The role of apoptosis in response to photodynamic therapy: what, where, why, and how

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Photodynamic therapy (PDT), a treatment for cancer and for certain benign conditions, utilizes a photosensitizer and light to produce reactive oxygen in cells. PDT is primarily employed to kill tumor and other abnormal cells, so it is important to ask how this occurs. Many of the photosensitizers currently in clinical or pre-clinical studies of PDT localize in or have a major influence on mitochondria, and PDT is a strong inducer of apoptosis in many situations. The purpose of this review is to critically evaluate all of the recently published research on PDT-induced apoptosis, with a focus on studies providing mechanistic insights. Components of the mechanism whereby PDT causes cells to undergo apoptosis are becoming understood, as are the influences of several signal transduction pathways on the response. Future research should be directed to elucidating the role(s) of the multiple steps in apoptosis in directing damaged cells to an apoptotic *vs.* necrotic pathway and for producing tumor ablation in conjunction with tissue-level mechanisms operating *in vivo*.

1   Introduction to photodynamic therapy and apoptosis

Photodynamic therapy (PDT) is a novel treatment for cancer and certain non-cancerous conditions that are generally characterized by overgrowth of unwanted or abnormal cells.[1–10] The procedure requires exposure of cells or tissues to a photosensitizing drug followed by irradiation with visible light of the appropriate wavelength, usually in the red or near-infrared region and compatible with the absorption spectrum of the drug. Upon absorption of a photon, the photosensitizer undergoes one or more energy transitions and usually emerges in its excited triplet state. The triplet can participate in a one-electron oxidation–reduction reaction (Type I photochemistry) with a neighboring molecule, producing free radical intermediates that can react with oxygen to produce peroxy radicals and various reactive oxygen species (ROS). Alternatively, the triplet-state photosensitizer can transfer energy to ground state oxygen

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(Type II photochemistry), generating singlet molecular oxygen, a highly reactive form of oxygen that reacts with many biological molecules, including lipids, proteins, and nucleic acids.[11–16] Most photosensitizers for PDT are efficient producers of singlet oxygen in simple chemical systems, and it is assumed, although impossible to measure directly, that Type II photochemistry is the dominant mechanism for PDT in most circumstances in cells and tissues.[10,11]

The first PDT photosensitizer to win approval by regulatory agencies in several countries was Photofrin, a complex mixture of the more active porphyrin oligomers that comprised hematoporphyrin derivative (HpD).[17] Photofrin is now approved for treatment of lung and esophageal cancer in the US and for several other cancers worldwide.[10,16,18] The limitations of Photofrin, including its complexity, its poor absorption of tissue-penetrating red light, and its tendency to be retained in skin and thus to produce cutaneous photosensitivity,[16] have encouraged the development of many second-generation photosensitizers.[19,20] Although most of the newer photosensitizers are porphyrin-like macrocycles, such as benzoporphyrins, purpurins, texaphyrins, phthalocyanines, and naphthalocyanines, or endogenously generated photosensitive metabolites, such as protoporphyrin IX (PPIX), a few have other types of structures, *e.g.*, hypericin, rhodamine, methylene blue, and derivatives of these molecules (Fig. 1). In the case of PPIX, synthesis of this photosensitizing precursor of heme is greatly enhanced by supplying an earlier metabolic precursor, 5-aminolevulinic acid (ALA). In addition to Photofrin, regulatory approval has also been obtained for photodynamic treatment of age-related macular degeneration with Visudyne (formulated Verteporfin) and for treatment of actinic keratoses with Levulan (formulated ALA). In this review, we will attempt to identify the photosensitizer with which a particular observation was made, but it is not yet possible in many cases to assign a unique cellular response to a particular photosensitizer. Because investigators tend to study effects of one or at best a few photosensitizers, the conclusions that are drawn can sound as though the effect can be elicited only by that photosensitizer, when in fact, many of the responses that will be discussed here are a result of photodynamic damage to cells which can be produced by many different photosensitizers, so long as their amount and location and the fluence and wavelengths of light are appropriately adjusted. Emphasis will be on those factors, related both to the photosensitizer and to the cell properties, that can influence cellular responses to PDT.

In the vast majority of applications, the primary role of PDT is to kill unwanted cells. Since the term "apoptosis" was introduced in 1972,[21] cell killing mechanisms are generally classified as occurring through apoptosis or necrosis. Apoptosis is a normal physiological process essential for the control of tissue development and involution and for tissue homeostasis.[22] Apoptosis is a tightly regulated process of cell suicide, controlled by both intracellular and extracellular signals, terminating in a characteristic sequence of morphological and biochemical changes for the systematic dismantling of the cell and preparation of the residual cell components, known as apoptotic bodies, for engulfment by tissue macrophages or other neighboring cells. The process limits leakage of intracellular material to the immediate environment, and thereby prevents tissue inflammation. The loss of ability to regulate
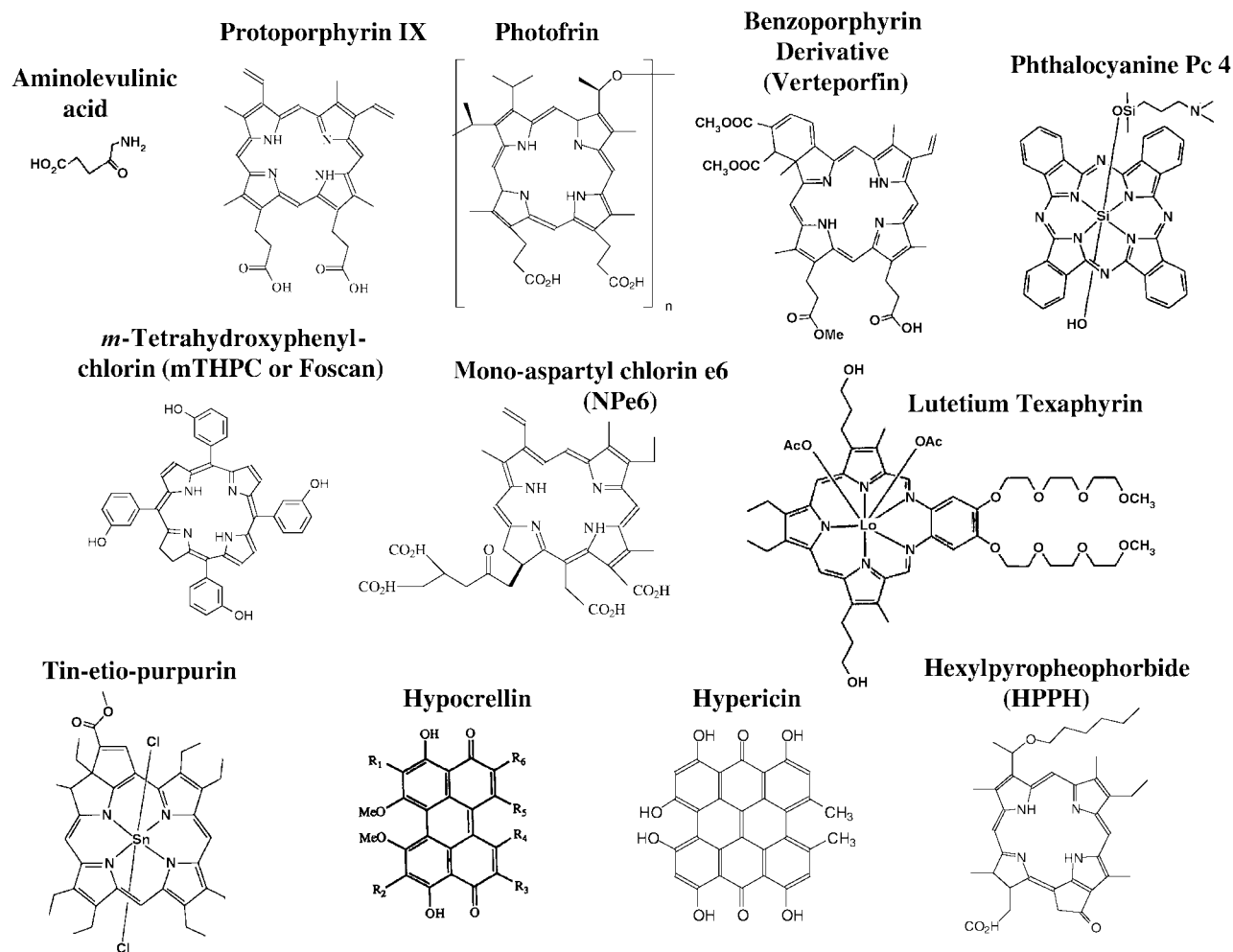

Fig. 1 Names and structures of selected photosensitizers in clinical or pre-clinical studies. Three of these have obtained regulatory approval for clinical PDT: Photofrin; benzoporphyrin derivative (Verteporfin); and aminolevulinic acid (Levulan), which is not a photosensitizer but a metabolic precursor of protoporphyrin IX.

apoptosis can lead to disease.[23–25] For example, many cancer cells have lost the ability to undergo apoptosis in response to normal signals or after damage from therapeutic interventions,[26,27] whereas unrestrained apoptosis of neurons may contribute to Alzheimer's and other neurodegenerative diseases.[28,29] In contrast, necrosis results from high levels of cell damage, in which plasma membrane integrity is lost, cells lyse, and tissue inflammation is triggered.[30] Although PDT can produce apoptosis or necrosis, or a combination of the two mechanisms, in many cases PDT is highly efficient in inducing apoptosis.[31] This property is of more than theoretical interest, because it implies that lower doses than those needed to produce necrosis are very effective in producing the desired cell killing result.

The past few years have produced a copious outpouring of research into the mechanisms of apoptosis, and many excellent reviews have appeared describing the steps involved in response to a wide variety of stimuli. An excellent series of five reviews appeared together in *Science* in 1998,[22,23,32–34] and several others have appeared since then.[24,25,30,35–37] Mechanisms of PDT have been reviewed as well, and the most recent contain brief capsules of research on PDT-induced apoptosis.[10,20,38–40] Two recent reviews have focused on signaling mechanisms of PDT and their role in PDT-induced apoptosis[41,42] and another two have reviewed PDT-induced apoptosis from the perspective of research on Verteporfin.[43,44] This report aims to provide a critical evaluation of the state of the science on PDT-induced apoptosis, with the primary emphasis on elucidation of mechanisms. Accordingly, recent papers that have reported observations of apoptosis but without new mechanistic insights have been ignored.

2 Mechanisms of apoptosis

Apoptotic cells have been recognized since the 19th century by their distinctive morphological appearance in tissues as shrunken cells with condensed nuclear chromatin, retracted from surrounding cells. Initiation of apoptosis in developing tissues is asynchronous, and the biochemical pathways triggering the process depend on the inducing agent. During physiological cell turnover, apoptosis is initiated by depletion of a growth factor or by the interaction of cytokines or other ligands with cell surface receptors.[22] However, cell damage by chemicals, radiation, heat, or other stresses can also trigger apoptosis.[45–48] After initiation, signaling pathways from the cell surface or from the site of cell damage converge onto a small number of central pathways that lead to the final "execution" phase of apoptosis, during which hydrolytic enzymes, including proteases and nucleases, are activated, and the characteristic morphological changes become evident.[25,34] Before discussing mechanisms of PDT-induced apoptosis, four general components of apoptosis mechanisms will be considered: (a) the role of caspases, (b) mechanisms initiated at the cell surface, (c) the role of mitochondria, and (d) the role of Bcl-2 family members.

2.1 Caspases

The central hydrolytic reactions of apoptosis are catalyzed by a family of proteases, now termed "caspases" for "*c*ysteine proteases acting on *asp*artic acid".[25,34,49,50] At least 12 of these enzymes are known. They have in common an active site cysteine and specificity for a short sequence of amino acids in their target site that terminates in aspartic acid. Caspases are synthesized as pro-enzymes that are activated by cleavage either in an autolytic fashion or by other caspases. For example, when multiple procaspase-9 molecules assemble on the apoptosomes, they can cleave one another to remove a leader sequence and generate a short and long peptide. Active caspases are composed of two short (~p10) and two long (~p20) peptides, *i.e.*, a tetramer. Caspases are often classified as "initiator" or

"effector" enzymes, depending on their placement in the pathway, either proximal to the initiating signal or at a late stage of cell disassembly, respectively. Effector caspases cleave and inactivate proteins that protect living cells from apoptosis, such as the DNA repair protein, poly(ADP-ribose) polymerase (PARP), ICAD/DFF45 (inhibitor of caspase-activated DNase, the nuclease responsible for DNA fragmentation), or the anti-apoptotic Bcl-2 proteins. Other actions of caspases in apoptosis include cleavage of cytoskeletal proteins, including the lamins, proteins forming the nuclear lamina; cytoplasmic intermediate filaments (vimentin, cytokeratins), and several proteins involved in cytoskeleton regulation (gelsolin, focal adhesion kinase and p21-activated kinase 2). This results in disassembly of cell structures that depend on the cytoskeleton.

2.2 Cell surface "death receptors"

Apoptosis is rapidly induced by activation of "death-inducing signaling complexes" (DISCs) at the plasma membrane[22,25] (Fig. 2). DISCs are formed when extracellular ligands interact with cell surface receptors belonging to the cysteine-rich tumor necrosis factor (TNF) receptor gene superfamily. The death receptors Fas (also called CD95 or Apo1) and TNFR1 (also called p55 or CD120a) are two of the best characterized. Fas and its ligand (FasL) are important for the elimination of activated mature T cells at the end of an immune response and of virus-infected cells, and for the attack of cancer cells by cytotoxic T cells and natural killer cells. Ligation of FasL to Fas causes the intracellular death domains of the receptor to cluster in groups of three. FADD (Fas-associated death domain) acts as the adaptor, binding the clustered death domains through its own death domain. Then, procaspase-8 is recruited to the adaptor, resulting in enzyme oligomerization and activation by self-cleavage. Caspase-8 activates downstream effector caspases, such as caspase-3, committing the cell to apoptosis. Alternatively, caspase-8 can cleave Bid, a Bcl-2 homolog, which then promotes apoptosis at the mitochondria (Fig. 2). Unlike Fas, the TNF pathway appears to require that protein synthesis be blocked for apoptosis to be induced. TNFα is produced by activated macrophages and T cells as a response to infection. Ligand binding causes trimerization of TNFR1 and a consequent association of the receptors' death domains. TRADD (TNFR-associated death domain) serves as the adaptor and binds through its own death domain to the clustered receptor death domains. TRADD is a platform adaptor, capable of recruiting several signaling molecules to activate divergent pathways that signal apoptosis or survival.

2.3 Mitochondria

Mitochondria have emerged as the central processing organelles in the majority of apoptotic pathways. Signals from cell surface death receptors or from damaged sites converge on mitochondria, leading to permeabilization of both mitochondrial membranes, dissipation of the inner membrane transmembrane potential ($\Delta \Psi_m$), and release of apoptosis-related proteins, such as cytochrome c, apoptosis-inducing factor (AIF), SMAC (second mitochondria-derived activator of caspases), and certain procaspases from the intermembrane space. Although these changes in mitochondria are implicated in most apoptotic pathways, their ordering in the pathway remains in dispute.[24,25,30,35–37,51]

One current model suggests that the release of apoptosis-related mitochondrial factors and the collapse of the mitochondrial transmembrane potential results from the opening of a large conductance channel known as the permeability transition pore complex (PTPC), which forms at contact sites of the outer and inner mitochondrial membranes. Models of the PTPC have been proposed in recent reviews.[35,36,52–54] The PTPC is thought to be comprised of at least three transmembrane proteins: (a) the 30 kDa inner membrane adenine
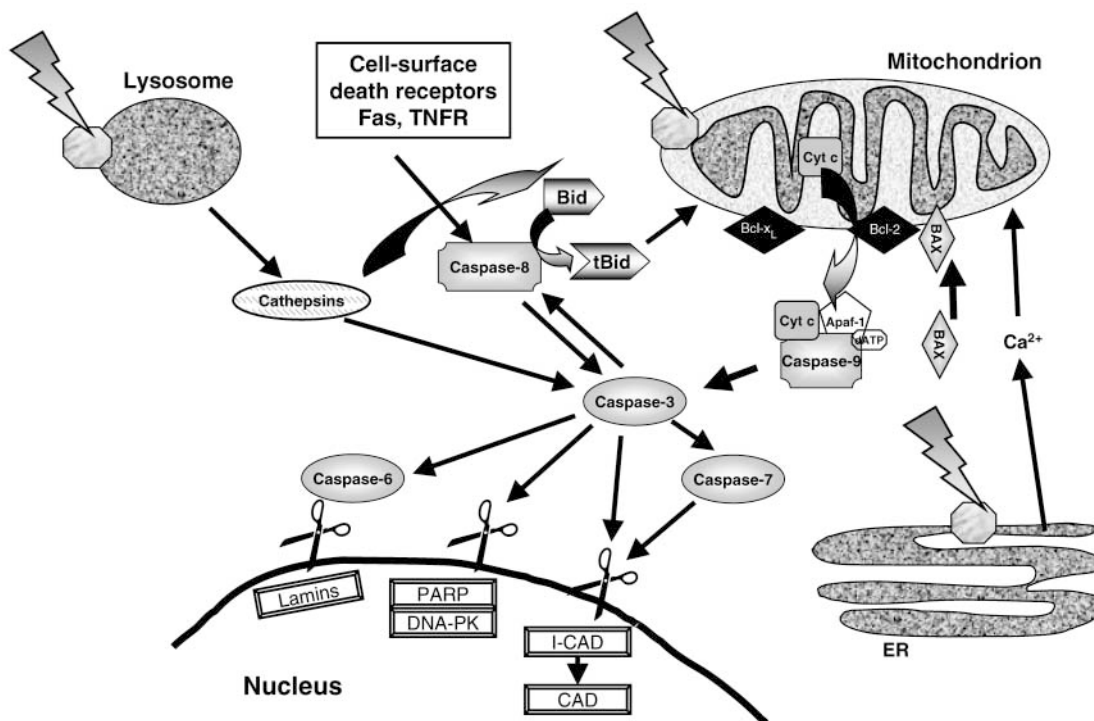
Fig. 2 Some PDT-associated apoptosis pathways involving plasma membrane death receptors, mitochondria, lysosomes and ER, caspases, and Bcl-2 family proteins. Most photosensitizers for PDT bind to mitochondria, lysosomes, and/or other intracellular membranes, including the ER. Photoactivation of a mitochondrion-localized photosensitizer causes release of cytochrome c, which may or may not be accompanied by loss of the mitochondrial membrane potential and opening of the PTPC. The released cytochrome c becomes part of the "apoptosome" complex to generate active caspase-9, which then cleaves and activates caspase-3. Caspase-3 is the major effector caspase and is responsible for cleavage and activation of other caspases, especially caspases-6, -7, and -8. The effector caspases cleave numerous proteins, including nuclear lamins, leading to nuclear breakdown; PARP and DNA-PK, resulting in inhibition of DNA repair; ICAD, releasing active CAD to degrade DNA; and other proteins that affect cell structure and adhesion. In cases where the cell surface death receptors Fas and TNFR participate, binding to their respective ligands leads to activation of caspase-8, which can result in the activation of caspase-3 independent of mitochondrial involvement. Caspase-8 cleaves the Bcl-2 homolog Bid to produce the pro-apoptotic fragment tBid, which can act on mitochondria to cause cytochrome c release. Photoactivation of lysosome-bound photosensitizers can cause the release of cathepsins, which can cleave Bid to promote apoptosis and caspase-3 to inhibit apoptosis. Damage to the ER by PDT causes release of Ca^{2+}, which can promote apoptosis. Apoptosis is controlled by members of the Bcl-2 family that either promote or inhibit the process. In addition to Bid, shown here are the anti-apoptotic proteins Bcl-2 and Bcl-xL, that block PDT-induced apoptosis by inhibiting caspase activation, and the pro-apoptotic Bax, that has been proposed to promote mitochondrial reactions, including cytochrome c release.

nucleotide translocator (ANT); (b) the 32 kDa outer membrane voltage-dependent anion channel (VDAC) or mitochondrial porin; and (c) the 18 kDa outer membrane peripheral benzo-diazepine receptor (PBR). There is also evidence for the association of other proteins with the PTPC: members of the Bax/Bcl-2 family, cyclophilin D, and enzymes of energy metabolism, such as hexokinase and mitochondrial creatine kinase.

The potential across the inner mitochondrial membrane is a consequence of the pumping of protons to the cytoplasmic side of the membrane using the energy of electron transport. Under normal conditions, the passage of molecules and ions across the inner mitochondrial membrane is tightly regulated. In response to changes in redox state, pH, and local concentrations of ATP/ADP and ions, such as Ca^{2+} and Mg^{2+}, the PTPC is thought to regulate membrane permeabilization. Opening of a non-selective channel in the inner membrane would allow ion equilibration between the matrix and intermembrane space, thus dissipating the H^+ gradient across the inner membrane and uncoupling the respiratory chain. Another possible consequence of PTPC opening is mitochondrial volume dysregulation, as normally excluded ions and water expand the matrix space. Since the folded cristae of the inner mitochondrial membrane have a larger surface area than the outer membrane, matrix space expansion then causes outer membrane rupture and consequent release of caspase-activating proteins located within the intermembrane space into the cytosol.[35,53]

Therefore, cytochrome c may be released from mitochondria through opening of the PTPC and bursting of the outer membrane or through the creation of more selective channels in the outer membrane or by another mechanism. Once in the cytosol, cytochrome c binds to cytoplasmic apoptosis-activating factor-1 (APAF-1) to form the apoptosome complex and initiate activation of the cascade of caspases that carry out the final stages of apoptosis.[37]

2.4 Bcl-2 family proteins

Bcl-2 family members regulate apoptosis induced by many apoptotic stimuli, primarily at the level of the mitochondria.[55] Anti-apoptotic members of this large protein family include Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1 and Boo; whereas pro-apoptotic members include Bax, Bad, Bok, Bcl-xS, Bak, Bid, Bik, Bim, Krk, and Mtd.[56] Apoptotic regulation by Bcl-2 family proteins has been extensively reviewed.[32,33,52,55–57] In the absence of a death signal, pro-apoptotic Bcl-2 family members are often sequestered by cytoskeletal elements or cytoplasmic proteins (*e.g.*, sequestration of phosphorylated Bad by 14–3–3 proteins) or are only loosely associated with membranes. In contrast, anti-apoptotic Bcl-2 family members are often integral membrane proteins found in the mitochondrial membrane, the nuclear envelope, and the endoplasmic reticulum.[52]

The activity of Bcl-2-related proteins is regulated through several mechanisms, including their levels of expression, sequestration, and post-translational modifications, such as phosphorylation, cleavage, and translocation.[52] Cleavage of Bcl-2 not only inactivates the protein but also produces a

fragment that promotes apoptosis.[58,59] Cytosolic Bid (p22) is cleaved by caspase-8 to generate truncated Bid (tBid), which then translocates to the mitochondria to integrate into the membrane and assist in the release of cytochrome c.[52] A recent report demonstrated that proteases released from lysosomes, cathepsins, can also cleave Bid to generate a mitochondrion-targeted pro-apoptotic protein.[60] In response to apoptotic stimuli, several pro-apoptotic proteins are translocated to the mitochondria, where they can interact with membrane-bound anti-apoptotic proteins, thereby inhibiting the survival functions of the latter.[52] Bcl-2, Bcl-xL, and Bax can form ion channels in artificial membranes, suggesting regulation of apoptosis *via* the formation of pores.[56,61] Other hypotheses for the inhibition of apoptosis by Bcl-2 include participation in an anti-oxidant pathway[62] and blockage of the release of cytochrome c.[52,55,63,64] Bcl-2 homologs often form homo- and hetero-dimers with each other and with other non-homologous proteins that are presumed to control the availability of their active forms.[52,57] The ratio of pro- to anti-apoptotic members has been suggested to regulate cell life or death.[32] Some examples of the interaction of these proteins at the mitochondrion to control apoptosis are shown in Fig. 2.

3   Measuring apoptosis

Quantification of the precise extent of apoptosis *vs.* necrosis and total cell death in cultured cells is difficult and several issues need to be considered. Apoptosis is a biochemical process that passes through several stages each with its own hallmarks and timing. The kinetics of the process depend upon a variety of factors, including the dose of the inducing agent and the ability of different cell types to carry out steps in the process. Thus, the percentage of cells in apoptosis will vary with time after initiation. Cells treated with doses higher than those needed to cause apoptosis may die without exhibiting the usual characteristics of apoptosis, and are generally regarded as undergoing necrosis. As noted above, during the later stages of apoptosis, cells disintegrate into membrane-enclosed apoptotic bodies that *in vivo* are phagocytosed by macrophages or adjacent tumor cells and further catabolized. Since phagocytic cells are not found in tissue cultures, apoptotic bodies may complete the breakdown of their cellular constituents or may simply become detached from the culture substrate. In addition, late apoptotic cells or bodies can eventually lose the ability to maintain their membrane pumps and as a result may be unable to exclude vital dyes, such as trypan blue. Such apoptotic cells or bodies would then appear as necrotic cells. Therefore, one cannot conclude that a particular cell type or treatment does not produce an apoptotic response without studying a range of doses of the initiating agent and assaying for apoptotic markers over a wide range of times.

There are numerous assays for apoptosis. The two most common endpoints of apoptosis are morphological changes (cell shrinkage, condensation of nuclear chromatin, formation of apoptotic bodies) and DNA fragmentation [to large 300 kbp and 50 kbp fragments and then to oligonucleosome-sized fragments (multiples of ~200 bp that appear as a "ladder" of DNA bands upon agarose gel electrophoresis)]. Although observation of these endpoints is an indicator of apoptosis, quantification of the percentage of cells in a population that are in apoptosis is difficult or impossible without more sophisticated methods. For this purpose, many investigators use the TUNEL assay (during which fluorescently tagged or biotinylated nucleotides are added to the ends of DNA fragments within fixed cells). The cells are then stained in their DNA with propidium iodide and can be viewed on a microscope stage, or they can be quantified by flow cytometry to indicate the presence of viable, apoptotic, necrotic, and late apoptotic cells. Apoptotic cells can also be detected with the protein Annexin V,

which binds in a highly selective manner to phosphatidyl serine; this phospholipid flips from the inner to the outer leaflet of the plasma membrane during apoptosis. This assay is also adaptable to quantification by flow cytometry or to imaging.

Since many of the enzymatic steps in apoptosis are known, it is often desirable to follow the process by monitoring those steps. Antibodies are available commercially for all of the common caspases, so that procaspase levels and their proteolytic processing can be monitored by western blot analysis. In addition, model peptide substrates can also be purchased for many of the caspases in fluorogenic or chromogenic forms, permitting assay of cell extracts for the presence and level of individual active caspases. Inhibitors based on active site peptide motifs can be used to test the participation of suspected caspases. However, because caspases are not completely specific for the amino acid motifs, the model substrates and inhibitors cannot provide absolute identification of the involved caspase. Caspase action can also be monitored by assessing the cleavage of intracellular protein substrates; *e.g.*, cleavage of PARP or lamin B is a common indicator of the action of caspase-3 or -7 in apoptosis. A listing of commercially available kits for these and other assays of apoptosis, including measurement of caspases, substrates, and inhibitors, can be found at the following web site: www.the-scientist.org.

With any of these or other assays, it is advisable to study sufficient doses and post-treatment times so that the progress of cell death may be observed. In addition, it is necessary to use more than one assay to confirm results. Finally, if some or all of the cells do not undergo apoptosis, one cannot conclude that the cells remain alive, without conducting an assay for total cell death. The best assay for this purpose is a clonogenic assay, which measures the ability of each cell in a population to grow, replicate, and form a colony. Unlike assays for apoptosis or for viability, such as dye exclusion or tetrazolium reduction, clonogenic assays register all cell death events integrated over time.[65]

4   Cell death in the response to PDT: apoptosis *vs.* necrosis

Since the first report of apoptosis in PDT-treated cells,[66] apoptosis has been found to be a prominent form of cell death in response to PDT for many cells in culture, as judged by assays measuring either the fragmentation of DNA or the condensation of chromatin. There are by now numerous examples in which the ability of PDT-exposed cells to initiate the apoptotic process differs depending on the cell line, the photosensitizer and its subcellular location, the overall dose, and other conditions. In an early study, He *et al.*[67] exposed three carcinoma cell lines to equitoxic doses of PDT with Photofrin and found apoptosis in two of the cell lines but not in the third. Noodt *et al.*[68] compared responses of V79 Chinese hamster fibroblasts and WiDr human colon adenocarcinoma cells to equitoxic doses of PDT with ALA/PPIX, *i.e.*, PPIX synthesized in the cells from exogenously supplied ALA. Using the TUNEL assay and flow cytometry to quantify the fraction of cells with fragmented DNA, they showed evidence for a dose- and time-dependent increase in the apoptotic fraction in PDT-treated V79 cells, with a maximum of 75–85% of the cells in apoptosis by 3–4 hours after a dose that resulted in 85% overall cell death. In contrast, under comparable conditions resulting in similar levels of overall cell death as judged by clonogenic assay, no evidence for apoptosis was found in WiDr cells. This study underscores the importance of verifying assays for apoptosis using an assay for total cell death.

In a study of the ability of PDT sensitized by Pc 4 to purge viruses from red blood cell concentrates, Ben-Hur *et al.*[69] found that T lymphocyte cell lines latently infected with HIV were more easily killed and more readily induced to undergo apop-

tosis than was a cell line with actively replicating virus. The authors attributed the enhanced elimination of HIV in latently infected cells to apoptosis. Lymphoid cell lines generally enter apoptosis more rapidly than those of non-lymphoid origin (*e.g.*,[66,70,71]). Furthermore, the rapid induction of apoptosis by PDT in murine lymphoma L5178Y-R cells appears to occur in all stages of the cell cycle, as revealed by flow cytometric analysis of TUNEL positivity *vs.* cell cycle stage,[72,73] and murine leukemia L1210 cells arrested in the cell cycle by crypto-phycin 52 were still able to undergo apoptosis within 60 min of exposure to CPO-sensitized PDT.[74] Murine T cells, activated by a 48-hour treatment with anti-CD3 monoclonal antibody, were slightly more sensitive to the induction of apoptotic DNA fragmentation by Verteporfin-PDT than were resting T lymphocytes, which could have resulted from increased uptake of the photosensitizer, a higher level of Bcl-2, or other noted changes, including increased IL-2 and transferrin receptors.[75] Moreover, murine thymocytes were modestly more sensitive to PDT than mature splenic T cells.[76]

The subcellular location of a photosensitizer has a strong influence on whether and to what extent cells undergo apoptosis in response to photoactivation (Fig. 2). Dellinger[77] found apoptosis in CV-1 cells if they were photoirradiated 24 hours after introduction of Photofrin, when the photosensitizer was internalized, whereas after only 1 hour, when Photofrin was primarily in the plasma membrane, necrosis was the predominant form of cell death. The photosensitizer methylene blue derivative (MBD) localizes in mitochondria of V79 cells, as revealed by fluorescence microscopy and by the photodynamic loss of cytochrome c oxidase activity when enzymes of other organelles remained intact.[78] When MBD was present at concentrations above 0.05 µg ml^{-1}, photoactivation of the cells caused early (within 3 hours) apoptosis as the dominant mechanism of cell death. At 0.05 µg ml^{-1}, the process was much slower, and apoptotic cells appeared only after 1 day. When PDT with the low concentration of MBD was combined with the inhibitor of glycolysis, 2-deoxyglucose, apoptotic cells appeared within 6 hours; in contrast, combination with the uncoupler of oxidative phosphorylation, CCCP, acted like PDT alone. The authors interpreted these data as indicating mitochondrial damage from MBD-PDT; at the higher concentrations, they suggested that damage to an apoptosis inhibitor, such as Bcl-2, might lead to release of apoptogenic factors, whereas at the low concentration of MBD, the induction of apoptosis may be indirect. Molecular evidence for the photochemical damage to Bcl-2 and for indirect pathways after low PDT doses has now been obtained (see Section 5.5).

With Rose Bengal, a photosensitizer that is distributed in cellular membranes, Kochevar et al.[79] observed extensive apoptosis in HL-60 cells, if the dye was photoactivated by visible light, producing predominately singlet oxygen; in contrast, if the dye was activated by UVA radiation, producing Rose Bengal-derived radicals in addition to singlet oxygen, there was no further increase in the yield of apoptosis, in spite of a much greater yield of lipid peroxidation products. These results demonstrated that different reactive species produced at the same sites of photosensitizer localization can have markedly different cellular effects. Lin et al.[80] studied the effects of confining Rose Bengal-PDT to the plasma membrane by irradiating bovine aortic endothelial cells attached to a glass substrate with evanescent wave visible radiation. In contrast to ordinary trans-illumination of the cells, evanescent waves penetrate only about 100 nm into the cell, limiting PDT to the plasma membrane and a very thin layer of cytoplasm. Under these conditions, Annexin V staining and nuclear morphology indicated that apoptosis was efficiently induced, demonstrating that singlet oxygen produced at the plasma membrane can induce apoptosis.

Kessel and Luo[81] studied a series of photosensitizers in L1210 leukemia and other cells and demonstrated that photo-sensitizers that bind to mitochondria induce apoptosis upon photoirradiation, whereas those that bind to the plasma membrane or lysosomes, but not to mitochondria, kill cells less efficiently and by a non-apoptotic mechanism.[81–83] The photosensitizer LuTex has been shown to bind to lysosomes of EMT6 cells *in vitro* and to produce apoptosis in EMT6 tumors *in vivo*, indicating that lysosomally bound photosensitizers can induce apoptosis upon photoactivation.[84] To further evaluate the role of the subcellular localization of photosensitizers in the killing of V79 cells by PDT, Noodt et al.[85] compared two lipophilic porphyrins (3THPP and Photofrin) that localize to intracellular membranes, including mitochondria, and two relatively hydrophilic sulfonated porphines (TPPS$_{2a}$ and TPPS$_4$), that are taken up into lysosomes by endocytosis. PDT with either of the membrane-localizing photosensitizers resulted in increasing numbers of cells becoming apoptotic (TUNEL positive) during the first 12 hours, but apoptotic bodies were not observed. In contrast, after photoactivation of the lysosome-localized photosensitizers, apoptotic cells were not detected until after 12 hours but extensive fragmentation of the cells into apoptotic bodies was found. These data provide evidence for at least two distinct pathways by which PDT can induce apoptosis.

In some cases, the initial sites of photosensitizer binding may not be the most sensitive targets. Kessel and Poretz[86] have reported that chlorin e6 triacetoxymethyl ester (CAME) accumulated within 10 minutes in acidic endosomes, but mitochondria were the preferential sites of photodamage at the early time. After hydrolysis and redistribution of CAME throughout the cells, which occurred by 18 hours, photodamage was found in mitochondria and lysosomes. The two conditions produced similar levels of apoptosis and loss of viability, although a higher concentration of CAME led to less apoptosis but more cell death, presumably by necrosis. This is in agreement with earlier evidence for a transition from apoptosis to necrosis with higher doses of PDT sensitized by any one of several photosensitizers, *e.g.*, hypericin, dimethyl tetrahydroxy-helianthrone (DTHe), or phthalocyanines.[87–90] Lavie et al.[90] also found that the human leukemia cell line Hut-78 was resistant to DTHe-sensitized PDT in terms of morphological apoptosis, a phenomenon that may have been due to crosslinking of nuclear lamin.

Although PDT can produce apoptosis or necrosis, or evoke a combination of the two outcomes, in many cases PDT is highly efficient in inducing apoptosis.[39] Thus, lower doses than those needed to produce necrosis may be very effective in eliciting cell killing. Furthermore, the efficient induction of apoptosis by PDT implies that PDT may be able to bypass mechanisms that make cells resistant to apoptosis in response to chemotherapeutic drugs and ionizing radiation.

A longstanding principle in studies of cell killing by many different types of toxic agents is that cell death occurs in those cells that are directly damaged by the treatment, whereas a neighboring cell that is not hit will remain alive. However, recent evidence has challenged this concept. A series of papers by Dahle et al.[91–94] demonstrate that MDCK II and other cells treated with a photosensitizer and light die in clusters rather than as random individuals, producing an overabundance of colonies where either all or none of the cells are dead. This effect is known as the "bystander effect". The experimental distribution of dead cells was best described by a mathematical model in which inactivated cells inactivated neighboring cells which could then inactivate their neighboring cells, *i.e.*, a "propagated inactivation" model. When Photofrin served as photosensitizer in these studies, very few of the cells died through apoptosis. In contrast, for PDT with photosensitizers other than Photofrin, apoptosis was the main mechanism of cell death. However, the bystander effect was greater when the cells were dying by necrosis, possibly because necrotic cells had a better chance of leaking toxic substances through their damaged membranes. When MDCK II cells were treated with

TPPS$_4$ and light to various levels of overall cell killing, the fraction of cells dying by apoptosis was greater when the overall level of cell killing was low, as found for PDT with other photosensitizers (see above), and confluent cultures, with greater possibility for cell-to-cell interactions, were more sensitive than sub-confluent ones. In a comparison of PDT-induced killing of MDCK II cells vs. WiDr human colon adenocarcinoma cells, with the lipophilic photosensitizer 3THPP, the degree of the bystander effect was greater when the normal cells died by necrosis than by apoptosis and greater for normal cells than for the cancer cells. This potentially important component of PDT response remains unexplained at the molecular level. One possible mechanism for the bystander effect with PDT is the interaction of Fas ligand produced on one cell with Fas on the plasma membrane of an adjacent cell; such a mechanism is suggested by experiments described below (see Sections 6.3 and 7).

5   The role of mitochondria *vs.* other cellular targets

5.1   Mitochondrial binding sites for photosensitizers

Numerous reports have implicated mitochondria as important targets of PDT. Photosensitizers that localize to mitochondria are reported to be more efficient in killing cells than those that localize at other cellular sites[81,82] (Fig. 2). Localization of drugs in mitochondria can be driven by the electrochemical potential gradient across the inner mitochondrial membrane. Examples of photosensitizers in this class are various cationic lipophilic compounds, such as the krytocyanine dye EDKC and rhodamine-123[95] as well as MBD.[78,96] Photosensitizers that are negatively charged, such as Photofrin, or neutral, such as Pc 4, have also been found to accumulate in mitochondria,[97,98] and are thought to bind to various mitochondrial constituents. For instance, the inner membrane of mitochondria is rich in cardiolipins. Wilson et al.[99] demonstrated the ability of a cardiolipin probe, 10N-nonyl acridine orange, to competitively inhibit the uptake of Photofrin into mitochondria, indicating that some photosensitizers might bind to cardiolipins of the inner mitochondrial membrane.

Several investigations have examined components of the permeability transition pore complex (PTPC) as potential targets for PDT. PK11195, a selective high-affinity isoquinoline carboxamide ligand for the PBR, has been used to test the role of the PBR in PDT-induced apoptosis. An extensive series of studies by Verma and Snyder[100–102] showed that porphyrins are endogenous ligands for the PBR; most prominent among these are dicarboxylic porphyrins, such as PPIX. Further, a porphyrin's binding affinity to the PBR correlated well with that porphyrin's ability to photoinactivate V79 cells, suggesting the PBR as an important molecular target for porphyrin-based photocytotoxicity. Morgan et al.[103] found that a pyropheophorbide, also localized to mitochondria, can compete with PK11195 for binding to the PBR. In contrast, Kessel et al.[104] demonstrated that PPIII and PPXIII, positional isomers of PPIX, had similar uptake and localization properties as PPIX and the same ability to photosensitize L1210 leukemia cells, as measured by a clonogenic assay, but significantly lower binding affinity to the PBR. Thus, a relationship between PBR affinity and photosensitizing efficacy may not extend beyond porphyrins with structures similar to PPIX. Ratcliffe et al.[105] demonstrated that PK11195 inhibited PDT-induced apoptosis in rat pancreatoma cells treated with ALA to produce PPIX. For the phthalocyanine Pc 4, which localizes preferentially to mitochondria,[97,98] competition with PK11195 for binding to the PBR demonstrated both mid- and low-affinity binding sites to the PBR. However, the observed inhibition of Pc 4-PDT-induced apoptosis of CHO cells by PK11195, which occurred as well in CHO cells overexpressing human Bcl-2, was likely due

to inhibition of a downstream step in apoptosis, rather than to blockade of Pc 4 binding to the PBR.[106]

Other components of the PTPC have also been considered as targets of photosensitization. Salet et al.[107] observed strong non-competitive inhibition of the ADP/ATP exchange carrier (ANT) and inhibition of PTPC opening in isolated rat liver mitochondria when challenged with hematoporphyrin-sensitized photodynamic action. Recently, Belzacq et al.[108] loaded liposomes with semi-purified PTPC or with the purified ANT and showed that Verteporfin-PDT caused permeablization of the proteo-liposomes, but not of protein-free liposomes. The ANT ligands, ADP and ATP, inhibited the response. PDT using hypericin was reported to cause the release of mitochondrion-bound hexokinase into the cytosol and to inhibit hexokinase activity.[109]

Inhibition of respiration and oxidative phosphorylation is also common with PDT induced by mitochondrion-localized photosensitizers.[78,110] Photoactivated Photofrin inhibited electron transport components, including succinate dehydrogenase and cytochome c oxidase, and also disrupted the mitochondrial electrochemical gradient.[111–114] Morgan et al.[115] compared PDT responses in a human ovarian carcinoma cell line 2008 and its mitochondrial DNA-deficient derivative ET3, which lacks several respiratory protein subunits. When photosensitizers that did not primarily localize in mitochondria (Nile Blue A or Photofrin after a short incubation) were studied, both cell lines responded similarly. For Photofrin after a long incubation, when it is found in various intracellular membranes including mitochondria, again the responses of the two cell lines were similar. However, with Victoria Blue BO (mitochondrial localization), ET3 cells were less sensitive than 2008 cells. The authors suggest that the study of cells deficient in mitochondrial DNA shows that inhibition of respiratory enzymes is a target of PDT with VBBO but much less so with the other photosensitizers. In response to PDT with various mitochondrion-bound photosensitizers, cytochrome c is released from the intermembrane space into the cytosol where it very likely forms a complex with cytoplasmic apoptosis-activating factor-1 (APAF-1) to initiate the activation of the cascade of caspases that carry out the final stages of apoptosis.[110,116–118] In a study of the inhibition of respiration in L5178Y-R murine lymphoma cells that were treated with Pc 4-PDT then permeabilized, Varnes et al.[110] were able to restore much of the loss in respiration with exogenous cytochrome c, suggesting that the inhibition of respiration by Pc 4-PDT primarily results from the release of cytochrome c, rather than from damage to the major enzyme complexes of the electron transport chain.

The importance of mitochondria as targets for the initiation of apoptosis by PDT has been convincingly demonstrated by Kessel and Luo.[81,82,118] These investigators tested a series of photosensitizers for their preferential intracellular binding sites as well as their ability to photosensitize cells to apoptosis and overall cell death. They found that those photosensitizers that bound to mitochondria induced apoptosis upon photo-irradiation, whereas those that bound to the plasma membrane or to lysosomes, but not to mitochondria, killed cells less efficiently and by a non-apoptotic mechanism. In a subsequent study, Kessel and Luo[119] showed that lysosomally bound photosensitizers cause the release of cathepsins from the damaged organelles; once in the cytosol, these proteases cleaved procaspase-3, thus blunting the apoptosis pathway. Since lysosomal proteases can also cleave Bid to generate a mitochondrion-targeted pro-apoptotic protein,[60] PDT damage to lysosomes can induce apoptosis as well as inhibit it.

Kessel et al.[120] have recently reported the ability of the bile acid ursodeoxycholic acid (UDCA) to greatly potentiate the apoptotic response to PDT with CPO and SnET2, two photosensitizers that bind to lysosomes as well as other cytoplasmic membranes. UDCA binds to mitochondrial membranes and

can protect cells from the apoptotic effects of a variety of cytotoxic agents. With PDT, UDCA potentiated the loss of mitochondrial membrane potential, release of cytochrome c into the cytosol, activation of caspase-3, and apoptotic cell death in murine leukemia L1210 or hepatoma 1c1c7 cells. Since UDCA did not enhance the intracellular accumulation of the photosensitizers, Kessel et al.[120] proposed that UDCA sensitized mitochondrial membranes to photodamage. There are important clinical implications of this discovery, because UDCA is currently used to treat biliary cirrhosis and other conditions in gastroenterology. If the interaction observed in cell culture occurs in PDT-treated tissue in vivo, UDCA may offer a means to promote the efficacy of PDT with minimal adverse effects.

A novel approach to enhancing the endogenous production of PPIX in mitochondria has been devised by Gagnebin et al.[121] With the ultimate goal of improving PDT targeting to tumor cells through gene therapy, these investigators generated an adenovirus vector that carries a mutated gene for ALA synthase, the rate-limiting enzyme in heme biosynthesis. ALA synthase is normally subject to feedback inhibition by heme through heme regulatory motifs (HRMs) that bind heme and prevent import of the proenzyme into mitochondria. The HRMs were mutated, permitting the virally expressed proenzyme to enter mitochondria and be processed to fully active enzyme independent of the heme concentration. Following infection of H1299 lung carcinoma or Saos-2 osteocarcinoma cells with the viral construct, PPIX production was increased and the cells became photosensitive with respect to the induction of apoptosis, as revealed by flow cytometric analysis of particles with less than the G1 content of DNA. As with all gene therapy approaches, the utility of this type of vector will depend upon the ability to target it selectively to tumors in patients.

5.2 Mitochondrial DNA

Mitochondria contain some 1000 proteins that are encoded in the nuclear DNA and are imported after synthesis on cytoplasmic ribosomes; only 13 proteins (and multiple RNAs) are encoded by mitochondrial DNA.[122,123] The proteins that are specified by mitochondrial DNA are specific peptide components of respiratory complexes I, III, IV, and the ATP synthase. In the absence of those peptides, respiration is slowed. However, mitochondrial DNA does not appear to be necessary for efficient induction of apoptosis by PDT with a mitochondrion-localizing porphycene (CPO), as L1210 murine leukemia cells depleted of their mitochondrial DNA by prolonged exposure to ethidium bromide were more sensitive to apoptosis induction and loss of clonogenicity with CPO and light.[124] With a lysosome-targeted photosensitizer, a chlorin e6 analog (LCP), L1210 cells with or without mitochondrial DNA showed equal sensitivity to PDT. The data suggest a protective role for mitochondrial DNA or its gene product(s) against the lethal effects of PDT, but only with photodamage directed to mitochondria. A different conclusion was drawn by Singh et al.[123] based on their studies of HeLa cells (Rho$^+$) and a mitochondrial DNA-deficient (Rho^o) derivative line. Using mesoporphyrin IX as photosensitizer, they reported a >2-log loss of clonogenicity for the PDT-treated Rho^o cells, as compared to no killing of the Rho$^+$ cells, but only in a single condition in the presence of 80 μg ml^{-1} suramin, but not with less suramin. The Rho^o cells were also more sensitive to adriamycin than were the Rho$^+$ cells. Although the report of Singh et al.[123] may be valid for adriamycin, which was the main subject of the study and which was active in the absence of suramin, the conclusion regarding PDT is untenable, because mesoporphyrin IX uptake into the two cell lines was not quantified, the subcellular localization(s) of the photosensitizer was not identified, and appropriate conditions for killing of both lines by PDT, with-

out the aid of suramin, were not established. Finally, the extent of apoptosis was low at 24 and 48 hours after introduction of adriamycin, but no data on apoptosis were reported for PDT. In another study, human B lymphoma cells (Namalwa) that were depleted of mitochondrial DNA were resistant to PDT with a boronated porphyrin.[125] Similarly, human ovarian carcinoma cells that were depleted of their mitochondrial DNA were less susceptible to overall cell killing by PDT with the mitochondrion-localizing photosensitizer Victoria Blue BO.[115] Thus, there is still insufficient information to predict the effect of mitochondrial DNA depletion on PDT response, which may vary with cell type and photosensitizer, and especially with the subcellular localization of the photosensitizer.

5.3 Chronology of the mitochondrial events in response to PDT

Often the first event observed in PDT-induced apoptosis is a dissipation of the mitochondrial transmembrane potential ($\Delta\Psi_m$). Kessel et al.[118] reported immediate loss of $\Delta\Psi_m$ after PDT with porphycenes in leukemia P388 cells. Similarly, PDT with hypericin or hypocrellin, also mitochondrion-localized photosensitizers, induced rapid collapse of $\Delta\Psi_m$.[126] The mechanisms responsible for the loss of $\Delta\Psi_m$ remain unclear, as inhibitors of PTPC opening have provided contradictory results. The decrease in membrane potential after hypericin-PDT was not prevented by specific PTPC inhibitors, such as cyclosporin A and bongkrekic acid.[127] In contrast, Lam et al.[98] showed that cyclosporin A produced a partial inhibition of PTPC opening in human epidermoid carcinoma A431 cells exposed to Pc 4-PDT, suggestive of a transient inhibitory effect. With a combination of cyclosporin A and trifluoperazine, PTPC opening and apoptosis were strongly inhibited.[98] In another study, dissipation of the membrane potential in isolated mitochondria exposed to PDT with Victoria Blue dyes was cyclosporin A-insensitive and was proposed to result from direct effects of the dye on inner membrane permeability to protons rather than on the mitochondrial permeability transition.[128]

Contradictory results have also been obtained regarding the timing of the release of cytochrome c from mitochondria into the cytosol relative to the dissipation of the transmembrane potential. Kessel et al.[118] demonstrated that cytochrome c was released simultaneously with the loss of $\Delta\Psi_m$, both events occurring immediately after PDT with porphycenes in leukemia P388 cells. In contrast, photodynamic treatment of HeLa cells with Verteporfin resulted in cytochrome c translocation from mitochondria into the cytosol and accessibility of the mitochondrial 7A6 epitope to the APO2.7 monoclonal antibody without any changes in transmembrane potential.[129] Release of cytochrome c was shown to occur immediately upon Verteporfin-PDT.[116] Interestingly, Chiu and Oleinick[130] found the relationship between these critical events for Pc 4-PDT to be dose-dependent in mouse lymphoma L5178Y-R cells. Although high irradiation fluences resulted in a rapid dissipation of membrane potential concurrent with cytochrome c release, lower fluences produced cytochrome c release without loss of transmembrane potential. Furthermore, using an immunohistochemical method which does not require cell fractionation to monitor cytochrome c release, Chiu et al.[131] demonstrated a dose-dependent and nearly complete release of cytochrome c about 15 minutes post Pc 4-PDT. In contrast, in A431 cells, Pc 4-PDT-induced efflux of cytochrome c from mitochondria followed the loss of $\Delta\Psi_m$.[98]

In the absence of mitochondrial depolarization, cytochrome c release appears to be independent of PTPC opening. However, transient opening of the PTPC as a result of mild (non-PDT) oxidative stress has been observed.[54] A transient opening of the PTPC may cause limited swelling of the mitochondria, sufficient to disrupt the outer membrane and release cytochrome c while maintaining the inner mitochondrial membrane

potential.[54] Cytochrome c is only one of many proteins of the mitochondrial intermembrane space that are released into the cytosol during apoptosis.[36,37,42] A recent study[132] demonstrated the release of apoptosis-inducing factor (AIF) along with cytochrome c in smooth muscle cells exposed to Verteporfin-PDT.

5.4 Caspase activation in response to PDT

As noted above, photosensitizers that localize to the mitochondria are most efficient for inducing apoptosis following PDT. Cytochrome c released from the mitochondrial intermembrane space probably binds with cytoplasmic Apaf-1 and procaspase-9, leading to auto-cleavage and self-activation of this caspase. Caspase-9, in turn, activates caspase-3, which initiates the activation of the cascade of caspases. Activation of caspase-9 was reported following PDT with Verteporfin or Pc 4.[133,134] Caspase-3, considered the major executioner caspase, is activated in most cases of PDT-induced apoptosis with a number of different photosensitizers.[117,135–140] Strikingly, MCF-7 human breast cancer cells that lack endogenous procaspase-3 showed no significant difference in the degree of cell death induced by Pc 4-PDT, as measured by clonogenic assay, in comparison to MCF-7 cells stably transfected with procaspase-3.[134] Cytochrome c release was similar in the two cell lines. However, in the absence of caspase-3, MCF-7 cells were not able to carry out the early apoptotic program involving caspases-9 and -3; but, they underwent delayed apoptosis, which suggests alternate mechanisms following release of cytochrome c or other apoptogenic mitochondrial factors. Activation of other caspases, such as 2, 6, 7, and 8, was also shown to occur downstream of cytochrome c release in response to Verteporfin photosensitization of HeLa cells or human endothelial cells.[116,141] Caspase-8 was activated in response to Rose Bengal-sensitized PDT as well.[142] However, HeLa cells overexpressing the serpin-like protease inhibitor CrmA, a selective inhibitor of caspases-1 and -8, still underwent significant apoptosis following PDT with hypericin, but not in response to TNFα.[88,137] Ahmad et al.[143] recently reported evidence for the increased expression and secretion of Fas protein into the medium, engagement of Fas Ligand and FADD, and cleavage of procaspase-8, providing evidence that this pathway may be linked to apoptosis in A431 human epidermoid carcinoma cells exposed to Pc 4-PDT. Inhibition of the pathway by recombinant human Fas:Fc fusion protein, which blocks Fas-dependent steps, or by z-VAD-fmk, a general caspase inhibitor, partially prevented the loss of cell viability, as monitored by the MTT assay. Thus, current evidence suggests that the most common pathway for apoptosis in PDT-treated cells is that involving cytochrome c, caspase-9, and caspase-3; however, other pathways, especially that through caspase-8, may also contribute, particularly in situations where the dominant pathway is suppressed.

5.5 Role of Bcl-2 family proteins in PDT response

The Bcl-2 family of proteins exercises the most proximal and important controls over apoptosis. Some, like Bcl-2 and Bcl-xL, are anti-apoptotic, while others, like Bax and Bad, promote apoptosis. The focus of action of Bcl-2 family proteins is the mitochondrion, and depending upon the initiating agent and the cell type, different members are involved in the regulatory process. A role for Bcl-2 in suppressing PDT-induced apoptosis was first shown in 1996 by He et al.[144] In that study, CHO cells overexpressing human Bcl-2 were found to be more resistant to apoptosis and to loss of clonogenicity caused by Pc 4-sensitized PDT than were the companion cells expressing only the endogenous hamster Bcl-2. Granville et al.[145] subsequently confirmed the ability of overexpressed Bcl-2 to suppress PDT-induced apoptosis in HL60 cells treated with BPD-MA (Verteporfin) and light. This group also found that overexpressed Bcl-2 or Bcl-xL did not prevent the release of cyto-

chrome c from mitochondria but instead blocked the activation of several caspases.[129,146] In contrast to results from studies in various cell-free systems,[53] cytochrome c release in response to PDT, at least with photosensitizers that preferentially target mitochondria, appears to be unaffected by the presence of Bcl-2 or Bcl-xL.

The importance of Bcl-2 in regulating PDT-induced apoptosis has been tested by transfecting an antisense Bcl-2 sequence in a retrovirus vector into human gastric adenocarcinoma MGC803 cells followed by treatment with the photosensitizer 2-BA-2-DMHA and light.[147] The antisense-treated cells retained about 20% of the level of Bcl-2 protein found in the control cell lines and were more sensitive to PDT, as determined by MTT assay and by the generation of oligonucleosomal DNA fragments, than were untransfected cells or those transfected with the neo gene. Antisense Bcl-2 also sensitized A431 cells to Pc 4-PDT.[148]

It has been proposed that a major influence over a cell's propensity to undergo apoptosis in response to a toxic stimulus is the ratio of Bax to Bcl-2;[32] thus, elevated Bax promotes apoptosis even in the presence of Bcl-2. Kim et al.[89] overexpressed Bcl-2 in human breast epithelial MCF-10A cells and found that overexpression was accompanied by the upregulation of Bax. A similar observation was made by Srivastava et al.[148] in A431 cells exposed to Pc 4-PDT. When MCF-10A or A431 cells were exposed to AlPc- or Pc 4-sensitized PDT, respectively, in contrast to expectations, the cells overexpressing Bcl-2 were more sensitive to the induction of apoptosis than were the parental cells.[89,148] However, the CHO cells overexpressing human Bcl-2 that were used in the study by He et al.[144] were found to contain the same level of Bax as the cells from which they were derived,[149] and there are other instances when Bax upregulation did not follow upon Bcl-2 overexpression.[150] Thus, the conditions that result in the apparent control of Bax levels by Bcl-2 remain to be defined. Interestingly, in the study by Kim et al.,[89] the level of Bcl-2 detected on western blots of the PDT-treated cells was much reduced in comparison to the untreated cells; this effect was found for both cell lines. The authors interpreted their results in terms of possible photodamage to Bcl-2, thereby increasing the ratio of the pro-apoptotic Bax to anti-apoptotic Bcl-2. In a recent study of Lewis lung carcinoma (LLC) cells, Usuda et al.[151] found that stable overexpression of IL-6 conferred increased sensitivity to NPe6-mediated PDT, as measured by the MTT assay. Furthermore, apoptosis was observed only in those cells expressing IL-6, but not in various control cell lines. Interestingly, the IL-6-expressing cells contained elevated amounts of both Bax and cytochrome c and similar amounts of Bcl-2, as compared to untransfected LLC cells; at 6 and 12 hours post-PDT, Bcl-2 was lost and the level of cytochrome c was markedly reduced in both cell lines, whereas Bax expression was further elevated only in IL-6-overexpressing cells, implicating Bax in the increased sensitivity to PDT. Further study is needed to determine if the ability of Bcl-2 overexpression to produce PDT resistance vs. sensitization may be a function of the degree of Bax upregulation and if photodamaged Bcl-2 may promote apoptosis by more efficiently recruiting Bax to the mitochondria.

Xue et al.[149] have studied the destruction of Bcl-2 by PDT in greater detail. They found that photodamage to Bcl-2, as observed on western blots, could be induced by Pc 4-PDT in several different cell lines, including human tumor lines. The effect was observed in cell samples recovered immediately after PDT and from cells photoirradiated in the cold. Study of an epitope-tagged Bcl-2 chimeric protein revealed that the entire protein is lost, rather than the photodamage being limited to the epitope for the anti-Bcl-2 antibody. In addition to a loss of the great majority of Bcl-2, a small amount of a 23 kDa cleavage product could be detected, as well as the apparent generation of high-molecular weight products that may be the

result of the photochemical crosslinking of Bcl-2 to itself or to other proteins. The 23 kDa Bcl-2 fragment has been observed in various cells at late times (hours to days) after exposure to a variety of (non-PDT) apoptosis inducers;[59,152–156] in some cases, it was shown that generation of the fragment was dependent on caspase-3. However, in the case of Pc 4-PDT, the fragment was found in MCF-7 cells that do not express this caspase.[149] Additional evidence against the participation of a protease in the loss of Bcl-2 was obtained with a series of protease inhibitors, none of which blocked the response. Thus, it appears that the Bcl-2 protein is a target of PDT, at least with certain photosensitizers that localize in mitochondria,[89,149] and probably with photosensitizers that bind to multiple cytoplasmic membranes.[157] The damage appears to be photochemical, perhaps through the oxidation of key amino acid residues by photochemically generated singlet oxygen. Thus far, it appears that only Bcl-2, and not Bcl-xL, Bad, or Bax, is destroyed by PDT. Several other mitochondrial proteins remained intact after Pc 4-PDT: cytochrome c, a subunit of cytochrome oxidase, the voltage-dependent anion channel (VDAC1), and the adenine nucleotide translocator (ANT).[149] However, as mentioned above, inhibition of ANT function as a pore-forming protein has been found in ANT-containing proteo-liposomal preparations treated with Verteporfin and light.[108]

Pro-apoptotic members of the Bcl-2 family may contribute to promotion of apoptosis in PDT-treated cells. During the first few hours after exposure of human umbilical vein endothelial cells (HUVEC) to Verteporfin-PDT, Bax was found to disappear from the cytosol, but not from whole cell lysates, suggesting it had redistributed to mitochondria.[141] This response occurred simultaneously with the redistribution of cytochrome c from mitochondria to the cytosol. At the same time, Bid cleavage was observed, such that at times later than 2 hours post-PDT, Bid could not be detected in the cells. Bid cleavage was also reported for HL60 cells treated with Rose Bengal and light.[158] This latter photosensitizer is thought to bind preferentially to the plasma membrane, so it would appear that apoptosis initiated at that site can also proceed through modification of Bcl-2 family members. Bid is usually cleaved by caspase-8, which was activated by Rose Bengal-sensitized PDT; the product of this cleavage, truncated Bid (tBid), is known to promote the release of cytochrome c from mitochondria. However, Bid can also be cleaved by lysosomal proteases,[60] providing a potential mechanism for cases in which apoptosis occurs after PDT with lysosomally bound photosensitizers.

Ultimately, it will be important to determine the importance of the level of expression of Bcl-2 family members in the response of tumors to PDT. Kawaguchi et al.[159] examined a series of squamous cell carcinomas of the bronchus that were treated with PDT (with Photofrin I or II) for their level of Bcl-2 and p53 expression, as determined immunohistochemically. Their analysis showed no correlation of expression of either protein with PDT response or cure. In the case of p53, the results of Gomer and co-workers (see below) would predict the observed result, i.e., no correlation. In the case of Bcl-2, all analyses were carried out on pre-treatment biopsies; thus, it is not known if the treatment eliminated some or all of the Bcl-2. If PDT destroys Bcl-2, then the pre-treatment level of this apoptosis suppressor may not be relevant to the ability of the tumor cells to undergo apoptosis in response to the treatment. The level of Bcl-2 has also been measured in biopsies of esophageal tumors treated with Photofrin-sensitized PDT.[160] Biopsies were taken before delivering the photosensitizer, two days later before exposure to laser light, and then three days after that. Although the study was not designed to reveal early effects of PDT on Bcl-2, there was no apparent relationship between tumor response and level of Bcl-2. A recently reported study of tissue from esophageal cancer patients treated with

HpD-sensitized PDT[161] provides interesting data suggesting that tumors expressing higher levels of Bcl-2 were actually more sensitive to PDT. Although the study was small, Bcl-2 expression levels, but not p53 levels, were positively correlated with better relapse-free and overall survival of the patients. The critical question of the relevance of the Bcl-2 proteins to human tumor response to PDT needs considerable additional data from clinical studies.

6 Signaling pathways that can regulate PDT-induced apoptosis

PDT has been found to upregulate numerous signaling pathways (reviewed in ref. 39 and 42). The induction of apoptosis by most physiological stimuli or toxic agents proceeds through series of signaling pathways. Thus, DNA-damaging agents, such as ionizing radiation, require the signaling of damage by p53-dependent steps that block cell cycle progression and upregulate transcription of multiple genes eventually leading to apoptosis after one or more cell divisions.[162,163] In contrast, Fas ligand acts by binding to its receptor and recruiting components of death-inducing complexes that directly activate caspase-8 (see above). PDT activates many signaling reactions that have been characterized in response to other oxidative stresses or physiological stimuli. It is therefore appropriate to ask which of the many reactions are mediators or promoters of apoptosis in PDT-treated cells, which are stress responses whose function is to promote repair or tolerance of damage, and which have no obvious relationship either to the cell death process or to its prevention. In order for a reaction to be a mediator of apoptosis, certain criteria must be met. First, the circumstances under which both apoptosis and the reaction are induced must be defined and agree with respect to dose and timing. Second, a simple demonstration of a correlation is insufficient to prove that the reaction is a cause or an effect of apoptosis. To demonstrate that an enzymatic reaction is necessary for PDT-induced apoptosis, the ability of PDT to induce apoptosis must be studied after inhibition of the enzyme either chemically or using dominant-negative, knock-out, or anti-sense technology. The goal in this review is to focus on those pathways that have been demonstrated to affect PDT-induced apoptosis. Therefore, reactions that are enhanced or inhibited by PDT but where no data are available to suggest an interplay of the pathway with apoptosis in PDT-treated cells will not be considered here.

6.1 Ca^{2+}

PDT causes both transient and more sustained increases in intracellular free calcium concentration [Ca^{2+}]i, which can come from release of intracellular stores of Ca^{2+}, mostly in the endoplasmic reticulum, and from an influx of extracellular Ca^{2+} (reviewed in ref. 164). The first demonstration of an association of a rapid, transient increase in [Ca^{2+}]i with apoptosis was made by Agarwal et al.,[165] studying PDT with the photosensitizer AlPc in mouse lymphoma L5178Y-R cells. The pulse of Ca^{2+}, as observed with the Ca^{2+}-sensitive fluorescent probe, Indo-1, reached a maximum within 1 min after PDT, followed an earlier increase in phospholipase C (PLC) activity, and was prevented by a PLC inhibitor but not by chelation of extracellular Ca^{2+} with EGTA. Inhibition of PLC also prevented the induction of apoptosis in these cells. The data showed that Ca^{2+} release from the endoplasmic reticulum was a result of PDT-induced stimulation of PLC activity and was necessary for the resultant apoptosis. In contrast, the addition of the chelator of intracellular free Ca^{2+}, BAPTA-AM, did not alter the appearance of apoptotic DNA fragmentation in Pc 4-PDT-treated HIV-infected cells.[69]

Several recent papers have further explored the relationship between the rise in [Ca^{2+}]i and the induction of apoptosis by

PDT.[117,166,167] In human squamous cell carcinoma HSC-2 cells, PDT with Photofrin produced a small increase in $[Ca^{2+}]i$ by 30 min and a strong increase by 1–2 h post-PDT, as observed by confocal microscopy.[166] The latter changes coincided with a dramatic increase in apoptotic cells, quantified by flow cytometry, which reached a maximum at 2 h. Inanami et al.[117] observed a similar time-course for apoptosis in Chinese hamster V79 cells exposed to PDT with the photosensitizer pheophorbide a (PPa). In addition to detection of DNA fragmentation by flow cytometry and by agarose electrophoresis, these investigators documented the release of cytochrome c from mitochondria and the activation of caspase-3. Apoptosis was inhibited by azide, a scavenger of $^1O_2$, by the $[Ca^{2+}]i$ chelator, BAPTA, by Ac-DEVD-CHO, an inhibitor of caspase-3-like activity, and by forskolin, which can increase the cellular level of cyclic AMP. Because increases in $[Ca^{2+}]i$ by a calcium ionophore can activate the apoptosis pathway through release of cytochrome c, these investigators concluded that $[Ca^{2+}]i$ was responsible for that mitochondrial step. However, because forskolin did not inhibit cytochrome c release, it was felt that cyclic AMP and Ca^{2+} were acting through different pathways to regulate apoptosis.

In a recent study by Rück et al.,[167] apoptosis was induced in rat bladder epithelial cells by PDT with $AlPcS_4$, and $[Ca^{2+}]i$ was followed by confocal microscopy using the calcium-sensitive dye Fluo-3. Under conditions in which the photosensitizer was located mainly in the endoplasmic reticulum (24 h incubation), low light fluences promoted cell growth, while higher fluences caused apoptosis, as indicated by Annexin V labeling of the plasma membrane. A transient elevation of $[Ca^{2+}]i$, especially in the nucleus, occurred during the irradiation period, followed by a sustained increase. The antioxidant PDTC blocked the changes in $[Ca^{2+}]i$ and the appearance of apoptosis, indicating reactive oxygen species are involved in both endpoints. Thus, the available information implicates $[Ca^{2+}]i$ as one signal contributing to the induction of apoptosis by PDT with several different types of photosensitizer.

6.2 Lipid-derived second messengers: ceramide

Stress-induced activation of sphingomyelinase leads to generation of ceramide, an important lipid mediator of apoptosis.[168] Recent evidence suggests that ceramide also plays a role in PDT-induced apoptosis. A series of studies by Separovic et al.[72,169,170] showed that Pc 4-PDT resulted in elevated levels of ceramide in L5178Y-R cells that were rapid and correlated well with the fraction of cells undergoing apoptosis. Increased levels of ceramide following Pc 4-PDT were also shown for CHO and U937 cells, and the addition of N-acetylsphingosine, a cell-permeable C2-ceramide, mimicked the apoptotic response of PDT in both cell lines. Niemann-Pick disease lymphoblasts, which are deficient in acid sphingomyelinase, the enzyme that catalyzes the initial step in the sphingomyelin pathway, failed to respond to Pc 4-PDT with ceramide accumulation and apoptosis. Combined treatment of the cells with bacterial sphingomyelinase and Pc 4-PDT restored ceramide production and apoptotic response. Nevertheless, bacterial sphingomyelinase is not cell permeable and thus acts at the cell membrane. As Pc 4 does not localize to the plasma membrane,[97,98] the mechanism by which the combined treatment restored apoptosis remains unclear.

Matroule et al.[171] reported acid sphingomyelinase activation, ceramide accumulation, and NF-κB activation after photosensitization of human colon carcinoma HCT-116 cells with pyropheophorbide-a methyl ester (PPME), again suggesting that acid sphingomyelinase may be another target for the cellular response to PDT. Using chloroquine, an acid sphingomyelinase inhibitor, these authors showed significant inhibition of PPME-PDT-induced activation of the NF-κB transcription factor, suggesting that sphingomyelinase activity and gener-

ation of ceramide are responsible for NF-κB activation. Nevertheless, these authors did not report chloroquine effects on the level of ceramide. Of note, chloroquine is a cell-permeable base that prevents acidosis which often precedes apoptosis in response to many stimuli (IgM anti-Fas antibody, cycloheximide, or ultraviolet irradiation)[172] and also inhibits phospholipase A_2 activity;[173] hence non-specific effects cannot be excluded.

Nagy et al.[174] demonstrated a rapid increase in ceramide levels in A431 cells exposed to Pc 4-PDT that correlated well with induction of apoptosis. Interestingly, a simultaneous inhibition of acid sphingomyelinase activity was also reported. With the aid of fumonisin B1, an inhibitor of ceramide synthase, the enzyme that catalyzes de novo synthesis of ceramide, the authors showed that despite complete inhibition of ceramide production, apoptosis following PDT was not blocked. Nevertheless, fumonisin B1 is also non-specific and can alone induce apoptosis.[175] Embryonic fibroblasts from FADD-knockout mice were as efficient as those from mice with wild-type FADD in generating ceramide in response to Pc 4-PDT, although the FADD−/− cells were deficient in ceramide production in response to TNFα.[176] Further, ceramide production was found to accompany but to be non-essential for apoptosis after Pc 4-PDT or TNFα.[176] Although evidence to date is suggestive, the role of ceramide in PDT-induced apoptosis remains to be elucidated.

6.3 Cell surface death receptors

It is well established that PDT induces expression and secretion of TNFα.[39,42] Recently, there has been some research into the possible involvement of the Fas or TNFR-mediated pathways in the induction of apoptosis in response to PDT. Jiang et al.[76] found that either Verteporfin-PDT or anti-Fas antibody selectively depleted CD4+CD8+ cells from a pool of mature thymocytes, and the combined treatments augmented apoptosis in the pooled population, as assessed by caspase-3 activity, PARP cleavage, and DNA fragmentation. Ahmad et al.[143] found a marked increase in expression of Fas in A431 cells during the first hour after Pc 4-PDT; this was followed by a decrease that corresponded with an increase of Fas in the culture medium. Evidence was also provided for the multimerization of Fas within the cells, for an increase in FasL and FADD expression, for the interaction of FasL with FADD, and for the activation of caspase-8. Exposure of the cells to either a blocking antibody or the general caspase inhibitor zVAD-fmk reduced the level of cell killing detected by the MTT assay within 6 or 12 hours post-PDT. It should be noted that caspase-8 is also activated by PDT downstream of cytochrome c release, presumably by caspase-3-dependent cleavage (see section 5.4). In a study of Hep-2 carcinoma cells, Yslas et al.[177] demonstrated that PDT sensitized by 5,10,15,20-tetrakis(4-methoxyphenyl)porphyrin led to apoptosis, under which conditions Fas antigen was also expressed in the cells. These data argue for participation of Fas-mediated apoptosis in the response to PDT.

In A431 cells, Azizuddin et al.[178] found that Pc 4-PDT induced TNFα production but that recombinant human TNFα did not interact with PDT in the induction of apoptosis. A test of the importance of the death receptor pathways was conducted by Nagy et al.,[176] using primary embryonic fibroblasts derived from mice expressing wild-type FADD and from FADD knock-out mice. They found that Pc 4-PDT induced similar levels of apoptosis (TUNEL-positive cells) and caspase-3 activity in both types of cells, although the FADD knock-out cells were resistant to the induction of apoptosis by TNFα, arguing that PDT can induce apoptosis in the absence of functional FADD. Thus, the role of pathways for apoptosis initiating with cell surface death responses in the response of cells to PDT is still controversial. It has been suggested that

these pathways may be upregulated in response to cell damage and regulate stress responses (see Section 6.4).

6.4 Nuclear factor-kappa B (NF-κB)

NF-κB is a transcription factor responsible for promoting expression of genes for several proteins involved in immuno-regulatory and proinflammatory processes.[179,180] Evidence for regulation of apoptosis through transcription of anti-apoptotic genes has also been obtained. NF-κB normally exists as a dimer residing in the cytoplasm in association with an inhibitory sub-unit, one of several IκB factors. Stimulation by TNFα leads to phosphorylation of specific serine residues of IκB, ubiquitin-ation of specific lysine residues, and targeting of the protein for degradation by the 26S proteasome. Upon release of the inhibitory subunit, the remaining dimer can enter the nucleus to promote transcription of genes containing NF-κB binding motifs. The most common NF-κB dimer is composed of a p50 and a p65 (RelA) species, of which there are several variants. One of the genes whose transcription is stimulated is that for IκB; resynthesis of the inhibitory subunit thus restores NF-κB to its baseline state.

PDT produces an oxidative stress that can result in activation and translocation of NF-κB to the nucleus, as originally shown for treatment of L1210 murine leukemia cells with Photofrin and light.[181] Although NF-κB activation was not observed in HeLa cells exposed to Photofrin-PDT,[182] PDT with methylene blue was demonstrated to activate and translocate NF-κB to the nucleus.[183] A similar NF-κB response was also found for treatment of HIV-infected lymphoid ACH-2 cells by photo-sensitization with the DNA-intercalating drug proflavine.[184]

Recently, new insights have been obtained into the impor-tance of the NF-κB response in PDT-induced apoptosis. Using PPME as photosensitizer, Matroule et al.[171] demonstrated that PDT recruits the IL-1 receptor-dependent signaling pathway to activate NF-κB in HCT-116 human colon cancer cells. This group then developed a new aminopyropheophorbide (APP) derived from PPME and showed that it bound to membranes but not to mitochondria of HCT-116 cells.[185] Using an electro-phoretic mobility shift assay with an oligonucleotide containing the NF-κB binding motif, they demonstrated that following PDT, nuclear extracts contained elevated levels of material that could retard migration of the oligonucleotide. The increase in nuclear NF-κB activity was biphasic, as demonstrated by an early (10–30 min) activation of NF-κB, which was followed by a decrease at 1 hour and a second wave of activity at 2–24 hours after PDT. Competition by various peptides confirmed that the activity was the p50/RelA heterodimer. To study the relation-ship of the NF-κB response to the induction of apoptosis, Matroule et al.[185] constructed HCT-116 cell lines expressing mutated IκB; one such mutant (Y42F) expressed IκB with tyro-sine 42 changed to phenylalanine, and the other (S32,36A) expressed IκB in which serines at positions 32 and 36, the serines known to be phosphorylated as a prerequisite for inacti-vation of the inhibitor, were converted to alanine. Activation of NF-κB by PDT was equally efficient in the Y42F-expressing line as in the parental cells, but there was no significant activation in the S32,36A-expressing line, demonstrating that phosphorylation of the two essential serines was necessary for release of the active NF-κB heterodimer from inhibition by IκB. HCT-116 S32,36A cells were much more sensitive to the induction of apoptosis by APP-PDT than were the parental cells, as demonstrated by caspase-3 activity, TUNEL assay, and chromatin condensation. Finally, activation of a κB-luciferase reporter construct provided further evidence for the PDT-induced activation of NF-κB. Thus, these studies confirmed that NF-κB provides an anti-apoptotic signal for cells exposed to PDT with a photosensitizer that does not localize in mitochondria.

In a follow-up study, Granville et al.[133] extended the results to human promyelocytic leukemia HL-60 cells using the materials produced for the study of Matroule et al.[185] and the photo-sensitizer Verteporfin. They also showed that NF-κB levels were increased in the nuclei of cells undergoing apoptosis, but that cellular IκB levels decreased; however, in spite of demonstrated activation of caspases-9 and -3 in the PDT-treated cells, it is unlikely that IκB was cleaved by caspase-3, since the loss of this inhibitory subunit was not prevented by the general caspase inhibitor zVAD-fmk. This may indicate that degradation of IκB occurred through the 26S proteasome.

A recent detailed study of the role of NF-κB activation in the response of HCT-116 cells to PPME-sensitized PDT has been reported.[186] In this system, N-acetylcysteine and PDTC pro-tected against apoptosis, but D_2O (which enhances the lifetime of $^1O_2$) increased necrosis but not apoptosis, implicating ROS other than $^1O_2$ in the induced apoptosis. Evidence for the participation of mitochondria (activation of caspases-9 and -3, PARP cleavage, DNA fragmentation) was obtained; however, in spite of the localization of PPME in lysosomal and ER/Golgi membranes, no evidence was found for the participation of Ca^{2+}, Bid, Bap31, or caspase-12. Likewise, IL-1 receptor signaling and ceramide appeared not to participate in PPME-PDT-induced apoptosis. Although Bcl-2 was converted by PDT to a more slowly migrating (phosphorylated?) form, transient transfection of the cells with either wild-type Bcl-2 or either of two dominant negative Bcl-2 mutants did not affect the appearance of apoptotic endpoints. This study confirms the earlier conclusion that NF-κB is activated by PPME-PDT but is distinct from the pathway leading to apoptosis.

6.5 Mitogen-activated protein kinases (MAPKs)

The MAPKs belong to a well-studied family of signaling path-ways that function in all cells to convert various extracellular stimuli into intracellular responses, generally through regula-tion of genes controlling cell growth and responses to stress.[187] There are at least three major pathways, each named for the terminal MAPK of a cascade of three kinases: ERK 1/2, SAPK/JNK, and p38/HOG. Each MAPK is phosphorylated on threonine and tyrosine by a dual-specificity MAP kinase kinase, which is activated on serine and threonine by a MAP kinase kinase kinase, itself activated through a variety of upstream signals. Extracellular signal-regulated kinase (ERK) is activated when certain growth factors engage their membrane-localized receptors; this pathway proceeds through sequential activation of Ras, Raf, and MEK, culminating in activation of ERK1 and ERK2. The other two major pathways are activated by a variety of cell stresses, including UV or ionizing radiation, osmotic stress, and withdrawal of certain growth factors. The stress-sensitive pathways are those culminating in stress-activated protein kinase (SAPK), also known as c-Jun N-terminal kinase (JNK), and p38/HOG (hyperosmotic glycerol) kinase. The upstream kinases in the SAPK/JNK pathway are MKK4/SEK1 and MKK7, whereas p38/HOG kinases are activated by MKK3 and 6.[187] The identified substrates of each of the MAPKs are transcription factors: c-Jun, ATF2, and Elk1 in the case of SAPK; ATF2, Max, CREB, and Elk1 in the case of p38; and Elk1 and Stat3 in the case of ERKs.

A link between the SAPK and p38 pathways and apoptosis has been suggested in several studies.[188–190] For PDT, there is evidence linking the p38 pathway with apoptosis. The rapid activation of both the SAPK and p38 pathways, but not ERKs, has been observed in cells responding to PDT with BPD-MA,[191] Pc 4,[189] PPIX synthesized endogenously from ALA,[192] and Rose Bengal.[158] In three cases, the relationship of p38 acti-vation to PDT-induced apoptosis was tested using an inhibitor of p38 phosphorylation. Inhibition of p38/HOG by SB202190 was found to partially inhibit apoptosis in L5178Y-R and CHO cells photosensitized with Pc 4.[189] In contrast, Klotz found that inhibition by SB202190 promoted apoptosis in response to

PPIX-PDT.[192] With Rose Bengal as photosensitizer, PDT-induced activation of p38 led to activation of caspase-8 and cleavage of Bid, which suggests a mechanism for the inter-section of this stress kinase pathway with the mitochondrial pathway for apoptosis.[158] However, as with all chemical inhibitors, there is a question concerning their specificity. In the future, it will be necessary to address the role(s) of the stress kinases using genetic methods for eliminating selected activities in the stress kinase pathways.

Among the many known signal transduction pathways, phosphatidylinositol-3 kinase (PI3-kinase) has been shown to promote cell survival and resistance to apoptosis.[193,194] The role of this pathway in apoptosis in response to Pc 4-PDT was investigated by modulating the activity of epithelial and endo-thelial derived tyrosine kinase (Etk), a newly discovered tyro-sine kinase that is a substrate of PI3-kinase.[195] Parental LNCaP human prostate cancer cells and two derivative cell lines, one overexpressing wild type Etk (Etkwt) and the other expressing a dominant-negative Etk (EtkDN), were used to study the function of Etk. As measured by DNA fragmentation and by cleavage of PARP, the extent of apoptosis was much reduced in Etkwt cells as compared to LNCaP or EtkDN cells. Assay of overall cell viability confirmed that Etkwt cells were con-siderably less sensitive to PDT than were the parental LNCaP or EtkDN cells. The results suggest that the PI3-kinase/Etk pathway is involved in the protection of prostate carcinoma cells from apoptosis in response to PDT. The mechanism for the protection is not yet known.

The phosphorylation of multiple cellular proteins has been observed on western blots using an anti-phosphotyrosine anti-body. Two major phosphoprotein bands (p80 and p55) were observed in extracts of L5178Y-R cells immediately after Pc 4-PDT, one of which (p80) was identified as HS1, a substrate of nonreceptor-type *src* protein tyrosine kinases.[73] Two other pro-teins (p>200 and p15) were found in murine P815 mastocytoma cells immediately following Verteporfin-PDT.[196] In neither case could the protein tyrosine phosphorylations be linked directly with apoptosis, but evidence obtained with the protein tyrosine kinase inhibitor genistein suggested that the events in the L5178Y-R cells may be part of a stress response.[73]

6.6   p53

The response of many cells to DNA-damaging agents, such as ionizing radiation, is controlled by the product of the p53 tumor suppressor gene. Thus, in response to DNA damage, cells with wild-type p53 either become delayed in progression through the cell cycle to undergo repair of the damage or are directed into apoptosis. Cells with mutant or no p53 are deficient in these responses. With respect to PDT, it is known that cell lines with mutant p53 or lacking p53 can undergo apoptosis (*e.g.*, L5178Y-R cells;[66] V79 cells[117]). In order to determine whether the sensitivity of cells to the lethal effects of PDT or their ability to undergo apoptosis was affected by the expression of p53, Fisher *et al.*[197–199] compared responses within several sets of closely related human tumor cells. The presence of functional p53 was demonstrated by the accumulation of p53 protein in PDT-treated cells, by the p53-mediated upregula-tion of p21, and by the dephosphorylation of the Rb protein. Human promyelocytic leukemia HL60 cells expressing wild-type p53 were more sensitive to cell killing by PDT, with either Photofrin or SnET2 as the photosensitizer, than were HL60 cells in which the p53 genes were deleted or inactive. All of these cell lines underwent rapid apoptosis in response to PDT.[197] A human colon carcinoma cell line expressing wild-type p53 (LS513) was more sensitive to PDT than another colon carcinoma cell line with mutated p53.[198] Although those studies provided evidence that p53 can sensitize cells to PDT-induced apoptosis, introduction of the viral oncoprotein E6 to abrogate wild-type p53 function of the LS513 cells or of MCF-7 cells

did not alter their PDT sensitivity to loss of clonogenicity or induction of apoptosis, leading to the conclusion that PDT sensitivity is not p53-dependent.[199]

The role of p53 in PDT response was further explored by Zhang *et al.*[200] in the p53-null HT29 human colorectal carcinoma cell line using a hypocrellin A derivative as photo-sensitizer. When wild-type p53 was transfected into these cells, it caused growth inhibition but did not induce apoptosis; how-ever, the transfected cells were more sensitive to the induction of apoptosis after PDT. As judged by an MTT assay, the p53-expressing cells were approximately two-fold more sensitive to PDT (*i.e.*, 50% loss of viability required 9 J cm^{-2} *vs.* 18 J cm^{-2} for the non-transfected cells or for cells transfected with the *neo* gene). However, in the absence of a clonogenic assay, it would appear that the apparent increase in sensitivity to killing of the p53-transfected cells may reflect a reduced rate of death that parallels their slower growth kinetics. Recently, Tong *et al.*[201] reported a comparison of normal human fibroblasts that express wild-type p53, immortalized fibroblasts from a patient with Li-Fraumeni syndrome, in which the only p53 allele was mutated, and the Li-Fraumeni cells expressing wild-type p53 introduced by an adenovirus vector. The normal cells were significantly more sensitive than the Li-Fraumeni cells to PDT with Photofrin, as determined by clonogenic assay; transfection of p53 modestly enhanced the sensitivity of Li-Fraumeni cells. In this system, there was no apoptosis in the normal fibro-blasts in response to PDT, and a low level of apoptosis in the Li-Fraumeni cells, which must have occurred through a p53-independent mechanism. The major conclusion from these data is that neither PDT-induced cell killing nor PDT-induced apoptosis has marked dependence on p53 function.

6.7   p21 and cell cycle controls

Mukhtar and co-workers have investigated a series of signaling reactions as potential explanations for the difference in response of two cell lines to Pc 4-PDT: murine RIF-1 fibro-sarcoma cells, which often die without expressing apoptotic characteristics, and human A431 epidermoid carcinoma cells, which undergo apoptosis. The two cell lines were treated with the same external dose of Pc 4 and the same red light fluence. Gupta *et al.*[202] found an early (maximum at 5 min) increase in nitric oxide formation that was greater in A431 cells than in RIF-1 cells, as well as a greater increase in the activity of nitric oxide synthase (NOS) in A431 cells after PDT. When they assayed for the amount of constitutive NOS on western blots, both cell lines revealed a strong upregulation of the protein in cells exposed to Pc 4 only, but little if any additional change upon photoirradiation. Further studies of A431 cells showed that the chosen PDT condition results in slow entry of the cells into apoptosis, such that the majority was apoptotic by 12 hours after treatment.[203] During the progression into apoptosis, changes in the cell cycle distribution of the population were noted, *i.e.*, an increase in the G0–G1 phase at the expense of cells in S phase by 12 hours. These changes were preceded by an increase in p21/WAF1/CIP1 and downregulation at both the protein and activity levels of cyclins D1 and E and cyclin-dependent kinases cdk2 and 6. Since these data suggested that Pc 4-PDT-treated A431 cells experienced a delay in passing the G1–S cell cycle checkpoint, Ahmad *et al.*[204] explored sub-sequent steps in the pathway for regulating that checkpoint and found a decrease in the hyperphosphorylated form of pRb and downregulation of five members of the E2F family of transcription factors and their heterodimeric partners DP1 and DP2. Collectively, the data suggest that when A431 cells are exposed to Pc 4-PDT, the cells upregulate a pathway for delay-ing cell cycle progression prior to the G1–S border. The timing of the responses was consistent with the cells needing to delay at the G1 checkpoint prior to entering the final stages of apoptosis. It will be of interest in the future to study cells in

Table 1 Apoptosis in PDT-treated model tumors

Tumor	Photosensitizer	Reference
RIF-1 fibrosarcoma	Pc 4	210
HeLa cell xenografts	HpD	206
Chemically induced squamous papillomas	Pc 4	213
Colon-26	ALA-PPIX	214
MS-2 fibrosarcoma	Zn(II) Pc	215
EMT6	LuTex	84
OVCAR-3 xenografts	Pc 4	209
Human pancreatic carcinoma xenografts	PP*a*	216
A673 sarcoma xenografts	Photofrin	211
RIF-1 fibrosarcoma, murine skin papillomas	Pc 4	205
VX2	Photofrin, SnET2, ALA/PPIX, *m*-THPC	212
SW480 xenografts	Pc 4	208
NR-S1 squamous carcinoma	Photofrin	207

which steps in the pathway are abrogated, *e.g.*, by knockout of one or more of the proteins, to determine if the cells are prevented from undergoing apoptosis and further if they are rescued from overall cell death by disabling the pathway. Finally, Kalka *et al.*[205] demonstrated that the glycoprotein clusterin was also upregulated in dying A431 cells but not in RIF-1 cells. Thus, this series of papers presents evidence for the activation of several pathways in response to Pc 4-PDT during the process of apoptosis. Which of these pathways are causative for the observed apoptosis is not clear. It is possible that all participate in sub-populations of the cells, or that they are stress responses turned on by lethally damaged cells in an attempt to recover from the damage.

7 Apoptosis in PDT response in experimental tumors *in vivo*

Evidence for the occurrence of apoptosis in PDT-exposed tumors has been obtained for many different tumor types and photosensitizers, including human tumors grown as xenografts in athymic nude mice (Table 1). In addition to observation of apoptotic cells in stained tumor sections by light or electron microscopy, apoptosis has been verified by morphometric analysis,[206] by TUNEL-positivity,[174,207,208] by DNA fragmentation observed after agarose electrophoresis of extracted tumor DNA,[84,209,210] and by western blot analysis of tumor proteins for PARP cleavage.[208,209] Although the time course for the occurrence of apoptosis varies with the tumor, the photosensitizer, and the PDT dose, generally apoptosis has been found within the first several hours following PDT, and is considered an early event, preceding the appearance of extensive necrosis and tumor regression.

Recently, there have been several attempts to more carefully quantify and locate apoptotic cells in tumors following PDT. In human A673 sarcoma xenografts in athymic nude mice, Engbrecht *et al.*[211] monitored the responses following Photofrin-sensitized PDT. They observed progressive blood vessel occlusion, as has been found for other tumors treated with Photofrin-PDT,[10] and progressively increasing numbers of apoptotic (TUNEL-positive) cells from 4–36 hours after treatment. Patches of necrosis that were present before treatment did not increase during the same post-treatment period. The earliest evidence of apoptosis in this tumor model was associated with endothelial cells or in the vicinity of occluded vessels. Since Photofrin-PDT primarily targets the tumor vasculature, Engbrecht *et al.*[211] suggested that tumor cell apoptosis was a secondary consequence of the hypoxia produced when blood flow was interrupted.

Yokota *et al.*[207] exposed mouse NR-S1 squamous carcinomas implanted in C3H/HeNCrj mice to PDT sensitized by Photofrin. They excised, fixed, and sectioned the tumors at various times up to 48 hours after treatment, and identified apoptosis by the TUNEL assay and Fas and FasL by immunohisto-

chemistry, in addition to locating areas of necrosis. Under the chosen conditions, PDT increased the area of necrosis, the number of TUNEL-positive tumor cells, and the number of tumor cells expressing Fas or FasL, reaching maxima at 24, 12, and 12 hours post-treatment, respectively. The time course was interpreted as suggesting that necrotic areas may derive from apoptotic ones. The Fas-expressing cells were found in the same non-necrotic tumor areas as the TUNEL-positive cells, and FasL-expressing cells were found in tumor cells surrounding the TUNEL-positive cells. A significant increase in FasL-positive lymphocytes was also observed at 12 hours, but these cells only rarely infiltrated into areas of TUNEL-positive tumor cells. Yokota *et al.*[207] suggest that the Fas–FasL system may mediate the apoptotic response of tumors to PDT in an autocrine or paracrine fashion; however, in the tumor model, lymphocytes seemed to play only a minor role, if any.

The findings of Yokota *et al.*[207] are important for relating *in vivo* observations to mechanisms of PDT response and deserve to be examined in other models and with other photosensitizers. Interestingly, the observation of a high incidence of apoptosis in areas of the tumor surrounding necrotic areas confirms similar findings by Lilge *et al.*,[212] who quantified the incidence of apoptosis in normal brain and intracranial VX2 tumor 24 hours following PDT with 5 different photosensitizers (Photofrin, ALA/PpIX, AlPc, SnET2, and *m*-THPC). After staining apoptotic cells by the TUNEL assay, confocal microscopy was used to quantify the local incidence of apoptosis and its spatial distribution. The incidence of apoptosis was confirmed by histopathology. PDT with each photosensitizer except AlPc produced apoptotic cells near the region of coagulative necrosis by 24 hours. The apoptotic response did not appear to be related to photosensitizer dose. The results suggested that areas receiving high PDT doses became necrotic and surrounding tissue went through apoptosis.

Mechanisms for the induction of apoptosis by PDT in tumors *in vivo* may involve certain signal transduction pathways being identified *in vitro*. Studying human tumor xenografts in athymic nude mice, Colussi *et al.*[209] found p21 upregulation in response to Pc 4-PDT in human ovarian cancer OVCAR-3 xenografts that express wild-type p53. However, in human colon cancer SW480 xenografts, that are p53 null, Whitacre *et al.*[208] demonstrated rapid post-Pc 4-PDT phosphorylation (activation) of the p38 stress kinase, but no response of p21. Both tumors responded well to PDT. Thus, in agreement with data obtained on other tumor cells *in vitro*,[197,199] p53-dependent responses may occur but appear to be unnecessary to elicit apoptosis in response to PDT *in vivo*.

8 Conclusions

1. It is unlikely that there is a single universal mechanism for the response of cells to PDT. The responses vary with the cell type and its genetic and metabolic potential, as well

as the photosensitizer and, importantly, its sub-cellular localization. The responses also vary with the overall dose of PDT (*e.g.*, sub-lethal *vs.* lethal *vs.* supra-lethal), since gene induction that requires many hours post-PDT is unlikely to occur or be relevant to cells that undergo apoptosis within a few hours.

2. PDT stimulates multiple signal transduction reactions, some but not all of which have been discussed above. Current evidence implicates certain signals as promoters of cell death (*e.g.*, Ca^{2+}, p38), whereas others seem to provide a protective mechanism for cells (*e.g.*, NF-κB). For still others, the roles appear to depend on the context. It is possible that multiple pathways can influence cell fate. Based on the available data, we have grouped the various responses and signals discussed above into 3 categories (Fig. 3): (a) those that are direct targets or
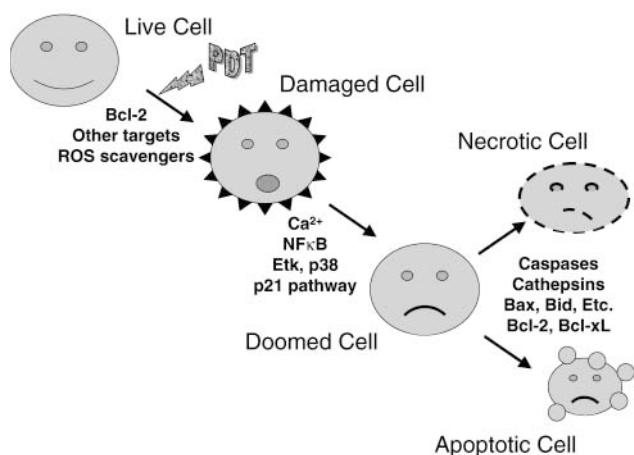

Fig. 3 A model of the controls over PDT-induced cell death. PDT produces initial photodamage to molecular targets, one of which can be Bcl-2. Scavengers of reactive oxygen species and other factors that can alter the amount or distribution of initial photoproducts determine the extent of the initial cellular damage. If damage is sufficiently high, the cell is doomed to die. However, various pathways (*e.g.*, NF-κB) can influence tolerance or repair of the damage that may allow some of the cells to survive the damage. Once a cell is doomed, it may die by apoptosis or necrosis, and the choice will depend upon the presence of the needed caspases, Bcl-2 family members, and other enzymes of apoptosis. Some pathways indicated as affecting the conversion of a damaged to a doomed cell may also affect the choice of pathway for eliminating the doomed cell.

modifiers of oxidative damage produced by PDT; (b) those that can potentially alter the ability of a PDT-damaged cell to live or die; and (c) those that determine whether a lethally damaged, "doomed" cell undergoes apoptosis or necrosis. Since the data needed to assign roles to these reactions are still limited, it is possible that certain reactions have more than one role; *e.g.*, reactions in group b may also affect the choice of cell death pathway.

3. There are now numerous examples of cells that die in response to PDT without undergoing apoptosis. When very high PDT doses are involved, it is likely that excessive damage has occurred such that cells lyse or that components of the apoptotic pathway are damaged, preventing the process. In a limited number of cases, *e.g.*, MCF-7 cells that lack the gene for procaspase-3, a component of the apoptosis pathway is known to be defective and accounts for the relative resistance to apoptosis. Even in this case, other caspases are present and may be responsible for the delayed appearance of apoptotic cells. Furthermore, it is well established that PDT can activate multiple caspases, including caspases-2, -6, -7, and -8, and these may be responsible for the apoptosis that can occur in the absence of caspase-3.

4. Photoactivation of a sensitizer in or influencing the mitochondria leads to rapid apoptosis, presumably as a result of the release of cytochrome c into the cytoplasm where it can interact with APAF-1 and activate the apoptotic caspases. In many cases, this appears to be the central pathway for induction of apoptosis by PDT. However, the observations of multiple pro- and anti-apoptotic pathways that are elicited by PDT suggest that the central pathway can be modulated strongly by such conditions as the relative levels of pro- and anti-apoptotic Bcl-2 homologs and by changes in components of mitochondrial membranes, such as the PTPC. For photosensitizers localized in lysosomes, cathepsins that are released upon photodamage can cleave Bid, which promotes apoptosis at the mitochondria, and/or cleave caspase-3, which inhibits apoptosis.

5. Resistance to apoptosis is not necessarily the same as resistance to death. Many workers in the field equate apoptosis with cell death. If that is true, than cells blocked in the ability to undergo apoptosis should remain alive, not just for a few days but for an unlimited time. In the few cases where this idea has been tested, the results suggest that apoptosis is not the process by which PDT-treated cells die, but rather the process by which lethally damaged cells are dismantled. Whether this conclusion is true for all photosensitizer and cell combinations is not clear at present. In order to evaluate the importance of any apoptosis-related reaction to overall cell death, knowing the total number of killed cells is important, and this requires using clonogenic assays for quantification of cell killing whenever possible, as viability assays are influenced by growth delays and cell loss post-treatment and are unreliable when the level of killing exceeds ~90%.

6. PDT appears to be able to overcome or bypass many of the blocks to cell killing that limit the effectiveness of ionizing radiation and chemotherapy. One hypothesis to explain the apparent success of PDT in killing otherwise resistant cells is that PDT triggers apoptosis at a late stage in the process, often by directly damaging mitochondria. An important question for PDT is: what is the critical PDT-induced lesion(s) or step(s) which kill the cell and from which it cannot recover? One molecular target of PDT *in vitro* is the Bcl-2 protein. Photodamage to Bcl-2, which includes photocrosslinking as well as photo-induced cleavage, may limit the effectiveness of this important anti-apoptotic control. The direct targeting of Bcl-2 by PDT with certain photosensitizers can explain the ability of PDT to overcome the resistance to apoptosis afforded by overexpression of Bcl-2. For photosensitizers that do not cause photodamage to Bcl-2, there must be other (as yet unidentified) molecular targets. In some cases, control of PDT-induced apoptosis may depend on the ratio of Bax to Bcl-2 protein, although the universality of this notion is in question. Certainly, where cytochrome c release from mitochondria is an immediate response to PDT, a slower recruitment of Bax to the mitochondria cannot drive that response. However, when Bcl-2 is a phototarget, tumor cells with abnormally high levels of Bcl-2 may be sensitized to PDT even though they are resistant to most chemotherapeutic agents and radiation.

7. There are limited data from clinical studies to test the role of apoptosis and Bcl-2 or other molecular targets in PDT response. Where the dominant effect of PDT is vascular, the levels of molecular targets within the malignant cells may not be relevant; however, where some or all of the tumor response depends upon direct photodynamic action on the malignant cells, the possible sensitization of tumor cells to PDT by Bcl-2 may offer a distinct advantage. Because of the potential of PDT to overcome treatment resistance of tumors, further work on apoptosis and the identification of specific target molecules, such as Bcl-2, in PDT-treated human tumors may be exceptionally fruitful. It is suggested that pre-treatment clinical samples need to be compared to samples taken as soon after treatment as possible, so that the early responses can be recorded before major changes in the cellular content of the tumors blurs those responses.

Table 2 Abbreviations

AIF	Apoptosis inducing factor
ALA	5-Aminolevulinic acid
ANT	Adenine nucleotide translocator (carrier)
APAF-1	Apoptosis-activating factor-1
APP	Aminopheophorbide
2-BA-2-DMHA	2-Butylamino-2-demethoxy-hypocrellin A
tBID	Truncated bid
BPD-MA	Benzoporphyrin derivative monoacid ring A (Verteporfin)
CAME	Chlorin e6 triacetoxymethyl ester
CCCP	Carbonylcyanide-m-chlorophenylhydrazone
CPO	9-Capronyloxytetrakis(methoxyethyl)porphycene
DISC	Death-inducing signalling complex
2-DOG	2-Deoxyglucose
DTHe	Tetrahydroxyhelianthrone
EDKC	N,N'-Bis(2-ethyl-1,3-dioxolanyl)kryptocyanine
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FADD	Fas-associated death domain
FasL	Fas ligand
HIV	Human immunodeficiency virus
HOG	Hyperosmotic glycerol (kinase)
HRM	Heme regulatory motifs
ICAD/DFF45	Inhibitor of caspase-activated dnase/dna fragmentation factor 45
IL	Interleukin
IκB	Inhibitor of κB
JNK	C-jun N-terminal kinase
LuTex	Lutetium texaphyrin
MAPK	Mitogen-activated protein kinases
m-THPC	$meta$-Tetrakis(hydroxyphenyl)chlorin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MBD	Methylene blue derivative
MDCK	Madison-Darby canine kidney cells
NF-κB	Nuclear factor-κB
PARP	Poly(ADP-ribose) polymerase
PBR	Peripheral (mitochondrial) benzodiazepine receptor
PDTC	Pyrrolidine dithiocarbamate
PPa	Pheophorbide a
Pc	Phthalocyanine
PDT	Photodynamic therapy
PLC	Phospholipase C
PPIX	Protoporphyrin IX
PPME	Pyropheophorbide-a methyl ester
PTPC	Permeability transition pore complex
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SMAC	Second mitochondrial activator of caspases
SnET2	Tin ethyl etiopurpurin
3THPP	Tetrakis(3-hydroxyphenyl)porphyrin
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TPPS$_{2a}$	Disulfonated $meso$-tetraphenylporphine
TPPS$_4$	Tetrasulfonated $meso$-tetraphenylporphine
TUNEL	Terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling
TRADD	TNF receptor-associated death domain
UDCA	Ursodeoxycholic acid
VDAC	Voltage-dependent anion channel

9 Abbreviations

Abbreviations are given in Table 2.

10 Acknowledgements

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