10^8 $M^{-1}s^{-1}$ in cells. This imposes requirements on the photosensitizer triplet lifetime, τ_T. For example, the highest Φ_Δ that a photosensitizer with $\tau_T=10$ μs can attain in a tissue at the limit of tumour hypoxia is $\Phi_\Delta=0.04$.[68] This restriction is alleviated with photosensitizers with longer τ_T. Redaporfin has $\tau_T\approx50$ μs, which leads to an estimated $\Phi_\Delta=0.17$ in the same conditions.

Porfimer sodium and temoporfin, together with most of the porphyrin and chlorin photosensitizers, favour type II reactions in cells.[69-70] As long as the laser is on, the cycle of light absorption, excited state energy transfer and generation of singlet oxygen is only limited by the photodecomposition of the photosensitizer. In principle, one photostable photosensitizer molecule could generate an infinite amount of singlet oxygen molecules. In practice, all photosensitizers have finite Φ_{pd} and bleach. The values of Φ_{pd} reported for porfimer sodium and temoporfin in organic solvents or in aqueous:organic solvent mixtures decrease when oxygen is removed from the solution,[47] which indicates that singlet oxygen contributes to photodecomposition.

Padeliporfin has a contrasting behaviour because it photo-oxidizes rapidly in aqueous solutions and does not generate singlet oxygen.[71] The photodynamic efficacy of padeliporfin in aqueous media is based exclusively on type I reactions, with superoxide ion and hydroxyl radical generation concomitant with formation of the $WST11^{\bullet+}$ radical cation. The photodecomposition is lowered by the addition of human serum albumin (HSA) to the solution, because WST11 binds to this plasma protein and forms a complex that seems to support 15 cycles of light-induced electron transfer from the amino acid residues of the protein to $WST11^{\bullet+}$ regenerating WST11, which then transfers an electron to molecular oxygen, before the complete degradation of WST11.

The lifetime of the hydroxyl radical in cells is 1 ns, which limits to 1 nm the range of distances over which the hydroxyl radical can effect damage.[72] On the other hand, lifetime of intracellular singlet oxygen is 2 μs and the radial diffusion distance of singlet oxygen is estimated to reach 150 nm.[73] Thus, $OH^\bullet$ radical has a shorter half-life than singlet oxygen in biological systems and this can limit the range of oxidative damage by the radical. But both species are clearly highly-reactive agents, capable of modifying properties of any biologic molecules in the vicinity of their formation. The strong oxidative stress generated by hydroxyl radicals accentuates the oxidation of the photosensitizers that generate them, and limits the cycles of light absorption and ROS generation. The photodecomposition quantum yield of a "photostable" photosensitizer, Φ_{pd}, defined as (initial rate of disappearance of photosensitiser molecules)/(initial rate of absorption of photons), tells that ca. 10^5 photons may be initially absorbed before the first photosensitiser molecule is bleached in solution. In cells bleaching tends to be much faster but

thousands of singlet oxygen molecules can be generated by one photosensitizer molecule before it bleaches. For sufficiently high photon fluxes the cells antioxidant mechanisms will be unable to deal with the singlet oxygen photogeneration and it also becomes cytotoxic.

Photosensitizers such as verteporfin and redaporfin have the ability to participate both in type I and type II reactions.[33,74] The competition between energy and electron transfer from the photosensitizer to molecular oxygen favours the first reaction both for verteporfin and redaporfin. The difference with respect to WST11 is certainly related to the higher triplet state oxidation potentials of verteporfin and redaporfin, which lower the electron transfer rates and increase the photostability.[33,75] The photodecomposition quantum yields of verteporfin and redaporfin in aerated ethanol are 6.8×10^{-5} and 6.9×10^{-7}, respectively. These very low Φ_{pd} are probably favoured by the ability of ethanol to quench hydroxyl radicals, but Table 1 shows that redaporfin is also remarkably photostable in micelles. The generation of both singlet oxygen and hydroxyl radical in the tumour tissue was proposed to bring about synergistic effects that increase phototoxicity.[76] This and the strong absorption of redaporfin in a region of increased tissue transparency, shown in Figure 3, motivated a detailed investigation of the relation between treatment regimens and the outcome of PDT with redaporfin.

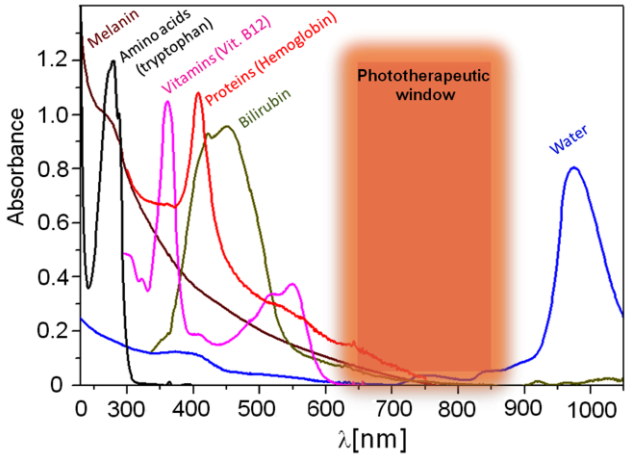

Fig. 3 Phototherapeutic window: the electromagnetic radiation between 650 and 850 nm of the spectral region possesses a high penetration power into human tissues and where these tissues are the most transparent.

Drug and light dosing

The optical penetration depth (δ) of light is defined as the depth at which its intensity of light is reduced to $1/e$ of the value at the surface. The value of δ for the skin increases from 1.8 mm at 633 nm, to 2.0 mm at 660 nm and reaches 2.3 at 750 nm.[77] These values are the basis for the calculation of the radiant exposure attenuation with the tissue penetration depth z,

$$R = R_0 \exp(-z/\delta) \quad (1)$$

where R_0 is the radiant exposure at the tissue surface, usually expressed in J/cm^2. The oxidative stress generated by PDT can be estimated, in the absence of photobleaching, in terms of the total amount of ROS produced per unit volume of tissue[78]

$$[\mathrm{ROS}] = F_{ROS}(1000/hcN_A)\,\beta[\mathrm{photosensitizer}]_{local} \quad (2)$$

The threshold ROS dose that produces tissue necrosis depends on physiologic factors. Values between [ROS]=1[79] and 10 mM[80] have been reported and probably covers a wide range of cases. The dependence of the depth of tumour necrosis on the properties of photosensitizers can be illustrated with the series of *meta*(tetrahydroxyphenyl)porphyrin (mTHPP) and the corresponding chlorin (mTHPC, temoporfin) and bacteriochlorin (mTHPBC). Using their photophysical properties[25] and assuming that they all attain the concentration of 70 µM in the tumour 24 h post-intraperitonial administration of 0.26 mg/kg of photosensitizer, as observed for mTHPC.[81] Eq. (2) for a radiant exposure of 10 J/cm^2 gives [ROS]<5 mM for mTHPP even for z=1 mm, whereas for mTHPC this limit is reached for z>4 mm, and for mTHPBC it occurs for z>8 mm. This estimate of photonecrosis is in good agreement with the observations for mTHPP[82] and for mTHPC.[81] However, the depth of photonecrosis observed for mTHPBC is only 5.2 mm,[83] significantly less than expected from Eq. (2). This discrepancy is readily explained by the photodecomposition of mTHPBC during the irradiation, because this bacteriochlorin has a Φ_{pd} comparable to that of WST11.[33]

The major advantage of using bacteriochlorins to treat deep-seated tumours is in the high molar absorption coefficient of the absorption band in the 740-780 nm region. However, it is only possible to fully exploit this advantage using bacteriochlorins that are as photostable as temoporfin. This photostability was thoroughly explored in protocols designed to treat BALB/c mice with subcutaneously implanted CT26 with redaporfin vascular-PDT. An objective method to evaluate the practical limits of the photostability is, for a given drug dose, to escalate the radiant exposure and see if the outcome of the therapy improves with the escalating drug dose. Figure 4 uses data from a recent publication[84] where the median survival time of the control group is 11 days and remains unchanged in the Redaporfin-PDT group 5 using a drug dose of 0.52 mg/kg followed 15 minutes after intravenous administration by the illumination of the tumour with a radiant exposure of 39 J/cm^2.

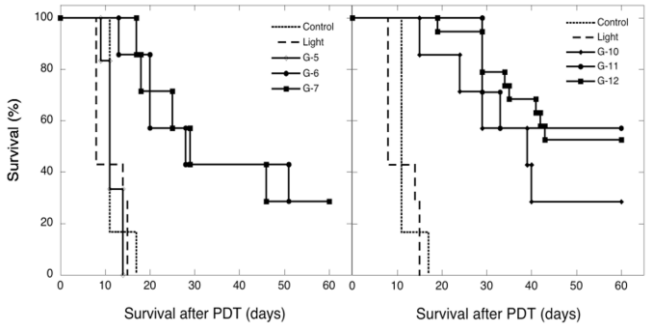

Fig. 4 Kaplan-Meier plots of Redaporfin-PDT with DLI=15 min, where G-5, G-6 and G-7 are treatment groups with 0.52 mg/kg drug dose and ascending radiant exposures of 39, 56 and 69 J/cm^2, and G-10, G-11 and G-12 are treatment groups with 0.75 mg/kg drug dose and ascending radiant exposures of 39, 53 and 74 J/cm^2.

Increasing the radiant exposure to 56 J/cm^2 for the same drug dose and DLI, the median survival time increases to 28 days. However, a further increase in the radiant exposure to 69 J/cm^2 does not significantly increases the median survival time, which stays at 29 days.

The lack of response to the increased radiant exposure suggests that redaporfin was appreciably bleached in the irradiated zone. Increasing the drug dose of 0.75 mg/kg offered a wider range of effective radiant exposures. Groups 10, 11, and 12 used a drug dose of 0.75 mg/kg and radiant exposures of 39, 53 and 74 J/cm^2, respectively. The median survival time with 39 J/cm^2 was 39 days and for the other two light doses, the cure rates were higher than 50%. Further improvement of the cure rates required an increase in the surface area irradiated to a circle of 13 mm concentric with the 5 mm tumours used in these treatments. A cure rate of 86% were obtained with DLI=15 min, a redaporfin dose of 0.75 mg/kg and a radiant exposure of 50 J/cm^2 (i.e., total light dose of 67 J).[84]

The margin of 4 mm that optimized the treatment must be regarded as a 3-dimensional margin. Assuming that the tumour is 5 mm thick, the successful treatment should correspond to a treatment of a depth of 9 mm. Eq. (2) can be applied to estimate this margin using the photophysical properties of redaporfin[33] and $[redaporfin]_{local}$. However, at DLI=15 min the drug is entirely in the vascular compartment and the example discussed above is a case of vascular-PDT. The concentration of redaporfin in the blood is $[redaporfin]_{blood}$=29 μM at DLI=15 min for an intravenous injection of 2 mg/kg,[37] and can be expected to be ca. 10 μM for a drug dose of 0.75 mg/kg. Using Φ_{ROS}=0.59 to account for the generation of both singlet oxygen and superoxide ion with redaporfin, $[redaporfin]_{local}$=10 μM at DLI=15 min R_0=50 J/cm^2, we calculate [ROS]<5 mM for z>9 mm. Although various assumptions are involved in these calculations, they nevertheless reveal the importance of a using a drug and light doses that produce a necrotic volume all around the tumour with a margin of 4 mm, which is also a typical surgical margin. Protocols with longer DLI have also been tested with redaporfin.[33,37,66,84] The clearance from the body requires the use of larger drug and light doses at longer DLI, and cures have been observed in mice with subcutaneously implanted tumours using DLI=72 h. However, the therapeutic index, assessed as the range of doses between the observation of the first cures and the onset of PDT-induced lethality, tends to be narrower for DLI=72 h than for DLI=15 min.

A blunt evaluation of these results may lead to the conclusion that vascular-PDT is comparable to surgery: they both eliminate solid tumours with a margin of healthy tissue. PDT can claim that it is much less invasive than surgery because optical fibres can reach virtually all parts of the body with little discomfort, and that it can be repeated without loss of efficacy or increased in side effects. Although this is uncontroversial, the most important difference between PDT and surgery may on their impact in the immune system. In order to understand this impact, it is necessary to give a perspective of the pharmacodynamics of PDT photosensitizers.

Localization of photosensitizers

Localization of the photosensitizer in the tumour is affected by several factors such as: charge of the photosensitizer, degree of aggregation, solubility (hydrophilic, hydrophobic or amphiphilic character), administration vehicle, and time between administration and irradiation (DLI).[9] If the photosensentizer is sufficiently stable and is not completely eliminated from the body in a few hours, it can be used either in vascular-targeted photodynamic therapy (V-PDT, DLI≤15 min) or cellular-targeted photodynamic therapy (C-PDT, DLI≥12 h). In the later case, the intracellular localization of the photosensitizer is most relevant for the outcome of the therapy.[85]

The diffusion distance of singlet oxygen in a cell is ca. 550 nm, much smaller than the 10-30 μm diameter of typical eukaryotic cells.[73] The more reactive $OH^{\bullet}$ radical is expected to have an even smaller diffusion distance. Hence, the space probed by these ROS is a small fraction of the cell volume and the intracellular localization of the photosensitizers will restrict their primary targets. Most porphyrins and their derivatives localize at the level of the cell membranes, including cytoplasmic, mitochondrial and lysosomal membranes, of the Golgi apparatus and of the endoplasmic reticulum (ER).[9,86] An exception is meso-tetra(4-N-methylpyridyl)porphine, a cationic porphyrin, which was found to localize at the nuclear level in cultured cells.[87-88] The high polarity of hydrophilic and anionic photosensitizers may restrict their passive diffusion across the plasma membrane, and such photosensitizers are typically internalized by endocytosis. Consequently, they are primarily localized in lysosomes. A classic example is tetrasulfonated meso-tetraphenylporphyrin,[89-91] but talaporfin and padeliporfin also tend to localize in lysosomes.[92-94] On the other hand, more lipophilic photosensitizers can diffuse across the plasma membrane and redistribute between the membranes of cellular organelles. Association of lipophilic photosenstitizers with low-density lipoproteins (LDL) may facilitate cellular uptake, but it is believed that the photosensitizer leaves the LDL particle at the plasma membrane and diffuse into the cytoplasm.[95] After prolonged incubation with A431 cells, Photofrin® enters mainly the endoplasmic reticulum (ER)/Golgi apparatus as to a less extent can be found at other perinuclear sites.[96-97] It is also established that Foscan® after 3 h of incubation with MCF-7 cells can be found both in the ER and in the Golgi apparatus, but after 24 h it extrudes from the Golgi and is essentially in the ER, with only a weak distribution in the mitochondria.[98] Interestingly, tolyporphin, a highly phototoxic porphyrin derivative extracted from cyanobacteria, also localizes preferentially in the ER and this was associated with its PDT efficacy.[99] Verteporfin localizes in the mitochondria and also in perinuclear area where the nuclear membrane and the ER are located.[100-101] Pc 4 also localized preferentially in mitochondria and ER/Golgi membranes.[102] On the contrary, its derivatives bearing hydroxyl axial ligands which are less aggregated than Pc 4, are localized in the lysosomes and are more efficient than Pc 4.[102] There are some reports claiming that photosensitizers

localized in the ER or in the mitochondria are more efficient at generating phototoxicity by direct induction of apoptosis,[103] but in fact there are multiple determinants of photokilling and the best choice may depend on the tumor phenotype. There are multiple sites of localization with some agents having a broad spectrum of localization sites (e.g. Photofrin), while other photosensitizers are far more specific. Photodamage to lysosomes can inhibit autophagy, a potentially cytoprotective effect. The loss of autophagy could therefore improve the overall photodynamic effect in cells capable of initiating an apoptotic program in response to photodamage.

On the other hand, localization in mitochondria or the ER could cause photodamage to Bcl-2 or release of cytochrome c, also with pro-apoptotic consequences. Halogenated sulfonamide bacteriochlorins, including redaporfin, are preferentially located in the endoplasmic reticulum and can also be found in the mitochondria.[33,76] Figure 5 presents fluorescence microscopy images of the intracellular distribution of redaporfin in A549 cells.

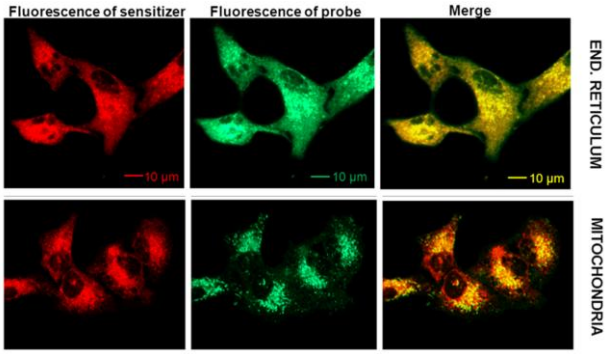

Fig. 5 Intracellular distribution of redaporfin in A549 cells studied by fluorescence microscopy high localization of evaluated photosensitizer in endoplasmic reticulum and mitochondria.

The intracellular localization of the photosensitizers determines where in the cell the oxidative stress attains its peak and this has implications on the mechanism of cell death. Arguably the most important implication is in the guidance towards non-immunogenic versus immunogenic cancer cell death.[104] It is becoming increasingly clear that immunogenic cell death requires both endoplasmic reticulum stress and ROS production,[105-106] which is a combination remarkably achieved with redaporfin.

Photodynamic effect mechanisms

The direct photodynamic effect toward tumour cells results from the interaction of ROS with various biological targets, which are prone to destruction due to the oxidation, disruption of homeostasis, changes in lipid metabolism and ion transportation. The cellular response to oxidative stress also leads to activation of protein kinase pathway, the expression of transcription factors and cytokines and release a number of mediators responsible for the process of cell death, which can occur by apoptosis and/or necrosis (Fig. 6).[107]

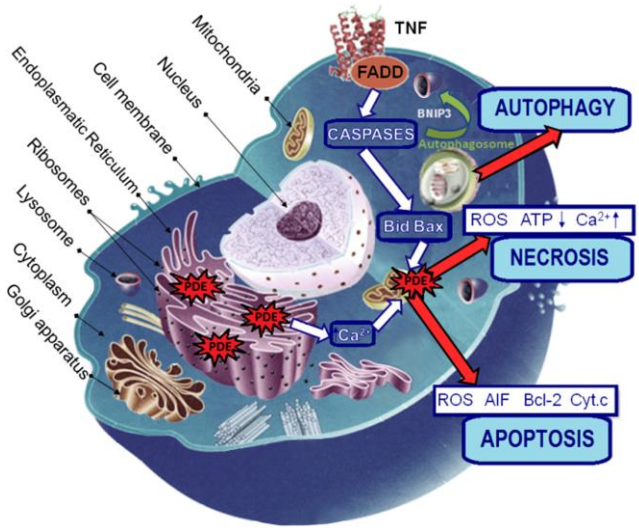

Fig. 6 Schematic illustration of types of the organelles dependent pathways in PDT that might lead to the cell death, and indication of localized photodynamic effects (PDE).

The third possible pathway illustrated in Figure 6 that might lead to the cell death, but at the same time can be cytoprotective, is called autophagy. These pathways are not mutually exclusive and may occur simultaneously in the same cell population.

Apoptosis (programmed cell death) is a complex physiological process that determines the proper functioning of the body through the elimination of unwanted cells without causing disturbances in their integrity or eliciting inflammatory responses.[108] Changes in cellular morphology, such as shrinkage, cell surface blebbing, chromatin condensation and DNA fragmentation triggered by Apoptosis Inducing Factor (AIF) are typical indicators of apoptosis. Depending on the type of cellular organelles involved in this process, apoptosis can occur according to specific pathways: receptor pathway (cell membrane), the mitochondrial pathway (mitochondria), pseudo receptor pathway (T cells, NK cells) and the stress-induced sfingomyelin-ceramide pathway (ER). A common element to all these programmed cell death pathways is the activation of caspase cascades. Their activation triggers a number of biochemical processes that execute apoptosis. The best-known molecular mechanisms of apoptosis are extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway.[109] Operation of the external pathway is based on the membrane receptors and ligands. Among various membrane receptors, several necrosis factor (TNF) receptors were identified. After binding of the respective ligands to the receptors, signal transduction of death to protein FADD occurs and has the ability to interact with caspases. The mitochondrial pathway is activated by an increase of ROS, ion transport disruption or an increase the concentration of Ca^{2+} in the cytoplasm.[102] The primary role of mitochondria in apoptotic signaling pathway is the regulation of the release of pro-apoptotic molecules, such as cytochrome c into the cytoplasm. This release is controlled by proteins of the Bcl-2 family. When two pro-apoptotic members of the Bcl-2 family called Bax and Bak, are activated, they oligomerize, insert into

the mitochondrial membrane and form channels through which cytochrome c and other proteins escape to the cytosol. Cytochrome c, a key protein in mitochondrial electron transport, once in the cytosol activates caspase-9 that eventually leads to the formation of apoptosome and further cascades of caspases. The ability of cytochrome c in the cytosol to activate caspases play a critical role in loss of cells functionality and apoptotic cell death during PDT.[107] The protein Bid, a natural substrate of caspase-8, is the nexus between extrinsic and intrinsic pathways. Bax overexpression is a consequence of a Bcl-2 transfection resulting in simultaneous overexpression of Bax.[110-113]

Necrosis is a pathological process leading to a loss of membrane integrity and complete degradation of the cell causing the release of cellular contents into the intercellular space. The disintegration of intracellular organelles results in a strong response from the immune system and the onset of inflammation. Necrosis takes place above the threshold of resistance of cells treated with non-physiological disturbances, thus it is often observed after PDT with high light and photosesensitizer doses.[10] Necrosis is associated with a significant decrease in ATP level resulting from ions imbalance due to depolarisation of the mitochondrial membrane. The volume of the cell is increased and the membrane integrity is lost due to the damage, which causes the passive influx of Ca^{2+}, Na^{+} and water to its interior. Typical necrotic changes are also a high concentration of Ca^{2+} arising from their influx from extracellular environment and the outflow from the endoplasmic reticulum undergoing destruction. Activation of many nucleases of DNA and release of lysosomal hydrolytic enzymes consequently leads to the total cell lysis.[107,110-113]

The last possible pathway illustrated in Figure 6 that might lead to the cell death is autophagy. It is a process of degeneration of the macromolecular components of the cytoplasm and organelles, which are surrounded by a double membrane forming autophagosome. Following the merger of the lysosome with autophagosome, its content is degraded. This type of cell death also allows cells to survive under oxidative stress. In particular, cancer cells may be involved in their survival or death. The role of PDT-triggered autophagy in cancer therapy resistance or susceptibility is recently reviewed.[93] Nevertheless it should be noted that it is not yet clear how to reconcile the few examples of 'cell death by autophagy' with the large number of studies indicating a pro-survival function of autophagy. It is possible that autophagy is not always a cell death impostor, but the majority of studies suggest that the primary function of autophagy lies in cytoprotection and promotion of cellular and organismal health.[114] Nevertheless, this process can play an important role in the functioning of the immune system.[115-117] Thus, antigens exposed by the action of autophagy on malignant cell types after photodamage could evoke additional immunologic elements of cancer control.[115]

Various factors govern the mechanism of cell death induced by PDT: the fluence rate of the light source, the radiant exposure, the type of photosensitizer, its physicochemical properties, subcellular localization and local concentration, and the concentration of oxygen in biological targets.[113] The relevance of the intracellular localization of the photosensitizers is revealed by their *in vitro* phototoxicity. The accumulation of photosensitizer molecules in organelles such the mitochondria is thought to efficiently trigger cell death upon illumination.[118] The oxidative stress in mitochondria not only induced by the primary photodegenerated ROS, but also by superoxide anion generated as a secondary product due to photodamage of components of the electron transport chain.[119] Photosensitizers targeting the endoplasmic reticulum have been reported to mediate both types necrosis and apoptosis. In contrast, photosensitizers preferentially localized in lysosomes and in the plasma membrane are not generally thought to be as phototoxic.[103]

In vivo other factors come into play. We have previously emphasised the importance of producing a necrotic volume concentric with the tumours, but the attenuation of the radiant exposure with the depth of the tissues necessarily generates a range of radiant exposures. For example, considering that the optical penetration depth of light is 2.3 mm at 750 nm,[77] a fluence rate of 130 mW/cm^2 at the tumour surface is reduced to 1 mW/cm^2 at a depth of 11 mm. Thus, PDT *in vivo* is characterized by a distribution of fluence rates, and this is further complicated by the intracellular distribution of the photosensitizer and the heterogenous concentration of oxygen in tissues. The mechanism of cell death is dependent on oxidative stress and, consequently, on the photon flux, photosensitizer local concentration and oxygen availability. The oxidative stress is very high close to the surface of the tumour and necrosis is expected to be the predominant cell death mechanism, with a shift to predominant apoptosis when the oxidative stress is lowered deeper in the tumour and further way from blood vessels, and eventually to autophagy in the regions where very few ROS are generated.

It was recently shown that some cytotoxic agents can induce immunogenic cell death and that this is related to the presence of calreticulin (CRT) and disulphide isomerase ERp57 at the surface of the cells within a few hours after the treatment, well before the cells manifest signs of apoptotic cell death.[104,120] These proteins are usually contained in the ER and their exposure at the cell surface required the induction of oxidative stress in the ER. In treatments with cytostatic agents such as anthracycline or oxaliplatin some elements of the apoptotic machinery must be activated very early, such as the caspase-8/Bax/Bak module, but in PDT with ER-localized photosensitizers the involvement of caspase-8 is dispensable.[106] Interestingly, PDT with the ER-localized hypericin photosensitizer led to surface exposure of CRT that was not accompanied by co-translocation of ERp57 to the surface of the cell.[106] Moreover, ATP secretion and CRT surface exposure were both photosensitizer- and light-dose dependent, and were both compromised by the absence or depletion of the ET-transmembranal kinase PERK. This suggests that CRT and ATP can share the same plasma membrane trafficking pathway after PDT. Interestingly, CRT exposure is observed as soon as 30 min post-PDT.

CRT exposure is an "eat-me" signal required but not sufficient for immunogenic cell death. It is also required that ATP is secreted during the blebbing phase of apoptosis.[105-106] Extracellular ATP released by dying cells is one of the most prominent "find-me" signals for macrophage and dendritic cell precursors. Finally, a third requirement for immunogenic cell death is the release of high mobility group protein BA (HMGB1), which operates as a potent pro-inflammatory stimulus in the extracellular milieu.[121] The elucidation of the mechanisms of pre-apoptotic translocation of CRT from the ER to the cell surface and of immune system stimulation by cancer cells with CRT exposed at the surface may change the paradigm of cancer therapy. Particularly interesting for PDT is the notion that ER-localized photosensitizers may produce oxidative stress in the critical location to trigger immunogenic cell death.

Managing intracellular oxidative stress

A most interesting feature of PDT is that it can be combined with other cancer therapies. Indeed, PDT efficacy can be potentiated by the combination of drugs targeting tumour cell pathways and/or the tumour microenvironment with the phototoxicity of PDT photosensitizers. Particularly attractive are approaches that target cellular antioxidant systems.

Cell responses to PDT depend on the type and quantity of ROS and the presence and activity of the antioxidant, melanin, heat shock proteins and the immune response.[122] Endogenous ROS production occurs primarily as a ubiquitous by-product of both oxidative phosphorylation and a myriad of oxidases necessary to support aerobic mechanisms.[123] Whereas high ROS levels are lethal to the cell, a moderate increase in ROS can promote cell proliferation and differentiation.[124] ROS homeostasis is normally maintained in the cells by antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidases, which can decompose superoxide anion and hydrogen peroxide. Small molecules such as glutathione, vitamin E and vitamin C (ascorbate) complement the intracellular control of ROS. It has been remarked that, compared with their normal counterparts, many types of cancer cells have increased levels of ROS.[125-126] The intrinsic oxidative stress of cancer cells led to the hypothesis that they should be more dependent on antioxidants for cell survival and, therefore, more vulnerable to further oxidative insults induced by ROS-generating agents or by compounds that abrogate the key antioxidant systems in cells.[126] Ascorbate is best known as a highly reducing antioxidant, and its incorporation in diets is believed to reduce the risk of cancer. Pharmacological concentrations of ascorbate (exposure of ca. 10 mM ascorbate for 2 h), that can only be achieved by parenteral administration, were shown to reduce the growth of tumours subcutaneously implanted in mice.[127] In general, ascorbate exposure is more cytotoxic to cancer cell than for normal cells, and this was related to the pro-oxidant effect of high concentrations of ascorbate. The pro-oxidant effect was mediated by the generation of H_2O_2 via ascorbate radical formation from ascorbate as the electron donor.[127-128] High concentrations of ascorbate in cell cultures generate H_2O_2 that may increase the oxidative stress of cancer cells and contribute to the selectivity of cancer treatments. Interestingly, we found that high concentrations of ascorbate can decrease the photodecomposition of photosensitizers used in PDT and allow them to perform more cycles of ROS photogeneration.[76] Hamblin and co-workers showed how the combination of PDT with ascorbate can have opposite effects the same cell line when two different photosensitizers are employed: the photosensitizer more likely to accept an electron from ascorbate became more phototoxic in the presence of ascorbate.[129] The combination between PDT and ascorbate can either enhance or suppress the efficacy of PDT, depending on the concentration of ascorbate, the intrinsic cell resistance to oxidative stress and the ability of the photosensitizer to accept an electron from ascorbate. The potentiation of PDT cytotoxicity has also been attempted with SOD, catalase and glutathione synthesis inhibitors.[130] The inhibitor of glutathione synthesis, l-buthionine sulfoximine, showed to have the largest augmentation of PDT efficacy in vitro. This suggests that glutathione is the major ROS-scavenging system in the cells, and that H_2O_2 plays a pivotal role in phototoxicity.[130] Cyclooxygenase (COX) is responsible for the maintenance of homeostasis (COX-1) and for the regulation of inflammation and mitogenesis (COX-2). Increased levels of COX may have an adverse effect on PDT efficacy. Hence, it was proposed to combine PDT with COX inhibitors to enhance PDT efficacy.[131-132] The combination of one photodynamic treatment with porfimer sodium with multiple injections of a clinically approved COX-2 inhibitor, celecoxib, in mouse model of mammary carcinoma resulted in an increase in the cure rate from 22% for PDT alone to 90% for the combination.[131] This result was interpreted in terms of decreased angiogenesis when PDT is combined with celecoxib. The presence of heme oxygenase in cells (OH-1) can also result in lowering the PDT efficacy[133-134] because this enzyme decomposes heme and reduces the amount of oxygen in a tissue. Moreover, its activity results in the production of bilirubin that has antioxidant properties. It has been shown that inhibition of OH-1 enhances the photodynamic effect.[135] PDT also affects the activity of the heat shock proteins (HSP), which act as molecular chaperones by assisting the correct folding of nascent and stress-accumulated misfolded proteins. The inhibition of high molecular weight HSP90 combined with PDT improved long-term tumouricidal responses when compared with individual treatment protocols.[136] On the other hand, HSP90 and the high molecular weight HSP70 may translocate to the plasma membrane and exhibit immunostimulatory activity.[137] The PDT-induced surface exposure of HSP70 has been reported to occur very shortly after PDT with porfimer sodium[138] or hypericin.[139] The cell response to the accumulation of misfolded proteins in the ER due to oxidative damage may involve the degradation of such proteins by proteasomes. It was hypothesised that the combination of PDT with proteasome inhibitors, such as bortezomib, could lead to the accumulation of carbonylated proteins in the ER, aggravate ER stress and potentiate

phototoxicity.[140] This hypothesis was verified with mice bearing subcutaneous tumours, which had a significantly higher cure rate in treatments combining bortezomib with PDT than with PDT alone.

Vascular effects in PDT

PDT also effectively targets tumour blood vessels.[141-146] Damage of endothelial cells causes a disturbance in the construction of the endothelium and the creation of a clot blocking the blood flow in the vessels. As a consequence, vessel obstruction leads to an inhibition or a significant reduction in the supply of nutrients to the tumour cells. The result of this process is the death of cancer cells, because they are not provided with adequate amount of oxygen and nutrients. Vascular-targeted photodynamic therapy (V-PDT), with DLI<1 h, was shown to be a very effective therapeutic procedure with various photosensitizers.[6,66,146-147] Occlusion of blood vessels and hypoxia can occur within a few minutes after the beginning of PDT. However, the increase of blood viscosity in hypoxic tissues reduces perfusion of hypoxic areas,[148] and the consumption of oxygen may limit the efficacy of V-PDT. In order to avoid that oxygen consumption becomes a limiting factor in PDT, various authors proposed the fractionation of light irradiation, with short dark periods (150-200 s), to allow for tissue re-oxygenation.[149-151] However, even when treatment protocols are selected to allow blood vessels to stay intact and functioning immediately after treatment, irreversible vascular damage and collapse develops within hours, leading to hemorrhage and tissue necrosis.

The decrease in tumour oxygenation, or increase in hypoxia, has been observed in V-PDT with padeliporfin[145-148] and redaporfin.[66] Figure 7 presents representative changes in pO_2 observed for mice subject to V-PDT and C-PDT with redaporfin.

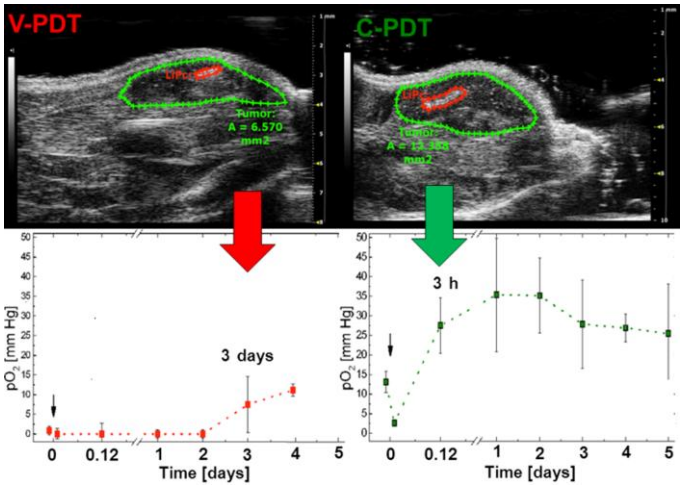

Fig. 7 Oxygen changes in tumours after vascular (V-PDT) and cellular (C-PDT) targeted PDT.[66] The figure is adopted from ref. 66 and modified with copyright to © Elsevier 2014.

It is very striking that the tissue pO_2 level recorded immediately after V-PDT, and for at least the subsequent 24 h, decreased to nearly zero. In contrast, C-PDT led to a transient decrease in pO_2, lasting usually no more than 3 h, followed by a recovery of pO_2. We have suggested that a very strong (<2 mmHg) and prolonged (> 1 day) hypoxia is a good predictor of the therapy outcome. Partial dysfunction of the blood flow does not guarantee a good long-term tumour response to PDT and, conversely, might even have a stimulatory effect on tumour growth. Extremely low pO_2 lasting for several days (0-2 mm Hg, i.e. chronic, extreme hypoxia) after V-PDT are correlated with long-term tumour responses, in contrast to mild and transient hypoxia, that in C-PDT lead to strong pO_2 compensatory effects (up to 10-12 mm Hg) and frequent tumour re-growths.

It is interesting to note that longitudinal studies of blood flow and tumour oxygenation after V-PDT, with the same animals, led to apparently conflicting results: although the hypoxia persisted for more than two days post-PDT, the blood flow exhibited only a transient decrease and recovered to flows higher than the pre-treatment ones within two days of the treatment.[66] These data can be rationalized considering that the blood flow measured by laser Doppler perfusion imaging is a flux obtained as the product between the average speed of blood cells and their number in the region of interest. The acute inflammatory response after V-PDT may increase the number of blood cells probed and give higher blood flows, but nevertheless V-PDT disrupts the tumour microvasculature and limits tumour oxygenation.

The first successful uses of V-PDT employed verteporfin. Interestingly, a comparison between the changes in the 20-30 µm diameter of the arterioles and venules in cremaster muscle versus those of vessels of the tumour vasculature with the same diameter, revealed that PDT with verteporfin using DLI=30 min produced complete or nearly complete occlusion of tumour microvasculature while normal blood vessels were spared.[152] This selectivity was related to the increased accumulation of verteporfin by tumour endothelial cells with high levels of low-density lipoproteins (LDL) receptors, or the rapid internalization of agents bound to the LDL receptor on proliferating endothelial cells compared to quiescent endothelial cells. However, the highest PDT efficacy was obtained with DLI=5 min, when the tissue selectivity is lower and a margin of normal tissue is destroyed. This is consistent with the observation that tumour cures with PDT require destroying a considerable margin of normal tissue surrounding the tumour, although this is not related with the infiltration of tumour cells beyond the visible tumour margin. Rather, tumour cures depend on the ability to establish a circulation-free zone surrounding the tumour that prevents re-supply of nutrients to tumour cells that survived the illumination.[153] Current PDT protocols with verteporfin favour DLI=15 min, which cause complete blocking of blood flow in the neovasculature.[154]

V-PDT has important advantages: (i) it uses photosensitizers that clear rapidly form the organism and minimize skin photosensibility, (ii) it gives the highest PDT efficacies, and (iii) it can be performed in one short session. The most promising photosensitizers for V-PDT currently in clinical trials are padeliporfin and redaporfin. V-PDT with

padeliporfin (4 mg/kg, 92 J/cm^2) led to congestion and destruction of blood vessels and multifocal haemorrhages within 1 h of the irradiation, followed by the development of necrosis in the following 24-48 h.[155] V-PDT with redaporfin (0.75 mg/kg, 50 J/cm^2) produces essentially the same effect.[156] V-PDT will lead to some necrosis of normal tissue in the region where tumour and normal tissue meet, but this is likely to acceptable for most organs. Fortunately, most tumours of hollow organs have more collagen than the normal tissue from which they arose, and collagen is largely unaffected by PDT.[157] Hence, normal tissues may heal safely and the mechanical integrity of the organ is maintained after V-PDT.

A final concern with V-PDT is in its ability to stimulate immune responses of the host system. As will be shown below, there is sufficient evidence to believe that V-PDT is not just a local treatment of a solid tumour and can control tumour metastasis.

Immune responses induced by PDT

PDT alters the tumour microenvironment and activates different immune responses. The mechanism of stimulation involves the release of pro-inflammatory cytokines, blood neutrophilia and recruitment of neutrophils at the tumour bed together with mast cells and macrophages,[158] induction of acute phase proteins, activation of the complement system,[159] and the subsequent activation of anti-tumour adaptive immunity.

A most remarkable increase in the expression of interleukin-6 (IL-6), peaking at 4-6 h post-PDT with porfimer sodium and related photosensitizers, was reported in the tumour and in the sera of mice.[158-161] V-PDT with redaporfin also leads to a very significant increase in IL-6 in the peripheral blood.[153] This proinflammatory cytokine has been implicated in the release of acute-phase proteins, complement activation and neutrophil migration. More recently, IL-6 was recognized as critical for resolving innate immunity and promoting adaptive immune responses.[162]

It was mentioned above that ER stress and ROS production may induce damaged/dying cells to expose intracellular molecules that can trigger immune responses. The damage-associated molecular patterns (DAMPs) observed after PDT in vitro are: the surface exposure of CRT, the secretion of ATP and the release of HMGB1.[105] The spectrum of DAMPs generated in vivo may be different but one of their most relevant functions is to enable the stimulation of dendritic cells (CDs).[106,163] DCs are the most potent antigen-presenting cells known and effective inducers of adaptive immunity. However, tumours lack an abundance of DCs. This suggested a strategy to improve the outcome of PDT that consisted in injecting DCs directly into PDT-treated tumours. The intratumoural injection of naïve DCs led to tumours regression at distant sites,[164] including lung metastasis.[165] A related strategy was to generate tumour cell lysates with PDT and test them as antitumour vaccines. It was found that such lysates activate DCs and stimulate tumour-specific T-cells that provide protection against subsequent re-challenges with the same tumour.[160,166]

The importance of T-lymphocytes to obtain cures with PDT was recognized nearly 20 years ago.[167] The outcome of PDT with porfimer sodium in wild-type BALB/c mice was much better than in the corresponding scid or nude mice models, and adoptive transfer of splenic virgin T lymphocytes from immunocompetent mice into scid mice delayed the recurrence of treated tumours. V-PDT also gave higher cure rates with immunocompetent BALB/c mice than with nude or scid mice, both using padeliporfin[168] or redaporfin.[84] Moreover, BALB/c mice bearing subcutaneous CT26 tumours were significantly protected from metastasis and from re-challenge with the same tumour model when treated with V-PDT using padeliporfin or redaporfin.[84,168] On the other hand, V-PDT with verteporfin did not cure BALB/c mice with CT26 tumours, and required the use of CT26.CL25 cells, that express the tumour antigen β-galactosidade, to attain the same level of cures and protection from re-challenges.[169] Interestingly, V-PDT with verteporfin of antigen positive CT26.CL25 tumours let to a significant increase in tumour necrosis factor alpha (TNFα) and interferon gamma (IFNϒ), but no changes in these signalling proteins were observed after the treatment of CT26 tumours.[169] On the other hand, V-PDT of BALB/c mice with CT26 tumours using redaporfin as photosensitizer led to a significant increase of IFNϒ in the peripheral blood,[156] which may explain the difference between the photosensitizers. IFNϒ can activate macrophages, inhibit the production of immunosuppressive molecules, enhance the secretion of antiangiogenic chemokines, and inhibit tumour cell proliferation.[170]

A meta-analysis of clinical data on tumour-infiltrating lymphocytes found in tumours revealed that the presence in tumours of CD3+, a general T-lymphocyte marker, is associated with a survival advantage.[170] CD3+ lymphocytes infiltration in CT26 tumours subcutaneously implanted in BALB/c mice was observed within 24 h of V-PDT with padeliporfin or redaporfin.[156,168]

It has been shown that CD8+ T cells mediate the control of tumours growing outside the treatment filed following PDT.[171-172] V-PDT with redaporfin also leads to an increase in CD8+ T cells in the peripheral blood shortly after the treatment, and this is concomitant with a significant increase in CD4+ T cells.[156] CD4+ T cells help make the initial CD8+ T cells response bigger and programs the differentiation of responding CD8+ T cells into a long-lived, protective memory.[173]

The knowledge of the immune responses elicited by PDT also inspired approaches to combine immunotherapies with PDT. A clinically approved methyltransferase inhibitor 5-aza-20-deoxycytidine used in combination with PDT improved the induction of adaptive immune response towards well-defined tumour antigen leading to the long-term survival of tumour-bearing mice.[174] PDT combined with low-dose cyclophosphamide, a cytotoxic drug that depletes regulatory CD4+FoxP3+ cells (T-regs) in mice, led to 70% permanent cures

presumably because it weakens the immunosuppressive microenvironment of the tumour.[175-176]

Outlook

Two decades after the first approval of a photosensitizer for PDT of cancer, this therapy has yet to move into the centre stage of oncology. Most of the limitations found with the first generation of PDT photosensitizers have now been overcome with the latest photosensitizers. The depth of the treatment with bacteriochlorin derivatives can be programmed to reach 10 mm and the use of interstitial illumination definitively resolves the issue of light penetration. The selectivity of the treatment can be planned as a function of the field of illumination. Skin photosensitivity after the treatment is now only a concern for a very short period of time (1-2 days). Vascular-PDT offers the opportunity to develop cost-effective protocols that can be performed in a short period of time and in an outpatient basis. However, it is difficult to change the perception that PDT is a local therapy and that it is mostly useful for palliative care in advanced cancer. PDT faces a tough competition with surgery for small tumours and with chemotherapy for metastatic disease. Hopefully, the tremendous advances in the understanding of immunogenic cell death, systemic antitumor protection and the potential to combine PDT with other therapeutic strategies, will eventually change old perceptions and promote the widespread use of PDT.

Particularly promising are the combinations of PDT with chemotherapy and immunotherapies. The increasingly better understanding of the biological mechanism of PDT offer a fertile ground to cultivate approaches that potentiate the effects of the various therapies involved. PDT offers the advantage of the unique targeting offered by laser light and optical fibres to direct the strongest effect to the primary target and yet the ability to potentiate the systemic effects of chemotherapies and immunotherapies. Moreover, PDT photosensitizers that fluoresce in the near infrared offer the opportunity for imaging of the tumours.[177]

The drug-device combination required for PDT poses additional regulatory challenges and has also been a deterrent to its development by pharmaceutical companies. Admittedly, PDT is not a "single product" and requires unconventional development strategies. Its development requires multidisciplinary teams with physics, chemistry and pharmacology backgrounds. Most likely, as PDT and immunotherapies advance, they will find more common grounds and the therapeutic approaches may become increasingly sophisticated. Nevertheless, the efforts to combine knowledge from these areas will eventually lead to a therapy with a strong and immediate effect on a primary tumour and systemic protection against metastasis, that may improve life expectancy without significant adverse effects for an increasingly larger fraction of the population.

Acknowledgements

The work from the authors' laboratories was supported by grants: FCT/PTDC/QUI-QUI/120182/2010, FCT/UID/QUI/00313/2013, NCN/2013/11/D/ST5/02995, and MNiSW/0085/IP3/2015/73.

References

1P. Agostinis, K. Berg, K. A. Cengel, T. H. Foster, A. W. Girotti, S. O. Golinick, S. M. Hahn, M. R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piette, B. C. Wilson, J. Golab, *CA Cancer J. Clin*., 2011, **61**, 250-281.
2T. J. Dougherty, C. J. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan, Q. Peng, *J. Natl. Cancer Inst*., 1998, **90**, 889-905.
3D. E. Dolmans, D. Fukumura, R. K. Jain, *Nat. Rev. Cancer*, 2003, **3**, 380-387.
4W. M. Sharman, C. M. Allen, J. E. van Lier, *Drug Discovery Today*, 1999, **4**, 507-517.
5D. Phillips, *Photochem. Photobiol. Sci*., 2010, **9**, 1589-1596.
6B. Krammer, *Anticancer Res*., 2001, **21**, 4271-4277.
7D. Preise, A. Scherz, Y. Salomon, *J. Photochem. Photobiol. Sci.*, 2011, **10**, 681-688.
8H. Pass, *J. Natl. Cancer. Inst*., 1998, **85**, 443–456.
9A. P. Castano, T. N. Demidowa, M. R. Hamblin, *Photodiag. Photodyn. Ther.*, 2004, **1**, 279–293.
10A. P. Castano, P. Mroz, M. R. Hamblin, *Nature Rev. Cancer*, 2006, **6**, 535-545.
11B. C Wilson, M. S. Patterson, *Phys. Med. Biol*., 2008, **53**, 61-109.
12Y. Chen, G. Li, R. K. Pandey, *Current Organic Chemistry*, 2004, **8**, 1105-1134.
13 K. E. Borbas, Ch. Ruzié, J. S. Lindsey, *Organic Letters*, 2008, **10**, 1931-1933.
14S. O. Gollnick, C. M. Brackett, *Immunol. Res*., 2010, **46**, 216-226.
15M. G. H. Vicente, *Current Med. Chem. Anticancer Agents*, 2001, **1**, 175–194.
16N. P. F. Gonçalves, A. V. Simoes, A. Abreu, A. J. Abrunhosa, J. M. Dąbrowski, M. M. Pereira, *J. Porphyrins Phthalocyanines,* 2015; **19**, DOI: 10.1142/S1088424615500728.
17M. Ethirajan, Y. Chen, P. Joshi, R. K. Pandey, *Chem. Soc. Rev*., 2011, **40**, 340-362.
18J. M. Dąbrowski, M. M. Pereira, L. G. Arnaut, C. J. P. Monteiro, A. F. Peixoto, A. Karocki, K. Urbanska, G. Stochel, *Photochem. Photobiol*., 2007, **83**, 897-903.
19A. V. C. Simoes, A. Adamowicz, J. M. Dąbrowski, M. J. F. Calvete, A. R. Abreu, G. Stochel, L. G. Arnaut, M. M. Pereira, *Tetrahedron*, 2012, **68**, 8767 – 8772.
20M. Kempa, P. Kozub, J. Kimball, M. Rojkiewicz, P. Kuś, Z. Gryczyński, A. Ratuszna, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 2015, **146**, 249-254.
21E. F. F. Silva, F. A. Schaberle, C. J. P. Monteiro, J. M. Dąbrowski, L. G. Arnaut, *Photochem. Photobiol. Sci*., 2013, **12**, 1187–1192.
22A. Szurko, M. Rams, A. Sochanik, K. Sieroń-Stołtny, A. M. Kozielec, F. P. Montforts, R. Wrzalik, A. Ratuszna, *Bioorg. Med. Chem*., 2009, **17**, 8197-8205.
23J. M. Dąbrowski, L. G. Arnaut, M. M. Pereira, C. J. P. Monteiro, K. Urbanska, S. Simões, G. Stochel, *ChemMedChem*, 2010, **5**, 1770-1780.
24J. M. Dąbrowski, M. Krzykawska, L. G. Arnaut, M. M. Pereira, C. J. P. Monteiro, S. Simões, K. Urbanska, G. Stochel, *ChemMedChem*, 2011, **6**, 1715-1726.

25R. Bonnett, P. Charlesworth, B. D. Djelal, D. J. McGarvey, T. G. Truscott, *J. Chem. Soc*., 1999, **2**, 325-328.
26V. A. Privalov, A. V. Lappa, O. V. Seliverstov, A. B. Faizrakhmanov, N. N. Yarovoy, E. V. Kochneva, M. V. Evnevich, A. S. Anikina, A. V. Reshetnicov, I. D. Zalevsky, Y. V. Kemov, *Proc. SPIE*, 2002, **4612**, 178-189.
27J. A. Hargus, F. R. Fronczek, M. G. H. Vicente, K. M. Smith, *Photochem. Photobiol*., 2007, **83**, 1006-1015.
28M. Pineiro, A. M. d. A. R. Gonsalves, M. M. Pereira, S. J. Formosinho, L. G. Arnaut. *J. Phys. Chem*., 2002, **106**, 3787-3795.
29S. V. Dudkin, E. A. Makarova, L. K. Slivka, E. A. Lukyanets, *J. Porphyrins Phthalocyanines,* 2014, **18**, 107.
30M. M. Pereira, C. J. P. Monteiro, A. V. C. Simoes, S. M. A. Pinto, A. R. Abreu, G. F. F. Sa, E. F. F. Silva, L. B. Rocha, J. M. Dąbrowski, S. J. Formosinho, S. Simoes, L. G. Arnaut, *Tetrahedron*, 2010, **66**, 9545-9551.
31Y. Y. Huang, P. Mroz, T. Zhiyentayev, S. K. Sharma, T. Balasubramanian, C. Ruzié, M. Krayer, D. Fan, K. E. Borbas, E. Yang, H. L. Kee, C. Kirmaier, J. R. Diers, D. F. Bocian, D. Holten, J. S. Lindsey, M. R. Hamblin, *J. Med. Chem*., 2010, **53**, 4018-4027.
32P. Mroz, Y. Y. Huang, A. Szokalska, T. Zhiyentayev, S. Janjua, A.-P. Nifli, M. E. Sherwood, C. Ruzié, K. E. Borbas, D. Fan, M. Krayer, T. Balasubramanian, E. Yang, H. L. Kee, C. Kirmaier, J. R. Diers, D. F. Bocian, D. Holten, J. S. Lindsey, M. R. Hamblin, *FASEB J*., 2010, **24**, 3160-3170.
33L. G. Arnaut, M. M. Pereira, J. M. Dąbrowski, E. F .F. Silva, F. A. Schaberle, A. A. Abreu, L. B. Rocha, M. M. Barsam, K. Urbanska, G. Stochel, C. M. A. Brett, *Chem. Eur. J*., 2014, **20**, 5346–5357.
34Y.-Y. Huang, T. Balasubramanian, E. Yang, D. Luo, J. R. Diers, D. F. Bocian, J. S. Lindsey, D. Holten, M. R. Hamblin, *ChemMedChem*, 2012, **7**, 2155-2167.
35J. M. Dąbrowski, K. Urbanska, L. G. Arnaut, M. M. Pereira, A. R. Abreu, S. Simões, G. Stochel, *ChemMedChem*, 2011, **6**, 465-475.
36J. M. Dąbrowski, L. G. Arnaut, M. M. Pereira, K. Urbanska, G. Stochel, *Med. Chem. Commun*., 2012, **3**, 502-505.
37R. Saavedra, L. B. Rocha, J. M. Dąbrowski, L. G. Arnaut, *ChemMedChem*, 2014, **9**, 390-398.
38B. G. Ongarora, X. Hu, H. Li, F. R. Fronczek, M. G. H. Vicente, *Med. Chem. Commun*., 2012, **3**, 179–194.
39A, D. Quartarolo, D. Pérusse, F. Dumoulin, N. Russo, E. Sicilia, *J. Porphyrins Phthalocyanines*, 2013, **17**, 980-988.
40D. Lafont, Y. Zorlu, H. Savoie, F. Albrieux, V. Ahsen, R. W. Boyle, F. Dumoulin, *Photodiagn. Photodyn. Ther*., 2013, **10**, 252-259.
41W. Liu, T. J. Jensen, F. R. Fronczek, R. P. Hammer, K. M. Smith, G. H. Vicente, *J. Med. Chem*., 2005, **48**, 1033-1041.
42P. Mroz, A. Pawlak, M. Satti, H. Lee, T. Wharton, H. Gali, T. Sarna, M. R. Hamblin, *Free Radic. Biol. Med*., 2007, **43**, 711-719.
43M. Krieg, J. M. Bilitz, M. B. Srichaiand, R. W. Redmond, *Biochim. Biophys. Acta*, 1994, **1199**, 149-156.
44Z. Diwu, J. W. P. Lown, *Free Radic. Biol. Med*., 1993, **14**, 209-215.
45K. Szaciłowski, W. Macyk, A. Drzewiecka-Matuszek, M. Brindell, G. Stochel, *Chem. Rev*., 2005, **105**, 2647-2694.
46A. E. O'Connor, W. M. Gallagher, A. T. Byrne, *Photochem. Photobiol*., 2009, **85**, 1053-1074.
47R. Bonnett, G. Martinez, *Tetrahedron*, 2001, **57**, 9513-9547.
48Y. Vakrat-Haglili, L. Weiner, V. Brumfeld, A. Brandis, Y. Salomon, B. Mcllroy, B. C.Wilson, A. Pawlak, M. Rozanowska, T. Sarna, A. Scherz, *J. Am. Chem. Soc*., 2005, **127**, 6487-6497.
49R. R. Allison, G. H. Downie, R. Cuenca, Xin-Hua Hu, C. Childs, C. H. Sibata, *Photodiag. Photodyn. Ther.*, 2004, **1**, 27-42.
50A. F. Mironov, A. S. Seylanov, J. A. Seylanov, V. M. Pizhik, I. V. Derughenko, A. J. Nockel, *J. Photochem. Photobiol*. *B:Biol.,* 1992, **16**, 341.
51C. Tanielian, C. Schweitzer, R. Mechin, C. Wolff, *Free Radic. Biol. Med.*, 2001, **30**, 208-212.
52S. I. Moriwaki, J. Misawa, Y. Yoshinari, I. Yamada, M. Takigawa, Y. Tokura, *Photodermatol. Photoimmunol. Photomed*., 2001, **17**, 241–243.
53M. Mori, I. Sakata, T. Hirano, A. Obana, S. Nakajima, M. Hikida, T. Kumagai, *Jpn. J. Cancer Res*., 2003, **91**, 753-759.
54Inc AP. Photofrin® – Product Monograph. In: Inc AP, editor.: Axcan Pharma Inc; 2005.
55http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_Product_Information/human/000318/WC500024398.pdf
56T. J. Kinsella, E. D. Baron, V. C. Colussi, K. D. Cooper, C. L. Hoppel, S. T. Ingalls, M. E. Kenney, X. Li, N. L. Oleinick, S. R. Stevens, S. C. Remick, *Front. Oncol*., 2011, **1**, 14.
57Q. Peng, J. Moan, L. W. Ma, J. M. Nesland, *Cancer Res*., 1995, **55**, 2620-2626.
58R. Whelpton, A. T. Michael-Titus, S. S. Basra, M. F. Grahn, *Photochem. Photobiol*., 1995, **61**, 397–401.
59M. C. Berenbaum, R. Bonnett, E. B. Chevretton, S. L. Ajande-Adebakin, M. Ruston, *Lasers Med. Sci*., 1993, **8**, 235-243.
60J. M. Houle, A. Strong, J. *Clin. Pharmacol*., 2002, **42**, 547-557.
61H. Lui, L. Nobbs, W. D. Tope, P. K. Lee, C. Elmets, N. Provost N, *Arch. Dermatol*., 2004, **140**, 26-32.
62M. T. Huggett, M. Jermyn, A. Gillams, R. Illing, S. Mosse, M. Novelli, *Br. J. Cancer*., 2014, **110**, 1698-704.
63O. Mazor, A. Brandis, V. Plaks, E. Neumark, V. Rosenbach-Belkin, Y. Salomon, *Photochem. Photobiol*., 2005, **81**, 342-351.
64A. R. Azzouzi, E. Barret, C. M. Moore, A.Villers, C. Allen, A. Scherz, G. Muir, M. de Wildt, N. J. Barber, S. Lebdai, M. Emberton, *Br. J. Urology Int*., 2013, **112**, 766-774.
65E. G. Azenha, A. C. Serra, M. Pineiro, M. M. Pereira, J. Seixas de Melo, L. G. Arnaut, S. J. Formosinho, A. M. d. A. R. Gonsalves, *Chem. Phys*., 2002, **280**, 177-190.
66M. Krzykawska-Serda, J. M. Dabrowski, L. G. Arnaut, M. Szczygiel, K. Urbanska, G. Stochel, *Free Radic. Biol. Med*., 2014, **73**, 239-251.
67E. F. F. Silva, B. W. Pedersen, T. Breitenbach, R. Toftegaard, M. K. Kuimova, L. G. Arnaut, P. R. Ogilby, *J. Phys. Chem. B*, 2012, **116**, 445-461.
68L. G. Arnaut, S. J. Formosinho, *Pure Appl. Chem.*, 2013, **85**, 1389-1403.
69R. V. Bensasson, E. J. Land, T. G. Truscott, *Oxford: Oxford Univ. Press*, 1993.
70K. Berg, *Mechanisms of cell damage in photodynamic therapy*. In: H. Hönigsmann, G. Jori, A. R. Young, eds. *The Fundamental Bases of Phototherapy*. Milano: OEMF spa; 1996, 181-207.
71I. Ashur, R. Goldschmidt, I. Pinkas, Y. Salomon, G. Szewczyk, T. Sarna, A. Scherz, *J. Phys. Chem. A.,* 2009, **113**, 8027-8037.
72J. C. Liao, J. Roider, D. G. Jay, *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 2659-2663.
73P. R. Ogilby, *Photochem. Photobiol. Sci.*, 2010, **9**, 1543-1560.
74C. Hadjur, G. Wagnières, F. Ihringer, P. Monnier, H. van den Berg, *J. Photochem. Photobiol. B: Biol.,* 1997, **38**, 196-202.
75E. Yang, J. R. Diers, Y. Y. Huang, K. Aravindu, M. R. Hamblin, J. S. Lindsey, D. F. Bocian, D. Holten, *Photochem. Photobiol.,* 2013, **89**, 605-618.
76 J. M. Dąbrowski, L. G. Arnaut, M. M. Pereira, K. Urbańska, S. Simões, G. Stochel, L. Cortes, *Free Radic. Biol. Med.*, 2012, **52**, 1188–11120.
77 A. N. Bashkatov, E. A. Genina, V. I. Kochubey, V. V. Tuchin, *J. Phys. D: Appl. Phys*. 2005, **38**, 2543-2555.

78AAPM. Photodynamic Therapy Dosimetry. Madison, WI, USA: American Association of Physicists in Medicine, 2005.
79T. J. Farrel, B. C. Wilson, M. S. Patterson, R. Chow, *Proc. SPIE*, 1991, **1426**, 146-155.
80I. Georgakoudi, M. G. Nichols, T. H. Foster, *Photochem. Photobiol*, 1997, **65**, 135-144.
81H. B. Ris, H. J. Altermatt, B. Nachbur, J. C. M. Stewart. Q. Wang, C. K. Lim, R. Bonnett, U. Althaus, *Int. J. Cancer*, 1993, **53**, 141-146.
82M. C. Berenbaum, S. L. Akande, R. Bonnett, H. Kaur, S. Ioannou, R. D. White, U. J. Winfield, *Br. J. Cancer*, 1986, **54**, 717-725.
83R. Bonnett, R. D. White, U. J. Winfield, M. C. Berenbaum, *Biochem. J.*, 1998, **261**, 277-280.
84L. Rocha, L. Gomes-da-Silva, J. M. Dabrowski, L. G. Arnaut, *Eur. J. Cancer*, 2015, DOI: 10.1016/j.ejca.2015.06.002.
85C. Abels, *Photochem. Photobiol. Sci*., 2004, **3**, 765–771.
86H. Mojzisova, S. Bonneau, C. Vever-Bizet, D. Brault, *Biochim. Biophys. Acta – Biomembranes*, 2007, **1768**, 2748-2756.
87A. Juarranz, A. Villanueva, V. Díaz, M. Cañete, *J. Photochem. Photobiol. B: Biol.,* 1995, **27**, 47-53.
88M. K. Kuimova, M. Balaz, H. L. Anderson, P. R. Ogilby, *J. Am. Chem. Soc.,* 2009, **131**, 7948-7949.
89K. Berg, A. Western, J. C. Bommer, J. Moan, *Photochem. Photobiol.,* 1990, **52**, 481-487.
90Z. Malik, I. Amit, C. Rothmann, *Photochem. Photobiol.,* 1997, **65**, 389-396.
91R. Sailer, W. S. L. Strauss, H. Emmert, K. Stock, R. Steiner, H. Schneckenburger, *Photochem. Photobiol.,* 2000, **71**, 460-465.
92L. Liu, Z. Zhang, D. Xing, *Free Radic. Biol. Med.,* 2011*,* **51**, 53-68.
93A. D. Garg, H. Maes, E. Romano′ P. Agostinis, *Photochem. Photobiol. Sci.*, 2015, DOI: 10.1039/C4PP00466C.
94D. H. Kessel, M. Price, J. J. Jr. Reiners, *Autophagy*, 2012, **8**, 1333–1341.
95K. Berg, J. Moan, *Photochem. Photobiol.,* 1997, **65**, 403-409.
96Y. J. Hsieh, C., W. C. C. J. Chang, J. S. Yu, *J. Cell. Physiol.* 2003, **194**, 363-375.
97Y. J. Hsieh, J. S. Yu, P. C. Lyu, *J. Cell. Biochem.,* 2010, **111**, 821-833.
98S. Marchal, A. François, D. Dumas, F. Guillemin, L. Bezdetnaya, *Br. J. Cancer,* 2007, **96**, 944-951.
99P. Morlière, J.-C. Mazière, R. Santus, C. D. Smith, M. R. Prinsep, C. C. Stobbe, M. C. Fenning, J. L. Golberg, J. D. Chapman, *Cancer Res.* 1998, **58**, 3571-3578.
100J. M. Runnels, N. Chen, B. Ortel, D. Kato, T. Hasan, *Br. J. Cancer.* 1999, **80**, 946-953.
101T. Osaki, S.Takagi, Y. Hoshino, M. Okumura, T. Fujinaga, *Cancer Lett.,* 2006, **243**, 281-292.
102M. E. Rodriguez, P. Zhang, K. Azizuddin, G. B. Delos Santos, S. M. Chiu, L. Y. Xue, J. C. Berlin, X. Peng, H. Wu, M. Lam, A. L. Nieminen, M. E. Kenney, N. L. Oleinick, *Photochem. Photobiol*., 2009, **85**, 1189–1200.
103L. Benov, *Med. Princ. Pract.* 2015, **24** Suppl. 1, 14-28.
104M. Obeid, A. Tesniere, F. Ghiringhelli, *Nature Medicine*, 2007, **13**, 54-61.
105D. V. Krysko, A. D. Garg, A. Kaczmarek, O. Krysko, P. Agostinis, P. Vandenabeele, *Nat. Rev. Cancer.* 2012, **12**, 860-875.
106A. D. Garg, D. V. Krysko, T. Verfaillie, A. Kaczmarek, G. B. Ferreira, T. Marysael, N. Rubio, M. Firczuk, C. Mathieu, A. J. M. Roebroek, W. Annaert, J. Golab, P. de Witte, P. Vandenabeele, P. Agostinis, *EMBO J*., 2012, **21**, 1062–1079.
107P. Mroz, A. Yaroslavsky, G. B. Kharkwal, M. R. Hamblin *Cancers,* 2011, ***3,*** 2516-2539.
108A. E. Edinger, C. B. Thompson, *Current Opinion in Cell Biology*, 2004, **16**, 663–669.
109A. C. Rego, C. R. Oliveira, *Neurochem. Res*. 2003, **28**, 1563-1574.
110A. M. Chinnaiyan, K. O'Rourke, M. Tewari, V. M. Dixit, *Cell*, 1995, **81**, 505-512.
111D. Carmona-Gutierrez, T. Eisenberg, S. Buttner, C. Meisinger, G. Kroemer, F. Madeo, *Cell Death and Differentiation,* 2010, **17**, 763–773.
112P. Agostinis, E. Buytaert, H. Breyssens, N. Hendrickx, *Photochem. Photobiol. Sci.,* 2004*,* **3**, 721-729.
113A. P. Castano, T. N. Demidova, M. R. Hamblin, *Photodiag. Photodyn. Ther.,* 2005, **2**, 1-23.
114 B. Levine, G. Kroemer, *Cell*, 2008, **132**, 27-42.
115A. D. Garg, D. V. Krysko, A. Kaczmarek, O. Krysko, P. Agostinis, P. Vandenabeele, *Autophagy*, 2013, **9**, 1292–1307.
116H. Maes, N. Rubio, A. D. Garg, P. Agostinis, *Trends Mol. Med.,* 2013, **9**, 428-446.
117D. J. Puleston, A. K. Simon, *Immunology*, 2014, **141**, 1-8.
118J. Morgan, A. R. Oseroff, *Advanced Drug Delivery Reviews*, 2001, **49**, 71–86.
119S. Wu, F. Zhou, Y. Wei, W. Chen, Q. Chen, D. Xing, *Antioxid Redox Signaling*, 2014, **20**, 733-746.
120T. Panaretakis, O. Kepp, U. Brockmeier, A. Tesniere, A. C. Bjorklund, D. C. Chapman, M. Durchschlag, N. Joza, G. Pierron, P. van Endert, J. Yuan, L. Zitvogel, F. Madeo, D. B. Williams, G. Kroemer, *EMBO J*., 2009, **28**, 578–590.
121G. Kroemer, L, Galluzzi, O. Kepp, L. Zitvogel, *Annu Rev. Immunol*., 2013, **31**, 51-72.
122H. Pelicano, D. Carney, P. Huang, *Drug Resist. Updat.*, 2004, **7**, 97-110.
123J. Boonstra, J. A. Post, *Gene*, 2004, **337**, 1-13.
124F. Q. Schafer, G. R. Buettner, *Free Radic. Biol. Med.*, 2001, **30**, 1191-1212.
125T. P. Szatrowski, C. F. Nathan, *Cancer Res.*, 1991, **51**, 794-798.
126D. Trachootham, J. Alexandre, P. Huang, *Nature Rev. Drug Discovery*, 2009, **8**, 579-591.
127Q. Chen, M. G. Espey, A. Y. Sun, C. Pooput, K. L. Kirk, M. C. Krishna, D. B. Khosh, J. Drisko, M. Levine, *Proc. Natl. Acad. Sci. USA*, 2008, 105, 11105-11109.
128Q. Chen, M. G. Espey, M. C. Krishna, J. B. Mitchell, C. P. Corpe, G. R. Buettner, E. Shacter, M. Levine, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 13604-13609.
129F. F. Sperandio, S. K. Sharma, M. Wang, S. Jeon, Y.-Y. Huang, T. Dai, S. Nayka, S.C.O.M. de Sousa, L. Y. Chiang, M. R. Hamblin, *Nanomed. Nenotech. Biol. Med.*, 2013, **9**, 570-579.
130S. G. Kimani, J. B. Phillips, J. I. Bruce, A. J. MacRobert, J. P Golding, *Photochem. Photobiol.*, 2012, **88**, 175–187.
131A. Ferrario, A. M. Fisher, N. Rucker, C. J. Gomer, *Cancer Res.*, 2005, **65**, 9473–9478.
132E. H. Harvey, J. Webber, D. Kessel, D. Fromm, *American Journal of Surgery,* 2005, **189**, 302–305.
133E. R. Gomes, R. D. Almeida, A. P. Carvalho, C. B. Duarte, P. Carvalho, *Photochem. Photobiol.*, 2002, **76**, 423–30.
134D. Nowis, M. Legat, T. Grzela, J. Niderla, E. Wilczek, G. M. Wilczynski, E. Głodkowska, P. Mrówka, T. Issat, J. Dulak, A. Józkowicz, H. Waś, M. Adamek, A. Wrzosek, S. Nazarewski, M. Makowski, T. Stokłosa, M. Jakóbisiak, J. Gołab, *Oncogene,* 2006, **25**, 3365–3374.
135J. Frank, M. R. Lornejad-Schäfer, H. Schöffl, A. Flaccus, C. Lambert, H. K. Biesalski, *Int. J. Cancer*, 2007, **31**, 1539–1545.
136A. Ferrario, C. J. Gomer, *Cancer Lett.,* 2010, **289**, 188–194.
137A. D. Garg, D. Nowis, J. Golab, P. Vandenabeele, D.V. Krysko, P. Agostinis, *Biochim. Biophys. Acta*, 2010, **1805**, 53–71.
138M. Korbelik, J. Sun, I. Cecic, *Cancer Res*., 2005, **65**, 1018-1026.
139A. D. Garg, D. Nowis, J. Golab, P. Vandenabeele, D. V. Krysko, P. Agostini, *Cancer Immunolog. Immunother.*, 2012, **61**, 215-221.
140A. Szokalska, M. Makowski, D. Nowis, G. M. Wilczyński, M. Kujawa, C. Wójcik, I. Młynarczuk-Biały, P. Salwa, J. Bil, S.

Janowska, P. Agostinis, T. Verfaillie, M. Bugajski, J. Gietka, T. Issat, E. Głodkowska, P. Mrówka, T. Stokłosa, M. R. Hamblin, P. Mróz, M. Jakóbisiak, J. Gołab, *Cancer Res.*, 2009, **69**, 4235-4243.
141 R. Bhuvaneswari, Y. Gan, S. S, Lucky, W. Chin, S.M. Ali, K.C. Soo, M. Olivo, *Molecular Cancer*, 2008, **7**, 56.
142 M. R. Horsman, J. Winther, *Acta Oncologica*, 1989, **28**, 693-697.
143 H. Lepor, *Reviews in Urology*, 2008, 10, 254–61.
144 M. W. Reed, T. J. Wieman, D. A. Schuschke, M. T. Tseng, F. N. Miller, *Radiat. Res.*, 1989, **119**, 542–552.
145 J. Trachtenberg, R. A. Weersink, S. R. Davidson, M.A.Haider, A. Bogaards, M. R. Gertner, A. Evans, A. Scherz, J. Savard, J. L. Chin, B. C. Wilson, M. Elhilali, *Br. J. Urology Int.*, 2008, **102**, 556–562.
146 J. Zilberstein, S. Schreiber, M.C. Bloemers, P. Bendel, M. Neeman, E. Schechtman, F. Kohen, A. Scherz, Y. Salomon, *Photochem. Photobiol.,* 2001, **73**, 257–266.
147 B. Chen, B. W. Pogue, P. J. Hoopes, T. Hasan, *Int. J. Rad. Oncol. Biol. Phys.,* 2005, **61**, 1216-1226.
148 B. D. Kavanagh, B. E. Coffey, D. Needham, R. M. Hochmuth, M. W. Dewhirst, *Br. J. Cancer*, 1993, **67**, 734–741.
149 S. G. Gross, A. Gilead, A. Scherz, M. Neeman, Y. Salomon, *Nat. Med.,* 2003, **9**, 1327-1332.
150 A. Curnow, J. C. Haller, S. G. Bown, *J. Photochem. Photobiol. B: Biol.,* 2000, **58**, 149-155.
151 B. W. Pogue, T. Hasan, *Radiat. Res.,* 1997, **147**, 551-559.
152 V. H. Fingar, P. K. Kik, P. S. Haydon, P. B. Cerrito, M, Tseng, E. Abang, T. J. Wieman, *Br. J. Cancer,* 1999, **79**, 1702-1708.
153 V. H. Fingar, B. W. Henderson, *Photochem. Photobiol.,* 1987, **46**, 837–841.
154 K. Kurohane, A. Tominaga, K. Sato, J. R. North, Y, Namba, N. Oku, *Cancer Lett*., 2001, **167**, 49-56.
155 N. V. Koudinova, J. H. Pinthus, A. Brandis, O. Brenner, P. Bendel, J. Ramon, Z. Eshhar, A. Scherz, Y. Salomon, *Int. J. Cancer*, 2003, **104**, 782–789.
156 L. C. Gomes-da-Silva, P. Santos-Rodrigues, A. Cabrita, M. S. Rosa, L. G. Arnaut, submitted.
157 J. H. Woodhams, A. J. MacRobert, M. Novelli, S. G. Bown, *Int. J. Cancer*, 2006, **118**, 477–482.
158 S. O. Gollnick, S. S. Evans, H. Baumann, B. Owczarczak, P. Maier, L. Vaughan, W. C. Wang, E. Unger, B. W. Henderson, *Br. J. Cancer*, 2003, **88**, 1772-1779.
159 I. Cecic, K. Serrano, M. Gyongyossy-Issa, M. Korbelik, *Cancer Lett.*, 2005, **225**, 215–223.
160 S. O. Gollnick, X. Liu, B. Owczarczak, D. A. Musser, B. W. Henderson, *Cancer Res.,* 1997, **57**, 3904–3909.
161 L. H. Wei, H. Baumann, E. Tracy, Y. Wang, A. Hutson, S. Rose-John, B. W. Henderson, *Br. J. Cancer*, 2007, 97, 1513-1522.
162 S. A. Jones, *J. Immunol*., 2005, **175**, 3463-3468.
163 A. D. Garg, D. V. Krysko, P. Vandenabeele, P. Agostinis. *Photochem. Photobiol. Sci.*, 2011, **10**, 670-680.
164 A. Jalili, M. Makowski, T. Świtaj, D. Nowis, G. M. Wilczyński, E. Wilczek, M. Chorąży-Massalska, A. Radzikowska, W. Maśliński, Ł. Biały, J. Sieńko, A. Sieroń, M. Adamek, G. Basak, P. Mróz, I. W. Krasnodębski, M. Jakóbisiak, J. Gołąb, *Clin. Cancer Res.*, 2004, **10**, 4498-4508.
165 H. Saji, W. Song, K. Furumoto, H. Kato, E. G. Engleman, *Clin. Cancer Res.*, 2006, **12**, 2568–2574.
166 M. Korbelik, B. Stott, J. Sun, *Br. J. Cancer*, 2007, **97**, 1381-1387.
167 M. Korbelik, G. Krosl, J. Krosl, G. J. Dougherty, *Cancer Res.*, 1996, **56**, 5647–5652.
168 D. Preise, R. Oren, I. Glinert, V. Kalchenko, S. Jung, A. Scherz, Y. Salomon, *Cancer Immunol. Immunother.*, 2009, **58**, 71–84.
169 P. Mroz, A. Szokalska, M. X. Wu, M. R. Hamblin, *PLoS One*, 2010, **5**, e15194.
170 G. L. Beatty, Y. Paterson, *J. Immunol.*, 2001, **166**, 2276-2282.
171 M. J. Gooden, G. H. de Bock, N. Leffers, T. Daemen, H. W. Nijman, *Br. J. Cancer*, 2011, **105**, 93–103.
172 E, Kabingu, L, Vaughan, B, Owczarczak, K. D. Ramsey, S. O. Gollnick, *Br. J. Cancer.*, 2007, **96**, 1839–1848.
173 M. J. Bevan, *Nat. Rev. Immunol.,* 2004, **4**, 595-602.
174 M. Wachowska, M. Gabrysiak, A. Muchowicz, W. Bednarek, J. Barankiewicz, T. Rygiel, L. Boon, P. Mroz, M. R. Hamblin, J. Golab, *Eur. J. Cancer*, 2014, **50**, 1370-1381.
175 A. P. Castano, P. Mroz, M. X. Wu, M. R. Hamblin, *PNAS,* 2008*,* **105**, 5495-5500.
176 E. Reginato, P. Mroz, H. Chung, M. Kawakubo, P. Wolf, M. R, Hamblin, *Br. J. Cancer*, 2013, **109**, 2167-2174.
177 J. P. Celli, B. Q. Spring, I. Rizvi, C. L. Evans, K. S. Samkoe, S. Verma, B. W. Pogue, T. Hasan, *Chem. Rev.*, 2010, **110**, 2795-2838.