In Vivo Studies on the Utilization of Mono-L-aspartyl Chlorin (NPe6) for Photodynamic Therapy

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ABSTRACT

The in vivo photosensitizing efficacy of mono-L-aspartyl chlorin has been studied by determining the percentage of BALB/c mice cured at varying doses of drug. Using an EMT-6 tumor model, animals received i.p. injections of mono-L-aspartyl chlorin (0.5–100 mg/kg) and were subsequently exposed to light at 664 nm. Tumor biopsies were taken from selected animals sacrificed at 24 h after treatment and routine histopathological sections made. The other animals remained in the dark for a period of 6 weeks to determine the cure rate.

Our results show that mono-L-aspartyl chlorin is an effective tumor localizer that brings about the selective degradation of tumor tissue following light exposure.

INTRODUCTION

The attack on cancer with drugs has been based on the thesis that it should be possible to target cancer cells while having only little or tolerable effects in normal cell populations. Many compounds have been screened for such activity during the past 40 years. Unfortunately, most common solid cancers respond either not at all or to a limited extent to these selective agents.

Several photosensitizing porphyrins are selectively retained in solid tumors and other rapidly growing tissues in humans and other animals. During the past several years, there has been an increasing interest in the use of porphyrins for tumor localization and therapy. Effective use of porphyrin photosensitizers for antitumor therapy has been documented at several clinical centers and has been used on several thousand patients with generally encouraging results (1–4). Although the history of porphyrins and their role as a diagnostic and therapeutic modality is relatively recent, a wide variety of human tumors with varying histological types have been treated. Good results with PDT2 have been reported with cancers of the skin (5), female genital tract (6), lung (7), esophagus (8), bladder (9), eye (10), breast (11), and oropharynx (12).

This photochemotherapeutic procedure which has become known as photodynamic therapy is based on the conclusion that some porphyrins, including HpD, are to some degree selectively retained by tumor tissue (13). On subsequent illumination with light of the appropriate wavelength absorbed by the porphyrin, tumors can be destroyed with relatively little damage to the surrounding normal tissue.

The properties of tumors that result in selective accumulation and retention of porphyrins remain to be elucidated. In cell culture, there appears to be no preferential uptake of porphyrins by either neoplastic versus normal cells (14–16) or as a function of malignancy in different cell lines (17). What is clear is that porphyrins are potent photosensitizers which result in the rapid necrosis of tumor tissue upon exposure to red light at 630 nm (the longest wavelength absorption peak of the porphyrin).

The major limitation of HpD-PDT is that it is primarily restricted to thin superficial tumors readily accessible to light. The attenuation of a light beam propagating in tissue is determined by the scattering and absorption characteristics of the tissue being exposed. It has been demonstrated that the efficiency for excitation of a drug molecule in a collimated beam of 630 nm light will be reduced to 10% at a location less than 1 cm from the tissue surface (18). Generally, absorption, which depends on specific chromophores in the absorbing molecules, increases with increasing wavelength. Furthermore, compounds with higher molar extinction coefficients may have a significant advantage in capturing any photons that penetrate into the tissue. It is anticipated that the development of other photosensitizers that utilize longer wavelengths with stronger absorption bands in the red and higher extinction coefficients will enhance the versatility of PDT.

In the present study, we have examined the effectiveness of NPe6 as a photosensitizer for selective tumor necrosis (Fig. 1). This compound has a strong absorption band at 664 nm, a wavelength that has a greater depth of tissue penetration than the 630 nm wavelength used with HpD.

MATERIALS AND METHODS

NPe6 and Photofrin II. NPe6 (Porphyrin Products, Logan, UT) was received as a dark blue-green powder and was stored in the dark at −70°C. The powder was reconstituted in Dulbecco's PBS (final pH 7.0–7.20) to a final concentration of 2.5 mg/ml and stored at −20°C until used. Photofrin II was obtained from Photomedica Inc., (Raritan, NJ) as an aqueous solution at a concentration of 2.5 mg/ml and stored in the dark at −20°C until used. An absorption spectrum of NPe6 was obtained with a Beckman DU-7 Spectrophotometer on PTK2 cells (rat kangaroo epithelial, American Type Culture Collection no. 56, kidney marsupial, Potorus tridactylus) incubated for 24 h with 25 μg/ml NPe6. Cells were washed three times in PBS and sonicated prior to being scanned from 350 to 700 nm.

Animal and Tumor System. Ten- to 12-week old BALB/c mice were used. They weighed between 30 and 35 g at the time of treatment. The tumor system used was the EMT-6 undifferentiated sarcoma obtained from the Frederick Cancer Research Institute, Frederick, MD. The EMT-6 tumor was obtained as an in vitro culture. Tumor cells were harvested from tissue culture flasks and a heavy inoculum was injected into the right flank of the mice. When the tumors attained a size of 1–2 cm in diameter, they were excised and minced in PBS. The resulting suspension of tumor cells was filtered through sterile fine-mesh gauze, washed twice in PBS and resuspended in RPMI media (GIBCO, Grand Island, NY) at a concentration of 5 × 10^6 viable cells/ml. Cell viability was assessed by the ability to resist cell lysis and exclude Trypan Blue dye (GIBCO). Tumors were initiated by injecting 0.1 ml of fresh tumor suspension i.p. into 10–12-week old BALB/c mice. Tumor cells were washed three times in PBS and resuspended in RPMI media (GIBCO). Tumors were initiated by injecting 0.1 ml of fresh tumor suspension i.p. into 10–12-week old BALB/c mice.

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2 To whom requests for reprints should be addressed, at University of California, Irvine, Beckman Laser Institute, Department of Surgery, 1002 Health Science Road East, Irvine, CA 92717.

3 The abbreviations used are: PDT, photodynamic therapy; HPD, hematoporphyrin derivative; NPe6, mono-L-aspartyl chlorin; PBS, phosphate buffered saline; EMT-6, experimental-mammary-tumor.
UTILIZATION OF NPe6 FOR PHOTODYNAMIC THERAPY

Table 1 NPe6 dose response

Animals treated with 100 J/cm² at a power density of 100 mw/cm² 24 h after injection with NPe6.

<table>
<thead>
<tr>
<th>Dose of NPe6 (mg/kg)</th>
<th>Complete response (cured)</th>
<th>Partial response</th>
<th>No response</th>
<th>% Cured</th>
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Dose Response Study. A total of 192 light-treated tumors were examined: 12 animals each at NPe6 doses of 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 40, 60, 80, and 100 mg/kg. Two animals were sacrificed at 24 h after treatment and their tumors removed for histopathological analysis as described above. The remaining 10 animals in each group were returned to the dark for a period of 6 weeks to determine the percentage of animals cured. Control experiments were also carried out: 12 animals with NPe6 (100 mg/kg) without light and 12 animals with 100 J/cm² light without NPe6.

Skin Photosensitivity Study. Animals were given i.p. injections of 10 mg/kg of either NPe6 or Photofrin II. An isolated patch of skin on the right hind leg of the animal measuring 1 cm² was subsequently exposed to light (664 nm for NPe6; 630 nm for Photofrin II) 24 h later. A total of 60 light-treated animals were examined: three animals each for NPe6 and Photofrin II at doses of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 J/cm². Control experiments were also carried out: three animals with 664 nm light at 1000 J/cm² and three animals with 630 nm light at 1000 J/cm² without drugs. The animals were returned to the dark and a clinical evaluation of the skin photosensitivity was made daily for 10 days posttreatment.

RESULTS

The absorption spectrum of NPe6 in cells demonstrated the main absorption peaks at 202, 284, 400, 502, and 664 nm (Fig. 2). The 664-nm peak was selected for treatment because of the improved tissue transmittance of light at this longer wavelength. The effect of NPe6 and activating red light at 664 nm on 192...
Fig. 3. Photomicrographs of EMT-6 tumor treated with NPe6 (8 mg/kg) and 100 J/cm² light at 24 h postinjection. Biopsies shown were taken 24 h after laser irradiation. In a, note the vast areas of hemorrhagic necrosis throughout the entire tumor; b, high power magnification of a showing tumor cells in varying stages of cell death and disintegration in a sea of red blood cells. a, 100 ×; b, 570 ×.

Table 2. Photofrin II versus NPe6 skin photosensitivity
Animals treated with 10 mg/kg of each drug and irradiated 24 h later at a power density of 100 mw/cm².

<table>
<thead>
<tr>
<th>Dose of light (J/cm²)</th>
<th>Photofrin II (630 nm)</th>
<th>NPe6 (664 nm)</th>
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<tbody>
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<td>100</td>
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*0, no response; +, erythema; ++, blistering; ++++, crusting/erosion; ++++, necrosis.

Tumors in mice is summarized in Table 1. With 100 J/cm² of light exposure at a power density of 100 mw/cm², we obtained a 100% cure rate at NPe6 doses of 8–100 mg/kg. Cure is defined as no palpable tumor mass at least 6 weeks after treatment. Without treatment, 100% of these animals died within 1 month of tumor inoculation. In the experimental animals, there was complete disappearance of palpable tumor mass with biopsy confirmed tumor necrosis beginning 24 h after light treatment. Tumor tissue biopsy of these animals at 24 h posttreatment revealed massive hemorrhagic necrosis with frank extravasation of red blood cells into the surrounding tumor stroma. The tumor cell architecture is characterized by nuclear pyknosis and karyorrhexis, with only minimal preservation of the basic cellular shape permitting recognition of the cellular outline in a sea of amorphous granular debris (Fig. 3). The entire tumor mass disintegrated into a nonpalpable scab within a few days after treatment and eventually, the skin completely healed with varying degrees of hair regrowth. At NPe6 doses of 5–7 mg/kg, all animals responded to the treatment with varying results of partial to complete necrosis. However, regrowth of the noncured tumors (partial necrosis) generally was apparent within 7–8 days and usually occurred around the periphery of the original tumor. These tumors grew as rapidly as the nontreated tumors and these animals subsequently died of tumor bulk at the same time as the untreated controls. Between doses of NPe6 (2–4 mg/kg) there was partial necrosis with no cures and at doses of 0.5–1 mg/kg no response.

Additionally, we attempted to determine the isolated skin
photosensitivity of animals receiving NPe6 (10 mg/kg) versus Photofrin II (10 mg/kg) at light doses of 100–1000 J/cm². Animals that received Photofrin II (10 mg/kg) and light up to 1000 J/cm² (630 nm) showed severe blistering with subsequent skin slough at 100–200 J/cm² and frank skin necrosis at 300 J/cm² or more. Animals that received NPe6 (10 mg/kg) and light to doses as high as 1000 J/cm² (664 nm) showed no signs of erythema (Table 2). Control animals with 1000 J/cm² of light at either the 630 nm or 664 nm wavelengths showed no signs of erythema.

**DISCUSSION**

During the past 10 years, the selective photoactivation of malignant tissue by hematoporphyrin derivative has proved to be a promising new therapeutic modality in the treatment of cancer. In some cases, it may be a viable alternative to debilitating surgery, while in others, it may be the treatment of choice. However, despite its broad experimental application in clinical oncology, efforts have been hampered by the lack of a complete understanding of what active component in the HpD complex is responsible for tumor uptake and retention. The most active component has been described as a dihematoporphyrin ether by Dougherty (19) or as a dihematoporphyrin ester by Kessel (20). Even though there is an increased tumor:neighbor tissue porphyrin content ratio following HpD administration, the amount retained by normal tissues such as the skin, liver, spleen, and kidney is significant. This is sufficient to cause nonspecific skin necrosis following PDT of cutaneous malignancies as well as photocutaneous side effects which can persist up to 4 to 6 weeks after HpD administration. These problems as well as a relatively weak porphyrin absorption band and low tissue transparency at 630 nm resulting in inefficient phototoxicity have resulted in considerable effort being devoted to developing new and more efficient tumor localizing photosensitizers for PDT.

The chloriners are known to have strong absorption bands with high molar extinction coefficients at wavelengths greater than 650 nm thus providing an advantage over the lower tissue penetration of 630 nm used for HpD. Our study was undertaken to evaluate the photosensitizing potential of NPe6 with a significant absorption band located at 664 nm.

This study describes the first successful “cures” resulting in long term animal survival with NPe6 and light. The results of our study suggest that NPe6 is an effective tumor photosensitizer in vivo. Our study shows that 100% cure rates can be obtained at an NPe6 dose as low as 8 mg/kg. In no animals has the tumor reappeared after 6 weeks following treatment. We can expect a 30–50% cure in animals receiving 5–7 mg/kg. Animals that received 2–4 mg/kg of NPe6 exhibited no cures but did have some observable response. Animals that received NPe6 (0.5–1 mg/kg) showed no response. The amounts of light energy used in this experiment have been shown by our group (21–23) and others (24) to effectively destroy the EMT-6 tumor in conjunction with HpD. Further studies on the uptake and excretion of NPe6 by proliferating tumor cells are needed to determine the optimal time for light exposure after injection.

As mentioned previously, a major drawback of this form of therapy is the potential for drug-induced sensitivity to sunlight. These effects are not trivial and may result in symptoms ranging from slight erythema and edema to extensive skin damage and necrosis. Ideally, the photosensitizer should be modified to avoid the deleterious effects on normal skin tissue upon light exposure. In the experiment reported here, animals that received NPe6 (10 mg/kg) and 100–1000 J/cm² of light showed no deleterious side effects whereas the HpD animals suffered adverse skin reactions such as blistering with subsequent skin slough and necrosis. This lack of skin photosensitivity which minimizes undesired side effects is clearly an advantage of NPe6. These results suggest that NPe6 may provide a realistic approach to the treatment of tumors where exposure to sunlight is unavoidable or even desirable.

While our study is promising, the studies on NPe6 are still at an early phase. Unanswered questions include delineation of light and drug dosimetry parameters, mechanisms of tumor localization, possible uptake in other organs such as liver, intestine, spleen, and kidney as well as determination of PDT cytotoxicity. It is hoped that future investigation will address these questions so that the role of NPe6 in the management of cancer can be fully defined.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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