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Review

Direct tumor damage mechanisms of photodynamic therapy✪

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Photodynamic therapy (PDT) is a clinically approved and rapidly developing cancer treatment regimen. It is a minimally invasive two-stage procedure that requires administration of a photosensitizing agent followed by illumination of the tumor with visible light usually generated by laser sources. A third component of PDT is molecular oxygen which is required for the most effective antitumor effects. In the presence of the latter, light of an appropriate wavelength excites the photosensitizer thereby producing cytotoxic intermediates that damage cellular structures. PDT has been approved in many countries for the treatment of lung, esophageal, bladder, skin and head and neck cancers. The antitumor effects of this treatment result from the combination of direct tumor cell photodamage, destruction of tumor vasculature and activation of an immune response. The mechanisms of the direct photodamage of tumor cells, the signaling pathways that lead to apoptosis or survival of sublethaly damaged cells, and potential novel strategies of improving the antitumor efficacy of PDT are discussed.

Keywords: photodynamic therapy, photosensitizer, apoptosis, tumor

Photodynamic therapy is a minimally invasive therapeutic modality approved for the treatment of neoplastic and vascular diseases. It consists of three elements: a photosensitizer that is applied topically or administered systemically; light usually in the visible range, commonly generated by laser sources, and molecular oxygen, which in the photodynamic reaction generates singlet oxygen ($^1O_2$) and reactive oxygen species. After a period necessary for efficient accumulation of the photosensitizer in the tumor, a light of appropriate wavelength is precisely delivered to the target tissue. Since light used in PDT is of low power there is no tissue heating and connective tissues containing collagen and elastin fibers are not damaged. Excellent cosmetic results in the treatment of skin cancers are therefore an important advantage of PDT over surgery or other invasive procedures.

LIGHT AND LIGHT SOURCES FOR PDT

The wavelengths used in PDT are in the red or infrared range of electromagnetic wave. For a photobiological reaction to occur light must be absorbed by the photosensitizer. This is possible when the wavelength of light matches the electron absorption spectrum of the photosensitizer (Hsi *et al*., 1999). For clinical use the activating light is usually between 600 and 900 nm. This is because endogenous dyes, mainly hemoglobin, strongly absorb light below 600 nm and longer wavelengths are energetically insufficient to produce $^1O_2$, which is the most important although not the only cytotoxic effector of PDT.

A critical parameter for consideration in discussing the efficacy of PDT is the depth of light penetration through tissues, which is dependent

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Abbreviations: ANT, adenine nucleotide translocator; COX, cyclooxygenase; EGFR, epidermal growth factor receptor; HIF, hypoxia inducible factor; HO, heme oxygenase; HRE, hypoxia response element; HPD, hematoporphyrin derivative; HSP, heat shock protein; IP_3, inositol 1,4,5-trisphosphate; PDT, photodynamic therapy; PG, prostaglandin; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PTPC, permeability transition pore complex; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

on several processes including: (i) reflection; (ii) scattering; (iii) transmission; (iv) absorption; or (v) a combination of these. Therefore, the excitation of the photosensitizer is dependent on the overall characteristics of the tissue as well as its thickness. Pigment-rich tissues, such as those of melanoma, are resistant to PDT. Tissue penetration is also affected by the wavelength of the light. Longer wavelengths of visible light penetrate tissues better than shorter ones, and for most PDT application the lower limit is 580 nm largely because of strong absorption by hemoglobin (Moore *et al*., 1997). Typically, the depth of penetration is from 3 to 8 mm for light in the range from 630 to 800 nm. Nonetheless, it was observed that tumors of up to 1 cm of depth can be effectively eradicated by PDT, an effect that can be explained by the concomitant activation of local immune response (Moan *et al*., 2003).

The light sources used by many investigators in experimental research frequently include relatively inexpensive incoherent lamps including halogen, fluorescent, tungsten or xenon lamps. Optimal illumination, however, is obtained with laser light which has certain features that distinguish it from other light sources, predominantly: collimation, coherence and monochromacity (Hsi *et al*., 1999; Mang, 2004). Although laser sources can be a substantial expenditure their real cost depends on the intensity of use. The overall cost of treatment is significantly lowered by the possibility of performing PDT procedures in an out-patient (ambulatory) setting.

BASIC PHOTOCHEMISTRY

The absorption of light energy by a photosensitizer increases the energy of its electrons rendering the photosensitizer excited. The excited photosensitizer can relax back to its ground state by emitting fluorescence, or to a triplet state through a process called intersystem crossing, from which it can relax by emitting phosphorescence. This phenomenon is currently being exploited for the development of photodiagnostic procedures (Stringer *et al*., 2004). In the triplet state, the excited photosensitizer can transfer its energy to molecular oxygen, one of the rare compounds which have triplet ground state, and the two molecules relax to respective singlet states. In the singlet state molecular oxygen, $^1O_2$, is excited, highly reactive and thereby responsible for the majority of lesions generated during PDT (Sharman *et al*., 2000). Excited photosensitizer on transferring its excess energy returns to its ground state to accept further photons or becomes photochemically degraded (used) in a process referred to as photobleaching. Alternatively, an excited photosensitizer may react directly with biomolecules to form free radicals that further react with molecular oxygen producing superoxide radical anion, hydrogen peroxide or hydroxyl radical. Superoxide radical anion is generated for instance by excitation of porphyrins in the presence of reducing substances (Sharman *et al*., 2000).

PHOTOSENSITIZERS

Currently, over 30 different photosensitizers are used in preclinical studies, all of which are tetrapyrrole derivatives (Allison *et al*., 2004). The most extensively studied photosensitizers are porphyrins that were identified over 150 years ago. One of their good features is minimal toxicity in the dark and a lack of pharmacological interactions with other drugs, making PDT a safe procedure in oncological combination treatments. For a long time most preclinical studies were dominated by the use of hematoporphyrin derivatives (HPD). The best clinical experience has been obtained with Photofrin which is a mixture of monomers, dimers and oligomers derived from hematoporphyrin. It was the first clinically approved photosensitizer.

Photofrin consists of over 60 compounds and is difficult to be reproducibly synthesized. Additionally, its molar absorption coefficient is relatively low and requires higher doses as well as fluence rates to produce similar effects as new generation photosensitizers. Furthermore, Photofrin is not particularly selective for tumor tissue and is retained by normal cells for prolonged periods. For this reason, it causes long-lasting cutaneous photosensitivity that requires sunlight avoidance for 4–6 weeks. These factors have stimulated research leading to the development of second generation photosensitizers. The ideal photosensitizer should meet several criteria: chemical purity, preferential tumor retention, fast tumor accumulation and rapid clearance, activation by light with good tissue penetration, high absorption coefficient, no dark toxicity, minimal or absent skin photosensitivity. No photosensitizer with such characteristics has been found but these criteria delineate the area for development.

ALA (5-aminolevulinic acid, Levulan) and its methylester (Metvix) are precursors of the photosensitizer protoporphyrin IX (PpIX) which is generated in mitochondria and can be activated with 635 nm light. Cells with increased metabolism generate PpIX at an increased rate. ALA is approved for the treatment of actinic keratosis and basal-cell carcinoma of the skin. It can be applied topically as a cream or an emulsion or in the case of internal malignancy, systemically

m-THCP (*meta*-tetrahydroxyphenylchlorin, Foscan), a photosensitizer with a 652 nm absorption peak, has been approved for the treatment of head and neck cancers in the European Union. This chlo-

Table 1. Selected photosensitizers and their short characteristics

Photosensitizer	Trade name	Wavelength for PDT	Time before illumination	Comments
Porfimer sodium	Photofrin	630 nm	24–48 h	Most frequently used photosensitizer, approved for clinical use in treatment of many cancers
Bnezoporphyrin derivative monoacid ring A, BPD-MA,verteporfin	Visudyne	690–692 nm	1–2.5 h	Sufficient tumor concentration can be achieved after 30 min, rapid clearance, low skin photosensitivity, approved for treatment of age-related macular degeneration
m-THPC, temeporfin	Foscan	652 nm	72–96 h	Skin photosensitivity for only 1–2 weeks, extremely high phototoxicity requiring very low drug and light doses
5-ALA	Levulan	635 nm	4–12 h	Precursor of photosensitizer converted in mitochondria into protoporphyrin IX
Methyl ester ALA	Metvix	635 nm	4–12 h	ALA ester with improved skin penetration
Benzyl esther ALA	Benzvix	635 nm	4–12 h	ALA ester with improved skin penetration
Hexyl ester ALA	Hexvix	635 nm	4–12 h	ALA ester with improved skin penetration
Tin ethyl etiopurpurin, $SnET_2$	Purlytin	660–665 nm	24 h	Increased cutaneous photosensitivity for up to 1 month
Hypericin	Hypericin	595 nm	24 h	Shallow light penetration, used mainly in psoriasis and superficial skin cancers
Silicon-based phthalocyanines, Pc4, Pc10, Pc12, Pc18	CGP55847, Photosense	670 nm	3 h	Deeper tissue penetration, low dark toxicity, fast accumulation in tumors, high tumor selectivity
Chloro-aluminum sulfonated phthalocyanine, CASPc		670–675	3 h	Deeper tissue penetration, low dark toxicity, fast accumulation in tumors, high tumor selectivity
Lutetium texaphyrin, motexafin lutetium, lutex	Lutrin, Antrin	720–760	2–4 h	Increased stability, very deep tissue penetration, advanced clinical trials in cardiology (treatment of atheromatous plaques), virtually no skin photosensitivity
N-Aspartyl-chlorin e6,	Npe_6	660–665	4 h	Rapid accumulation in tumor, short skin photosensitivity

rin derivative is an extremely potent photosensitizer that requires very low drug doses (typically less than 0.1 mg/kg) and low fluence rates (10 J/cm^2).

Another clinically approved photosensitizer is the benzoporphyrin derivative monoacid A (BPA-MA, Verteporfin, activated by light of 690 nm) which is used in the treatment of wet-type macular degeneration, the leading cause of blindness in elderly people. However, despite preclinical development this agent has not yet gained clinical approval in oncology.

Other photosensitizers include different chlorin derivatives, phthalocyanines, texapyrins, and less well characterized, porphycens, antracens, chlorophyll derivatives, purpurins, hypocrellins and hypericin.

CLINICAL APPLICATIONS OF PDT

The first clinical approval for Photofrin-mediated PDT was in Canada as an adjuvant treatment to resection of papillary bladder tumors. The US FDA has approved Photofrin for use in PDT to relieve symptoms of obstructive esophageal cancer and to treat both early and advanced non-small cell lung cancer. In 2003, the FDA approved Photofrin for the treatment of precancerous lesions in patients with Barrett's esophagus (Tang *et al*., 2004). Similar approvals have also been granted in several European countries, Canada and Japan (Kato *et al*., 2004). Temeporfin-based PDT has received regulatory approval in the European Union for the treatment of head and neck cancers, and ALA and its esters have been approved in Europe for the treatment of numerous cutaneous cancers (Brown *et al*., 2004; Fukuda *et al*., 2005). Outstanding results with Photofrin were also obtained in patients with head and neck cancers (mainly cancers of the larynx, pharynx and oral cavity) (Biel, 1998), nonresectable cholangiocarcinoma (Ortner *et al*., 1998; 2004), breast (Allison *et al*., 2004) and skin cancers (Dougherty, 2002). Moreover, Photofrin-PDT is investigated as adjuvant following resection of primary and recurrent astrocytomas and glioblastomas, malignant pleural mesothelioma, and intraperitoneal tumors (mainly ovarian carcinoma) (Moskal *et al*., 1998; Pass *et al*., 1994; Popovic *et al*., 1996; Tochner, 1994).

MECHANISM OF PDT ACTION

PDT is frequently regarded as a dual specificity treatment. The selectivity is achieved by an increased photosensitizer accumulation within the tumor as compared to normal tissues and by the fact that illumination is limited to a specified location. Several possible mechanisms of selective photosensitizer retention within tumors include greater proliferative rates of neoplastic cells, a lack of or poor lymphatic drainage, high expression of LDL receptors on tumor cells (many photosensitizers bind to LDL), low pH (which facilitates cellular uptake), increased vascular permeability, abnormal structure of tumor stroma characterized by large interstitial space and increased production of collagen that binds porphyrins, or tumor infiltration by macrophages that are efficient traps for hydrophobic photosensitizers (Moan *et al*., 1992; 2003). The selectivity can be even further enhanced by binding of photosensitizers to molecular delivery systems such as growth factors or monoclonal antibodies that are characterized by high affinity for target tissues (Konan *et al*., 2002). The local nature of PDT is both a drawback and an advantage. A limitation of PDT is that it cannot be a curative procedure for large and disseminated tumors. Nonetheless, even for an advanced disease it can improve the quality of patients' life and prolong survival. For small and localized diseases it can be a curative procedure with minimally invasive and excellent cosmetic results. By using up-to-date fiberoptic systems and modern types of endoscopy, light can be delivered accurately to any part of the body. Interstitial light-delivering electrodes can be used in the treatment of large-volume tumors. Because of these developments PDT is now rarely rejected due to difficulties in the delivery of light.

Due to the very short half-life of $^1O_2$, measured in nanoseconds, this cytotoxic molecule can diffuse only up to 20 nm in cells (Moan *et al*., 1991). Therefore, the subcellular localization of the photosensitizer determines which organelles are primarily damaged. Only rarely, however, do the photosensitizers localize selectively in particular organelles. Photofrin for example is usually regarded as targeting mitochondria. However, it can also be found at other sites including plasma membrane, endoplasmic reticulum and Golgi complex at concentrations seemingly sufficient for mediating cytotoxicity. It is frequently assumed that photosensitizers that localize in mitochondria are more likely to induce apoptosis and those targeting plasma membrane or mitochondria primarily induce necrosis. In practice, such differential cell death mechanisms induced by PDT can be mostly observed *in vitro*, at defined photosensitizer concentration and fluence rates of light. *In vivo* the situation is more complex and tumor destruction is likely to result from a mixture of apoptotic and necrotic cell death.

MECHANISMS OF CELL DEATH FOLLOWING PDT

The antitumor effects of PDT result from three interdependent processes: direct tumor cell kill, damage to the vasculature, and activation of a nonspecific immune response (Golab *et al*., 2000; van Duijnhoven *et al*., 2003; Abels, 2004). While all of these contribute to the overall outcome of the treatment their relative contribution is difficult to pinpoint. By manipulating light fluence rate, timing of illumination following photosensitizer administration or a combination of these it is possible to modulate the role played by each of these factors in the final damage towards the tumor. This review will mostly discuss the direct influence of PDT on tumor cells and the molecular mechanisms exploited by tumor cells to resist lethal damage. Increasing amount of experimental data indicate that at optimal PDT conditions (sufficient photosensitizer concentration and light exposure) tumor cells die by necrosis. During illumination the concentration of the photosensitizer decreases due to photobleaching, vascular stasis, tumor edema etc. At these conditions as well as in deep tumor regions (illuminated with suboptimal light from the very beginning of treatment) the effectiveness of PDT is decreased and tumor cells have a chance to either oppose or repair damage induced by the treatment. If they manage to do so they will survive the treatment and will contribute to tumor relapse (if not damaged by lethal ischemia or activation of immune response). In all other cases tumor cells should undergo apoptosis.

Induction of apoptosis

Apoptosis is a tightly controlled, energy-consuming process of suicidal cell death involving activation of hydrolytic enzymes such as proteases and nucleases leading to DNA fragmentation and degradation of intracellular structures (Reed, 2000). Morphologically, the crucial elements of the process are chromatin condensation, cell shrinkage and production of apoptotic bodies which are engulfed by surrounding cells and phagocytes.

Apoptosis has been shown to be a rapid and dominant form of cell death following photodynamic therapy in multiple experimental settings utilizing various photosensitizers and cell types (Oleinick *et al*., 2002). Apoptosis was, however, the only form of cell death investigated in the majority of studies and it is likely that some cells may also undergo necrosis after PDT. Factors that promote necrosis include extra-mitochondrial localization of photo-

sensitizer, high dose of PDT, and glucose starvation (Almeida *et al*., 2004; Dellinger, 1996; Kiesslich *et al*., 2005; Oberdanner *et al*., 2002). Also the cell genotype may influence the form of cell death following PDT (Wyld *et al*., 2001).

Photodynamic therapy induces apoptosis *via* two major pathways: mitochondria-mediated or intrinsic pathway, and death receptor-mediated or extrinsic pathway (Almeida *et al*., 2004). One of the initial oxidative stress-associated stimuli for apoptosis induction might include carbonylation of a selected set of proteins (Magi *et al*., 2004).

Mitochondria-mediated apoptosis

The mitochondrial apoptosis pathway occurs mainly when photosensitizers localizing in these organelles are used. Nonetheless, the intrinsic pathway may also be activated when other cell structures are direct targets of photodynamic action (Almeida *et al*., 2004). The first stages of the pathway are disruption of mitochondrial transmembrane potential and release of cytochrome *c* to the cytosol. This allows formation of a complex called apoptosome and activation of hydrolytic enzymes — caspases. The pathway is strongly influenced by the Bcl-2 family of proteins (Oleinick *et al*., 2002).

Disruption of mitochondrial transmembrane potential and cytochrome *c* release

Some data suggest that loss of mitochondrial transmembrane potential and the release of cytochrome *c* to the cytoplasm result from opening of a large conductance channel located in the mitochondrial membrane, known as permeability transition pore complex (PTPC). This is a large protein complex comprising at least three transmembrane subunits. In A431 human epidermoid carcinoma cells cyclosporin A plus trifluoperazone, potent inhibitors of PTPC opening, prevented the loss of mitochondrial transmembrane potential and cytochrome *c* release (Lam *et al*., 2001). The direct mechanism of PTPC opening after PDT is not known, but some data exists showing that adenine nucleotide translocator (ANT) one of PTPC subunits, may be the target of photodynamic damage (Belzacq *et al*., 2001).

There is also some evidence that the release of cytochrome *c* and later events in mitochondria-mediated apoptosis may occur independently of PTPC opening. In cells photosensitized with hypericin or hypocrellins PTPC-opening inhibitors did not prevent transmembrane potential disruption (Chaloupka *et al*., 1999) and for some doses of Verteporfin-based PDT cytochrome *c* was released to cytosol without any loss of mitochondrial transmembrane potential (Chiu *et al*., 2001). Recently it has been suggested that cytochrome *c* release may be caused by mitochondrial cardiolipin peroxidation (Kriska *et al*., 2005).

Activation of caspases

When present in the cytoplasm, cytochrome *c* binds Apaf-1 and procaspase-9 to form a protein complex called apoptosome leading to auto-cleavage and self-activation of caspase-9. Active caspase-9 cleaves and activates procaspase-3. A number of caspases (caspase 2, -3, -6, -7, and -8) have been shown to be activated following PDT in various experimental models. Activation of these hydrolytic enzymes leads to the cleavage of multiple cellular proteins, DNA fragmentation and eventually cell death (Almeida *et al*., 2004).

Activation of procaspase-3 after PDT has been demonstrated in multiple experimental settings. However, its role in eliciting cell death seems to be redundant. Studies with MCF-7 (breast cancer cells lacking caspase-3) and MCF-7c3 cells (stably transfected with a procaspase-3 gene) revealed equal sensitive to PDT-induced death measured by clonogenic assay but, surprisingly, DNA fragmentation and poly(ADP-ribose) polymerase cleavage were observed only in MCF-7c3 cells (Xue *et al*., 2001a).

Role of Bcl-2 family of proteins

Proteins of the Bcl-2 family can be divided into two groups depending on the influence on apoptotic pathways: Bcl-2, Bcl-X_L, Bcl-w, Mcl and A1 are antiapoptotic while Bax, Bok, Bfm, Bcl-X_S and others promote apoptosis (Cory *et al*., 2003). In various experimental settings (different photosensitizers, various light sources and doses) transfection of tumor cells with Bcl-2 genes has been shown to protect cells from PDT-induced apoptotic death (He *et al*., 1996; Carthy *et al*., 1999). Accordingly, studies with antisense Bcl-2 oligonucleotides demonstrated that reduction of Bcl-2 protein level sensitizes tumor cells to PDT-induced apoptosis (Zhang *et al*., 1999; Srivastava *et al*., 2001).

In contrast with these findings, there are reports suggesting that overexpression of Bcl-2 may have no effect on apoptosis following PDT (Klein *et al*., 2001) or may even promote cell death (Kim *et al*., 1999). Bcl-2 overexpression resulted in greater loss of mitochondrial transmembrane potential, enhanced release of cytochrome *c* and reduced cell viability. This phenomenon may be explained by enhanced expression of the proapoptotic Bax protein in Bcl-2-overexpressing cells. While PDT caused significant reduction of Bcl-2 protein level in both transfected and parental cell lines, Bax levels were unaffected making the cells more susceptible to apoptosis (Kim *et al*., 1999). This and other observations indicate that Bcl-2 protein can be directly photodamaged during PDT. Importantly, not only Bcl-2 but also Bcl-

X_L photodamage was observed in various cell lines (Xue *et al*., 2001b; 2003). In some other systems Bcl-2 has been shown to undergo rapid phosphorylation thereby restricting premature apoptosis of photodamaged cells (Vantieghem *et al*., 2002).

In the Bax-negative DU-145 cells no cytochrome *c* release was observed indicating that Bax expression is necessary for the early steps in mitochondria-mediated apoptosis (Usuda *et al*., 2002). All the aforementioned data suggests that the influence of the balance between the pro- and antiapoptotic members of the Bcl-2 protein family significantly influences the susceptibility of cancer cells to apoptosis following PDT.

Death receptor-mediated apoptosis

Death receptor-mediated apoptosis is considered to occur preferentially when photosensitizers targeting the cell membrane are used. It is triggered by multimerization of cell membrane receptors belonging to the tumor necrosis factor (TNF) receptor superfamily. Of all the members of the family, Fas receptor is thought to play a major role in PDT induced apoptosis. Multimerization of Fas receptors allows formation of a "death inducing signaling complex" consisting of Fas, FADD adaptor protein and procaspase-8. In these conditions, procaspase-8 activates itself proteolytically and activates downstream effector caspases (Oleinick *et al*., 2002).

PDT-induced death-receptor mediated apoptosis was observed in many cell lines and experimental settings. In A431 cells Fas protein level was found to be elevated as early as 5 min after PDT (Ahmad *et al*., 2000). Such overexpression was also observed *in vivo* in tumor-bearing mice (Yokota *et al*., 2000). In natural conditions Fas multimerization is dependent on Fas ligand (FasL) binding. It has been suggested that in PDT treated cells Fas multimerization may occur in both ligand-dependent and -independent manner (Almeida *et al*., 2004). Formation of Fas-FADD complexes and activation of caspase-8 was also observed following photodynamic therapy (Ahmad *et al*., 2000). Some data indicate that caspase-8 may trigger the intrinsic apoptotic pathway when membrane-bound photosensitizers are used for PDT, as inhibition of this caspase resulted in decreased cytochrome *c* release and caspases-3 activation in cells photosensitized with Rose Bengal (Zhuang *et al*., 1999). This effect is probably mediated by Bid protein which is proteolytically activated by caspases-8 to promote cytochrome *c* efflux from the mitochondrial membrane. Although inhibition of some mediators of the extrinsic apoptotic pathway has been shown to produce partial resistance to PDT-induced apoptosis and cell death, FADD null mouse embryonic fibroblasts were capable of undergoing apoptosis when sensitized with Pc 4 (Ahmad *et al*., 2000; Nagy *et al*., 2001). This indicates that at least in some cell types the extrinsic pathway is not necessary for apoptosis following PDT.

PDT-induced apoptosis is also influenced by cellular signaling pathways other than the intrinsic and extrinsic apoptotic pathway, such as calcium homeostasis, ceramide formation and MAPK kinases. These and other signaling pathways are described further in this review.

SIGNALING MECHANISMS IN TUMOR CELLS EXPOSED TO PDT

Lipid metabolism

Most hydrophobic photosensitizers accumulate in the plasma membrane, making it the target for phototoxic events (Moor, 2000). PDT causes plasma membrane damage which is manifested by swelling, bleb formation, shedding of vesicles containing plasma membrane, cytosolic and lysosomal enzymes, reduction of active transport, increased permeability and depolarization of plasma membrane (Dougherty *et al*., 1998). Three different signaling pathways may be involved in the photodynamic reaction originating at the plasma membrane, including activation of phospholipase C (PLC), phospholipase A_2 (PLA_2) and ceramides (Moor, 2000; Almeida *et al*., 2004). PLC cleaves phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 participates in the release of calcium ions from intracellular stores. DAG activates protein kinase C (PKC), which is engaged in the induction of cell death or survival (Almeida *et al*., 2004). At least in two different cell lines inhibition of PKC resulted in an increase in cell survival following PDT (Agarwal *et al*., 1993; Rasch *et al*., 1997). However, the role of PKC in the cellular response to PDT remains controversial, as in another study inhibition of this kinase resulted in augmentation of cell death following PDT (Zhuang *et al*., 1998).

Phospholipase A_2 is an enzyme involved in the production of arachidonic acid metabolites (Almeida *et al*., 2004), which are further metabolized by cyclooxygenases (COX) into prostaglandins. PDT with HPD (hematoporphyrin derivative) induced PLA_2 activation and this phenomenon was supposed to play a crucial role in the tumor cells' survival, probably due to increased production of PGE_2 (Penning *et al*., 1993). Pretreatment of tumor cells with PGE_2 improved cell survival while inhibition of PGE_2 production with indomethacin resulted in cell death (Penning *et al*., 1993).

Ceramides are a group of potent apoptosis inducers generated from sphingomyelin by sphingomyelinidases (SMases) (Moor, 2000). Ceramide-dependent induction of apoptosis was observed in

different PDT models (Separovic *et al*., 1997; 1998; 1999). Interestingly, in RIF-1 cells an increase in ceramide production following PDT did not augment cell death, suggesting a dual role of these substances in the tumor cell metabolism (Separovic *et al*., 1998). Niemann-Pick (N-P) human lymphoblasts that lack SMases exposed to Pc4-mediated PDT showed an increased survival rate. Furthermore, addition of bacterial SMases resulted in improved PDT cytotoxicity (Separovic *et al*., 1999).

Secondary messengers — calcium ions, cyclic nucleotides and nitric oxide (NO)

Calcium ions are involved in many signaling cascades. PDT has been reported to induce an increase in the concentration of intracellular calcium ions (Ben-Hur *et al*., 1991; Penning *et al*., 1992). The consequences of this rise are still unclear. Some investigators suggest a proapototic role of calcium ions (Penning *et al*., 1992) while others propose an opposite mechanism (Hubmer *et al*., 1996). Chelation of Ca^{2+} ions with BAPTA in various tumor cells improved survival and decreased their apoptosis (Ogata *et al*., 2003; Thibaut *et al*., 2002). Different photosensitizers induce Ca^{2+} release form distinct sources (endoplasmatic reticulum, cell environment *via* the plasma membrane Ca^{2+}-ATPase and Ca^{2+} ion channels). This diversity may be the cause of a dual role of calcium ions in the cell survival following PDT (Almeida *et al*., 2004).

An increase in the intracellular amount of cAMP seems to have a protective effect in cells exposed to different kinds of stress (Almeida *et al*., 2004). In T24 cell line PDT induced a rapid but transient increase of cAMP which was diminished by the treatment of the cells with a COX inhibitor (Penning *et al*., 1993). COX is involved in the production of different kinds of prostaglandins, among which PGE_2 seems to have the greatest cytoprotective role (Hendrickx *et al*., 2003). PGE_2 increases the intracellular level of cAMP, which is supposed to be the mechanism of cytoprotection (Hendrickx *et al*., 2003).

Nitric oxide is a crucial mediator of tumor growth. NO causes smooth muscle relaxation leading to vasodilatation and improvement in tumor blood supply (Almeida *et al*., 2004). There is evidence that NO not only stimulates tumor growth but also facilitates formation of metastases (Gomes *et al*., 2002). Production of NO is also affected by PDT treatment (Almeida *et al*., 2004). Intratumoral expression of NO negatively correlates with the sensitivity of tumors to PDT (Korbelik *et al*., 2000). NO was shown to have a protective effect against PDT-induced tumor death. Treatment of tumor cells with NO donors decreased the cytotoxic effects of phthalocyanine-induced PDT (Gomes *et al*., 2002). Moreover, the protective effect of NO was reversed by incubation of PDT-treated cells with an inhibitor of protein kinase G (PKG), suggesting a cGMP-dependent mechanism of this protection (Gomes *et al*., 2002). NO may also play a role in tumor cell survival *via* induction of HO-1, HSP70 and Bcl-2 expression (Suschek *et al*., 1999) (see below).

Protein kinases

PDT of tumor cells leads to a rapid and extensive inhibition of protein phosphorylation. However, there are several pathways that involve active tyrosine or serine/threonine phosphorylation that are induced by PDT. These include ERK1/2 and stress kinases as well as PI3-K and AKT.

The family of mitogen activated protein kinases (MAPKs) consists of three members: the extracellular signal regulated kinases (ERK1/2), the c-Jun N-terminal kinases/stress activated protein kinases (JNK/SAPK) and the p38 MAPK. All of them are serine/threonine protein kinases, which need dual phosphorylation on a threonine and tyrosine residues for their activation (Chang *et al*., 2001). The ERK, JNK/SAPK and p38 MAPK signaling pathways are closely related. They are activated by extracellular signals and finally regulate expression of a wide panel of genes. Depending on cell type, the type of cellular stress and a number of other variables activation of ERK, p38 MAPK or JNK can lead to cell survival or participates in the induction of apoptosis. Studies of the ERK signaling pathway in cells resistant and sensitive to PDT revealed that sustained ERK1/2 activation protects cells from Photofrin-mediated phototoxicity and that the duration of ERK1/2 activation is regulated by mitogen-activated protein kinase phosphatase (MKP-1) (Tong *et al*., 2002). In some cells activation of only JNK and p38 kinases but not ERK was observed (Klotz *et al*., 1998; Assefa *et al*., 1999; Hendrickx *et al*., 2003). Treatment of the cells with specific SAPK inhibitors resulted in the augmentation of the cytotoxic effects of PDT (Assefa *et al*., 1999). By contrast, CHO cells treated with Pc-4-mediated PDT in the presence of specific SAPK/JNK inhibitors survived in a higher percentage than without inhibition of this MAPK pathway (Xue *et al*., 1999). Additionally, transient transfection of HeLa cells with a dominant negative mutant of p38 MAPK had no effect on cell survival following Photofrin-mediated PDT (Tong *et al*., 2003). All the above experiments suggest that the protective or proapoptotic role of different MAPK kinases may me depend on the type of a cell line and on the mode of photosensitization (Almeida *et al*., 2004).

PDT treatment using two different photosensitizers induced downregulation of EGFR (epidermal growth factor receptor) expression and irreversibly prevented its phosphorylation (Ahmad *et al*., 2001;

Agostinis *et al*., 1995; de Witte *et al*., 1993). Similar inhibitory processes were observed in the case of SHC protein, one of EGFR downstream effectors (Kalka *et al*., 2000).

It has also been reported that in cells exposed to PDT activation of PI-3K and its downstream effectors AKT and ETK/BMX takes place (Xue *et al*., 1999a; 1999b). The latter seems to protect prostate cancer cells form PDT-induced apoptosis (Xue *et al*., 1999).

Transcription factors

NF-κB

The term NF-κB (nuclear factor kappa B) covers a family of inducible transcription factors that regulate the host immune and inflammatory responses and cell growth properties (Yamamoto *et al*., 2001). The NF-κB family mediates the transcription of over 180 target genes, including genes for cell adhesion molecules, cytokines, chemokines and antiapoptotic factors (Aradhya *et al*., 2001). In a stable state, NF-κB binds IκB which is an inhibitory molecule that sequesters NF-κB in the cytoplasm in an inactive state. When phosphorylated by IKK (IκB kinases), IκB is degraded by 26S proteasome allowing free NF-κB to enter the nucleus (Dixit *et al*., 2002). NF-κB has been shown to either promote or inhibit apoptosis of tumor cells (Perkins, 2004). The effect is dependent on the cell type and the type of the inducer of NF-κB activity (Almeida *et al*., 2004). PDT with different photosensitizers has been shown to induce NF-κB in different cellular systems (Moor, 2000). In HL-60 human promyelocytic leukemia cell line treated with Verteporfin and light a decreased cellular level of IκBα was observed (Granville *et al*., 2000). Expression of a dominant negative mutant of IκBα increased the cell death after PDT. This may suggest an anti-apoptotic role of genes regulated by NF-κB (Matroule *et al*., 1999). Moreover, activation of NF-κB is crucial for the induction and modification of immune responses, which are extremely important mechanisms of PDT activity (Granville *et al*., 2000). Further studies are still needed to evaluate the role of NF-κB induction in cells exposed to PDT.

AP-1

AP-1 is a protein complex composed of Jun, Fos, Maf and ATF-family proteins. This transcription factor is activated in cells exposed to various kinds of stress of either physical or chemical nature. AP-1 recognizes cAMP response elements (CRE) or TPA response elements. Similarly to NF-κB, AP-1 is involved in the regulation of transcription of genes responsible for either cell death or survival (Almeida *et al*., 2004). PDT of HeLa cells using Photofrin resulted in a strong and prolonged induction of c-Jun and c-Fos and increased AP-1-DNA binding activity (Kick *et al*., 1996). Similar results have been shown in different experimental models (Almeida *et al*., 2004). AP-1 induction can be diminished using protein kinase inhibitors, suggesting a possible role of upstream MAPK kinases in AP-1 activation (Luna *et al*., 2000).

Rb and E2F

The retinoblastoma (Rb) and E2F proteins are transcription factors important for the regulation of transition from G1 to S phase of the cell cycle. The involvement of these factors in G0/G1-cell cycle arrest and induction of apoptosis following PDT was shown (Ahmad *et al*., 1999). PDT has also been shown to increase the cellular level of the cyclin kinase inhibitor $p21^{WAF1/CIP1}$ and its binding to cyclin D1 and CDK6 (Ahmad *et al*., 1999). Complexes of cyclins and corresponding cyclin-dependent kinases are involved in the phosphorylation (activation) of Rb protein during the cell cycle. The decrease in Rb phosphorylation following PDT may be due to changes in the binding properties of cyclins. Hypophosphorylated Rb associates and inhibits the activity of E2F transcription factors and a decrease in the E2F, DP1 and DP2 proteins has been shown in cells exposed to Pc4-mediated PDT (Ahmad *et al*., 1999).

Hypoxia inducible factor (HIF)

It was reported that PDT with Photofrin as a photosensitizer resulted in overexpression of HIF-1α (Ferrario *et al*., 2000). HIF-1α and HIF-2α are key proteins regulating cellular response to hypoxia (Koukourakis *et al*., 2001). HIF-1α binds to HIF-1β thus forming the HIF-1 transcription factor complex (Almeida *et al*., 2004). HIF-1β is a stable and constitutively expressed subunit of HIF-1, while HIF-1α is rapidly degraded in normal oxygen concentration. Hypoxia results in HIF-1α stabilization and activation of HIF-1 transcription functions (Almeida *et al*., 2004). HIF-1 migrates to the nucleus and binds to HRE (hypoxia response element) in the promoters of a wide panel of genes, e.g. VEGF (vascular endothelial growth factor) gene (Almeida *et al*., 2004). Since VEGF is a strong mitogen, treatment of murine mammary tumor with agents decreasing the expression of this growth factor resulted in augmented response to PDT (Ferrario *et al*., 2000). The effectiveness of PDT is strongly dependent on the appropriate amount of oxygen in the tumor site. Improvement of tumor oxygenation by correction of chemotherapy-induced anemia with erythropoietin resulted in an augmentation of the antitumor activity of PDT in C-26 tumor bearing mice (Golab *et al*., 2002).

High expression of HIF-1α and Bcl-2 in early esophageal cancers in patients correlated with poor tumor response to PDT (Koukourakis *et al*., 2001).

Proteins overexpressed after PDT

Analysis of mRNA expression with cDNA arrays is a very useful method to investigate changes in gene activation in cells exposed to PDT. It was assessed in three different cell lines: C-26 (murine colon carcinoma) (Makowski *et al*., 2003), HT29 (human colon adenocarcinoma) (Wang *et al*., 2002) and A431 (human squamous cell carcinoma) (Verwanger *et al*., 2002). Surprisingly, only a few genes were induced after PDT, among them cyclooxygenase-2, heme oxygenase-1, aldehyde dehydrogenase, cytochrome P450, RhoB, HSP27 and HSP70.

Heme oxygenase-1 (HO-1)

Heme oxygenase-1 is the rate limiting enzyme involved in the degradation of heme into carbon monoxide, free iron and biliverdin. Biliverdin is converted into bilirubin by biliverdin reductase (Dulak *et al*., 2003). Being potent antioxidants, biliverdin, as well as bilirubin may play a very important role in cytoprotection of cells exposed to the oxidative stress induced by PDT. Induction of HO-1 by PDT was described in the CHO cell line using Photofrin as well as Rose Bengal as photosensitizers (Gomer *et al*., 1991a). Even dark incubation of human fibroblasts with two different photosensitizers induced mRNA for HO-1 (Bressoud *et al*., 1992). The gene for heme oxygenase contains an AP-1 binding site and thus may be easily activated in different stress conditions (Almeida *et al*., 2004).

Heat shock proteins (HSPs)

The term heat shock proteins covers a family of proteins involved in the protection of cells against different kinds of stress, e.g. oxidative, heat, osmotic stress as well as growth factors deprivation. In normal growth conditions cellular levels of HSPs are stable and low. If a cell is exposed to any kind of stress, the levels of HSPs dramatically and rapidly increase (Pockley, 2003). PDT induces a wide panel of different HSPs. In murine RIF-1 cells PDT using chlorine or purpurin derivatives resulted in an increased expression of HSP70 (Gomer *et al*., 1996). Interestingly, Photofrin-mediated PDT induced overexpression of HSP70 only in *in vivo* model (Gomer *et al*., 1996). In HT29 human colon carcinoma cells upregulation of HSP27 plays a protective role against PDT-induced cytotoxicity and in cell lines resistant to PDT HSP27 is constantly overexpressed (Wang *et al*., 2002). Elevated levels of mRNA as well as a significant increase in different GRP (glucose-related protein) protein levels were observed in fibrosarcoma cells exposed to porphyrin-mediated PDT (Gomer *et al*., 1991b). In different PDT models overexpression of HSP60 (Hanlon *et al*., 2001) and GRP78 (Matsumoto *et al*., 2000) has been reported. In contrast, HSP47 which is associated with collagen type I metabolism, is up-regulated only after hyperthermia. PDT does not induce collagen damage and hence does not change the expression of HSP47 (Verrico *et al*., 1997). The regulation of HSPs expression is nowadays well described. In normal (meaning: non-stress) conditions HSP70 is associated in the cytoplasm with HSF (heat shock factor). When any kind of stress occurs and results in production of damaged proteins, HSP70 serves as a chaperone and binds the damaged macromolecules. This results in dissociation of HSF form HSP70. HSF then trimerizes and migrates to the nucleus where it binds HSE (heat shock elements) leading to HSPs overexpression (Morimoto, 1993). HSP70 and HSP90 may play a protective role against PDT-induced apoptosis. HSP70 prevents the recruitment of procaspase-9 to the apoptosome complex, while HSP90 inhibits formation of an active apoptosome (Almeida *et al*., 2004).

Induction of HSPs could potentially be used for therapeutic purposes. The promoter of GRP78 has been exploited as a PDT-inducible molecular switch for the controlled expression of herpes simplex virus thymidine kinase (HSV-tk) suicide gene. Administration of ganciclovir potentiated the antitumor effects of PDT against tumor cells stably transfected with this inducible construct (Luna *et al*., 2000). As some HSPs function as endogenous adjutants that by unknown mechanism stimulate the adaptive immune response PDT has been combined with administration of immature dendritic cells, the most potent antigen presenting cells. Such combined treatment was superior, as compared with all other treatment regimens, in terms of both local control of the illuminated tumor as well as in eradication of tumor foci growing at a distant site (Jalili *et al*., 2004).

Cyclooxygenase-2 (COX-2)

It was reported that PDT using Photofrin as a photosensitizer induced expression of COX-2, but not COX-1 (Ferrario *et al*., 2002; Hendrickx *et al*., 2003). Furthermore, inhibition of COX-2 with NS-398 augmented the antitumor activity of PDT (Ferrario *et al*., 2002). In studies in the C-26 cell line *in vitro* no synergistic effects were observed in a combined treatment with Photofrin-mediated PDT and three different COX-2 inhibitors (Makowski *et al*., 2003). Using a COX-2 inhibitor in the sensitization model in mice bearing C-26 tumors did not result in a potentiation of the antitumor activity of PDT either (Makowski *et al*., 2003). Prolonged treatment with a COX-2 inhibitor started right before the tumor illumination caused an augmented antitumor response (Makowski *et al*., 2003). The data obtained suggest that COX-2 inhibition may act as an antiangiogenic factor and thus potentiate PDT-mediated tumor damage (Makowski *et al*., 2003). Activation of the

NF-κB and p38 MAPK signaling pathways may be involved in COX-2 induction following PDT (Hendrickx *et al*., 2003; Volanti *et al*., 2005).

Vimentin and clusterin

PDT induces vimentin expression. Vimentin is a major cytoskeletal protein, whose cleavage with caspases precedes poly(ADP-ribose) polymerase (PARP) degradation (Belichenko *et al*., 2001). Transfection of Jurkat cells with caspase-resistant vimentin delayed and attenuated apoptosis induced by Pc-4-PDT (Belichenko *et al*., 2001).

Clusterin is a glycoprotein of unknown function which is ubiquitously expressed in almost all tissues (Almeida *et al*., 2004). It was reported than Pc-4-mediated PDT induced clusterin overexpression in the A431 cell line. By contrast, in the apoptosis-resistant RIF-1 cells no change in clusterin level was observed (Kalka *et al*., 2000). The significance of this phenomenon remains to be elucidated.

Superoxide dismutase (SOD) and the complex of glutathione

Superoxide ion ($O_2^{\cdot -}$) is one of the main products of phototoxic reactions. Superoxide generation increases several-fold after Photofrin-mediated PDT (Salet *et al*., 1997), leading to lipid peroxidation, DNA cross-linking and changes in protein conformation (Martinez-Cayuela, 1995). A constitutive (Cu,Zn-SOD) as well as an inducible (Mn-SOD) isoform of superoxide dismutase is engaged in the superoxide ion scavenging (McCord, 2002). PDT increases the expression of Mn-SOD but not Cu,Zn-SOD (Golab *et al*., 2003). Treatment of cancer cells with an SOD mimetic as well as trasfection of the cells with an Mn-SOD gene-containing vector decreased the effectivity of Photofrin-mediated PDT (Golab *et al*., 2003). Furthermore, use of 2-methoxyestradiol, an Mn-SOD inhibitor, significantly augmented the antitumor activity of PDT in *in vitro* as well as in *in vivo* models (Golab *et al*., 2003). All the data mentioned above suggest an important protective role of an inducible isoform of superoxide dismutase in tumor cells exposed to PDT.

Gamma-glutamyl cysteine synthetase is the rate limiting enzyme in the biosynthesis of glutathione. The complex of glutathione plays an extremely important role in the prevention of oxidative stress-mediated cell damage. In SNB-19 cell line exposed to hypericin-mediated PDT an overexpression of mRNA for gammaglutamyl cysteine synthetase was observed, suggesting a protective role of the complex of glutathione in cell damage following PDT (Miccoli *et al*., 1998). Furthermore, stable transfection of MCF-7 cell line with phospholipid hydroperoxide glutathione peroxidase cDNA protected the cells from PDT-induced cytotoxicity (Wang *et al*., 2001). Phospholipid hydroperoxide glutathione peroxidase removes singlet oxygen-induced lipid hydroperoxides from cell membranes (Wang *et al*., 2001).

p53

p53 tumor suppressor protein, also called 'guardian of the genome', is constitutively present in healthy cells and functions as a detector of DNA damage induced by different kind of stress. p53 stops cell cycle progression in S phase allowing DNA repair. If a cell fails to repair its DNA, p53 serves as an inducer of apoptosis. Some tumor cells acquire p53 mutations leading to its malfunction. In such a way tumor cells may accumulate DNA mutations still divide. There is strong evidence that p53 plays an important role in the response of tumor cells to chemo- and radiotherapy (Tong *et al*., 2000). Tong and coworkers compared the sensitivity to Photofrin-mediated PDT of normal human fibroblasts expressing wild-type p53 with immortalized Li-Fraumeni syndrome cells expressing a p53 mutant only. The latter seemed to be much more resistant to PDT induced damage (Tong *et al*., 2000). Similar results were obtained in wild-type and mutant p53 human colon carcinoma cell lines (Fisher *et al*., 1998). Although treatment of tumor cells with PDT results in overexpression of p53, the cell death following PDT seems to be p53-independent (Almeida *et al*., 2004).

THE INFLUENCE OF PDT ON DNA

Since DNA encodes genetic information, any lesions that occur in the genome create an extremely dangerous situation for the cell. DNA damage can be caused either by endogenous or exogenous factors. The number of DNA-damaging events in any given cell exceeds 10 000 per day, therefore sophisticated mechanisms have developed to respond to such a threat (Barzilai *et al*., 2004). This rapid reaction is termed DNA damage response and includes sensing and correcting the damage. The mechanisms of DNA damage induced by photodynamic therapy are not well understood. PDT can cause base oxidation, cross-linking of DNA strands or sister chromatid exchange (McNair *et al*., 1997; Haylett *et al*., 2003; Woods *et al*., 2004). Two aspects of DNA damage response in PDT should be distinguished. The first issue is what kind of DNA damage PDT inflicts on tumor cells and how tumor cells deal with PDT-induced lesions. A separate issue concerns the possible DNA damage to surrounding normal cells. PDT generates through the photosensitizer either reactive oxygen species — ROS (such as hydroxyl ion, hydrogen peroxide and superoxide) — or singlet oxygen. The latter has a very limited range (< 0.1 μm) and life-span (less than 1 s) so the probability of sin-

glet oxygen-induced DNA damage is low, unless it is generated in close proximity of a DNA strand. On the other hand, ROS can cause oxidative damage to DNA and are believed to be the major endogenous toxic agents, although at the physiological level they play a vital role in several cellular processes. However, current studies point at ROS-induced damage to cytoplasmic proteins and mitochondria, rather than specific DNA damage, as a cause of cell death after PDT. Further studies are necessary to determine the role of PDT-induced DNA damage in the effectiveness of this treatment. Good news is that normal cells seem to cope adequately with PDT-induced DNA damage, although this may depend on the type of the photosensitizer (Woods *et al*., 2004).

CONCLUSIONS AND FUTURE DIRECTIONS

Photodynamic therapy is among the most promising therapeutic options that have appeared in oncology within the last 30 years. Despite enormous number of research articles published on this topic in recent years, we are still far from thorough understanding of the molecular mechanisms of tumor cell killing. Published reports are full of conflicting results. One of the possible reasons for these discrepancies is the extremely complex interaction between light, photosensitizer, molecular oxygen and intracellular structures. Definitely, the use of various photosensitizing drugs, different cell lines, and various light sources contributes to the complexity of PDT studies. Future research should be more complex and should include analysis of multiple contributing factors and outcomes of the treatment. We can be sure that elucidation of the mechanisms of PDT action will provide us with more effective treatment regimens and a better future for people suffering from cancer.

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