Photochemistry and Photobiology, 2008, 84: 407–414

Apoptosis Mechanisms Related to the Increased Sensitivity of Jurkat T-cells *vs* A431 Epidermoid Cells to Photodynamic Therapy with the Phthalocyanine Pc 4†‡

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Received 27 October 2007, accepted 4 December 2007, DOI: 10.1111/j.1751-1097.2007.00278.x

ABSTRACT

To examine the clinical applicability of Pc 4, a promising second-generation photosensitizer, for the photodynamic treatment of lymphocyte-mediated skin diseases, we studied the A431 and Jurkat cell lines, commonly used as surrogates for human keratinocyte-derived carcinomas and lymphocytes, respectively. As revealed by ethyl acetate extraction and absorption spectrophotometry, uptake of Pc 4 into the two cell lines was linear with Pc 4 concentration and similar on a per cell basis but greater in Jurkat cells on a per mass basis. Flow cytometry showed that uptake was linear at low doses; variations in the dose–response for uptake measured by fluorescence supported differential aggregation of Pc 4 in the two cell types. As detected by confocal microscopy, Pc 4 localized to mitochondria and endoplasmic reticulum in both cell lines. Jurkat cells were much more sensitive to the lethal effects of phthalocyanine photodynamic therapy (Pc 4-PDT) than were A431 cells, as measured by a tetrazolium dye reduction assay, and more readily underwent morphological apoptosis. In a search for molecular factors to explain the greater photosensitivity of Jurkat cells, the fate of important Bcl-2 family members was monitored. Jurkat cells were more sensitive to the induction of immediate photodamage to Bcl-2, but the difference was insufficient to account fully for their greater sensitivity. The antiapoptotic protein Mcl-1 was extensively cleaved in a dose- and caspase-dependent manner in Jurkat, but not in A431, cells exposed to Pc 4-PDT. Thus, the greater killing by Pc 4-PDT in Jurkat compared with A431 cells correlated with greater Bcl-2 photodamage and more strongly to the more extensive Mcl-1 degradation. Pc 4-PDT may offer therapeutic advantages in targeting inflammatory cells over normal keratinocytes in the treatment of T-cell-mediated skin diseases, such as cutaneous lymphomas, dermatitis, lichenoid tissue reactions and psoriasis, and it will be instructive to evaluate the role of Bcl-2 family proteins, especially Mcl-1, in the therapeutic response.

INTRODUCTION

Photodynamic therapy (PDT) has been gathering acclaim as an effective mode of treatment for a variety of diseases outside of those already accepted by the United States Food and Drug Administration (FDA). Approved for the treatment of esophageal and lung carcinomas in the United States, PDT has been evaluated to treat other neoplasms, including transitional cell carcinomas of the bladder, head and neck tumors and brain lesions as adjuvant therapy (1–3). Along with malignant neoplasms, PDT has been approved for premalignant (actinic keratosis and Barrett's esophagus) and nonmalignant (age-related macular degeneration) conditions, and tested for others, such as benign prostatic hypertrophy, atherosclerosis and arthritis (1,4–8). Results have been favorable, and it seems only a matter of time before approval for other treatments is gained. Approved in Canada for the prophylactic treatment of papillary bladder tumors in 1993, PDT has already been accepted in other countries for the treatment of a variety of diseases (1–3,5,9,10).

†This paper is part of a special issue dedicated to Professor Hasan Mukhtar on the occasion of his 60th birthday.
‡Presented in part at the Society for Investigative Dermatology, May 15–18, 2002, Los Angeles, CA, USA.
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As exposure to visible light is essential to photodynamic action, skin-related disorders, given their convenient exposure to any light source, are natural targets for PDT. Evaluation of PDT as a potential treatment for primary and secondary skin cancers, such as basal and squamous cell carcinomas, cutaneous T and B cell lymphoma, Kaposi's sarcoma and metastatic breast cancer are under way. Nonmalignant skin disorders have also been evaluated, from the treatment of virus-induced lesions to inflammatory disorders like psoriasis (5,11,12). The first FDA-approved photosensitizer to have shown promise in the treatment of psoriasis is Photofrin®. However, disadvantages inherent in this complex mixture of porphyrin oligomers preclude its practical use in treating patients. As Photofrin® tends to localize to the skin, prolonged cutaneous photosensitivity hinders patients from performing their activities of daily living for weeks after therapy. Photofrin also requires activation by wavelengths of light (~630 nm) suboptimal for skin penetration. Other alternative photosensitizers, such as protoporphyrin IX (PPIX) synthesized from topically applied aminolevulinic acid and other porphyrin-related macrocyles have been introduced in hopes of optimizing the therapeutic effect of PDT in the skin (3,13,14). The silicon phthalocyanine, Pc 4, has shown such promise.

Pc 4 has a defined structure, containing a dimethylaminopropyl side chain bound *via* a siloxy bridge in the axial position to a silicon phthalocyanine ring (Fig. 1). The interval of cutaneous photosensitivity in murine models exposed to Pc 4 was significantly less than in those exposed to Photofrin® (15). The far-red absorption peak of Pc 4 has a high extinction coefficient ($>2 \times 10^5$), allowing efficient light absorption. Lastly, activation of Pc 4 at 675 nm may allow for deeper tissue penetration of light than is possible with the 630 nm light used with Photofrin® or PPIX.

In cells, Pc 4 localizes to mitochondria and endoplasmic reticulum in preference to other membranes (16–18). Photoirradiation of Pc 4-loaded cells produces prompt photodamage to the antiapoptotic proteins Bcl-2 and Bcl-xL, loss of the mitochondrial membrane potential, release of cytochrome *c* into the cytosol, and induction of the intrinsic pathway of apoptosis in cells expressing all of the requisite factors (reviewed in 19,20).

Pc 4 has shown excellent treatment response in mouse skin tumors and is currently in a Phase I clinical trial of PDT for the treatment of cutaneous tumors (13,15). Its efficacy in the treatment of inflammatory disorders has not been explored. Other photosensitizers have been tested for their ability to treat psoriasis and certain other inflammatory disorders, with results suggesting good potential of PDT (21–24). In order to examine this clinical application for Pc 4-sensitized PDT, we chose two cell lines: A431 cells as an *in vitro* model for keratinocytes and Jurkat cells as a model for lymphocytes, positing that if Jurkat cells were more sensitive to Pc 4-PDT than were A431 cells, then there might be a favorable therapeutic window in the treatment of T-cell-mediated skin diseases with this modality. We compared these two cell lines with respect to the dose–response relationships for uptake of Pc 4, photocytotoxicity and sensitivity of the antiapoptotic proteins Bcl-2, Bcl-xL and Mcl-1 to loss by photodamage or proteolysis.

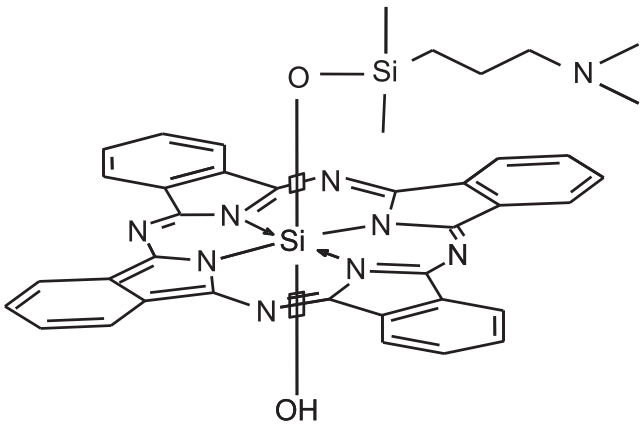

Figure 1. Structure of the silicon phthalocyanine Pc 4.

MATERIALS AND METHODS

Photosensitizer. The silicon phthalocyanine Pc 4 (25) (Fig. 1) was supplied by Dr. Malcolm E. Kenney (Department of Chemistry, Case Western Reserve University). A stock solution of 0.5 mM Pc 4 was prepared in dimethylformamide and stored in the dark at 4°C. Pc 4 was serially diluted in complete culture medium to attain final concentrations ranging from 0.1 to 3000 nM.

Cell culture. The human epidermoid carcinoma cell line A431 (American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium with high glucose and L-glutamine (Cellgro) and supplemented with 10% fetal bovine serum, penicillin (50 U/mL) and streptomycin (50 μg/mL) at 37°C in a 5% CO_2 humidified incubator. Seventy to ninety percent confluent cultures were used in all experiments. Suspension cultures of the human acute T-cell leukemia cell line Jurkat (American Type Culture Collection) were maintained in RPMI 1640 (Cellgro) medium supplemented with 10% fetal bovine serum, penicillin (50 U/mL) and streptomycin (50 μg/mL) at 37°C in a 5% CO_2 humidified incubator.

Extraction of Pc 4 and quantification by absorption spectrophotometry. A431 or Jurkat cells (2×10^6) were grown in 100 mm cell culture plates in 12 mL of their respective complete media and Pc 4 at varying concentrations (0–3000 nM) for 18 h in the dark at 37°C. For A431 cells, the medium was discarded, and the cells were trypsinized and collected in fresh medium. For Jurkat cells, suspensions were directly transferred to centrifuge tubes. Cells were immediately centrifuged at 1200 rpm for 6 min, and cell pellets were resuspended in 200 μL Hanks' Balanced Salt Solution (HBSS) and sonicated at low speed on ice for three cycles. Ethyl acetate was added to the lysates at a 3:1 ratio of solvent to HBSS, and the mixture was vortexed and centrifuged at 3000 rpm for 20 min at 4°C. The organic layers containing Pc 4 were collected for absorption spectrophotometry ($\epsilon = 2.3 \times 10^5$ at 670 nm).

Fluorescence of cell-bound Pc 4 measured by flow cytometry. A total of 6×10^5 A431 cells or 1×10^6 Jurkat cells were incubated in the dark at 37°C for 18 h in 6 mL complete medium with Pc 4 at various concentrations from 0 to 3000 nM. Cells were collected as above, and Pc 4 content was analyzed by flow cytometry (Elite Flow Cytometer, λ_{ex} = 334–364 nm with Innova 305 multiline output laser, λ_{em} = 675 nm with bandpass filter). The mean channel fluorescence (MCF) was obtained for 15 000 events per dose level. To minimize systematic variations in data, both cell lines were analyzed in each experiment.

Localization of Pc 4. A431 cells were plated at 2×10^5 cells per 35 mm glass-bottom coverslip dish, and Jurkat cells were plated at 6×10^5 cells per 60 mm tissue-culture dish. Following overnight growth, cells were exposed to 200 nM Pc 4 for 2 or 18 h and then with 75 nM MitoTracker Green (Molecular Probes, Eugene, OR) for 40 min. Cells were washed with PBS before imaging. Jurkat cells were concentrated by centrifugation and pipetted onto slides. Images were taken with a 63× NA 1.4 oil-immersion planapochromat objective on a Zeiss LSM 510 fluorescence confocal microscope. Excitation of Pc 4 was achieved with the 633 nm He–Ne2 laser, while emission was

collected with a 650 nm long-pass filter. Excitation of MitoTracker Green was with 488 nm light from an argon–krypton laser, and emission was collected with a 500–550 nm band-pass filter. Images shown are of cells exposed to Pc 4 for 18 h; the respective 2 h cells revealed identical Pc 4 localization.

Photodynamic treatment. 100 μL of A431 or Jurkat cells were seeded into separate 96-well microculture plates at 1.2×10^4 cells/well or 4×10^4 cells/well, respectively. After 18 h, Pc 4 concentrations ranging from 0 to 3000 nM were added to the extracellular medium, and after allowing 2 h for Pc 4 uptake, the plate was irradiated with an EFOS light-emitting diode array (EFOS, Mississauga, ON, Canada) delivering red light (λ_{max} = 675 nm). All irradiations were performed at room temperature. Fluences are given in the figure legends.

Determination of cytotoxicity. Following irradiation, the cells were incubated for an additional 24 h at 37°C. Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated in 50 μL freshly prepared MTT solution for 4 h. The formazan crystals formed by viable cells were dissolved in dimethyl sulfoxide, and the absorbance at 540 nm was measured. Cytotoxicity was determined with reference to untreated control cultures. The LD_{50} was defined as the concentration of Pc 4 that reduced formazan formation to 50% of the control value for a constant light fluence. All of the data were then combined. Multiple regression and analysis of covariance were used to compare regression lines of Ln(LD_{50}) *vs* Ln(Fluence) for the A431 and Jurkat cell lines, in order to compare LD_{50} levels controlling for fluence.

Quantification of apoptotic cells. A431 cells were plated in 60 mm tissue-culture plates containing glass coverslips at 2×10^5 cells/dish and allowed to grow overnight. Jurkat cells were seeded at 6×10^5 cells/25 cm^2 flask and grown overnight in complete medium. After a 2 h incubation of Pc 4 in fresh growth medium at the indicated concentrations, cells were irradiated with red light (200 mJ/cm^2). Cells were harvested at the indicated times by removing coverslips from the A431 cultures or removing an aliquot of the Jurkat cell suspension. Cells were fixed in 3.7% formaldehyde for 30 min at room temperature. Hoechst 33342 (Molecular Probes) was diluted in 0.2% Triton X-100 in PBS to a final concentration of 5 μg/mL and used to stain nuclei for 30 min at room temperature. Slides were prepared and viewed in a fluorescence microscope. Cells were scored as apoptotic based on their morphology (chromatin condensation, margination and fragmentation).

Evaluation of photodamage to or cleavage of Bcl-2 family proteins. After photoirradiation, some cultures were further incubated at 37°C for up to 23 h, whereas others were harvested immediately. Cells were lysed and sonicated as described previously (26). An equal volume of 2x SDS sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% mercaptoethanol and 20% glycerol) was added to the whole cell lysate. Equivalent amounts of protein were loaded onto polyacrylamide gels, subjected to electrophoresis, transferred to a polyvinylidene fluoride membrane and incubated with the following antibodies: monoclonal hamster anti-human Bcl-2 (6C8) from PharMingen (San Diego, CA, Cat. No. 15131A; used at 1:1000 dilution), polyclonal anti-Bcl-xL affinity-purified antipeptide antiserum from Cell Signaling (Beverly, MA, Cat. No. 2762; used at 1:1000 dilution), anti-Mcl-1 (S-19, Santa Cruz Biotechnology, Santa Cruz, CA; used at 1:500 dilution), anti-Bax (N-20, Santa Cruz Biotechnology; used at 1:200 dilution), or antiactin from NeoMarkers (Fremont, CA, Cat. No. MS-1295; used at 1:1000 dilution). For secondary antibodies, goat antihamster antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA, Code No. 127-035-099) and antirabbit antibody was from Calbiochem (San Diego, CA, Cat. No. 401352). The immune complexes were detected by ECL system (Amersham, Arlington Heights, IL).

RESULTS

Uptake of Pc 4 into A431 and Jurkat cells

To evaluate the relative ability of the two cell types to take up Pc 4, cells were incubated with the photosensitizer for 18 h and then collected. Pc 4 in the cell pellet was extracted into ethyl acetate and quantified by absorption spectrophotometry. This analysis revealed a dose-dependent increase in uptake by both cell lines (Fig. 2a). Pc 4 uptake per cell was similar for A431 and Jurkat cells in the dose range from 100 to 3000 nM. For concentrations less than 100 nM, the method was not

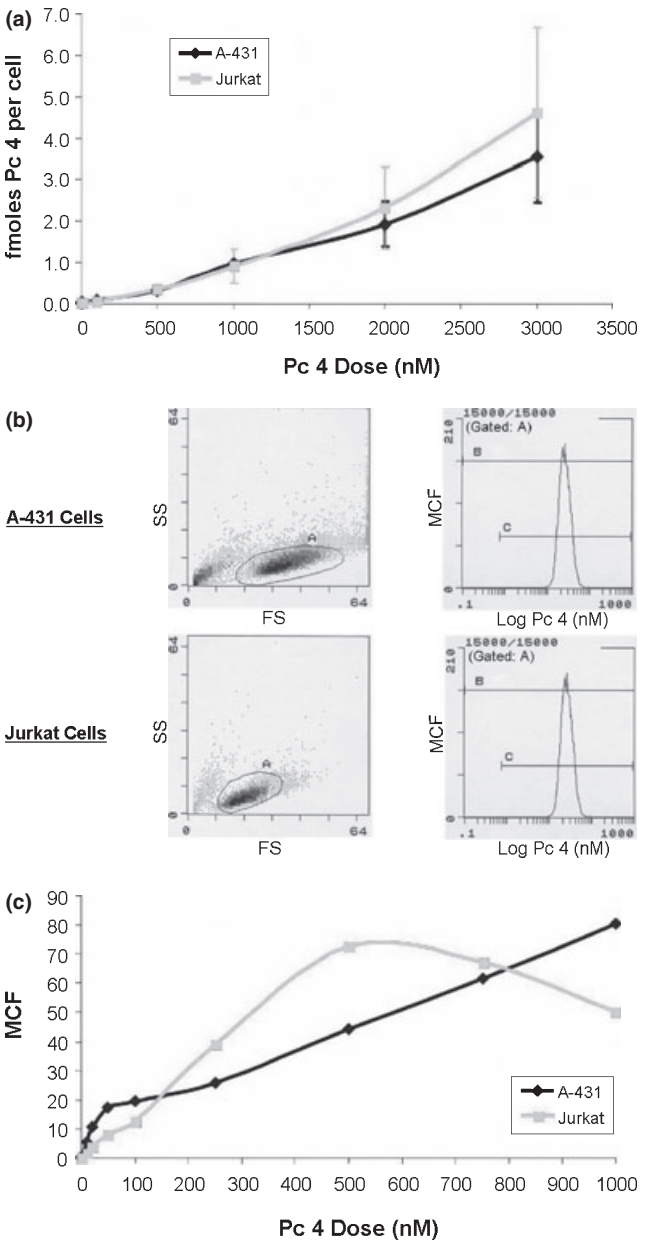

Figure 2. Uptake of Pc 4 into A431 and Jurkat cells. (a) Measurement of cellular content of Pc 4 by extraction in ethyl acetate followed by quantification by absorption spectrophotometry. Cells were incubated with the indicated concentrations of Pc 4, collected and counted. Cell-bound Pc 4 was extracted into ethyl acetate and quantified by its absorbance. Data are the mean of 2 (for 100 and 3000 nM) or 3 (for 500, 1000 and 2000 nM) independent determinations; error bars indicate the range or standard deviation, respectively. Where no error bars appear, they were smaller than the symbol. (b) Representative flow cytometry histograms of A431 and Jurkat cells after exposure to 250 nM Pc 4. Mean channel fluorescence (MCF) was measured in the gated populations representing single, viable A431 or Jurkat cells, respectively. (c) Flow cytometric analysis of Pc 4 uptake measured as the MCF for each cell type exposed to a series of Pc 4 concentrations. Data are from a single experiment that was repeated twice more with similar results.

sufficiently sensitive to detect significant amounts of Pc 4 in the cells.

Using flow cytometry, Pc 4 fluorescence was measured in each cell of the gated population and displayed as a histogram of 15 000 events (Fig. 2b), expressed as the MCF. Pc 4 uptake measured by this method revealed reproducible dose-dependent differences between the two cell lines (Fig. 2c). At low to moderate doses of Pc 4 (1–100 nM), uptake appeared greater in A431 cells than in Jurkat cells. At higher Pc 4 doses (250–750 nM), the Jurkat cells were observed to have a higher MCF than the A431 cells. However, at doses of 1000–3000 nM, A431 cells once again displayed greater fluorescence than Jurkat cells.

In a test of the stability of the uptake data, it was found that measurements at 2 and at 19 h after addition of Pc 4 to the culture medium did not differ by more than 5% for A431 cells and 20% for Jurkat cells. These data are in agreement with observations on Pc 4 uptake in other cells in which uptake reached a maximum in 1–2 h and did not change thereafter for the next 16–18 h (27). The average sizes of A431 and Jurkat cells were also measured against standard sizing beads. A431 cells were found to be approximately 23 μm in diameter, while Jurkat cells were approximately 1/3 smaller in diameter at 15 μm. Thus, assuming spherical cells, the average A431 cell is about 3.6 times larger in volume than the average Jurkat cell.

Differences in uptake values or photosensitivity might be explained if Pc 4 binds to different subcellular sites in the two cell lines. To test this possibility, A431 and Jurkat cells were exposed to Pc 4 for 2 or 18 h and viewed by confocal microscopy (Fig. 3). In both cell lines, the Pc 4 image partially colocalized with that of MitoTracker Green. Therefore, Pc 4 bound to mitochondria and other intracellular membranes of both cell lines, with no obvious accumulation on the plasma membrane or in the nucleus. The images of Fig. 3 are those obtained after 18 h in Pc 4, but essentially the same localization was observed after 2 h in Pc 4. These results are similar to those obtained in prior studies (16–18).

Cytotoxicity of PDT

After incubation of the two cell lines in varying doses of Pc 4, the cells were photoirradiated, and the efficiency of cell killing was monitored by the MTT assay. Although this assay may miss delayed deaths and overestimate repairable damage, it was chosen because both cell lines could be monitored, and it is likely to capture the majority of responses. Figure 4a shows a representative experiment comparing the two cell lines exposed to varying doses of Pc 4 and 200 mJ/cm^2 of red light. For each fluence tested, the Pc 4 dose producing 50% loss of formazan formation (LD_{50}) was determined. All of the cytotoxicity data are combined and presented in Fig. 4b. Throughout the range of Pc 4 concentrations and light fluences tested, Jurkat cells were found to be far more susceptible to Pc 4-PDT-induced cell killing than A431 cells (Fig. 4a and b). For example, at a low fluence of 50 mJ/cm^2, the average Pc 4 dose required to achieve an LD_{50} was 726 nM for A431 cells, but only 107 nM for Jurkat cells. Jurkat sensitivity was even greater at 200 mJ/cm^2, where A431 cells required an average Pc 4 dose of 294 nM, while the Jurkat cells required less than 10% of the dose at 23 nM. Overall, the regression lines (Fig. 4b) differed significantly ($P < 0.0001$); estimated mean LD_{50} values

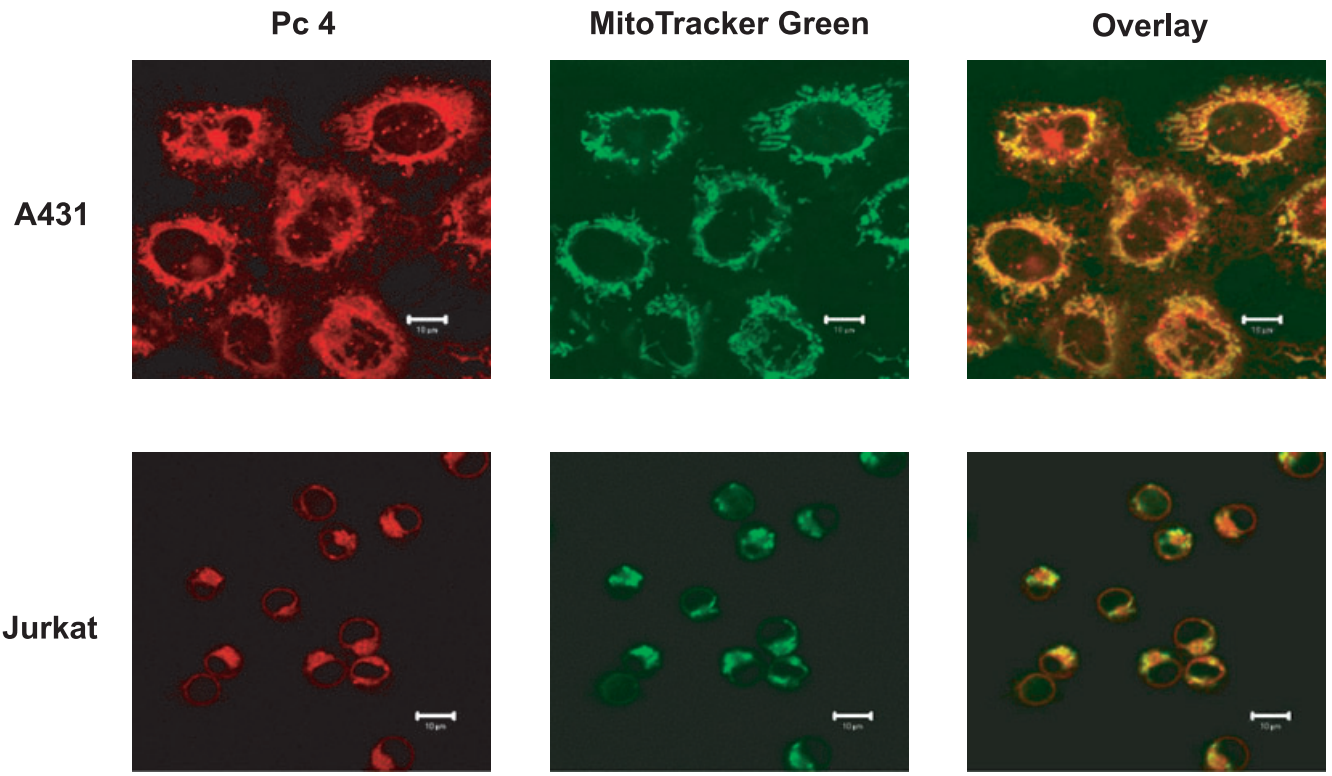

Figure 3. Subcellular localization of Pc 4 in A431 and Jurkat cells. Cells were incubated with 200 nM Pc 4 for 18 h and with MitoTracker Green for 30 min. Localization was imaged by confocal microscopy, as described in Materials and Methods. Yellow in the merged image indicates overlap of the two dyes. Cells loaded with Pc 4 for 2 h showed a localization pattern similar to the respective 18 h image (not shown).

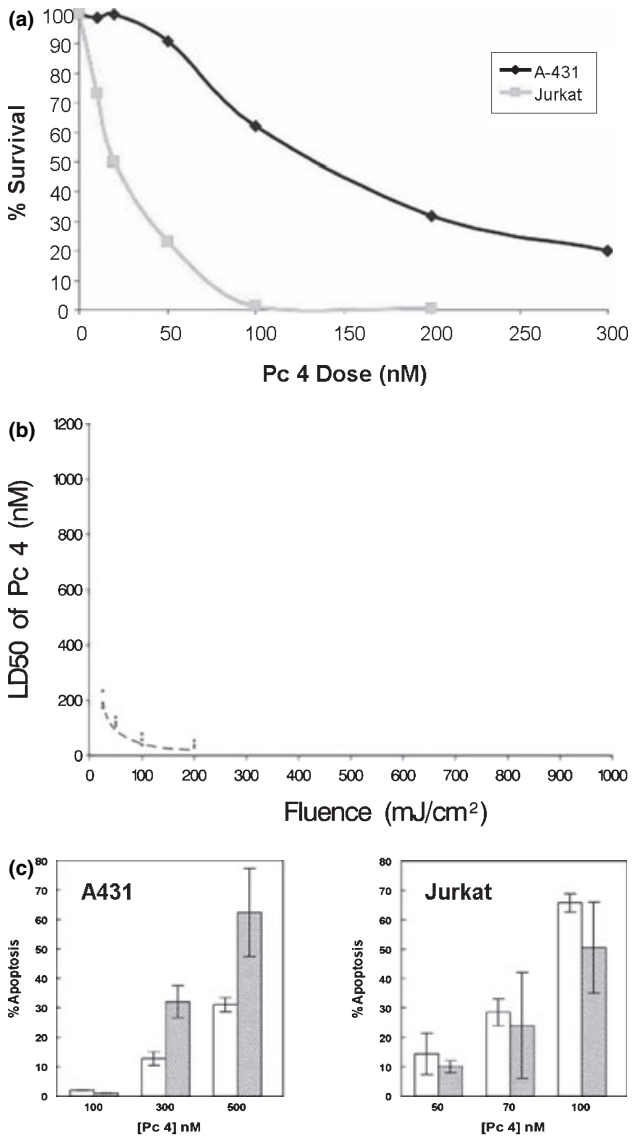

Figure 4. Sensitivity to Pc 4-PDT. (a) Example of a cell cytotoxicity study using the MTT assay. Cells of each line were plated in 96-well plates, exposed to a series of Pc 4 concentrations followed by red light at 200 mJ/cm^2, and returned to the 37°C incubator for 24 h. Cytotoxicity was assayed with MTT, and the LD_{50} determined (in this case, 20 nM for Jurkat cells and 140 nM for A431 cells). (b) Plot of LD_{50} of Pc 4 *vs* fluence, for experiments on A431 (diamonds) and Jurkat (squares) cell lines. Solid and dashed lines are least-squares regression lines obtained by regressing Ln(LD_{50}) *vs* Ln(Fluence) for A431 and Jurkat cells, respectively. (c) Quantification of Pc 4-PDT induced apoptosis. A431 cells (left) and Jurkat cells (right) were incubated with Pc 4-loaded growth medium for 2 h, followed by irradiation with 200 mJ/cm^2 red light. After further incubation for 4 (open bars) or 18 (hatched bars) hours, cells were fixed in 3.7% formaldehyde for 30 min at room temperature, followed by staining with Hoechst 33342 (Molecular Probes) for 30 min at room temperature. Slides were viewed under a fluorescence microscope. Cells displaying morphological apoptosis were quantified and normalized to untreated control cells. Data represent an average of two to three independent experiments ± standard error or range, except for A431 cells at 100 nM Pc 4, which are from single experiments.

obtained from the fitted lines differed between cell lines at fluences of 25 mJ/cm^2 ($P = 0.05$), 50 mJ/cm^2 ($P = 0.02$) and at 100 and 200 mJ/cm^2 ($P < 0.01$).

Apoptosis in A431 and Jurkat cells

Both cell lines can undergo apoptosis in response to Pc 4-PDT; however, the dose–response and time course differed for the lymphoid *vs* epithelial cells. In order to quantify the extent of apoptosis, cells were exposed to various concentrations of Pc 4 for 2 h followed by red light and monitored for apoptotic morphology at 4 and 18 h post-PDT by counting Hoechst 33342-stained cells by fluorescence microscopy. As shown in Fig. 4c, apoptotic nuclei were more abundant in Jurkat cells at lower doses than in A431 cells at both times, although with sufficient time and after a relatively high dose, A431 cells with apoptotic morphology were apparent. The sensitivity to apoptosis was similar to the sensitivity to overall cell death. As morphological apoptosis and loss of ability to reduce the MTT reagent may not occur simultaneously, it is not expected that the absolute values for the two measures would be identical in all cases. Still, it is clear that Jurkat cells are more sensitive to cell killing by both criteria.

Effects of Pc 4-PDT on Bcl-2 family proteins of A431 and Jurkat cells

We have found that the antiapoptotic proteins Bcl-2 and Bcl-xL are major phototargets of Pc 4-PDT in all human cancer cell lines tested (18,26,28). Photodamage is observed immediately upon photoirradiation of Pc 4-loaded cells and for cells irradiated in the cold, indicating that the effect is due to photochemical damage to Bcl-2 and Bcl-xL rather than to subsequent enzymatic action (18,26). The extent of photodamage roughly correlates with the sensitivity of the cells to Pc 4-PDT, providing evidence for the importance of the photodynamic loss of these two proteins in triggering cell death. Because of the observed difference in photocytotoxicity of A431 and Jurkat cells, it was of considerable interest to determine whether or not the photosensitivity of the antiapoptotic proteins also differed. As shown in Fig. 5a, both proteins could be photodamaged in both cell lines, as revealed by the loss of the bands representative of the native proteins on western blots. Photodamage appeared to occur at lower PDT doses in Jurkat cells than in A431 cells. Whereas for a fluence of 200 mJ/cm^2, photodamage to Bcl-2 was nearly complete at 200 nM Pc 4 in Jurkat cells, there was only partial loss of Bcl-2 by 300 nM Pc 4 in A431 cells, and some residual Bcl-2 remained in A431 cells until PDT with 500 nM Pc 4. Bcl-xL has been found to be less sensitive than Bcl-2 to photodamage in certain cell lines (28). Jurkat, but not A431, cells appear to fall into that category. Thus, in comparing these two cell lines, there is a better correlation between sensitivity to photodynamic cell killing and sensitivity to protein photodamage in the case of Bcl-2 than in the case of Bcl-xL. However, the differential sensitivity of Bcl-2 in the two cell lines (Fig. 5a) is not as great as the differential cytotoxicity of the lines (Fig. 4). Notably, the level of the proapoptotic protein Bax observed on the western blots was not affected by any of the tested PDT doses in either cell line.

We have recently reported that another antiapoptotic homolog of Bcl-2, Mcl-1, behaves differently from either Bcl-2 or Bcl-xL in response to Pc 4-PDT (29). We found no evidence of prompt photodamage to Mcl-1 in any of the cell lines studied, including A431 and Jurkat, in spite of the

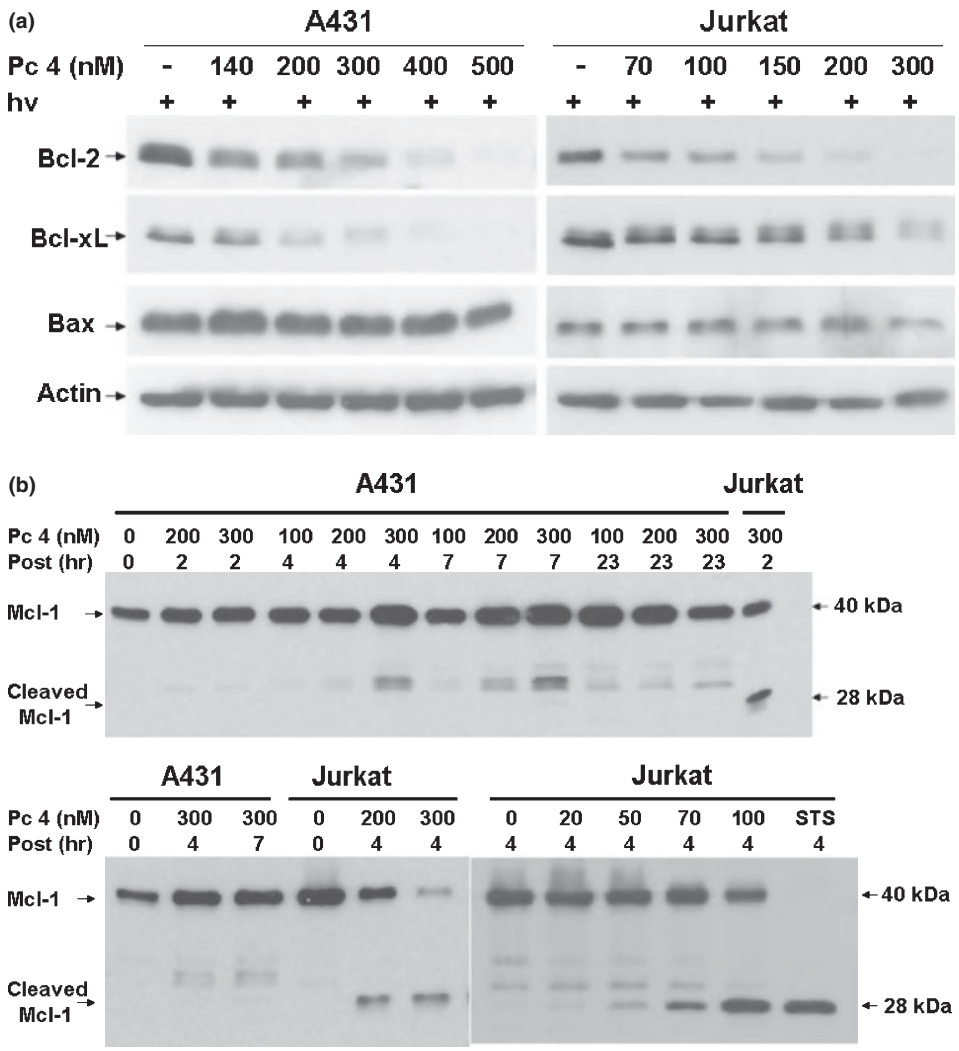

Figure 5. Fate of Bcl-2 family proteins following Pc 4-PDT. (a) Dose–response for loss of Bcl-2 and Bcl-xL in Jurkat and A431 cells. Cells were exposed to the indicated doses of Pc 4 and 200 mJ/cm^2 red light, then collected 10 min later. The solubilized proteins were subjected to electrophoresis and analysis by western blot for the presence of Bcl-2, Bcl-xL and Bax as well as actin as a loading control. (b) Dose–response and time course of changes in Mcl-1 levels and levels of a 28 kDa Mcl-1 cleavage fragment. Cells were exposed to the indicated doses of Pc 4 and 200 mJ/cm^2 red light, incubated for the indicated times after PDT, then collected. In one case, cells were exposed to staurosporine (STS, 1 μM) for 4 h as a positive control. The solubilized proteins were subjected to electrophoresis and analysis by western blot.

localization of this protein, at least in part, to the mitochondria and endoplasmic reticulum. However, in Jurkat cells, Mcl-1 was cleaved by caspases into a 28 kDa fragment during apoptosis induced by Pc 4-PDT. The cleavage of Mcl-1 occurred within 2–4 h of PDT, concomitantly with the activation of caspases-9, -8 and -3, and was blocked by the pan-caspase inhibitor z-VAD-fmk (29). It has been proposed that the 28 kDa Mcl-1 fragment is proapoptotic (30,31). In order to determine if that fragment was generated in A431 cells as efficiently as in Jurkat cells, the two cell lines were exposed to a range of doses of Pc 4-PDT and examined for Mcl-1 level by western blot analysis at various times thereafter (Fig. 5b). The early and extensive cleavage of Mcl-1 into the 28 kDa fragment was confirmed for Jurkat cells, as shown in the bottom panel of Fig. 5b. The dose–response for cleavage of Mcl-1 in Jurkat cells in the range of 20–100 nM Pc 4 + 200 mJ/cm^2 red light matches well with that for cytotoxicity, as observed in Fig. 4. In contrast, there was no evidence for generation of the same fragment in A431 cells at doses up to 300 nM Pc 4 + 200 mJ/cm^2 red light and at post-treatment times up to 23 h. Faint bands at intermediate sizes may represent limited cleavage of a small amount of Mcl-1 at different sites on the protein.

DISCUSSION

Pc 4-PDT has shown significant efficacy against murine tumors and human tumor cells growing as xenografts in athymic nude mice, and it is now in a clinical trial for the treatment of cutaneous malignancies (19). In the studies presented here, we demonstrate that the human lymphoid cell line, Jurkat, is 4–13 times more sensitive to killing by Pc 4-PDT than the human epidermoid carcinoma line, A431. These results suggest that Pc 4-PDT may have utility in the treatment of malignant and benign lymphocyte-mediated diseases, with relative preservation of epidermis.

Because one factor that can determine cell sensitivity to PDT is the extent of photosensitizer uptake, the comparative uptake of Pc 4 into the two cell types was evaluated. The extraction method provides a direct measure (by absorbance spectrophotometry) of overall Pc 4 levels in the cells, which appear to be approximately equal on a per cell basis. Because the method is not sensitive below about 100 nM Pc 4, uptake was also evaluated by flow cytometry. At the lowest concentrations studied, where distortions inherent in converting fluorescence in a biological environment to amount of fluorescing material were minimal, uptake was linear in both cell lines but appeared greater in A431 cells. At higher concentrations, likely aggregation of Pc 4 in cells produces deviations from a linear increase in fluorescence with concentration. MCF of the smaller Jurkat cells repeatedly revealed a leveling and even drop in apparent Pc 4 uptake at doses above 500 nM, while the MCF of the larger A431 cells appeared to steadily increase, not reaching a maximum until concentrations above 1 μM. The larger size of the A431 cells may have provided more independent binding sites for Pc 4 than the smaller Jurkat cells, reducing the tendency to aggregate. Aggregated photosensitizer would be expected to fluoresce poorly. In further considering the potential effect of the cell size, one would assume the amount of Pc 4 taken up per cell at a fixed dose would be greater in the larger A431 cells than in the smaller Jurkat cells. However, as shown by the extraction method, both cell lines displayed similar uptake per cell, implying greater uptake per cell mass in Jurkat cells (which are approximately 3.6 times smaller than A431 cells), a factor that may contribute to the greater sensitivity of Jurkat cells to PDT. Pc 4 was found to accumulate in mitochondria and endoplasmic reticulum in both cell lines (Fig. 3), so that at the level of resolution provided by confocal microscopy, differential localization of Pc 4 cannot account for the differential sensitivity of the cells.

The cell viability studies revealed Jurkat cells to be far more sensitive to PDT than A431 cells (Fig. 4a–c). This observation held true at all dose levels of light or Pc 4. For example, at fluences as low as 25 mJ/cm^2, the calculated Pc 4 dose to achieve an LD_{50} in A431 cells was 778 nM *vs* 186 nM Pc 4 in Jurkat cells. Alternatively, at a fixed Pc 4 dose of 100 nM, A431 cells required nearly a 15-fold greater light fluence than Jurkat cells to achieve an LD_{50}. Even considering the greater uptake of Pc 4 per cell mass for Jurkat *vs* A431 cells (3.6-fold) does not account for the far greater PDT sensitivity of Jurkat cells. Apoptosis also occurred more readily and at lower doses in Jurkat when compared with A431 cells (Fig. 4c). The greater killing effect in Jurkat *vs* A431 cells strongly suggests that lymphocytes are more sensitive to Pc 4-PDT than keratinocytes, and the greater sensitivity may prove effective in targeting inflammatory cells over normal keratinocytes in the treatment of T-cell-mediated diseases, such as cutaneous T-cell lymphoma or psoriasis.

We (18,26,28) and others (32,33) have identified the antiapoptotic protein Bcl-2 as a sensitive molecular target of PDT with certain photosensitizers that localize to mitochondria and endoplasmic reticulum. We found preferential localization of Pc 4 to those intracellular membrane systems in lymphoid (17) and epithelial-derived (16,18,34) tumor cells, including A431 and Jurkat cells (Fig. 3). Therefore, it was of interest to determine if the differential sensitivity of A431 and Jurkat cells to Pc 4-PDT was reflected in a similar differential response of Bcl-2 and its homolog Bcl-xL, which is also photodamaged by Pc 4-PDT (28). Figure 5a shows that Bcl-2 is lost at lower doses in Jurkat cells than in the more resistant A431 cells. Although the level of the proapoptotic protein Bax did not change with PDT dose, the ratio of Bax to native Bcl-2 increases with dose and may be one important factor promoting apoptosis (32). The data are consistent with a model wherein photodamage to Bcl-2 and Bcl-xL contribute to the greater sensitivity of Jurkat cells to the lethal effects of Pc 4-PDT by inducing cell death. However, the differences in protein photodamage (Fig. 5a) are not as large as those found for killing of the two cell types by Pc 4-PDT (Fig. 4). Thus, other factors likely contribute to the differential photosensitivity observed. One of those factors could be the pronounced ability of the antiapoptotic Bcl-2 homolog, Mcl-1, to be cleaved by caspases in Jurkat cells when compared with the absence of a 28 kDa cleavage product in A431 cells, even at doses and times where cell killing is manifest (Fig. 5b). The 28 kDa Mcl-1 fragment is thought to be proapoptotic (30,31), so that its early strong production in Jurkat cells may greatly promote the death of those cells following Pc 4-PDT. The relatively high levels of procaspases in T-cells and the ease with which they are activated may explain the differential responses of Mcl-1 in Jurkat *vs* A431 cells.

In summary, Jurkat cells, as exemplars of lymphoid cells, were more sensitive than A431 cells, as exemplars of epithelial cells, to Pc 4-PDT. The differences in photosensitizer uptake per cell mass may partially account for this greater sensitivity. We suggest that other important factors are the greater sensitivity of Bcl-2 to photodamage and the much greater sensitivity of Mcl-1 to PDT-induced proteolytic cleavage in Jurkat cells, inducing cell death at lower overall doses than in A431 cells. Regardless of the relative contributions of these mechanisms, these data show that Pc 4-PDT preferentially leads to lymphoid cell death, relative to epithelial cell death, providing a rationale for further evaluation in the treatment of T-cell-mediated skin disease.

Acknowledgements—The authors thank Michael Sramkoski and Megan Gottlieb for their knowledgeable, attentive assistance in the use of the Flow Cytometry Core Facility of the Case Comprehensive Cancer Center. This study was supported by NIH Grant 5P30AR39750 (NIAMS Skin Diseases Research Center Grant), NIH Grant KO8 ARO2063, VA Medical Service, and P01 CA48735 and P30 CA43703 from the US National Cancer Institute. M.S.K. was supported by a National Research Service Award.

REFERENCES

1. Dougherty, T. J. (2002) An update on photodynamic therapy applications. *J. Clin. Laser Med. Surg.* **20**, 3–7.
2. Dougherty, T. J., C. J. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan and Q. Peng (1998) Photodynamic therapy. *J. Natl Cancer Inst.* **90**, 889–905.
3. Oleinick, N. L. and H. H. Evans (1998) The photobiology of photodynamic therapy: Cellular targets and mechanisms. *Radiat. Res.* **150**, S146–S156.
4. Levy, J. G. (1994) Photosensitizers in photodynamic therapy. *Semin. Oncol.* **21**, 4–10.
5. Ibbotson, S. H. (2002) Topical 5-aminolaevulinic acid photodynamic therapy for the treatment of skin conditions other than nonmelanoma skin cancer. *Br. J. Dermatol.* **146**, 178–188.

6. Lang, K., P. Lehmann, K. Bolsen, T. Ruzicka and C. Fritsch (2001) Aminolevulinic acid: Pharmacological profile and clinical indication. *Expert Opin. Investig. Drugs* **10**, 1139–1156.
7. Szeimies, R. M., M. Landthaler and S. Karrer (2002) Non-oncologic indications for ALA-PDT. *J. Dermatolog. Treat.* **13** (Suppl. 1), S13–S18.
8. Shieh, S., A. S. Dee, R. T. Cheney, N. P. Frawley, N. C. Zeitouni and A. R. Oseroff (2002) Photodynamic therapy for the treatment of extramammary Paget's disease. *Br. J. Dermatol.* **146**, 1000–1005.
9. Hopper, C. (2000) Photodynamic therapy: A clinical reality in the treatment of cancer. *Lancet Oncol.* **1**, 212–219.
10. Sibata, C. H., V. C. Colussi, N. L. Oleinick and T. J. Kinsella (2000) Photodynamic therapy: A new concept in medical treatment. *Braz. J. Med. Biol. Res.* **33**, 869–880.
11. Calzavara-Pinton, P. G., R. M. Szeimies, B. Ortel and C. Zane (1996) Photodynamic therapy with systemic administration of photosensitizers in dermatology. *J. Photochem. Photobiol. B* **36**, 225–231.
12. Bissonnette, R., J. F. Tremblay, P. Juzenas, M. Boushira and H. Lui (2002) Systemic photodynamic therapy with aminolevulinic acid induces apoptosis in lesional T lymphocytes of psoriatic plaques. *J. Invest. Dermatol.* **119**, 77–83.
13. Gomer, C. J. (1991) Preclinical examination of first and second generation photosensitizers used in photodynamic therapy. *Photochem. Photobiol.* **54**, 1093–1107.
14. He, J., H. E. Larkin, Y. S. Li, D. Rihter, S. I. Zaidi, M. A. Rodgers, H. Mukhtar, M. E. Kenney and N. L. Oleinick (1997) The synthesis, photophysical and photobiological properties and in vitro structure-activity relationships of a set of silicon phthalocyanine PDT photosensitizers. *Photochem. Photobiol.* **65**, 581–586.
15. Anderson, C., S. Hrabovsky, Y. McKinley, K. Tubesing, H. P. Tang, R. Dunbar, H. Mukhtar and C. A. Elmets (1997) Phthalocyanine photodynamic therapy: Disparate effects of pharmacologic inhibitors on cutaneous photosensitivity and on tumor regression. *Photochem. Photobiol.* **65**, 895–901.
16. Lam, M., N. L. Oleinick and A. L. Nieminen (2001) Photodynamic therapy-induced apoptosis in epidermoid carcinoma cells. Reactive oxygen species and mitochondrial inner membrane permeabilization. *J. Biol. Chem.* **276**, 47379–47386.
17. Trivedi, N. S., H. W. Wang, A. L. Nieminen, N. L. Oleinick and J. A. Izatt (2000) Quantitative analysis of Pc 4 localization in mouse lymphoma (LY-R) cells via double-label confocal fluorescence microscopy. *Photochem. Photobiol.* **71**, 634–639.
18. Usuda, J., S. M. Chiu, E. S. Murphy, M. Lam, A. L. Nieminen and N. L. Oleinick (2003) Domain-dependent photodamage to Bcl-2. A membrane anchorage region is needed to form the target of phthalocyanine photosensitization. *J. Biol. Chem.* **278**, 2021–2029.
19. Miller, J. D., E. D. Baron, H. Scull, A. Hsia, J. C. Berlin, T. McCormick, V. Colussi, M. E. Kenney, K. D. Cooper and N. L. Oleinick (2007) Photodynamic therapy with the phthalocyanine photosensitizer Pc 4: The Case experience with preclinical mechanistic and early clinical-translational studies. *Toxicol. Appl. Pharmacol.* **224**, 290–299.
20. Oleinick, N. L., R. L. Morris and I. Belichenko (2002) The role of apoptosis in response to photodynamic therapy: What, where, why, and how. *Photochem. Photobiol. Sci.* **1**, 1–21.
21. Edstrom, D. W., A. Porwit and A. M. Ros (2001) Photodynamic therapy with topical 5-aminolevulinic acid for mycosis fungoides: Clinical and histological response. *Acta Derm. Venereol.* **81**, 184–188.
22. Hunt, D. W. and J. G. Levy (1998) Immunomodulatory aspects of photodynamic therapy. *Expert Opin. Investig. Drugs* **7**, 57–64.
23. Jiang, H., D. J. Granville, B. M. McManus, J. G. Levy and D. W. Hunt (1999) Selective depletion of a thymocyte subset in vitro with an immunomodulatory photosensitizer. *Clin. Immunol.* **91**, 178–187.
24. Jiang, H., D. J. Granville, J. R. North, A. M. Richter and D. W. Hunt (2002) Selective action of the photosensitizer QLT0074 on activated human T lymphocytes. *Photochem. Photobiol.* **76**, 224–231.
25. Oleinick, N. L., A. R. Antunez, M. E. Clay, B. D. Rihter and M. E. Kenney (1993) New phthalocyanine photosensitizers for photodynamic therapy. *Photochem. Photobiol.* **57**, 242–247.
26. Xue, L. Y., S. M. Chiu and N. L. Oleinick (2001) Photochemical destruction of the Bcl-2 oncoprotein during photodynamic therapy with the phthalocyanine photosensitizer Pc 4. *Oncogene* **20**, 3420–3427.
27. He, J., M. F. Horng, J. T. Deahl, N. L. Oleinick and H. H. Evans (1998) Variation in photodynamic efficacy during the cellular uptake of two phthalocyanine photosensitizers. *Photochem. Photobiol.* **67**, 720–728.
28. Xue, L. Y., S. M. Chiu, A. Fiebig, D. W. Andrews and N. L. Oleinick (2003) Photodamage to multiple Bcl-xL isoforms by photodynamic therapy with the phthalocyanine photosensitizer Pc 4. *Oncogene* **22**, 9197–9204.
29. Xue, L. Y., S. M. Chiu and N. L. Oleinick (2005) Differential responses of Mcl-1 in photosensitized epithelial vs lymphoid-derived human cancer cells. *Oncogene* **24**, 6987–6992.
30. Michels, J., J. W. O'Neill, C. L. Dallman, A. Mouzakiti, F. Habens, M. Brimmell, K. Y. Zhang, R. W. Craig, E. G. Marcusson, P. W. Johnson and G. Packham (2004) Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. *Oncogene* **23**, 4818–4827.
31. Zhou, P., L. Qian, K. M. Kozopas and R. W. Craig (1997) Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. *Blood* **89**, 630–643.
32. Kim, H. R., Y. Luo, G. Li and D. Kessel (1999) Enhanced apoptotic response to photodynamic therapy after bcl-2 transfection. *Cancer Res.* **59**, 3429–3432.
33. Kessel, D. and M. Castelli (2001) Evidence that bcl-2 is the target of three photosensitizers that induce a rapid apoptotic response. *Photochem. Photobiol.* **74**, 318–322.
34. Usuda, J., K. Azizuddin, S. M. Chiu and N. L. Oleinick (2003) Association between the photodynamic loss of Bcl-2 and the sensitivity to apoptosis caused by phthalocyanine photodynamic therapy. *Photochem. Photobiol.* **78**, 1–8.