Photodynamic therapy with the phthalocyanine photosensitizer Pc 4: The case experience with preclinical mechanistic and early clinical–translational studies

Janine D. Miller, Elma D. Baron, Heather Scull, Andrew Hsia, Jeffrey C. Berlin, Thomas McCormick, Valdir Colussi, Malcolm E. Kenney, Kevin D. Cooper, Nancy L. Oleinick

Department of Dermatology, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, OH 44106, USA
Department of Radiation Oncology, School of Medicine, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, OH 44106, USA
Department of Chemistry, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, OH 44106, USA
The Case Skin Diseases Research Center, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, OH 44106, USA
The Case Comprehensive Cancer Center, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, OH 44106, USA
Louis Stokes VA Medical Center, 10701 East Boulevard, Cleveland, OH 44106, USA

Received 4 October 2006; revised 6 January 2007; accepted 10 January 2007
Available online 15 February 2007

Abstract

Photodynamic therapy (PDT) is emerging as a promising non-invasive treatment for cancers. PDT involves either local or systemic administration of a photosensitizing drug, which preferentially localizes within the tumor, followed by illumination of the involved organ with light, usually from a laser source. Here, we provide a selective overview of our experience with PDT at Case Western Reserve University, specifically with the silicon phthalocyanine photosensitizer Pc 4. We first review our in vitro studies evaluating the mechanism of cell killing by Pc 4-PDT. Then we briefly describe our clinical experience in a Phase I trial of Pc 4-PDT and our preliminary translational studies evaluating the mechanisms behind tumor responses. Preclinical work identified (a) cardiolipin and the anti-apoptotic proteins Bcl-2 and Bcl-xL as targets of Pc 4-PDT, (b) the intrinsic pathway of apoptosis, with the key participation of caspase-3, as a central response of many human cancer cells to Pc 4-PDT, (c) signaling pathways that could modify apoptosis, and (d) a formulation by which Pc 4 could be applied topically to human skin and penetrate at least through the basal layer of the epidermis. Clinical–translational studies enabled us to develop an immunohistochemical assay for caspase-3 activation, using biopsies from patients treated with topical Pc 4 in a Phase I PDT trial for cutaneous T-cell lymphoma. Results suggest that this assay may be used as an early biomarker of clinical response.

Keywords: Photodynamic therapy; Silicon phthalocyanine; Pc 4; Cutaneous T-cell lymphoma; Apoptosis

Contents

Background: photodynamic therapy .................................................................................................................. 291
Overview of the case experience with PDT ........................................................................................................... 291
From DNA damage to caspases ............................................................................................................................. 292
The role of caspases in PDT-induced apoptosis .................................................................................................... 293
Bcl-2 family proteins in PDT-induced apoptosis .................................................................................................... 293
Signaling pathways and apoptosis in response to Pc 4-PDT ................................................................................ 294

* Corresponding author. Department of Radiation Oncology, School of Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4942, USA. Fax: +1 216 368 1142.
E-mail address: nlo@case.edu (N.L. Oleinick).

0041-008X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2007.01.025
Background: photodynamic therapy

Photodynamic therapy (PDT) is emerging as a promising non-invasive treatment for cancers (Babilas et al., 2005; Dougherty et al., 1998). It has been shown to induce favorable responses in the treatment of cutaneous squamous cell and basal cell carcinomas, as well as cancers of the head, neck, lung, esophagus, and bladder (Wolfisen, 2005; Sibata et al., 2001). This treatment involves either local or systemic administration of a photosensitizing drug, which preferentially localizes within the tumor, followed by illumination of the involved organ with light, usually from a laser light source. The light excites the photosensitizing drug, resulting in formation of reactive oxygen species, believed to be responsible for the cascade of cellular and molecular events in which the end result is selective tumor destruction (Dougherty et al., 1998).

PDT has many advantages over current cancer treatment modalities, such as surgery, chemotherapy, and radiation therapy (Dougherty et al., 1998; Sibata et al., 2001). The treatment is relatively non-invasive in that it usually requires mere illumination of the tumor site. It does not result in systemic immunosuppressive effects that would translate to a clinical opportunistic infection. PDT can also be repeated without detrimental consequences to the patient. However, PDT with the currently FDA-approved photosensitizers is not without adverse effects. For example, Photofrin®, the first systemic drug to be approved, is well known for causing an intense inflammatory and necrotic reaction at the treated site and prolonged widespread photosensitivity for up to several weeks post-PDT, thereby imposing severe limitations on the patient’s lifestyle (Dougherty et al., 1990; Moriwaki et al., 2001). Because of this and other drawbacks of Photofrin®, many additional photosensitizers have been synthesized, and a few of them have developed into FDA-approved drugs or are in clinical trials.

Notwithstanding the many positive results with PDT, both preclinical and clinical protocols are still being optimized to address the major reasons that PDT sometimes fails to eradicate the targeted tumor. Failure generally results from (a) inhomogeneous delivery of the photosensitizer within the tumor or minimal differential in photosensitizer level between tumor and surrounding normal tissue, (b) poor penetration of light to some parts of the tumor, and/or (c) inability to ensure that the entire tumor remains sufficiently well oxygenated during the full photoinactivation period. To improve light delivery, combinations of surface and interstitial illumination have been applied, or PDT has been preceded by tumor-debulking surgery (Vogi et al., 2004). To maintain oxygenation in the face of photochemical deactivation of molecular oxygen to generate singlet oxygen, the rate of light delivery (the fluence rate) can be reduced, thereby slowing the photodynamic utilization of oxygen to bring it in line with the diffusion of oxygen from the tumor blood supply (Foster et al., 1991; Henderson et al., 2004).

Photosensitizers are usually delivered by intravenous injection and are initially taken up by all tissues; earlier release from some normal tissues permits the tumor-to-normal tissue ratio to increase, but the ratios are rarely sufficient to offer optimal selectivity in the photodynamic effect, and some damage to normal tissues is inevitable. Furthermore, the retention of some photosensitizers, especially Photofrin, in the skin after systemic delivery is responsible for the lingering cutaneous photosensitivity experienced by patients undergoing PDT (Vogi et al., 2004; Castano et al., 2006). Topical delivery of a photosensitizer to accessible tumors offers the potential for improvements in selectivity and the elimination of cutaneous photosensitivity at sites distant from the targeted tumor.

Currently, topical PDT using amino-levulinic acid (ALA), or its esters (e.g., methyl-ALA), is used for certain cutaneous malignancies (e.g., superficial basal cell carcinoma), inflammatory diseases (e.g., acne), and photorejuvenation (Morton et al., 2002). Advantages of ALA-based PDT are the ease of administration of the ALA preparation and selectivity in the metabolism of ALA to the photosensitizer Protoporphyrin IX (PpIX) in malignant cells, pilosebaceous units, and other rapidly growing tissue. However, there are at least two disadvantages with the use of ALA. One disadvantage is the prolonged contact period (about 4 to 24 h) necessary before light activation can be performed. This is because ALA itself is not a photosensitizer, but a precursor that undergoes a biochemical conversion to PpIX. Secondly, significant burning and pain are often associated with ALA-PDT. The pain severity is such that patients who have multiple basal cell carcinomas (such as those seen in basal cell nevus syndrome) may need to receive the treatment under general anesthesia.

Now that multiple approvals of several photosensitizers have been obtained, and PDT is finding its place in the armamentarium for malignant and benign diseases, the next phase of development of PDT will derive from understanding the mechanisms of how cells and tissues respond after treatment.

Overview of the case experience with PDT

Research on PDT at Case Western Reserve University began in the mid-1980s with collaborations among faculty in several
departments, most notably Chemistry, Dermatology, and Radiation Oncology. These early studies led to the synthesis and characterization of novel silicon phthalocyanines that proved to be strong, active photosensitizers in cell cultures and model tumors. Phthalocyanines are structurally related to porphyrins but with a larger macrocycle ring system, and hence they absorb longer (tissue-penetrating) wavelengths of light (Fig. 1). Of the phthalocyanines synthesized and characterized, Pc 4 was selected for intensive study and further development (Oleinick and Evans, 1998).

One of the first contributions of our group to uncovering the mechanisms of PDT was the demonstration that PDT was a strong inducer of apoptosis (Agarwal et al., 1991). Interestingly, at that time, the scientific community did not have much interest in or appreciation for the significance of apoptosis in tissue development or tumor therapy. However, this changed rapidly over the next few years following the discovery and recognition of one of the most important proteins controlling apoptosis, Bel-2 (Raffeld et al., 1987; Tsujimoto et al., 1985; Vaux et al., 1988). Bel-2 was soon revealed as the founding member of a family of pro- and anti-apoptotic proteins that are critical regulators of the primary pathways of apoptosis (Adams and Cory, 1998; Green and Reed, 1998). By the mid-1990s, it was apparent that the later steps in apoptosis that were responsible for generating morphological and biochemical hallmarks of apoptotic cells were activated by a series of proteases, now termed caspases (Thomberry and Lazebnik, 1998). Both the Bel-2 family proteins and certain caspases proved to be important in defining PDT-induced apoptosis (Oleinick et al., 2002).

The following brief excursion through our experience in studying how PDT kills cells will focus on (a) how we learned about the roles of Bel-2, its homologues, and caspases in apoptosis induced by Pc 4-PDT, (b) our current model for the initiation of apoptosis by Pc 4-sensitized PDT, and (c) how we are now beginning to apply this knowledge to the evaluation of human cancer treated with Pc 4-PDT.

From DNA damage to caspases

Our earliest exploration into the mechanism of PDT with phthalocyanine photosensitizers focused on DNA damage, in particular, the formation of DNA single-strand breaks and DNA–protein crosslinks. In one study, we exposed L929 mouse fibroblasts to an LD90 dose of ionizing radiation (i.e., a dose causing 90% reduction of clonogenic survival) and/or a sub-lethal dose of PDT. Radiation-killed cells remained attached to the culture dish and repaired the initially produced strand breaks in their DNA. In contrast, cells exposed to both agents became detached from the culture dish and showed increased DNA breakage 1 h after exposure. Therefore, we learned that a dose of PDT that was too low to kill cells on its own was sufficient to cause radiation-killed cells to follow a pathway of morphological and biochemical changes similar to those killed by PDT alone (i.e., the cells lifted off of the monolayer, and the DNA became increasingly fragmented). Although we had no other evidence at the time, we suggested that this mechanism might be apoptosis (Ramakrishnan et al., 1990).

Apoptosis is a physiological mode of cell death that is important in normal tissue development and remodeling (Fadeel and Orrenius, 2005). It can also be triggered by many different types of cell stresses, including cancer therapies. Apoptosis is characterized by a series of morphological and biochemical changes. Of the two major pathways for induction of apoptosis, those triggered internally (intrinsic) and externally (extrinsic), the former is most commonly observed following PDT of cells in culture (Fig. 2). Early steps in the intrinsic apoptosis pathway include the release of cytochrome c from mitochondria and the loss of mitochondrial membrane potential. This is followed by activation of a group of intracellular proteases called caspases, which are the primary effector molecules of apoptosis. Caspase-3 is one of the key mediators of apoptosis, and the most widely studied; therefore, active caspase-3 levels have been used as an indicator of apoptosis (Xue et al., 2001a, 2003a; Whitacre et al., 2002; Wu et al., 2006; Granville et al., 1997). Cleavage of proteins by caspases ultimately leads to chromatin condensation and the degradation of DNA into oligonucleosome-sized fragments. All of these steps have been documented in cells exposed to PDT in vitro (Oleinick et al., 2002).

To directly test for apoptosis, we sized the DNA of PDT-treated murine lymphoma L5178Y cells by agarose gel electrophoresis and found oligonucleosome-sized DNA fragments very early after PDT (Ramakrishnan et al., 1990). In a subsequent study, it was established that caspase activity was necessary for the DNA to be cleaved (Xue et al., 2001a; He et al., 1998). It is now clear that the major caspase activated in the cells we have studied is caspase-3. The activation process (a

Fig. 1. The structure of (a) the porphyrin ring (b) the phthalocyanine ring, and (c) Pc 4.
specific cleavage of the pro-enzyme form by another caspase, caspase-9), occurs in the apoptosome, a cytosolic complex formed from procaspase-9, procaspase-3, apoptosis-activating factor-1 (APAF-1), dATP, and cytochrome c released from damaged mitochondria (Fig. 2). Active caspase-3 cleaves numerous substrates, including the inhibitor of caspase-activated DNase (ICAD), releasing CAD to attack chromatin between nucleosomes. Another substrate of caspase-3 is the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP). The cleavage of PARP is also a useful indicator of apoptosis (He et al., 1998). The presence of DNA fragments in cells undergoing apoptosis is the basis of another commonly used assay for apoptosis, the TUNEL assay. The TUNEL assay has been used to demonstrate apoptosis in model murine tumors treated with Pc 4-PDT (Agarwal et al., 1996; Zaidi et al., 1993).

Very early in our studies, we became interested in identifying the critical step(s) that trigger apoptosis and/or commit the cell to die following Pc 4-PDT. One way to frame the question was to ask (a) what role does apoptosis play in the response of cells to PDT; and (b) what are the earliest biochemical reactions following the initial photodynamic process?

The role of caspases in PDT-induced apoptosis

Addressing part a, we tested the relevance of the caspase-3-dependent steps in the killing of human breast cancer MCF-7 cells (Xue et al., 2001a; Whitacre et al., 2002). MCF-7 cells have a deletion in the CASP-3 gene and do not express procaspase-3 at all. We obtained two derivative cell lines from Dr. C. Froehlich (Northwestern University) that were stably transfected with human procaspase-3 (MCF-7c3 cells) or the empty vector (MCF-7v cells). These lines were exposed to various doses of Pc 4 and light, and several known steps in the apoptosis pathway were measured.

We found that the early steps in apoptosis (loss of mitochondrial membrane potential and the release of cytochrome c from mitochondria into the cytosol) occurred identically in the two cell lines. In contrast, activation of caspases-9 and -3, cleavage of PARP, DNA fragmentation, and formation of morphologically apoptotic cells with condensed chromatin were observed only in the MCF-7c3 cells. As measured by tetrazolium dye reduction (a measure of the number and activity of mitochondria in the cell culture), MCF-7c3 cells were more sensitive to PDT than MCF-7v cells. However, when overall cell death was measured by a clonogenic assay, the two cell lines displayed the same PDT dose dependence. This study revealed that the activity of caspase-3 was important for the cells to carry out the late steps in apoptosis, but overall cell death was not dependent upon these steps (Xue et al., 2001a).

Bcl-2 family proteins in PDT-induced apoptosis

Addressing part b, we explored the role of Bcl-2 and its homologues in PDT-induced apoptosis to evaluate the earliest identifiable biochemical changes post-PDT. Bcl-2 is a 26-kDa protein that resides in the outer mitochondrial membrane and the endoplasmic reticulum (ER), anchored via a C-terminal transmembrane (TM) domain. Bcl-2 contains four BH (Bcl-2 homology) domains (BH1-BH4). The other members of the family share one or more of these domains. We (Xue et al., 2001b) and the Kessel laboratory (Kim et al., 1999) found that PDT with a variety of photosensitizers resulted in the immediate loss of Bcl-2, as observed by western blot analysis of cellular protein. We subsequently demonstrated that Bcl-xL, a close homologue of Bcl-2, was also subject to “photodamage”, and this response involved a tight interaction of these proteins with adjacent membrane proteins and/or lipids, to form large complexes that migrate more slowly across the gel during electrophoresis relative to the native proteins (Xue et al., 2003a; Usuda et al., 2003a).

Although the precise chemistry of the Bcl-2/xL photoreaction is still unclear, several properties suggest its importance in the cellular response to PDT with Pc 4 and the other photosensitizers. First, Bcl-2 photodamage is an immediate photochemical response to photoactivation of Pc 4, occurring during photoirradiation, in cells kept on ice, and in the presence of inhibitors of a wide variety of proteases (Xue et al., 2001b).
Thus, the loss of Bcl-2 is not a result of downregulation or degradation by proteasomes or other cellular proteases. Second, the PDT dose dependence for photodamage closely follows that for cell killing, as defined by loss of clonogenicity (Xue et al., 2001b). Third, susceptibility of Bcl-2 to photodamage is dependent upon its transmembrane domain binding to mitochondria or ER and the presence of at least one of a pair of alpha-helices between the BH1 and BH2 domains of the protein (Usuda et al., 2003b). These structures (alpha-5 and alpha-6) are thought to form a secondary anchorage site to the membrane. Fourth, overexpression of Bcl-2 can protect cells from cell killing by PDT; in those cases, higher PDT doses eventually photodamage sufficient amounts of Bcl-2 to overcome the protection and lead to loss of clonogenicity (Usuda et al., 2003a).

As important as the photodamage to Bcl-2 and Bcl-xL appears to be, it is unlikely to be the sole determining factor in photocytotoxicity. Evidence has also accumulated for a role of certain pro-apoptotic members of the Bcl-2 family. Of these, Bax, Bak, and Bid have received the most attention in PDT studies (Oleinick et al., 2002). Bax is a cytosolic protein that translocates to mitochondria during apoptosis. There, it undergoes a conformational change and oligomerizes, resulting in the opening of the mitochondrial permeability transition pore complex to allow the release of cytochrome c and other pro-apoptotic factors. Bak, a close homologue of Bax, resides in the mitochondrial outer membrane but otherwise appears to carry out the same step in apoptosis as does Bax. Bid is a cytosolic protein that is cleaved during apoptosis to generate a truncated protein, tBid, which then translocates to mitochondria and promotes the activation of Bax and Bak.

Kessel and co-workers transfected Bcl-2 into human breast epithelial MCF-10A cells and found that cells in which Bcl-2 protein was overexpressed also contained increased levels of Bax. When these cells were exposed to PDT, Bcl-2 levels were decreased through photodamage, resulting in a greatly elevated ratio of Bax to Bcl-2 and greater cell killing than in the untransfected cells (Kim et al., 1999). Upregulation of Bax upon overexpression of Bcl-2 does not occur in any of the human cancer cell lines we have studied (Xue et al., 2003b); however, some of these lines (the human prostate cancer DU145 and human colon cancer HCT-116 lines) are naturally deficient in Bax (Chiu et al., 2003, 2005). We have addressed the role of Bak in PDT-induced apoptosis using the above Bax-negative cells, comparing them to corresponding Bax-positive cells (Chiu et al., 2003, 2005). In both cases, the critical lethal lesion appeared to occur upstream of the step in apoptosis controlled by Bax.

However, because all of these cell lines express Bak, it remained possible that Bak was responsible for susceptibility to photocytotoxicity when Bax was absent. To address the role of Bak, we have recently turned to murine embryonic fibroblasts, obtaining four cell lines expressing both Bax and Bak, Bax only, Bak only, or neither protein. PDT-induced apoptosis was found in the two lines expressing Bax but in neither line that was missing Bax, indicating that Bax is necessary for apoptosis after PDT and cannot be replaced by Bak (unpublished observations). However, all of the cell lines were similarly photosensitive when cell killing was evaluated by tetrazolium dye reduction (embryonic fibroblasts cannot be evaluated by clonogenic assay).

Our observations indicate that cells die after Pc 4-PDT whether or not they express Bax and Bak, whether or not they express caspase-3, and whether or not they are competent for apoptosis. Thus, the commitment to cell death following Pc 4-PDT occurs at an early step in the process. However, in cells with all of the components needed for apoptosis, the morphological and biochemical changes of apoptosis are very convenient for evaluating the response to PDT (Fig. 2).

Another anti-apoptotic Bcl-2 homologue, Mcl-1, reveals a different response to Pc 4-PDT. Although it has significant homology to Bcl-2 and Bcl-xL in the domains important for photodamage, Mcl-1 is not photodamaged. However, we found that it is proteolytically degraded in lymphoid cells undergoing apoptosis but not in human A431 keratinocytes (Xue et al., 2005). The degradation of Mcl-1 occurs in Jurkat and U937 cells and is dependent on caspase activity, as revealed by its inhibition by a broad-spectrum caspase inhibitor. The response of Mcl-1 is thus an indicator of lymphoid cells undergoing apoptosis. We are currently investigating whether Mcl-1 degradation can serve as an indicator of the response of malignant and/or normal T-cells to PDT after treatment of cutaneous T-cell lymphoma.

**Signaling pathways and apoptosis in response to Pc 4-PDT**

PDT has been found to upregulate numerous signaling pathways (Oleinick and Evans, 1998; Moor, 2000). For example, stress kinases such as SAPK/JNK and p38/HOG are strongly activated by PDT and may help to promote apoptosis (Xue et al., 1999). When Hasan Mukhtar was studying Pc 4-PDT at Case, he made several notable contributions to our understanding, including demonstrating the presence of apoptosis within PDT-treated murine tumors (Agarwal et al., 1996; Whitacre et al., 2000; Colussi et al., 1999; Zaidi et al., 1993). One focus of the research on Pc 4-PDT in the Mukhtar laboratory at Case was on the potential roles of cell-surface death receptors and cell cycle checkpoints. In A431 epidermoid carcinoma cells, they observed increased expression of Fas within the first hour after Pc 4-PDT followed by emergence of Fas in the culture medium (Ahmad et al., 2000). They also found multimerization of Fas within the cells, an increase in FasL and FADD expression, the interaction of FasL with FADD, and 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 h post-PDT. These data all suggested that activation of the extrinsic FAS-mediated pathway of apoptosis could augment cell death through the intrinsic pathway.

During the progression of A431 cells into apoptosis, an increase in cells in the G0-G1 phase at the expense of cells in S phase was noted (Ahmad et al., 1998). These changes were preceded by an increase in p21/WAF1/CIP1 and downregulation...
of cyclins D1 and E and cyclin-dependent kinases cdk2 and 6. Thus, it appeared that Pc 4-PDT-treated A431 cells experienced a delay in passing the G1/S cell cycle checkpoint. Exploring subsequent steps in regulating that checkpoint, Ahmad et al. found a decrease in hyperphosphorylated pRb and down-regulation of five members of the E2F family of transcription factors and their heterodimeric partners DP1 and DP2 (Ahmad et al., 1999). The timing of the responses was consistent with a delay at the G1 checkpoint prior to entering the final stages of apoptosis.

The possible role of cardiolipin in cell killing by Pc 4-PDT

Returning to our earlier question concerning the immediate critical reaction following PDT that commits cells to apoptosis (or cell death by another mechanism), we now describe some recent observations implicating mitochondrial lipids as key mediators of the initial photodynamic damage. In an attempt to use the high-affinity ligand of cardiolipin, nonyl-acridine orange (NAO), as a probe of cardiolipin oxidation, we were surprised to observe that NAO could undergo fluorescence resonance energy transfer (FRET) with Pc 4 (Morris et al., 2003). The observation of FRET from NAO to Pc 4 indicates that these two molecules must reside in mitochondria very close to one another (calculated as 7 nm). Because NAO binds specifically to cardiolipin, if Pc 4 is close to NAO, it must also be very close to cardiolipin. This phospholipid contains highly unsaturated fatty acids and is found only in the mitochondrial inner membrane and at the contact sites between the inner and outer membranes. Therefore, cardiolipin may be one of the immediate targets of singlet oxygen generated from photoactivation of Pc 4. In fact, Kriska et al. (2005) have demonstrated that cardiolipin, as well as other membrane phospholipids, can be oxidized by PDT sensitized by protoporphyrin IX.

What makes cardiolipin such an intriguing target is that it plays an important role in the anchoring of the respiratory complexes and cytochrome c to the mitochondrial inner membrane. When cardiolipin is oxidized, cytochrome c is released into the intermembrane space. It was recently reported that cytochrome c can catalyze the oxidation of cardiolipin to set itself free of the inner membrane (Kagan et al., 2005). Thus, we hypothesize that a (the?) critical lethal event caused by Pc 4-PDT is the oxidation of the cytochrome c–cardiolipin complexes on the inner mitochondrial membrane. We are currently testing this model (Fig. 3).

Clinical development of Pc 4 in photodynamic therapy at Case Western Reserve University and our translational mechanistic studies

While the mechanistic details of cell killing by Pc 4-PDT were being elucidated, steps were taken to bring Pc 4 into clinical studies. With the help of the National Cancer Institute’s (NCI) Drug Decision Network, pre-clinical pharmacokinetic, toxicity, and efficacy studies were carried out. Following these studies, the NCI made available formulated GMP (Good Manufacturing Practices)-grade Pc 4 for use in phase I clinical trials. Subsequently an Investigational New Drug Application was approved and a phase I trial opened at the Case Comprehensive Cancer Center for treatment of cutaneous cancers.

Fig. 3. Mitochondrial targets of Pc 4-PDT. Pc 4 may bind to both the outer and inner mitochondrial membranes. Colocalization of Pc 4 and NAO probably occurs within proximity of cardiolipin and key components of the mitochondrial inner membrane. Oxidation of cardiolipin may result in release of cytochrome c through opening of the permeability transition pore complex formed by the voltage-dependent anion channel (VDAC) and the adenine-nucleotide translocator (ANT). Photodamage to Bcl-2 may be an independent event or secondary to lipid oxidation.
Intravenous Pc 4-PDT trial

This first phase I trial of Pc 4-PDT involved intravenous administration of the photosensitizer followed 24 h later by laser irradiation of the involved skin sites. Preliminary findings revealed that the drug was well tolerated and early in the dose escalation of the trial, one patient with cutaneous T-cell lymphoma (CTCL) had a partial response to Pc 4-PDT. However, because accrual to this trial proved difficult, we explored the possibility of delivering Pc 4 topically to human skin lesions. Accordingly, we tested a variety of vehicles (e.g., olive oil, acetone, DMSO, ethanol, propylene glycol) for topical application of Pc 4 and found a combination that was capable of delivering the photosensitizer through the stratum corneum. Our data using human skin keratome biopsies showed that formulation of Pc 4 at concentrations ranging from 0.01 mg/mL to 0.1 mg/mL in an ethanol/propylene glycol vehicle permitted penetration of Pc 4 at least through the basal layer of the epidermis within 1 h of application (Swick et al., 2004).

Topical Pc 4-PDT trial

With that information in hand, a phase I clinical trial of topically applied Pc 4 to treat cutaneous malignancies was approved by the FDA, the Institutional Review Board of University Hospitals of Cleveland, and the Case Comprehensive Cancer Center. This trial is still accruing patients, and results will be reported elsewhere once it is complete. However, some patients have consented to provide biopsies for additional translational mechanistic studies. The availability of these biopsies has allowed us to investigate a variety of assays of PDT response, including those for apoptosis, in order to choose one or more that can be applied reliably to human skin biopsies in subsequent trials as a surrogate marker of activity. We hope to test the hypothesis that selective apoptosis of the target malignant cell population correlates with clinical improvement.

Because our pre-clinical results had indicated that T-cells appear to be more susceptible to Pc 4-PDT-induced apoptosis than were keratinocytes (Swick et al., 2004), we reasoned that cutaneous T-cell lymphoma (CTCL), mycosis fungoides (MF) type, might be an appropriate malignancy to target. Our reasoning centered around epidermotropism, an elevated concentration of malignant T-cells in the epidermis, which is a cardinal diagnostic feature of MF-type CTCL. The MF type is the most common form of CTCL and accounts for almost 50% of all primary cutaneous lymphomas (Kim et al., 1999; Willemze et al., 2005; Willemze and Meijer, 2006). Our center has an active multidisciplinary cutaneous lymphoma program, providing access to a well-characterized cohort of patients. Thus, patients with early-stage MF were included in the translational methodological study. These patients were enrolled in the topical Pc 4-PDT phase I clinical trial currently underway at University Hospitals of Cleveland. Early-stage disease was considered to include TNM classification IA, IB, and IIA. The patients were concurrently not using any other type of systemic or topical medication for their disease.

Patients were evaluated clinically pre-treatment, and three areas of involved skin were chosen for treatment (maximum 5 cm × 5 cm each area). These lesions were measured clinically by a graded 5-point scale, via chromometer, via ultrasound, and by global evaluation. Additional lesions were also evaluated in the same manner and served as control untreated lesions. Treated lesions were selected for biopsy along with untreated lesions that were matched both anatomically and morphologically.

The Pc 4 (NSC 676418) drug supply utilized in this study was provided by NCI/CTEP. An amount of the formulated Pc 4 sufficient to cover the skin area to be treated (approximately 10 μL/cm²) was pipetted onto the lesion surface, and applied by an investigator via a finger cot. Because this is a phase I study, the Pc 4 dose applied (0.01, 0.05, or 0.10 mg/mL) and light fluence were escalated sequentially in groups of three patients each. If no toxicity was noted at the lowest drug and light doses, the light dose was escalated in the next three patients until 100 J/cm² was reached, and then the drug dose was escalated while returning to the lowest light dose again. This will be repeated until a maximally toxic dose (MTD) is reached.

The treated areas were covered with plastic wrap and an opaque dressing for 1 h. After removal of the dressing, visible light of 675 nm was delivered (50–100 J/cm²; 100 mW/cm²) from an Applied Optronics diode laser (AOC Medical Systems, South Plainfield, NJ) coupled to a fiber optic cable terminating in a microlens. The entire Pc 4-treated area was irradiated. Twenty-four hours after treatment, both the treated and selected untreated (control) lesions of patients were evaluated using the same criteria, and two 6-mm biopsies were taken, one from a treated lesion, and one from an adjacent untreated lesion. The specimens were fixed in formalin for routine H&E and immunohistochemical staining. Based on our previous experience in detecting apoptotic T-cells in psoriatic skin induced by broad-band, narrow-band, or monochromatic UVB-radiation, we focused on TUNEL and caspase staining. TUNEL staining resulted in excessive background signal, whereas anti-active caspase-3 immunohistochemistry shows promise in this application.

The fixed biopsies were embedded in paraffin, and serially cut into 4-μm sections. The sections were mounted onto poly-L-lysine-coated slides and deparaffinized. After epitope retrieval and blocking, the sections were incubated with polyclonal anti-active caspase-3 antibody (dilution 1:250; Cayman Chemical, Ann Arbor, MI), followed by biotinylated anti-rabbit immunoglobulin, and then Vectastain ABC (avidin–biotin–peroxidase complex) (Vector Laboratories, Burlingame, CA). Areas positive for caspase-3 induction stained brown after development with diaminobenzidine. Slides were counterstained with Mayer’s Hematoxylin (Sigma-Aldrich, St. Louis, MO), rinsed with distilled water, allowed to dry, and then mounted for viewing purposes. Five 20× brightfield images were taken from each section using an AxioCam HR digital camera and AxioPhot microscope (Carl Zeiss, Germany).

To validate this technique, we preliminarily examined biopsies from a patient with a clear clinical response, and...
from a patient without a clinical response. All sections revealed some brown-stained areas that were considered caspase-3-positive against the blue-purple hematoxylin staining. Sample images from both treated and untreated lesions are shown in Fig. 4. The caspase-3 positivity of the untreated lesions was similar for the responding and non-responding patient. By contrast, for both patients, the treated lesions showed greater caspase-3 positivity than did the matched untreated lesions. Of interest, however, was that the increase in caspase-3 positivity was markedly greater in the sections from the therapy-responsive patient’s lesion (Fig. 4). The images are consistent with an increase in apoptosis in responding lesions, and serve to validate the use of anti-active caspase-3 for further testing in the clinical trial as a biomarker of PDT activity in vivo, and hopefully, as an early surrogate marker of clinical response.

In regard to clinical response, our preliminary experience is promising, although the trial is only partially complete. Of the 30 patients treated thus far in the phase I trial of topical Pc 4-PDT, no local or systemic toxicities have been reported. Even though only a single treatment was delivered to each lesion in this study, a subset of patients responded to therapy showing improvement in the Pc 4-PDT-treated lesions, as evidenced by thinning and decreased erythema. Both the responders and non-responders tolerated the treatment well, with no side effects or pain reported. We noted that the responders consistently reported a minor tingling sensation during laser irradiation.

Discussion and conclusions

Topical application is a convenient and safe method of specifically directing photosensitizers to accessible lesions, avoiding the widespread distribution that occurs following intravenous administration. This concept has been well studied for the administration of the porphyrin prodrugs, ALA and its esters. However, until now, little attention has been given to the delivery of active photosensitizers after the apparent failure of studies with porphyrins (Brown et al., 2004). Our clinical study suggests that topically applied Pc 4 can be efficacious for CTCL and perhaps other dermal malignancies, such as basal cell carcinoma, as well as non-malignant conditions of the skin, such as psoriasis. In the appropriate vehicle, Pc 4 may also be efficiently delivered into certain other accessible cancers, such as those of the head-and-neck, esophagus, or lung.

As mentioned in Background: photodynamic therapy, PDT has certain advantages over surgery for the removal of skin cancers, including focusing the damage on the tumor cells rather than on the collagen matrix; the presence of undisturbed matrix promotes re-epithelialization of the area with less scarring than produced by surgery (Triesscheijn et al., 2006). In addition to being much less invasive than surgery, PDT with a topically applied photosensitizer in our protocol is less intrusive in a patient’s life, less painful, and has a shorter recovery time. An important advantage of PDT over other cancer treatments is the ability to attack tumors through direct damage to the malignant

Fig. 4. Immunohistochemical identification of cells with caspase-3 protein. Biopsy sections are stained blue-purple with hematoxylin and brown for caspase-3. Four sections are shown: (a) untreated non-responder, (b) treated non-responder, (c) untreated responder, and (d) treated responder.
cells, damage to the tumor vasculature that secondarily kills malignant cells, and promotion of inflammatory and immune responses that can eliminate malignant cells missed by the first two mechanisms (Castano et al., 2006). In fact, PDT is being studied as a means to generate anti-tumor immunity by a vaccine-type mechanism (Gollnick et al., 2002; Korbelik, 2006). The ease of administration and potential efficacy of PDT with topically applied P4 makes this modality attractive for treatment of skin malignancies and for evaluating the importance of all three types of mechanism.

Apoptosis is commonly observed in cells in vitro as well as in many animal tumor models exposed to PDT with numerous photosensitizers. Although caspase-3 activity is not essential for cell death after PDT, it is activated in most human cancer cells and is a very useful measure of the induction of apoptosis. There is little information and very few studies on the incidence or relevance of apoptosis to human tumors responding to PDT (Oleinick et al., 2002; Lilge et al., 2000). Although PDT has been found to be beneficial in treating cancers and other skin conditions (Oleinick et al., 2002; Lui et al., 2004; Bissonnette et al., 2002), there is a lack of studies evaluating the mechanism behind improvement in skin lesions following PDT. It is also not known how well the incidence of apoptosis correlates with the response to PDT of patients with skin cancer. The preliminary results of our phase I clinical trial have been positive, and suggest that apoptosis may correlate with clinical improvement following P4-PDT.

Acknowledgments

Research on PDT in our laboratories has been supported by the following NIH grants: R01 CA83917 (to NLO), R01 CA-106491 (to NLO), P01 CA48735 (to NLO), P30 CA43703, AR-39750 (to KDC), T32 AR-007569 (to KDC), R01 AR-051498 (to KDC).

References


