Tissue Distribution and Photosensitizing Properties of Mono-L-aspartyl Chlorin e6 in a Mouse Model

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ABSTRACT

Mono-L-aspartyl chlorin e6 (NPe6) is a photosensitizer that possesses properties such as chemical purity and a major absorption band at 664 nm which are potentially exploitable for photodynamic therapy (PDT). The current investigation examined pharmacological and photosensitizing parameters of NPe6 in tumor and normal tissues in mice. [14C]NPe6 was used to obtain quantitative tissue distributions of the photosensitizer as a function of: (a) time following administration; (b) drug dose; (c) mode of drug administration; and (d) tumor size. The in vivo photosensitizing efficiency of NPe6 was compared directly to Photofrin II in experiments which evaluated tumor responses and induction of normal skin damage. Initial PDT experiments demonstrated that NPe6 was ineffective at inducing tumor cures when a 24-h time interval (between drug administration and light treatment) was used. However, PDT-induced tumor cures were obtained when NPe6 was administered 4-6 h prior to light exposure, and these NPe6-PDT treatment parameters were as effective as standard Photofrin II-mediated PDT. Interestingly, the level of PDT-induced normal skin damage was significantly greater for Photofrin II than for NPe6 at comparable drug and light doses. An analysis of pharmacological data and PDT time interval requirements suggests that plasma concentrations of NPe6 may be a more important predictive factor than tumor tissue levels of the photosensitizer for the production of PDT-mediated tumor cures. The results of this investigation indicate that NPe6 is an effective tumor photosensitizer with in vivo clearance properties that eliminate the side effect of prolonged normal skin photosensitization.

INTRODUCTION

PDT continues to be evaluated for its effectiveness in treating solid tumors (1). Phase III clinical trials are currently in progress for malignancies of the esophagus, bronchus, and bladder (2). In addition, encouraging preliminary results have been obtained when PDT is used to treat tumors of the skin, head and neck, cervix, eye, and brain (3). Unfortunately, Photofrin II (the photosensitizer used in clinical PDT) exhibits chemical, pharmacological, and photophysical properties which are not optimal for PDT (4). Photofrin II is a complex mixture of monomeric and aggregated porphyrins that possess weak absorption at wavelengths greater than 600 nm (5, 6). This drug is also retained in normal tissues for extended time intervals following administration (7). Therefore, problems related to drug purity as well as the use of wavelengths of light with suboptimal tissue transmission and minimum drug absorption properties are associated with current Photofrin II-mediated PDT procedures. The prolonged skin photosensitivity observed in patients treated with PDT has not been a major clinical problem but does represent an additional disadvantage of Photofrin II (3).

Interest in the synthesis and evaluation of new photosensitizers for use in PDT has grown as a result of both the encouraging initial clinical results of this therapy and the documented need to improve upon the limitations of Photofrin II. Classes of photosensitizers which are in various stages of preclinical evaluation include phthalocyanines, purpurins, chlorins, cationic cyanines, and bacteriochlorins (8–12). These "second generation" photosensitizers possess major absorption peaks at wavelengths above 650 nm and many of these compounds are chemically pure.

The current study was designed to document pharmacological and photosensitizing properties of NPe6 in a mouse-tumor model. Quantitative tissue distributions of [14C]NPe6 were obtained as a function of: (a) time following drug administration; (b) drug dose; (c) tumor size; and (d) method of drug administration. In addition, the in vivo photosensitizing efficiency of NPe6 was compared to Photofrin II in separate experiments which evaluated PDT-induced tumor response and normal skin photosensitivity. Results from our study indicate that NPe6 is an effective tumor photosensitizer with clearance properties that eliminate the problem of prolonged skin photosensitization. Our data also suggest that plasma concentrations of NPe6 may be a more important factor than tumor tissue levels of NPe6 for inducing PDT-mediated tumor cures.

MATERIALS AND METHODS

Photosensitizers. The tetrasodium salt of mono-L-aspartyl chlorin e6 and [14C]NPe6 were obtained from Porphyrin Products, Inc., Logan, UT. The 4C atoms in the radioisotopically labeled NPe6 were positioned exclusively on the ring structure of the chlorin molecule as shown in Fig. 1. High-performance liquid chromatographic analysis of the [14C]NPe6 (performed by Porphyrin Products, Inc.) demonstrated a single peak of radioactivity corresponding to the NPe6 molecule. The working solution of [14C]NPe6 had a specific activity of 72 μCi/mmol. Photofrin II (Quadralogics Technologies, Inc., Vancouver, British Columbia, Canada) was also used in PDT experiments involving tumor and normal skin response. All photosensitizers were administered in volumes ranging from 0.2 to 0.3 ml.

Animal and Tumor Models. Female C3H/HeJ mice (8–12 weeks old) and the BA mammary carcinoma (obtained from the NIH tumor bank) were utilized for both tumor response and pharmacology studies. s.c. tumors were generated by trocar transplantation of 1-mm3 pieces of the BA mammary carcinoma to the hind flank of C3H mice. Experiments utilizing this tumor were started when the largest diameter of the lesions was 6–7 mm (7–12 days following transplantation). Female Swiss Webster mice (8–12 weeks old) were used in experiments designed to document normal skin damage following PDT.

Analysis of Tissue Distribution of [14C]NPe6. Eighteen tissue and fluid samples from tumor-bearing mice were analyzed following [14C]NPe6 administration. These samples included: plasma, whole blood, tumor, brain, heart, liver, kidney, adrenal glands, spleen, skin, intestine, muscle, lung, esophagus, stomach, bladder, feces, and urine. Seventeen time points were assayed in experiments related to drug uptake as a function of time after a 5-mg/kg i.v. injection of [14C]NPe6. These time points included 2 min, 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h,
was measured three times per week and these measurements were 24-26 h after drug administration. Following treatment, each tumor prior to tumor light treatments which were performed either 4-6 or II via an i.v. tail vein injection. The mice were restrained immediately were used to evaluate NPe6 and Photofrin II-induced PDT responses. Intratumor temperatures were documented as a function of time during exposure to 75- and 100-mW/cm² irradiances. Average values for five mice were obtained for each dose rate of delivered light. A 21-gauge thermocouple was inserted 1.5 mm into the tumor at an angle perpendicular to the plane of a hypodermic needle (Omega Engineering, Inc., Stamford, CT) was in.

A single step quartz fiber interfaced to the dye laser was used to deliver light and a microlens (Cooper Lasersonics, Inc.). A quantitative skin scoring system (described in Table I) was utilized to document the degree of normal tissue damage induced by each treatment (15). The right hind foot of each animal was treated with PDT in the manner identical to that utilized in the tumor response studies. A 1-cm diameter treatment area was used during light treatments which were initiated 4-6 or 24-26 h following administration of either NPe6 or Photofrin II. A minimum of five mice were evaluated at each drug and light dose.

RESULTS

Table 2 presents data for [14C]NPe6 distribution in mouse tissues and fluids for time periods ranging from 2 min to 96 h following a 5-mg/kg i.v. injection. Excretion of the photosensitizer is primarily through the feces. The majority of NPe6 in the blood is associated with the plasma at early time intervals while an increasing proportion of NPe6 binds to blood cells at intervals greater than 24 h. Computer curve stripping analysis of NPe6 pharmacokinetics indicates that a two-compartment, biexponential model fits the current data. The first and second compartment half-lives for plasma are 0.5 and 11.6 h, respectively. The half-lives observed for all tissues were greater than those obtained for plasma. Liver, kidney, and spleen had the highest concentrations of [14C]NPe6 while the lowest levels were found in brain, muscle, and esophagus. Tumor tissue concentrations of the photosensitizer were higher than in all other tissues except liver, kidney, adrenal gland, and spleen at intervals greater than 4 h following drug administration. Figs. 2 and 3 document the tissue distribution of [14C]NPe6 at 4 and 24 h following drug dosages ranging from 1 to 50 mg/kg. An increase in tissue concentrations of NPe6 was observed with increasing doses of the administered drug. Table 3 documents tissue levels of [14C]NPe6 at 4 and 24 h following drug administration. Figs. 2 and 3 document the tissue distribution of [14C]NPe6 at 4 and 24 h following drug dosages ranging from 1 to 50 mg/kg. A linear increase in tissue concentrations of NPe6 was observed with

### Table 1: Grading system for acute skin photosensitization reactions

<table>
<thead>
<tr>
<th>Score</th>
<th>Observation</th>
<th>A. Reaction appearing</th>
</tr>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>1</td>
<td>Slight foot swelling</td>
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</tr>
<tr>
<td>2</td>
<td>Marked edema</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Erythema</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Breakdown of skin with moist desquamation</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Small area of necrosis and/or loss of toes</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Large area of necrosis and/or loss of foot</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>Score</th>
<th>Observation</th>
<th>B. Reaction subsiding</th>
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</thead>
<tbody>
<tr>
<td>4</td>
<td>Extensive scab formation</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Decreased scab and epilation</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Extensive papery skin</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Slight abnormal appearance:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slight dry desquamation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slight scab formation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduction in papery skin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sparse regrowth of hair</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Normal</td>
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</tr>
</tbody>
</table>

Plasma, whole blood, tumor, liver, and skin tissue levels of [14C]NPe6 were determined either 4 or 24 h after injection.

A dose-response analysis was examined in a fourth set of pharmacological experiments. [14C]NPe6 was administered to tumor-bearing mice via i.v. injection at dosages of 1, 5, 10, 25, and 50 mg/kg. The concentration of [14C]NPe6 was modulated so that the injected volume remained at 0.2-0.3 ml. Animals were sacrificed either 4 or 24 h after injection.

In Vivo Light Source and Delivery System. An argon pumped dye laser (Cooper Lasersonics, Inc., Santa Clara, CA) was utilized to generate light for all in vivo PDT experiments. The laser was tuned to 664 nm for NPe6-mediated PDT and to 630 nm for Photofrin II-mediated PDT. The wavelength of emitted light was documented with a spectroscope (Cooper Lasersonics, Inc.). A single step quartz fiber interfaced to the dye laser was used to deliver light and a microlens (Laserguide Inc., Buellton, CA) was fitted to the distal tip of the fiber to produce a uniform field of delivered light and the laser output was measured with a power meter (Coherent Radiation, Palo Alto, CA). The treatment area included a 1-mm margin of normal tissue.

Tumor Temperature Measurements. A separate group of mice were used to document tumor temperature profiles as a function of the irradiance (dose rate) of delivered light. A 21-gauge thermocouple hypodermic needle (Omega Engineering, Inc., Stamford, CT) was inserted 1.5 mm into the tumor at an angle perpendicular to the plane of delivery. Intratumor temperatures were documented as a function of time during exposure to 75- and 100-mW/cm² irradiances. Average temperatures for five mice were obtained for each dose rate of delivered light.

PDT Tumor Treatments. Tumor regrowth and tumor cure assays were used to evaluate NPe6 and Photofrin II-induced PDT responses. Prior to tumor treatment, mice were administered NPe6 or Photofrin II via an i.v. tail vein injection. The mice were restrained immediately prior to tumor light treatments which were performed either 4-6 or 24-26 h after drug administration. Following treatment, each tumor was measured three times per week and these measurements were continued until the tumors had regrown to an average diameter of 12 mm (at which time the animals were sacrificed) or until at least 40 days at which time animal cures were obtained (14). Mice which developed second primary tumors outside the treatment field (due to tracking during trocar injection of tumors) were excluded from calculations used to determine the percentage of cures. A 75-mW/cm² light dose rate was used for all PDT experiments and total light doses ranged from 0 (for controls) to 500 J/cm². A minimum of 10 mice were evaluated for each drug and light dose.

PDT-mediated Normal Skin Response. Normal skin response to PDT was evaluated in albino Swiss Webster mice treated with either Photofrin II or NPe6. A quantitative skin scoring system (described in Table I) was utilized to document the degree of normal tissue damage induced by each treatment (15). The right hind foot of each animal was treated with PDT in the manner identical to that utilized in the tumor response studies. A 1-cm diameter treatment area was used during light treatments which were initiated 4-6 or 24-26 h following administration of either NPe6 or Photofrin II. A minimum of five mice were evaluated at each drug and light dose.

![Chemical structure of [14C]NPe6 with positions of 14C designated by *](image-url)
drug administration is shown in Fig. 4. The tumors were grouped into size categories of 15–50, 70–100, or 125–300 mm³. Differential tumor sizes were obtained by having the transplanted tumors grow for increasing time intervals prior to entrance into experiments. Tumors were analyzed at 4 and 24 h following an i.v. drug dose of 5 mg/kg and [14C]NPe6 levels were comparable at all tumor sizes.

Fig. 5 shows the tumor temperature rise which is induced by PDT when 664-nm light was delivered at either 75 or 100 mW/cm². Five tumors were examined at each dose rate and the average temperature rise is plotted as a function of exposure time. Light delivered at an irradiance of 100 mW/cm² induced a 4°C rise while an irradiance of 75 mW/cm² induced a 2.5°C temperature rise. Subsequent PDT experiments were performed utilizing a 75-mW/cm² dose rate in an effort to minimize temperature rise. Subsequent PDT experiments were performed utilizing a 75-mW/cm² dose rate in an effort to minimize temperature rise.
Table 3: 14C[NPe6] tissue concentrations in C3H/HeJ mice transplanted with the BA mammary carcinoma as a function of method of drug administration

Mice received a 5-mg/kg dose of 14C[NPe6] by either i.p. or i.v. injection. Tissue levels of 14C[NPe6] were determined 4 or 24 h after drug delivery. Means ± SD are expressed in units of µg eq NPe6/g of tissue. N = five mice/group.

<table>
<thead>
<tr>
<th>Tissue Level</th>
<th>Plasma</th>
<th>Blood</th>
<th>Tumor</th>
<th>Brain</th>
<th>Heart</th>
<th>Liver</th>
<th>Kidney</th>
<th>Adrenal gland</th>
<th>Spleen</th>
<th>Skin</th>
<th>Intestine</th>
<th>Muscle</th>
<th>Lung</th>
<th>Esophagus</th>
<th>Stomach</th>
<th>Bladder</th>
<th>Feces</th>
<th>Urine</th>
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</thead>
<tbody>
<tr>
<td>4 h (mean ± SD)</td>
<td>4.44 ± 2.21</td>
<td>2.39 ± 1.21</td>
<td>2.59 ± 1.34</td>
<td>0.06 ± 0.12</td>
<td>1.21 ± 0.56</td>
<td>7.48 ± 5.56</td>
<td>2.92 ± 1.31</td>
<td>3.46 ± 1.57</td>
<td>2.05 ± 0.94</td>
<td>1.38 ± 0.47</td>
<td>1.75 ± 0.65</td>
<td>0.35 ± 0.15</td>
<td>1.49 ± 0.69</td>
<td>2.27 ± 1.50</td>
<td>1.79 ± 1.10</td>
<td>4.32 ± 1.95</td>
<td>42.57 ± 42.78</td>
<td>7.72 ± 3.43</td>
</tr>
<tr>
<td>24 h (mean ± SD)</td>
<td>4.07 ± 1.20</td>
<td>2.29 ± 0.48</td>
<td>3.25 ± 1.18</td>
<td>0.12 ± 0.07</td>
<td>1.19 ± 0.27</td>
<td>11.56 ± 3.02</td>
<td>3.76 ± 0.77</td>
<td>2.26 ± 0.61</td>
<td>2.65 ± 0.33</td>
<td>1.88 ± 0.54</td>
<td>2.22 ± 0.61</td>
<td>0.36 ± 0.60</td>
<td>1.95 ± 0.47</td>
<td>1.03 ± 0.29</td>
<td>1.27 ± 0.31</td>
<td>2.11 ± 0.37</td>
<td>167.14 ± 76.77</td>
<td>8.14 ± 3.31</td>
</tr>
</tbody>
</table>

* n = 4.
bp < 0.05 (two-tailed Student’s t test).

Fig. 4. Tumor concentrations of 14C[NPe6] as a function of tumor size. Mice received a 5-mg/kg i.v. injection of 14C[NPe6] and tumor levels of photosensitizer were assayed 4 or 24 h later. Each column represents the mean ± SD (bar) for five mice.

Fig. 5. Temperature rise in the BA mammary carcinoma as a function of time during exposure to 664-nm light delivered at irradiances (dose rates) of 75 or 100 mW/cm². Temperatures were measured at a 1.5-mm depth from the tumor surface and each point represents the average temperature from five tumors.

Fig. 6. Tumor response in mice treated with NPe6-induced PDT at a light dose of 500 J/cm². Animals received either a 15-, 25-, or 50-mg/kg dose of NPe6 24 h prior to light treatment. Each point represents the average response of 10 mice.

Fig. 7. Tumor response curves following NPe6-mediated PDT. Tumors were exposed to 664-nm light 4-6 h after a 2.5-, 5.0-, or 7.5-mg/kg i.v. dose of NPe6. Total light doses ranged from 0 to 500 J/cm² and the dose rate of delivered light was 75 mW/cm². Percentage of cures were determined from animals that were disease free 40 days following treatment. Each point represents the average of 10 animals.

Fig. 8. Tumor response curves following Photofrin II-mediated PDT. Tumors were exposed to 630-nm light 24-26 h after a 2.5-, 5.0-, or 7.5-mg/kg i.v. dose of Photofrin II. Other details as in Fig. 7.

when a 1-day time interval between NPe6 injection and light treatment was used. Figs. 7 and 8 present PDT-mediated tumor response curves (percentage cures as a function of light dose) for NPe6 administered 4–6 h prior to light treatment and for a standard Photofrin II-mediated PDT protocol in which the photosensitizer is administered 24–26 h prior to light treat-
PDT PARAMETERS OF A NEW CHLORIN PHOTOSENSITIZER

NORMAL SKIN PHOTOSENSITIZATION

PHOTOFRIN II vs NP6

INTERVAL BETWEEN DRUG AND LIGHT

DAYS POST TREATMENT

Fig. 9. Normal skin photosensitization in albino Swiss Webster mice treated with either Photofrin II or NP6-mediated PDT. Skin response as a function of time following treatment is plotted for mice exposed to light 4–6 h after drug administration. NP6 doses ranged from 5 to 15 mg/kg and Photofrin II doses were 5 mg/kg. 664-nm light (300–500 J/cm²) was used with NP6 and 630-nm light (100–300 J/cm²) was used with Photofrin II. The dose rate of delivered light was 75 mW/cm² and each point represents the average score for five mice.

DISCUSSION

NP6 belongs to a growing list of compounds which continue to show promise as photosensitizers for the treatment of solid tumors using PDT (4). This chlorin derivative is a pure compound and possesses a major absorption peak at 664 nm which implies that light transmission through tissue as well as photon absorption by the drug will be superior to that which is currently achieved with Photofrin II (16). Previous mechanistic investigations have shown that NP6 enters cells via endocytosis and is ultimately localized in lysosomes (17). In addition, electron microscopic analysis of NP6-mediated photosensitization of PTK2 cells indicates that lysosomes are preferentially damaged following treatment (18). Therefore, subcellular target sites for NP6 may be significantly different than for Photofrin II which is reported to enter cells via diffusion and localize in mitochondria (3, 4, 17).

Our current data demonstrate that NP6 is an effective tumor photosensitizer (as measured by the induction of tumor cures) only when light treatment is delivered at relatively short time intervals following drug administration. However, an earlier in vivo investigation reported 100% tumor cures in mice following NP6-mediated PDT when a 24-h time interval between drug administration (with doses as low as 8 mg/kg) and light treatments of 100 J/cm² were used (10). As noted in Fig. 6, a 100% tumor recurrence rate was observed in our initial experiments when 24 h was used as the time interval between NP6 administration (15–50 mg/kg) and 500-J/cm² light treatments. Our pharmacological data suggest that the differences observed in the two studies are not due to alterations in the mode of drug administration (i.v. versus i.p.) nor in the tumor size at the time of treatment. A possible explanation for the variations in treatment parameters required to produce tumor cures following NP6-mediated PDT may involve differences in the immunogenicity of the tumor models. The EMT-6 tumor used in the previous study has been reported to be strongly immunogenic while no such reports could be found for the BA mammary carcinoma used in our current investigation (19). Additional factors such as differences in tumor vascularity and photosensitizer uptake may be involved in the observed variations in tumor response in the two studies.

The pharmacological data obtained using [14C]NP6 provides the first quantitative information related to tissue localization and distribution of this photosensitizer. Initial high-performance liquid chromatographic analysis of tissue extracts suggest that NP6 does not undergo metabolic transformation in mice.4 Relative tissue affinities for NP6 appear to mimic published reports for hematoporphyrin derivative and Photofrin II (7, 20). Drug uptake is highest in tissues such as liver, kidney, adrenal gland, and spleen for all photosensitizers while the plasma half-life of NP6 is shorter than that which is observed for the porphyrin compounds. Theories regarding mechanisms of action can be proposed when the NP6 pharmacological data are combined with the tumor response results. Specifically, results from these experiments suggest that tumor tissue levels of NP6 may not be a primary factor in the PDT-mediated tumoricidal efficiency of this drug. Figs. 2 and 3 show that tumor concentrations of NP6 at 24 h following 25- and 50-mg/kg doses (with which tumor cures could not be obtained)

4 D. Kessel, private communication.
are 6.88 and 10.60 μg/g, respectively, while a 5-mg/kg dose of NPe6 analyzed 4 h following injection (at which time PDT-mediated tumor cures can be obtained) resulted in a tumor NPe6 concentration of only 3.23 μg/g. However, Figs. 2 and 3 also show that plasma concentrations of NPe6 at 4 h following a 5-mg/kg dose were 4.0 μg/ml while plasma concentrations at 24 h following 25- and 50-mg/kg doses were 0.55 and 1.10 μg/ml, respectively. Therefore, plasma levels of NPe6 at the time of light treatment may be more closely correlated with PDT tumor response than tumor concentrations. These observations suggest that vascular damage may be a primary target in NPe6-mediated PDT. This hypothesis agrees with a previous study in which the histological examination of tumor tissue following NPe6-mediated PDT indicated that direct tumor cell kill was secondary to destruction of the microvasculature (21). Our investigation did not include histological evaluation of tumors as a function of time following PDT but such experiments would be a logical extension of current studies.

In conclusion, NPe6 has been shown to be an effective tumor photosensitizer when short time intervals are utilized between drug administration and light treatment. In addition, the magnitude of prolonged skin phototoxicity induced by NPe6-mediated PDT is significantly less than that induced by Photofrin II. These observations demonstrate that NPe6 possesses properties essential for an effective and clinically useful photosensitizer.

ACKNOWLEDGMENTS

We thank Dr. Gerald Beckloff for helpful discussions and Dr. Jerry Bommer for supplying NPe6 and [14C]NPe6.

REFERENCES

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