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Cutaneous penetration of the topically applied photosensitizer Pc 4 as detected by intravital 2-photon laser scanning microscopy

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Abstract

The fundamental mechanism of photodynamic therapy (PDT)-induced cell death has been characterized, but early critical PDT events *in vivo* remain incompletely defined. With the recent development in advanced fluorescence imaging modalities, such as intravital 2-photon laser scanning microscopy (2P-LSM), researchers are now able to investigate and visualize biological processes with high resolution in real time. This powerful imaging technology allows deep tissue visualization with single-cell resolution, thus providing dynamic information on the 3-dimensional architectural makeup of the tissue. The main goal of this study was to determine the cutaneous penetration of a topically applied photosensitizer, the silicon phthalocyanine Pc 4, into the skin of live animals and to assess the effective absorption of Pc 4 through the skin barrier. Our 2P-LSM images indicate that Pc 4 penetrates to the epidermal/dermal junction of mouse skin. The data also indicate that the degree of Pc 4 absorption is dose dependent. These findings represent initial steps that may help in improving the clinical utilization of topical Pc 4-PDT.

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Keywords

Pc 4; Photodynamic Therapy (PDT); Intravital Microscopy; 2-Photon Laser Scanning Microscopy (2P-LSM)

Introduction

Photodynamic therapy (PDT), a rapidly advancing treatment modality with diverse clinical applications, involves exposure of tissues to a photosensitizing drug followed by irradiation with light of an appropriate wavelength (Dougherty, 2002; Sibata *et al.*, 2001). In the presence of molecular oxygen, the interaction of photosensitizer and light results in a cascade of events leading to the generation of singlet oxygen and other forms of reactive oxygen to exert cytotoxic effects on organelles such as mitochondria, endoplasmic reticulum and lysosomes, and eventually leads to the induction of apoptosis and tumor cell destruction (Dougherty *et al.*, 1998; Oleinick and Evans, 1998; Oleinick *et al.*, 2002). Photofrin®, the first US FDA-approved porphyrin-derivative photosensitizer, is administered intravenously prior to irradiation of the tumor or pathologic tissue with 633-nm light. Because Photofrin® remains present in tissues for up to several weeks, patients are advised to avoid natural sunlight or intense artificial light to minimize the likelihood of developing widespread photosensitivity reactions (Dougherty *et al.*, 1990; Moriwaki *et al.*, 2001). Occasionally, this may occur due to patients who have compliance issues and is considered one of the disadvantages of Photofrin®-PDT.

However, local or topical delivery of photosensitizers offers a more targeted approach, particularly in treating cutaneous lesions, either oncologic or non-oncologic, due to the accessibility of the skin. PDT has been shown to be effective for dermatopathologic conditions that include actinic keratosis, Bowen's disease, mycosis fungoides (MF), squamous and basal carcinoma and psoriasis (Bissonnette *et al.*, 2002; Ibbotson, 2002). Compared to surgical and other ablative therapies for dermatopathologic conditions, PDT has the advantage of being minimally invasive and non-scarring, with an excellent safety profile (Bissonnette *et al.*, 2002; Ibbotson, 2002). The only currently FDA-approved photosensitizers for dermatological conditions are aminolevulinic acid (ALA) or Levulan® and the methyl ester of ALA, Metvixia®, which are utilized for topical PDT of actinic keratosis, Bowen's disease, basal cell carcinoma, acne, photoaging, psoriasis, and cutaneous T-cell lymphoma (Coors and von den Driesch, 2004; Ibbotson, 2002; Szeimies *et al.*, 2002; Wang *et al.*, 2001). However, because ALA is a pro-drug that needs to undergo conversion to the active photosensitizer, protoporphyrin IX; an interval range between 4 and 24 hours is often required between ALA administration and light delivery, which can sometimes complicate the treatment schedule. Moreover, ALA-PDT is commonly accompanied by significant pain that may not be sufficiently alleviated with the use of local anesthetics (Holmes *et al.*, 2004).

These complications have led to the development of a second generation of photosensitizing agents that were created to ameliorate the disadvantages of their predecessor molecules. The silicon phthalocyanine Pc 4 (Fig. 1a) represents one of these second-generation agents that is a pure preformed photosensitizer with excellent potential for PDT *via* topical delivery. Topically applied Pc 4 is currently in a Phase I clinical trial for PDT of psoriasis at University Hospitals Case Medical Center. Data from an earlier *ex vivo* study using laser-scanning confocal microscopy of skin biopsy samples from human subjects indicated that Pc 4 uptake can be detected in the upper dermal region as early as one hour following topical treatment (Lam *et al.*). In addition, PDT with topically applied Pc 4 was demonstrated to be safe in an earlier Phase 1 clinical trial treating various neoplasms (Baron *et al.*).

Although topical PDT appears to hold great promise as a therapeutic, some factors require further improvement. These include the optimizing of regimens, the reduction of pain and other adverse effects, and most importantly increasing the success rate of eradicating the targeted dermatopathologic lesion. While mechanisms of PDT-induced cell death are commonly investigated and characterized *in vitro*, one remaining challenge in deciphering PDT function is to identify the critical early events *in vivo*. By understanding these events, we can focus on developing PDT that specifically targets malignant and benign diseases, but with the goal of preserving the integrity of surrounding tissues. As a first step, however, one must first be able to demonstrate sufficient absorption of the photosensitizer into the targeted tissue. In this study, using two-photon laser scanning microscopy (2P-LSM), we show effective penetration of clinically formulated Pc 4 to the epidermal/dermal junction of mouse skin. Our data also indicate that Pc 4 absorption is dose dependent.

Materials and methods

Formulation of Pc 4 for topical application

Pc 4 powder was dissolved in absolute ethanol at a rate of 1 mg/mL, and the exact concentration was determined by absorption spectrophotometry ($\epsilon = 2.4 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$; MW = 717.9 g/mol). The stock solution was further diluted in a vehicle of 70% ethanol/30% propylene glycol (v/v) to the desired final concentrations.

Animals

Female Balb/c mice (6-8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME) and housed and handled according to animal protocols which were approved by the Case Western Reserve University Institutional Animal Care and Use Committee and conformed to the American Association for Accreditation of Laboratory Animal Care guidelines. Upon arrival, mice were kept in controlled laboratory conditions for at least one week prior to their use in experimental procedures.

Intravital 2-photon laser scanning microscopy (2P-LSM)

For experiments, mice were anesthetized by inhaled isoflurane at 1.5% - 2.5%. The animals were immobilized in a specially designed apparatus for imaging the animals' ears or the back of their legs. If the leg was to be imaged, hair on the back of the right leg was removed by shaving and depilation with Nair. Ten to 20 μL of either Pc 4 solution (0.1 mg/mL) or vehicle were applied topically under reduced ambient light, allowed to remain for one hour, and then removed by an ethanol wash and followed by three rinses with phosphate-buffered saline (PBS). To illustrate the tissue architecture and to provide a guide in assessing the level of penetration, 5-10 μL of SYBR Safe DNA stain (1.0 mg/mL in DMSO, Invitrogen, Carlsbad, CA) was topically applied to the same site and left in place for at least 15 minutes. After the application period, the treated area was cleaned with ethanol, followed by three PBS rinses, and the mice in the restraining apparatus were transferred to the stage of a Leica SP5 confocal microscope fitted with a Chameleon near-infrared femtosecond pulsed laser (Coherent Inc., Santa Clara, CA) tuned to 800 nm. Intravital Pc 4 fluorescence images (pseudo green) were collected using a 625-675 nm band-pass emission filter. Images of SYBR Safe-stained nuclei (pseudo white) were collected using a 500-550 nm band-pass filter. The collagen image (pseudo blue) was acquired using a 400-495 nm band-pass filter detecting native second harmonic signal generation from collagen fibers. For detailed structural analysis, high resolution images were taken and analyzed using Imaris software (Bitplane, Inc., St. Paul, MN). Ten animals were studied for Pc 4 penetration with intravital 2P-LSM.

Measurement of Pc 4 uptake by confocal imaging

Pc 4 was topically applied to live animals as described above. To quantitatively assess the relative amount of Pc 4 absorption, different concentrations of Pc 4 (0.001 - 0.1 mg/mL) were topically applied onto mouse ears. Following a one-hour application period, animals were euthanized, and punch biopsies were collected, embedded in OCT, snap frozen and sectioned for confocal imaging. Prior to image acquisition, the specimens were stained with Hoechst 33342 (10 μ g/mL, Invitrogen, Carlsbad, CA) for 15 minutes at room temperature. All images were acquired using an Ultra *VIEW* VoX spinning disk confocal system (PerkinElmer, Waltham, MA, USA) mounted on a Leica DMI6000B microscope (Leica Microsystems, Inc., Bannockburn, IL, USA) equipped with a HC PLAN APO 20 $\times$ /0.7 objective. Confocal images of Pc 4 fluorescence and Hoechst 33342 were collected using solid-state diode lasers emitting 640-nm and 405-nm excitation light, respectively, and with appropriate emission filters. Images were then exported and quantitatively analyzed using MetaMorph Premier software (Molecular Devices Corporation, Sunnyvale, CA) and SigmaPlot (Systat Software, Inc., San Jose, CA). At least 3 animals were used for each of the Pc 4 concentrations applied; for each condition, 10 regions of interest at the epidermal-dermal junction were averaged for pixel intensity and then graphed as a function of applied Pc 4 dose.

Results

Cutaneous Pc 4 penetration as demonstrated by intravital 2-photon laser scanning microscopy

Pc 4 absorbs both in the UV ($\lambda_{\max}$ ~350 nm) and red ($\lambda_{\max}$ ~672 nm) regions of the absorption spectrum (Fig. 1b), and we exploited the UV peak for 2-photon intravital imaging of Pc 4 penetration into live mouse skin. As visualized by intravital 2P-LSM (Fig. 2b), Pc 4 uptake was apparent as early as one hour following a topical application. A gallery of Pc 4 fluorescence images was acquired in 2- μ m increments, starting from the stratum corneum (0.0 μ m) to the upper dermal region of the mouse ear skin (28.0 μ m, Fig. 2b). A maximum projection of this stack of images (pseudo green) illustrates the penetration of Pc 4 into the ear skin; there was no comparable fluorescence detected when the vehicle was applied, demonstrating the fluorescent signal originates from Pc 4 and the absence of autofluorescence under these conditions (Fig. 2c). We also examined Pc 4 penetration into skin on the back of the leg within the same one-hour time frame. In this case, a representative cross-sectional image is shown in Fig. 3a. Pc 4 fluorescence was observed in this anatomical skin location in addition to the ear. As a frame of reference for evaluating the depth of Pc 4 penetration in skin of the leg, we also stained the nuclei of keratinocytes, which are normally found predominantly in the epidermis, and those of other cell types in the dermis (Fig. 3b) by topically applying SYBR green (pseudo white). Additionally, the dermis was identified by recording the second harmonic signal of collagen (pseudo blue), as seen Fig. 3c. In the composition image of Fig. 3a, 3b and 3c (Fig. 3d), it is apparent that Pc 4 fluorescence was detected mainly in the epidermis, with minor amounts also in the superficial dermis. Finally, a hematoxylin- and eosin-stained tissue section (Fig. 3e) taken from the same area illustrates the tissue architecture and provides a guide in further validating the level of Pc 4 penetration in the mouse skin.

The effective concentration of Pc 4 in normal skin changed as a function of dose

To test whether the degree of Pc 4 absorption into the skin of live animals correlates with Pc 4 concentration, we topically applied Pc 4 at concentrations ranging from 0.001 to 0.1 mg/mL for one hour. The level of Pc 4 fluorescence in the tissue *in vivo* increased with increasing dose (Fig. 4a). To quantify the relative fluorescence intensities, we selected regions of interest displaying Pc 4 fluorescence and averaged the respective pixel intensities

(Fig. 4b). The data confirm that the relative Pc 4 fluorescence intensity or its absorption depends upon the Pc 4 dose applied.

Discussion

A prerequisite for effective topical treatment with a photosensitizer is sufficient delivery into the target layers of the skin within a practical timeframe to achieve optimum lesion response upon photoillumination. Data from our earlier *ex vivo* study using laser-scanning confocal microscopy of skin biopsy samples from normal human subjects suggested that Pc 4 uptake could be detected in the upper dermal region as early as one hour following topical treatment (Lam *et al.*). Intravital 2P-LSM has now become a widespread imaging modality to visualize immune cell trafficking in different locations throughout the body of anesthetized mice, as illustrated in Fig. 2a (Germain *et al.*, 2008; Miller *et al.*, 2002). Furthermore, this powerful imaging technique allows deep tissue visualization ($> 300 \mu\text{m}$) with single-cell resolution ($< 1.0 \mu\text{m}$), thus providing dynamic information on the three-dimensional architectural context of the tissue. By adapting this technique to study the fluorescence of Pc 4, we sought to investigate the efficiency of penetration of our currently formulated Pc 4 following topical application *in vivo*.

Interestingly, Pc 4 could be seen accumulating in the hair follicles (see white arrows in Fig. 3d). It also appears that Pc 4 penetration comes to a halt at the upper dermis. Upon closer examination, however, there might be a few fluorescent spots indicating Pc 4 in the dermis (see white arrows in Fig. 3a and the far right image in Fig 4a). It is possible that the limit of Pc 4 penetration within one hour has been reached.

The data of our study show that Pc 4, applied in the chosen vehicle to mouse skin, was able to penetrate into the epidermis and reach the dermal-epidermal junction within one hour of topical application, as indicated by the red dashed line in Fig. 3d. The significance of these findings is the demonstration that events critical for efficacy of PDT can be imaged in the living animal in real time with 2P-LSM. Next steps will be to exploit this powerful technology to examine the generation of reactive oxygen species (ROS) and calcium fluxes following photo-irradiation of the penetrated Pc 4 *in vivo*. The work presented here provides a first step into unraveling critical mechanisms which will aid in designing more advanced clinical trials using Pc 4-PDT.

In summary, this study marks the first demonstration of the effective penetration of a topically applied silicon phthalocyanine through the epidermal layer of living mouse skin utilizing intravital 2P-LSM imaging. This represents a major advance in the preclinical development of Pc 4 for topical PDT of cutaneous lesions. We demonstrated that Pc 4 can be found in significant concentrations in normal skin as early as one hour post application. In addition, the amount of Pc 4 absorbed, which was mainly evident in the epidermis with a lesser amount in the superficial dermis, is dose dependent.

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Figure 1. Structure of the silicon phthalocyanine Pc 4 (a) and its absorption spectrum in ethanol (b).

Figure 2a

Figure 2b

Figure 2c

Figure 2. Intravital 2P-LSM imaging of topically applied Pc 4. (a) Set-up for 2P-LSM imaging. Under anesthesia, Balb/c mice were immobilized in a specially designed apparatus shown here

prior to Pc 4 topical application on either a shaved leg or ear. (b) XY, XZ and YZ -axis representation of the mouse ear image (Top panel). Gallery images of Pc 4 fluorescence within mouse ear skin following a one-hour topical application of 0.1 mg/mL Pc 4 in 70% ethanol/30% propylene glycol. (c) XY, XZ and YZ -axis representation of the mouse ear image (left panel). Maximum projection images of fluorescence obtained from the stack of images of ear skin on which was applied Pc 4 (pseudo green; middle panel) or vehicle alone (right panel). Data for panels b and c represent one of 3 experiments with similar results. *Scale bar, 100 μ m.*

Figure 3.

Depth of Pc 4 penetration mouse leg skin, as revealed by intravital 2P-LSM. Topical Pc 4 application and intravital 2-photon image acquisition were done as described for Fig. 2. All intravital 2P-LSM images are projected in a cross-sectional view. (a) Pc 4 fluorescence (pseudo green) is observed as far as the dermal-epidermal junction of a shaved leg and is sporadically absorbed in the dermis (white arrows). (b) Images of nuclei (pseudo white) stained with SYBR green and (c) the second harmonic signal (pseudo blue) of collagen were simultaneously generated to illustrate the tissue architecture and provide a guide in assessing the level of penetration into mouse skin, as depicted in a (d) merged image; the red dashed line demarcates the junction between the epidermis (above) and dermis (below); Pc 4 could

be seen aggregating in the hair follicles (white arrows). (e) Hematoxylin and eosin stained section of normal mouse leg skin is also shown. Data for panel (a) is representative of one of 5 experiments with similar results; data for panels (b)-(d) represent one of 3 experiments with similar results. All *scale bars*, $100\ \mu\text{m}$.

Figure 4.

Pc 4 penetration into ear skin is a function of dose *in vivo*. Pc 4 (0.001 - 0.1 mg/mL in 30% propylene glycol, 70% ethanol) was topically applied onto different ear regions of an anesthetized mouse. After one hour to allow penetration, mice were euthanized, and punch biopsies were immediately collected, embedded in OCT, and snap frozen, prior to sectioning. (a) Confocal microscopy was used to image Pc 4 fluorescence (pseudo green) in a section, which was also counterstained with Hoechst 33342 (pseudo blue); *Scale bar*, 50 μ m. (b) Fluorescence within selected regions of interest (white boxes in panel a) were quantified. Values represent the relative average Pc 4 fluorescence intensity, expressed as mean $\pm$ standard error from 10 regions of interest encompassing the epidermal area for each Pc 4 concentration. Data are from three independent experiments.