Full Paper

Pilot Study of Topical Delivery of Mono-L-aspartyl Chlorin e6 (NPe6): Implication of Topical NPe6-Photodynamic Therapy

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Abstract. Photodynamic therapy (PDT) is an evolving cancer treatment with promising results in treating malignant tumors by photoactivation of a photosensitizer with a specific wavelength. The second generation photosensitizer mono-L-aspartyl chlorin e6 (NPe6) was reported to have significant efficacy in killing cancer cells in vitro and in vivo. Though topical application might yield a higher local concentration and less systemic side effect, no data concerning topical absorption of NPe6 is available even though the drug has already been used in clinical trial for several years. To evaluate the possibility of topical delivery of NPe6 via an animal model, escalated concentrations of NPe6 were applied to BALB/c mouse skin for a different time periods after barrier disruption with tape stripping. Since NPe6 fluorescence intensity and drug concentration in tissue was well correlated, we evaluated drug penetration depth with frozen sections of treated and non-treated skin under a fluorescence microscope. An on-line fluorescence imaging system was used to monitor the NPe6 fluorescence kinetics in the skin. The fluorescence microscope confirmed successful topical delivery of NPe6 in mouse skin with or even without barrier disruption. Orange to red NPe6 fluorescence appeared at the epidermis, dermis, and even the muscular layer when using 10 mg/ml NPe6 application. The fluorescence intensity peaked at 1 h and revealed a dose-dependent response pattern. NPe6 treated versus non-treated skin showed a statistically significant difference by Student's *t*-test ($P < 0.05$). The results described here suggest that topical delivery of NPe6 is possible. It showed fast and deep penetration into mouse skin. This implies that NPe6 might be useful as a topical photosensitizer for PDT in treating skin cancers.

Keywords: topical delivery, topical photodynamic therapy, mono-L-aspartyl chlorin e6 (NPe6)

Introduction

Topical photodynamic therapy (PDT) consists of applying a photosensitizer onto target skin and then this photosensitizer is activated by a selective visible light (1, 2). This procedure generates highly reactive oxygen intermediates and free radicals which lead to selective irreversible cell destruction (3). Topical delivery of photosensitizer yields the highest local tissue concentration in comparison to other routes and the photosensitizer can not be detected in plasma (4). This is more cost effective than other administration methods

and it has less systemic adverse reactions. The successful application of PDT in treating non-melanoma skin cancer and nononcologic dermatoses has bloomed for decades (1, 2). Porphyrin-based photosensitizers such as hematoporphyrin derivative (HpD), porfimer sodium, and 5-aminolevulinic acid have been widely used in PDT (1, 2). However, the disadvantages of these compounds include a low extinction coefficient at long wavelength that hinders sufficient light penetration or prolonged in vivo retention that increases the risk of skin phototoxicity.

Mono-L-aspartyl chlorin e6 (NPe6) is a member of the chlorin compounds that have many similarities to porphyrins in their ring structure (5). Intracellularly, it

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localizes in the lysosomal compartment and has in vivo tissue distribution properties similar to those of Photofrin (6, 7). Lysosomal damage is primarily responsible NPe6-PDT mediated cell death (8), while plasma membrane and mitochondria were the major targets for Photofrin (9). In vivo, NPe6 also targets vessels as Photofrin does even though the subcellular sites were differ for the two photosensitizers (10). NPe6 is a chemical pure hydrophilic photosensitizer possesses a major absorption peak of the Q band at 664 nm. The longer wavelength allows deeper light penetration within the tissue. NPe6 also appears to be less skin phototoxic (11, 12). PDT using NPe6 as photosensitizer has showed significant phototoxicity both in vitro (13) and in vivo (11, 14–17). A phase I clinical study revealed that NPe6 could accumulate in cancerous tissue after intravenous administration and induce regression of primary skin malignancy and recurrent adenocarcinoma of the breast with subsequent irradiation (12). It is thus reasonable to predict that NPe6 can accumulate in tumor tissue once it passes through the skin barrier. NPe6 is potentially exploitable for treating cutaneous malignancies, possibly as well as other benign dermatoses, in topical PDT. However, there is no available data regarding topical delivery of NPe6. In this pilot study, we demonstrated topical administration of NPe6 is possible in an animal model. It may be useful for the future clinical topical NPe6 base PDT.

Materials and Methods

Photosensitizer

NPe6 was provided by the Meiji Seika Kaisha, Ltd.,

Tokyo. It is a dark blue-green compound with a molecular weight of 799.70. It was reconstituted as 0.1, 1, and 10 mg/ml solution in physiological saline just before administration and kept in the dark.

Animal model

Four- to five-week-old BALB/c female mice (Funabashi Farm Co., Ltd., Chiba) weighting 15–19 g were used. They were randomly divided into three experimental groups treated with 0.1, 1, and 10 mg/ml of NPe6, respectively. NPe6 was applied for a different time period from 0, 1, 2, 4, 8 to 24 h (0 h represented the agent being washed off from skin immediately after application). All tests were performed in triplicate. During the experiment, the mice were kept anesthetized with pentobarbital sodium of around 30 mg/kg per hour, intraperitoneally. Hair on the right thoracic wall ($1 \times 1 \text{ cm}^2$) was carefully cut as short as possible with scissors instead of shaving to avoid damaging the skin (Fig. 1). Transdermal epidermal water loss (TEWL) of the mouse skin was measured with a Tewameter TM210 (Courage & Khazaka, Köln, Germany) with constant room temperature ($21–22^\circ\text{C}$) and relative humidity varied from 70–80%. During measurement, mice were kept in a clear plastic measuring box to lessen the environmental influences (18). The baseline of TEWL was between 3 to 6 g/m² per hour. To remove the stratum corneum, which was later confirmed histopathologically, the skin was repeatedly stripped 15–20 times with cellophane tape (Scotch) until the TEWL reached 2 times of the baseline. After barrier disruption, a same sized cotton pad saturated with 300 μl NPe6 was applied on the stripped area. Then it was covered

Fig. 1. A diagram of the on-line fluorescence imaging system.

with an air permeable, water-resistant polyurethane adhesive membrane (Tegaderm; 3M Health Care, St. Paul, MN, USA) and aluminum foil successively to prevent light exposure and evaporation of the agent. At different time points after application, mice were sacrificed by cervical dislocation. Hair on the adjacent skin ($1 \times 1 \text{ cm}^2$) was cut. The treated skin together with the adjacent skin ($2 \times 1 \text{ cm}^2$) was excised. To avoid interference of fluorescence signals from remaining NPe6 on the epidermal site, the skin was turned upside down with the dermal side facing upward and fixed on a wooden board, ready for on-line fluorescence examination (Fig. 1). Parts of the specimens were fixed in buffered formalin sectioned at $3\text{-}\mu\text{m}$ thickness and stained with hematoxylin and eosin for histology examination. To study the penetration depth of NPe6, the thoracic muscles with overlying skin were excised and frozen at -70°C for fluorescence microscopy ($n = 3$ for each concentration at 0 and 1 h). Mouse skin without treatment ($n = 6$) was excised and fixed as above to calculate the auto fluorescence. In addition, 3 mice with intact stratum corneum were treated as above with 10 mg/ml NPe6 and occluded for 1 h to test the influence of the integrity of stratum corneum on topical absorption of NPe6.

On-line NPe6 fluorescence imaging system

The fluorescence imaging system (Fig. 1) consisted of a stereoscope, an epifluorescence microscope, a video monitoring system, and a mercury light source as described previously (19). Briefly, the mercury light was filtered by a band pass filter to emit 405 nm (10 mW/cm^2 at skin surface) and employed for exciting NPe6. The diameter of the excited light beam was sufficient to cover the whole excised skin. Objective light shorter than 600 nm was filtered using a high pass filter mounted between the objective lenses. Red fluorescence of NPe6 with its peak at 664 nm, could be specifically observed in this manner. The fluorescence images were recorded with a video recorder through a connected cooled charge-coupled device (CCD). The images were later digitized by the MediaGrabber software (Apple Computer, Inc., Cupertino, CA, USA) and analyzed with the NIH Image 1.59 software (Wayne Rasband; National Institutes of Health, Bethesda, MD, USA).

Fluorescence images analysis

A circle with a 2-mm diameter in the center of treated skin ($n = 3$ in each subgroup) with bright NPe6 fluorescence was chosen to represent the amount of NPe6 absorption. The data was expressed as the mean $\pm$ S.E.M. after subtraction of the auto fluorescence. An

identical circle located in the center of the adjacent non-treated skin was used to assess the fluorescence in adjacent skin. Autofluorescence of mouse skin was calculated in the same way by measuring 6 untreated mice.

Statistical analyses

A two-way analysis of variance (ANOVA) was used to compare the mean fluorescence intensity of different NPe6 dose levels (0.1, 1, and 10 mg/ml) versus different time points (0, 1, 2, 4, 8, and 24 h). Multiple pairwise comparisons among individual groups were based on an adjusted alpha-level for 3 or 6 pairwise tests (Student-Newman-Keuls methods). Where necessary to meet the normal distribution requirement for the use of ANOVA, the data were log-transformed prior to the application of ANOVA. The paired *t*-test was used for the comparison of NPe6-treated skin versus adjacent non-treated skin. The Mann-Whitney test was used to compare whether the group with intact and disrupted stratum corneum could affect fluorescent intensity.

Results

Histology of mouse skin

The hematoxylin and eosin sections of mouse skin confirmed the absence of stratum corneum after stripping. All examined skin tissues showed no or minimal infiltrates of inflammatory cells. Under a Zeiss Axiovert 35 inverted epifluorescent microscope (Carl Zeiss Inc., Tokyo) coupled with a mercury arc lamp and a 530–585 nm excitation filter, no detectable red fluorescence was noted in mouse frozen skin sections without NPe6 application. When treated with different concentrations of NPe6 at 0 h, orange-red fluorescence was detected on the epidermis and skin appendages (not shown). The fluorescence intensity was increased with the drug concentrations and became red at the highest NPe6 concentration we used. At 1 h (peak) after application, bright orange-red fluorescence was detected in the whole layer of epidermis and dermis of the mice skin. The red fluorescence was detected in the thoracic muscles layer only at the highest NPe6 concentration (10 mg/ml) group (Fig. 2).

On-line NPe6 fluorescent intensity under different concentration of NPe6

The fluorescence intensity of NPe6 was achieved from the on-line fluorescence image system and expressed as the mean $\pm$ S.E.M. in each group after subtraction of auto fluorescence (Fig. 3). There was a statistically significant difference ($P < 0.00001$, two way ANOVA) in the mean values among the different levels

Fig. 2. Fluorescence analysis of BALB/c skin. Only background green fluorescence can be seen in mouse skin without topical NPe6 (left upper). Obvious orange to red fluorescence was noted in the epidermis and dermis after 1-h application of 0.1 mg/ml (right upper), 1.0 mg/ml (left lower), and 10 mg/ml (right lower) NPe6. The peaked fluorescence intensity increased with drug concentrations and turned red at 10 mg/ml NPe6. Note that red fluorescence appears in the deep muscular layer at the highest dose (right lower).

of time. Fluorescence intensity between 3 different concentrations revealed a dose-dependent pattern and was significantly different ($P < 0.05$, Student-Newman-Keuls methods). The peak intensity was reached at 1 h after drug application in NPe6-treated (T) and non-treated (N) skin in all 3 groups. The difference of fluorescence intensity between T and N also showed a significant difference ($P < 0.05$, paired Student *t*-test.) that implies a good selectivity of the drug.

Influence of different application time

Multiple paired comparison showed significant differences only between 0 h application and other time points ($P < 0.05$, Student-Newman-Keuls methods). The curves of T and N remained at a plateau after reaching their peaks at 1 h in all groups (Fig. 3). Further applied NPe6 did not alter the fluorescence intensity. This inferred that there was a saturated effect in the mouse skin.

Influence of the integrity of stratum corneum

The fluorescence intensity of mouse skin with intact stratum corneum treated with 10 mg/ml NPe6 for 1 h was 18.65 ± 1.73 (Fig. 4), about half of the same concentration with disrupted stratum corneum ($P = 0.05$, Mann-Whitney test).

Discussion

NPe6 is dark green solution after being dissolved in normal saline. It could stain skin and interfere with the fluorescence observation via the epidermal side. Therefore, we placed the skin with the dermal side facing upwards and used a less penetrating blue light (405 nm) for excitation during the on-line fluorescence examination. In this way, the detected fluorescence could be achieved mostly from the dermis and represent the topical delivery of the drug in a manner more like the real situation. Less difference was noted by using a 664 nm laser excitation in our system (data not shown), probably due to the deep penetration of the longer wavelength. However, the longer wavelength excitation might be useful in photodynamic diagnosis of a large tumor in vivo (14). The stratum corneum of skin is a natural barrier which prevents hazards from the surrounding environment as well as topical medications. NPe6 might be not easily pass through intact stratum corneum. Higher concentration of NPe6 might be needed to achieve the desired absorption in intact skin as we demonstrated in mice with an intact stratum corneum. Nevertheless, the common skin cancers including solar keratosis, Bowen's disease, squamous cell carcinoma and basal cell carcinoma might have a

Fig. 3. The kinetics of fluorescence intensity in BALB/c skin being treated with different concentrations of NPe6. Results are expressed as the means $\pm$ S.E.M. The asterisk indicates $P < 0.05$. Kinetics between treated (T) and non-treated (N) adjacent skin for the application of 0.1 mg/ml (A), 1.0 mg/ml (B), and 10 mg/ml (C) NPe6.

Fig. 4. The fluorescence intensity of NPe6 with disrupted barrier function vs normal barrier function. The asterisk indicates $P = 0.05$.

disrupted skin barrier such as parakeratosis, scale crusts, and even ulcerations (20). We imitated this situation by removing the stratum corneum by stripping and found that sufficient NPe6 could be absorbed quickly via this stripped skin model.

Several factors might influence fluorescence intensity such as NPe6 penetration profiles, thickness, and opacity of the irradiated tissue. The fluorescence measurements after excitation with 405 nm blue light could only represent a fluorescence integration of the superficial layers of the dermis near the fluorescence detector. When interpreting the data, one should keep in mind that the data were semi-quantitative. Nevertheless, a good correlation between the red fluorescence intensity of NPe6 and tissue concentration has been documented (14). The fluorescence intensity could represent a tissue concentration, at the least concentration of NPe6 of the outermost layers, although we did not extract the tissue NPe6. In case of disrupted stratum corneum, although we washed the mouse skin immediately after NPe6 application, obvious fluorescence could still be observed in all mice epidermis and skin appendages under the fluorescence microscope. Absorption of NPe6 might be very fast once it passed through the skin barrier. A saturation effect may exist in the skin after 1 h application since prolonged application did not enhance the fluorescence intensity. In topical aminolevulinic acid

PDT, it usually takes 4–6 h and even a longer time to reach the optimal photosensitizer in the tissue (21). Using systemic NPe6, Taber et al. (12) treated patients with superficial tumor 4 h after intravenous injection. They found less selectivity when the systemic NPe6 was higher than 2.5 mg/kg. In our study, the peak fluorescence was reached 1 h after drug application in mouse skin. It may more convenient for a patient and physician to have a shorter drug incubation time. We found a significant difference between treated and non-treated adjacent skin at all the different NPe6 concentrations we used. Better skin selectivity may be achieved via topical application. However, non-treated adjacent skin showed increase in fluorescence with concentrations and time. It implied that there was lateral diffusion of the drug from the treated area. After PDT, recurrence of the tumor usually occurs at the tumor margin (1, 2). A slightly lateral diffusion of the photosensitizer may be theoretically beneficial for tumor cells eradication. When NPe6 reached its peak level, obvious fluorescence was noted in the epidermis, dermis, and even muscular layer at the highest concentration. It infers that NPe6 may have more phototoxic effect on deeper tumors. On the other hand, although higher drug level in the reticular dermis might have a better tumor control, more scarring after PDT might be expected (1). The necessity of dermal damage during PDT for a long-term tumor control remains a controversial issue. Our study showed that topical application of a lower NPe6 concentration (0.1–1.0 mg/ml) resulted in epidermal and dermal fluorescence accumulation. It might be selected for treating superficial tumor involving mainly the epidermis and/or upper dermis such as actinic keratosis, Bowen's disease, superficial basal cell carcinoma (BCC), and superficial squamous cell carcinoma (SCC). Higher concentration (1.0–10.0 mg/ml) might be required for treating a nodular tumor such as nodular BCC, nodular SCC, and breast cancer with skin metastasis in which tumor cells may involve the epidermis, deep reticular dermis, and even muscular layer. Further studies are needed to optimize the dosage of topical NPe6. Although systemic NPe6 PDT has successfully treated different kinds of cancer (11, 12, 14–17), topical route NPe6 PDT has never been evaluated. It is reasonable to predict that once the photosensitizer passed the skin barrier, it could localize in tumor cells since tumor localization of NPe6 after systemic administration was well documented (11, 12, 14–17). However, further studies are needed to clarify this issue.

In summary, our pilot study demonstrated the success of topical delivery of NPe6 in stripped and non-stripped mouse skin. The drug can penetrate to the deep dermis and even the muscular level at the highest drug concen-

tration. After NPe6 was applied with continuous occlusion, the absorption peaked at 1 h. The better penetration, shorter incubation time in comparison to the first generation photosensitizers, and the good selectivity to non-treated skin made NPe6 a good candidate for further clinical application.

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