In Vivo Confocal Fluorescence Imaging of the Intratumor Distribution of the Photosensitizer Mono-L-Aspartylchlorin-e6

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Abstract
We present an in vivo fluorescence microscopic evaluation of intratumor distribution of the photosensitizer mono-L-aspartylchlorin-e6 (NPe6) in an intradermal mouse EMT6 tumor model. Although the identification of favorable photophysical and pharmacological properties has led to the development of new photosensitizers in photodynamic therapy, their intratumor distribution kinetics have remained relatively understudied. In this study, we used confocal fluorescence microscopy to follow the transport of NPe6 in vivo after systemic administration through the tail vein. Labeling of vasculature using fluorophore-conjugated anti-CD31 antibodies allows visualization of the uptake of NPe6 in tumor and normal vessels and its partitioning kinetics into the adjacent parenchyma for 3 hours after injection. During the initial 60 minutes after injection, the drug is predominantly confined to the vasculature. Subsequently, it significantly redistributes throughout the extravascular regions with no discernable difference in its extravasation rate between tumor and normal tissues. Further, we investigate the sensitizer’s altered intratumor distribution in response to photodynamic therapy irradiation and observe that treatment-induced changes in vessel permeability caused enhanced accumulation of NPe6 in the extravascular space. Our findings are of immediate clinical relevance and demonstrate the importance of an in vivo imaging approach to examine the dynamic process of intratumor drug distribution.

Introduction
Tissue response to photodynamic therapy (PDT) is understood to be a dynamic function of the photosensitizer concentration, the interval between photosensitizer administration and irradiation, the optical fluence, irradiance, and the local oxygen level. The complex relationships among these parameters have been studied extensively [1–3], and a recent modeling study has highlighted the interplay between intratumor photosensitizer distributions and oxygen concentrations in determining the microscopic deposition of singlet oxygen (1O2) dose [4]. Intracellular localization sites and bulk tissue pharmacokinetics have been thoroughly examined for most of the PDT sensitizers currently in clinical and preclinical evaluations. Investigation of intracellular localization is important because it determines potential subcellular targets for 1O2 and the cell death pathways they initiate [5]. Tissue pharmacokinetics report the temporal photosensitizer uptake and clearance in the blood and the bulk tissue, and these measurements have played a vital role in guiding the choice of drug-light intervals for PDT clinical trials based on criteria such as tumor versus normal tissue selectivity of the photosensitizer. For example, clinical trials with meso-tetrahydroxyphenyl chlorin (mTHPC) [6] have been informed by measurements of its time-dependent biodistribution, which evaluated the optimal drug-light interval based on maximum sensitizer selectivity [7].

One of the promising second-generation photosensitizers is mono-L-aspartylchlorin-e6, also known as NPe6, talaporfin sodium, MACE, laserphyrin, and LS11. Here, we will use the abbreviation NPe6. Successful phase I studies with NPe6 have been reported [8–10], and there is a large body of clinical experience with the drug in Japan that has

Abbreviations: BSA, bovine serum albumin; FOV, field of view; NPe6, mono-L-aspartylchlorin-e6; PDT, photodynamic therapy; ROI, region of interest
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demonstrated excellent antitumor effects [11]. NPe6-PDT is currently undergoing phase II trials for glioma and phase III trials for metastatic colorectal cancer and hepatoma (http://www.lsoncology.com/). The pharmacokinetics of NPe6 and its effectiveness in inducing tumor destruction with different drug-light intervals have been examined in preclinical models [12,13], and these studies have shown that the PDT efficacy of NPe6 is significantly greater at shorter drug-light intervals than those obtained with Photofrin-PDT, a US Food and Drug Administration–approved treatment. In addition, the skin photosensitivity induced by NPe6 is significantly less than that induced by Photofrin as reported in preclinical [12] and clinical studies [8]. At these shorter drug-light intervals, pharmacokinetic measurements have demonstrated that the drug is predominantly in the plasma bound to albumin and other serum proteins. This has led to the suggestion that NPe6 is a vascular targeting photosensitizer whose efficacy is augmented by factors such as its high extinction at 664 nm and a plasma protein binding that may contribute to minimal skin photosensitization [14]. In clinical trials of NPe6-PDT initiated by Light Sciences Oncology (Bellevue, WA), low-intensity light-emitting diodes are inserted interstitially, and photoactivating light is delivered for very long irradiation times. Although the drug-light interval is specified as 1 hour, low-irradiance irradiations of 5 to 11 hours are reported [9]. It is likely that significant redistribution of the sensitizer occurs during these long irradiation times.

With this as background, we decided to systematically investigate the intratumor distribution of NPe6 during drug-light intervals up to 3 hours using in vivo fluorescence imaging. The confocal fluorescence imaging experiments in this study were performed in intradermal tumors in live mice, which allowed us to monitor the fluorescence of NPe6 in vessels and its extravasation into tissue after i.v. administration. Quantitative tissue distributions of NPe6 were obtained in tumors and were compared to those in normal tissue. We observed that during a 3-hour period after administration, NPe6 in the vessels, as reported by the photosensitizer’s fluorescence, increases monotonically and that the drug partitions at similar rates from tumor and normal vessels into the interstitial space. In addition, we examined the distribution of the sensitizer during irradiation and found that the PDT-induced changes in tumor vessel permeability to NPe6 caused enhanced partitioning of the drug between the vessels and the extravascular space. This effect was significant, and it suggests caution in interpreting results of routine pharmacokinetic studies.

**Materials and Methods**

**Photosensitizer**

The sensitizer NPe6 (LS11) was kindly provided by Light Sciences Oncology in powder form. It was dissolved in saline and was stored in a −4°C freezer according to the manufacturer’s recommendations. The stock solution was thawed and equilibrated to room temperature before administering in animals.

**Animals and Tumor Model**

We investigated the intratumor distribution of NPe6 in EMT6 tumors grown intradermally in the ears of female BALB/c mice and in normal ears. Mouse mammary sarcoma EMT6 cells were cultured in Eagle’s basal medium with 10% fetal bovine serum (complete media) at 37°C. Using a 28-gauge needle, tumors were initiated by an injection of 2 × 10^6 EMT6 cells into the intradermal space of the rear pinna of 4- to 6-week-old mice. Approximately 10 days after inoculation of cells, the tumors reached a diameter of 3 to 5 mm. To minimize red fluorescence from a chlorophyll degradation product, during this period, mice were fed a chlorophyll-free diet prepared according to the recipe of Holmes et al. [15].

**In Vivo Confocal Imaging**

*In vivo* imaging of NPe6 distribution in the ears of anaesthetized live mice was performed using a custom-built inverted laser scanning confocal fluorescence microscope [16]. To image the distribution of NPe6 with respect to the vasculature, the vessels were rendered fluorescent by intradermal (i.d.) injection of a 30-μl solution of 0.1 μg/μl Alexa Fluor 647–conjugated anti–mouse CD31 antibodies (clone MEC13.3; Biolegend, San Diego, CA). Because i.d. injection requires the use of less antibody with no concerns regarding toxicity and enables labeling of vessels that may not be perfused, we adopted this method *versus* systemic administration for staining vasculature. Anti-CD31 was administered 24 hours before imaging to allow clearing of unbound label. An amount of 10 mg/kg NPe6 was injected i.v. through the tail vein, and the anesthetized mouse was positioned in a supine position with the tumor in contact with a coverslip mounted on the stage of the inverted microscope. Before imaging, hair was removed from the ear using a commercial depilating agent (Nair; Church & Dwight Co., Princeton, NJ). The intratumor distribution of the sensitizer was imaged during the first 3 hours after i.v. injection. We used sequential two-color excitation of identical fields of view (FOVs); NPe6 was excited with 514 nm from an argon ion laser (Coherent, Santa Clara, CA), and the Alexa Fluor 647 was excited with a 639-nm diode laser (Power Technology Inc., Alexander, AR). The NPe6 fluorescence was detected using a combination of a 525LP long pass filter (Chroma Technology, Rockingham, VT) and an RG645 long pass filter (Schott, Duryea, PA). The Alexa Fluor 647 emission was detected using a combination of a 647LP long pass filter (Semrock, Rochester, NY) and an RG665 long pass filter (Schott). The combination of a 100-μm-diameter pinhole and a 10×, 0.45 NA objective gave an optical section thickness of approximately 6 μm as determined by fluorescence edge response measurements. The images were acquired with a lateral resolution of 1 μm/pixel. Confocal fluorescence spectra of NPe6 were acquired using a 50-μm-core diameter multimode optical fiber that served as the confocal aperture, and the collected emission was dispersed by a grating onto a thermoelectric-cooled charge coupled device [16].

To visualize the distribution of albumin in normal and tumor tissues, 20 mg/kg of Alexa Fluor 555–labeled bovine serum albumin (BSA; Invitrogen, Carlsbad, CA) was administered to mice through tail vein injection. The extravasation of albumin from Alexa Fluor 647–labeled CD31-positive vessels was imaged up to 3 hours after administration. Alexa Fluor 555 was excited with the 514 nm line from the argon ion laser, and the emission was detected using a combination of 525LP (Chroma Technology) and OG550LP (Schott) long pass filters.

**PDT Treatment**

To study the kinetics of NPe6 redistribution in response to PDT treatment, tumors were subjected to 662-nm irradiation beginning 1 hour post–NPe6 injection. Irradiation was performed at an irradiance of 10 mW/cm². The treatments were briefly interrupted...
periodically to acquire a series of images of NPe6 intratumor distribution from the same FOV.

**Image Analysis**

The confocal fluorescence images were analyzed using the image processing software, ImageJ (http://rsb.info.nih.gov/ij/). ImageJ was used to read in and overlay the images acquired of the vessels stained by anti-CD31–Alexa Fluor 647 antibody and those of NPe6 or Alexa Fluor 555–BSA from identical FOV and to generate a dual-color image of each optical section. A stack of two-color images at each time point post-NPe6 and BSA administration was created, and by color segmenting CD31-positive images and thresholding over the background, the vessels were identified and the temporal kinetics of NPe6 and BSA distribution in the intravascular regions were obtained. This ensured that pixels from the extravascular space were excluded, and drug fluorescence intensity was measured only from regions that were bounded by CD31-positive signal. To quantify the extravasation kinetics of NPe6 in response to irradiation, at least five regions of interest (ROIs) were chosen in each of the tumor optical sections analyzed. The ROIs were selected beginning from but not including the boundary of a CD31-labeled vessel wall and had dimensions of at least 50 μm (height) × 50 μm (width). A width of 50 μm ensured that the fluorescence intensity was averaged over multiple cell layers to avoid any sampling errors introduced by analyzing extravascular regions that were in close proximity to the vessel wall.

**Results**

We labeled vessels with Alexa Fluor 647 fluorophore–conjugated anti-CD31 antibodies and visualized the distribution of the photosensitizer, NPe6, relative to the vasculature using in vivo imaging. Figure 1 shows a fluorescence image of anti-CD31–labeled tumor vessels at a depth of 100 μm in an EMT6 tumor in vivo. The image was acquired 24 hours after i.d. injection of fluorophore-conjugated anti–mouse CD31 antibody in the mouse ear. As illustrated, this antibody labeling technique enabled acquisition of high-quality images of the vasculature with excellent contrast. High signal-to-background ratio persisted up to 3 days after antibody injection (data not shown).

Shown in Figure 2, A–H, is a representative panel of images illustrating the time course of NPe6 accumulation in the same FOV of an EMT6 tumor after i.v. administration. The vasculature stained by Alexa Fluor 647–conjugated anti-CD31 and the NPe6 fluorescence are illustrated in red and green colors, respectively. During the first 60 minutes after injection, NPe6 is predominantly present in the vessels, and only a small fraction has extravasated. However, during the period between 60 and 180 minutes, there is a significant redistribution of the sensitizer, producing an approximately uniform fluorescence throughout the extravascular space in this FOV. At the 150- and 180-minute time points, the regions of fluorescence signal void correspond to lipid-rich sebaceous glands from which the water-soluble NPe6 is excluded.

Although in most of the imaged tumors, NPe6 was reasonably well distributed at 3 hours after injection, in some cases, it remained extremely nonuniform. The panel of images in Figure 3 shows a segmented two-compartment distribution pattern that is presumably due to the presence of well and poorly perfused vessels that are separated by only ~200 μm. The lower left quadrant of the image exhibits distribution kinetics similar to those observed in the images of Figure 2, whereas the lower right and upper half have extremely low levels of NPe6 fluorescence in the vessels and the extravascular regions even at 3 hours after administration. Although this phenomenon was not reproducible in all the animal tumors imaged, the observation demonstrates the complex heterogeneity of intratumor drug distribution even at early drug-light intervals.

To compare the distribution kinetics of NPe6 in tumor versus normal tissue, we imaged the vascular uptake and extravasation of NPe6 in normal ear tissue using the same methodology and imaging techniques. Figure 4 shows a panel of images illustrating the distribution of NPe6 in normal ear tissue at 15, 30, 60, and 120 minutes after administration. Similar to the distribution kinetics in tumors, at early time points, the majority of the drug is retained in the vessels, but at longer times, it partitions from the vasculature into the adjacent parenchyma. The pattern of severe spatial heterogeneity in NPe6 distribution as observed in some of the tumors (Figure 3) was not observed in any of the normal ears imaged.

The kinetics of NPe6 uptake in normal and tumor vessels and its distribution in the adjacent extravascular space are summarized in Figure 5, A and B, respectively. Figure 5A shows the mean NPe6 fluorescence intensity measured in vessels up to 3 hours after administration. We observe a monotonic increase in sensitizer levels in both normal and tumor vessels, thus indicating that NPe6 remains in circulation during this period. Interestingly, the accumulation of NPe6 occurs at a slightly but significantly faster rate in tumor versus normal vessels in the range of time points between 120 and 165 minutes. Figure 5B demonstrates a representative map of the temporal kinetics of extravascular NPe6 distribution in tumor as a function of distance from vessels. This distribution was obtained by superimposing an ROI (20 μm × 105 μm) on the tumor images of Figure 2 as illustrated in Figure 5C for the 180-minute time point. A significant redistribution of the sensitizer occurs during the initial 3-hour period.
after injection, and consistent with the panel of images illustrated in Figure 2, the sensitizer fluorescence signal near and remote from the vessels continues to increase with time during this interval.

Our imaging observations illustrate that NPe6 leaks out of both normal and tumor vessels into the adjacent parenchyma. If NPe6 is bound to albumin in the vessels as has been suggested [14], it would imply that both tumor and normal vessels are permeable to albumin. To test this hypothesis, we imaged the egress of Alexa Fluor 555–conjugated BSA from tumor and normal vessels. Shown in Figure 6, A and B, are images of Alexa Fluor 555–BSA (green) distribution in tumor and normal tissues, respectively, with the labeled CD31-positive vasculature in red, acquired ∼3 hours after i.v. albumin injection. The images provide strong evidence that BSA partitions from tumor and normal vessels and distributes into the adjacent interstitial space with spatial distribution patterns that are similar to those observed with NPe6 extravasation in tumor (Figure 2) and normal (Figure 4) tissues.

We have observed previously that the fluorescence spectrum of NPe6 is affected by binding to albumin. This offered the opportunity to use confocal fluorescence spectroscopy measurements to

Figure 2. Panel of in vivo confocal fluorescence images from the same field of view (FOV) of an EMT6 tumor acquired at different time points after i.v. administration of 10 mg/kg NPe6. The FOV in all the images is 600 μm × 600 μm, and the optical section thickness is 6 μm. NPe6 fluorescence is in green, and CD31-positive vessels labeled by Alexa Fluor 647–conjugated antibody are in red.

Figure 3. Panel of in vivo confocal fluorescence images from the same field of view (FOV) of an EMT6 tumor acquired at different time points after i.v. administration of 10 mg/kg NPe6. This representative panel of images highlights the severe heterogeneity of NPe6 distribution observed in some of the tumors that were imaged. The FOV in all the images is 800 μm × 800 μm, and the optical section thickness is 6 μm. In (H), the regions of fluorescence signal void correspond to lipid-rich sebaceous glands from which the water-soluble NPe6 is excluded.
further validate whether NPe6 is bound to serum proteins in extra-vascular tissue. Vascular and extravascular ROIs were identified in images, and \textit{in vivo} confocal fluorescence spectroscopy [16] was performed to acquire NPe6 emission spectra from those ROI. Figure 7A shows the confocal fluorescence spectrum of NPe6 (\textit{circles}) acquired from an extravascular region in the image shown in Figure 2. The spectrum has a peak at \(\sim 670\) nm and is almost identical to that obtained from a solution of NPe6 bound to BSA (Figure 7A, \textit{solid line}) but differs significantly from an emission spectrum of NPe6 in aqueous solution in the absence of BSA, which has a blue shifted peak at \(\sim 650\) nm (Figure 7B). Further, we note that the aqueous NPe6 (– BSA) spectrum has a full-width at half-maximum of \(\sim 28\) nm, whereas the spectra from both the tumor tissue and the BSA solution have a full-width at half-maximum of \(\sim 20\) nm. This spectral evidence therefore supports the postulation that NPe6 is bound to BSA or to other serum proteins as it distributes in the vessels and in the adjacent tissue region.

To determine whether PDT changes the kinetics of NPe6 extravasation, we imaged NPe6 distribution after the delivery of specific treatment fluences after a 1-hour drug-light interval. The panel of images in Figure 8 demonstrates that there is a significant increase in NPe6 fluorescence levels in the interstitial space after the delivery of 5 J/cm\(^2\), and the intensity appears to keep increasing even after 20 J/cm\(^2\). Quantification of the sensitizer partitioning from the vessels in response to PDT was performed by measuring NPe6 fluorescence in different ROIs adjacent to CD31-positive–labeled vessels. Figure 9 summarizes the mean enhancement of NPe6 fluorescence levels \textit{versus} unirradiated controls as a function of irradiation time corresponding to the delivery of fluences ranging from 3.5 to 20 J/cm\(^2\). The plot in Figure 9A shows that NPe6 levels increase by approximately two-fold after the delivery of 3.5 J/cm\(^2\) and by approximately five-fold after a 20-J/cm\(^2\) irradiation. Because the drug-light interval in irradiated
tumors is 1 hour, to make an appropriate comparison between the rates of NPe6 partitioning from irradiated versus unirradiated vessels, we analyzed the change in NPe6 fluorescence in extravascular regions of unirradiated tissue normalized to its levels at 1 hour after injection. As shown in Figure 9B, the five-fold increase in extravascular NPe6 accumulation observed in ~35 minutes in irradiated tumors requires approximately 120 minutes in unirradiated controls. Similar levels of increase in NPe6 extravasation were also observed in tumor tissue that was subjected to irradiation after a 3-hour drug-light interval (data not shown). Thus, the rate of NPe6 leakage from PDT-treated vessels is significantly faster than that observed from unirradiated vessels. These results therefore demonstrate that NPe6-PDT increases vascular permeability and potentiates the accumulation of extravascular sensitizer during irradiation.

In the panel of images of NPe6 distribution shown in Figure 8, we note that there is significant fluctuation of fluorescence levels in several intravascular regions in response to irradiation. Shown in Figure 10A is the mean temporal fluctuation in NPe6 amplitude measured from selected intravascular regions of the imaged tumor tissue illustrated in Figure 8. We find that NPe6 levels initially fall with irradiation due to bleaching; however, after 7.5 J/cm<sup>2</sup> fluence, the drug levels rise appreciably and show a decrease only after delivery of 20 J/cm<sup>2</sup>. Similar patterns of fluctuation in intravascular NPe6 fluorescence were observed in other tumors that were imaged during irradiation after a 1- or 3-hour drug-light interval. This observation provides support that NPe6 remains in circulation for an extended period of time and also suggests that PDT irradiation may be inducing changes in blood flow rate as has been reported previously by other investigators for Photofrin-PDT [17].

Discussion

Intratumor drug distribution is an extremely relevant aspect of PDT and has important implications for the choice of optimum drug-light interval. These microscopic-scale measurements can inform the rate and extent of drug distribution with respect to the vasculature, which is the source of both photosensitizer and oxygen. Fluorescence imaging is an ideal method for mapping the microscopic distribution of photosensitizers, because most of these drugs are fluorescent. However, with very few exceptions [18,19], their time-dependent intratumor distribution as a function of distance from a
blood vessel at the time of irradiation remains significantly understudied. Zhou et al. [18] explored the distribution of the sensitizer verteporfin as a function of distance from stained blood vessels in frozen tissue sections from subcutaneous and orthotopic rat tumors. In a recent study, we investigated the intratumor distribution of the sensitizer mTHPC with respect to perfused vasculature in optical sections of freshly excised tumor tissue at various times after systemic injection using a whole-mount technique [19]. We reported that mTHPC undergoes a remarkable reversal of its initial distribution pattern with increasing drug-light intervals. A point of view sometimes expressed in the PDT literature considers drug distribution in vivo to be either predominantly intravascular or extravascular, but this limited number of intratumor drug distribution studies have been instrumental in creating an appreciation that the situation is more complex, dynamic, and interesting.

Although the whole-mount imaging technique allows spatial mapping of microscopic distributions with preservation of minimally perturbed tumor architecture, these measurements are vulnerable to systematic error for early times after i.v. administration when sensitizer redistribution is likely to be relatively rapid. Therefore, for sensitizers that are irradiated at short drug-light intervals, it is especially important to image their initial distributions in vivo. A particular advantage of this approach is that, because in vivo imaging is noninvasive in nature, the same tumors can be imaged repeatedly during a period of several hours, providing raw unnormalized data from the same animal, enabling examination of the temporal kinetics in addition to the microscopic spatial information. Hamblin et al. [20] were the first to demonstrate the use of laser scanning fluorescence microscopy to follow the transport of a photosensitizer in vivo in an animal tumor model. Their microscope system used a low numerical aperture objective and a large detector aperture to acquire images in a nonconfocal mode. The in vivo system that we have used in this study is a laser scanning confocal fluorescence microscope that offers the advantage of optical sectioning to obtain three-dimensional visualization of the tissue architecture. Imaging depth in our system is limited by tissue scattering to \( \sim 130 \) \( \mu \)m in normal ear and tumor

\[ \text{Figure 8. Panel of in vivo confocal images illustrating the accelerated partitioning of NPe6 from the tumor vasculature into the tissue after irradiation. The first image is from a FOV in a tumor, acquired at 60 minutes after NPe6 administration. The subsequent images are from the same FOV obtained at different time points corresponding to the delivery of specific fluences ranging from 5 to 20 J/cm}^2. \]

\[ \text{Figure 9. (A) Temporal kinetics of NPe6 extravasation in tumor tissue demonstrating an approximately five-fold increase in NPe6 fluorescence in extravascular regions after treatment of up to 20 J/cm}^2 \text{ that corresponds to a total irradiation period of \( \sim 35 \) minutes at an irradiance of 10 mW/cm}^2 \text{ at 662 nm. The data points are an average of at least 20 measurements from four independent experiments, and the error bars represent SDs. (B) A similar fold increase in the partitioning of NPe6 from unirradiated vessels requires \( \sim 120 \) minutes.} \]
tissue in mice, but this depth is sufficient to visualize all the relevant drug distribution kinetics. To the best of our knowledge, no other imaging technique offers this ability to examine such a dynamic process at this level of spatial and temporal resolution.

As illustrated in the images of Figure 2, in the absence of irradiation at 2 to 3 hours after i.v. administration, NPe6 undergoes significant redistribution from the vasculature into the tumor tissue and therefore cannot be considered as a purely vascular targeting drug. This has important implications for the interpretation of preclinical studies [21,22] and clinical trials [9] with NPe6 that cite a 1-hour drug-light interval but use irradiation periods that are in the range of 2 to 5 hours. Further, we note that the drug levels in both the tumor and normal vessels increase monotonically during the first 3 hours (Figure 5A), thus indicating that the photosensitizer stays in circulation during a long period of time.

The approximately five-fold increase in intravascular NPe6 fluorescence after i.v. drug administration is an unexpected and surprising result. Indeed, previous animal studies have reported that plasma levels of $^{14}$C-labeled NPe6 decreased by approximately five-fold within the 2- to 4-hour period after i.v. injection [12,13]. We note, however, that a pharmacokinetic study of NPe6 plasma levels measured from cancer patients showed a slower decrease in drug fluorescence levels during the same period for a range of i.v. injection concentrations of 0.5 to 3.5 mg/kg [14]. This study reported that the plasma clearance rates dropped at the highest administered dose of 3.5 mg/kg, suggesting a saturation of the NPe6 elimination process from the plasma. We used a clinically relevant i.v. injection concentration of 10 mg/kg NPe6, and it is possible that the kinetics we observed through our fluorescence measurements may, therefore, have been influenced by this clearance saturation effect. Clearly, however, this could not account for the observed increase in fluorescence in the normal and tumor vasculature of the mouse ear. It is possible that this increase results from one or a combination of the following: a fraction of NPe6 in the plasma may initially be in an aggregated form, and with time, it may monomerize, consequently increasing the measured fluorescence; and/or the intravascular drug concentration kinetics in the peripheral circulation of the ear may be different from that determined by bulk plasma clearance measurements. The conclusion that high concentrations of NPe6 are present in the circulation for an extended period is also supported by our observation of a significant increase in intravascular drug fluorescence levels after an initial drop in intensity due to irradiation-induced photobleaching, as shown in Figure 10.

We observe that at 3 hours postadministration NPe6 is well distributed in the tumor tissue with fluorescence intensity at a distance of 75 μm from the vessel wall only approximately two-fold lower than the peak value at the vessel (Figure 5B). This distribution pattern is in sharp contrast to the severely nonuniform distribution observed with mTHPC at the same time point after injection, where drug levels at 75 μm from perfused vessels were approximately five-fold lower than at the vessel [19].

The problem of interstitial drug transport and the heterogeneity of intratumor drug distribution is a subject that has been extensively studied in the chemotherapy field. The same challenges are faced in PDT, where nonuniform photosensitizer delivery in tumors may contribute to the incomplete eradication of tumors after irradiation. In the images illustrated in Figure 3, we demonstrate the severity of the nonuniform drug distribution created within a relatively small area of 800 μm × 800 μm. Although such severe heterogeneity in NPe6 distribution was not observed in every tumor region that was imaged, the images reiterate an important point that the successful distribution of a sensitizer into tumor interstitium is not just governed by a passive diffusion process and that a feeding vessel’s perfusion status and the drug’s binding properties play an important role in its extravasation kinetics. The fact that similarly extreme heterogeneity in NPe6 distribution was not observed in normal ears may be attributed to the existence of chaotic vasculature and uneven microcirculation in tumors [23]. The phenomenon of intermittent tumor vessel perfusion and its relevance to tumor hypoxia have been recognized in the radiation biology literature for at least two decades [24].

Our observation of NPe6 partitioning into unirradiated tissue during time scales that correspond to PDT irradiation periods is further complicated by the finding that the rate of NPe6 extravasation is accelerated in response to treatment. The panel of images and summarized data in Figures 8 and 9, respectively, clearly indicate that after a 1-hour drug-light interval, PDT irradiation induces an increase in vascular permeability to NPe6, which results in enhanced drug levels in the extravascular tissue. PDT-induced vascular permeability has been investigated previously by several other investigators, and an increase in vascular leakiness has been reported post-PDT with...
different sensitizers. Fingar et al. [25] performed a detailed study using intravitral microscopy to examine the role of microvascular damage in PDT and found that Photofrin-PDT resulted in a significant increase of vascular permeability. Their study reported that increased vascular leakiness post-PDT, as measured by the leakage of fluorophore-labeled albumin from the blood vessels into interstitial space, occurred at discrete focal points rather than continuously along the vessel wall. This is consistent with our observations of NPe6 leakage from discrete regions of vessels during irradiation (data not shown). Interestingly, in another study by the same group [26], the authors reported that no significant change in tumor vascular permeability was observed after NPe6-PDT. Although the reasons for the discrepancy between their observations and our current results are not clear, it is possible that the differences in treatment protocol, such as irradiance, between the two studies could be attributing. Recent studies have also reported enhanced vascular leakiness in response to PDT irritation of verteporfin- and 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH)-sensitized tumors [27,28]. In fact, Snyder et al. [28] used the HPPH-PDT-induced vascular permeability to facilitate the improved delivery of the macromolecular therapeutic agent, Doxil, which resulted in increased tumor cures. Those authors proposed this combination treatment as a means to exploit increased vascular leakiness caused by PDT irradiation.

The data presented in this study show that vascular leakiness induced by NPe6-PDT results in increased partitioning of the photosensitizer from the vasculature. This is likely to augment the deposition of $O_2$ dose to tumor cells and potentiate direct tumor cytotoxicity. This implies that the delivery of a cytotoxic dose in NPe6-PDT at short drug-light intervals is not limited to the vasculature. Over the long periods of irradiation that are currently being used in NPe6 clinical trials, this effect would be amplified, and the PDT damage cannot be solely attributed to a vascular-shutdown phenomenon, as contribution from direct cell injury and/or death will be nontrivial. Further, as the increase in vascular permeability is observed within short irradiation time scales, it supports the hypothesis that vessel-permeabilizing effects are initiated by cytoskeletal responses induced by PDT damage to the endothelium that results in the rapid formation of large endothelial gaps [27,29].

In conclusion, we have presented an extensive in vivo imaging study of NPe6 intratumor distribution and showed evidence that the photosensitizer undergoes significant redistribution from the vessels into interstitial space and that the distribution kinetics are significantly accelerated when the tumor is subjected to PDT. These findings are of immediate clinical relevance, as NPe6-PDT is currently undergoing phase II/III clinical trials, and informed treatment plans based on knowledge of the sensitizer’s distribution is likely to improve the therapeutic outcome.

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