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Vascular targeting to the SST2 receptor improves the therapeutic response to near-IR two-photon activated PDT for deep-tissue cancer treatment

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Abstract

Background—Broader clinical acceptance of photodynamic therapy is currently hindered by (a) poor depth efficacy, and (b) predisposition towards establishment of an angiogenic environment during the treatment. Improved depth efficacy is being sought by exploiting the NIR tissue transparency window and by photo-activation using two-photon absorption (2PA). Here, we use two photon activation of PDT sensitizers, untargeted, targeted to SST2 receptors or EGF receptors, to achieve deep tissue treatment.

Methods—Human tumor lines, positive or negative for SST2r expression were used, as well as murine 3LL cells and bovine aortic endothelial cells. Expression of SST2 receptors on cancer cells and tumor vasculature was evaluated *in vitro* and frozen xenograft sections. PDT effects on tumor blood flow were followed using *in vivo* scanning after intravenous injection of FITC conjugated dextran 150K. Dependence of the PDT efficacy on the laser pulse duration was evaluated. Effectiveness of targeting to vascular SST2 receptors was compared to that of EGF receptors, or no targeting.

Results—Tumor vasculature stained for SST2 receptors even in tumors from SST2 receptor negative cell lines, and SST2r targeted PDT led to tumor vascular shutdown. Stretching the pulse from ~120 fs to ~3 ps led to loss of the PDT efficacy especially at greater depth. PDT targeted to SST2 receptors was much more effective than untargeted PDT or PDT targeted to EGF receptors.

General significance—The use of octreotate to target SST2 receptors expressed on tumor vessels is an excellent approach to PDT with few recurrences and some long term cures.

Keywords

Photodynamic therapy; Somatostatin receptor 2; Vascular shutdown; Laser pulse

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1. Introduction

Photodynamic therapy (PDT) is an approach to cancer treatment where a photosensitizer is introduced into tumor tissues, followed by activation with light of the appropriate wavelength [1, 2]. Provided that the sensitizer is raised to a sufficiently high-energy triplet state, excitation energy is transferred from the sensitizer to molecular oxygen, which, in turn, proceeds to generate singlet oxygen and other active oxygen species [2]. Singlet oxygen causes direct chemical damage to tumor tissues and initiates an inflammatory response [3]. Stimulation of anti-tumor immune responses has also been reported [4, 5].

Interestingly, the earliest known reference to a PDT-like treatment dates as far back as ancient Egypt [6]. PDT treatment is relatively gentle on patients [7,8] and it offers several other advantages, such as minimal risk of developing resistance even after repeated treatment [9]. PDT is often still effective after the tumors have already become resistant to chemotherapeutic agents [9]. In spite of these marked advantages, and notwithstanding its historical familiarity, PDT has so far failed to gain broader acceptance as a clinical treatment modality, mainly due to two remaining problems. These are: a) poor depth efficacy, and b) the establishment of an angiogenic environment following anoxia developing during the treatment. The angiogenic environment facilitates tumor recurrence and could favor tumor metastasis [6].

Currently approved PDT sensitizers are activated by light in the visible wavelengths ($\lambda < 750$ nm), where the light has a rather limited ability to penetrate tissues deeper than a few mm. The so-called NIR tissue transparency window ($\lambda \sim 780 - 1000$ nm) provides for much better tissue penetration, essentially due to reduced absorption and reduced scattering of the light. However, because the energy of the photon absorbed by the sensitizer is proportional to the inverse of the wavelength, above some critical wavelength the energy is no longer sufficient to activate the PDT process [7].

Our group, in collaboration with Dr. C.W. Spangler has designed, synthesized and characterized a series of porphyrin-based compounds with high two-photon absorption (2PA) cross sections [7]. By using a high peak intensity NIR laser we have demonstrated effective generation of singlet oxygen *in vitro* in solution [8] and showed that two low energy photons were effectively summed to provide the higher energy of a single, shorter wavelength, photon. Using this protocol, we were able to conduct PDT treatment of subcutaneous tumors through the body of the host mouse, and obtained good tumor regressions with some cures [7]. We use two-photon activation of our PDT sensitizers in all the studies described in this manuscript, verifying the protocol by the laser beam pulse requirements for good depth efficacy. A more recent approach to utilize the tissue transparency window is the use of upconversion luminescence nano particles (UCNPs)[10]. Here, NIR light is absorbed by the materials comprising the nanoparticle. Luminescence at a shorter, more energetic, wavelength then activates the sensitizer that is linked to the surface of the nanoparticle. Using UCNPs, direct irradiation of subcutaneous tumors by light of 980 nm wavelength led to largely curative PDT responses, while doing the same, with an 8 mm thick section of pork laid over the tumor, only delayed tumor growth[10]. The near-infrared tissue transparency region is also used to increase depth efficacy for other light driven medical technologies such as tumor cell imaging and photothermal therapy using gold nanoparticles [11, 12].

There have been several approaches to prevent PDT from generating an angiogenic environment [6]. The process of vascular regrowth after PDT was well described by Nowak-Sliwinska et al. using the chick chorioallantoic membrane [13]. These authors were also able

to demonstrate that the anti-VEGF antibody, Avastin®, inhibited the vascular regrowth [13]. In a more recent manuscript, these authors showed that the angiostatic kinase inhibitors, sorafenib, erlotinib and, in particular, sunitinib, also improved the results of PDT[14]. Ferrario et al. [15] combined treatment with two antiangiogenic peptides, either IM862 or EMAP-II, with photofrin PDT. Both combinations increased the effectiveness of PDT, and, in particular, led to more cures. Jiang et al. demonstrated that antibodies against VEGF receptors could also improve the results of PDT[16].

Rather than using combination therapies, it is also possible to target PDT sensitizers to tumor vascular tissues. Cheng et al. have used SnCe6 conjugated to factor VII in order to target tissue factor expressed on both the cancer cells and the angiogenic endothelial cells[17]. Their approach also led to improved PDT results. Many different tumor histotypes overexpress the somatostatin 2 receptor[18–20]. Proliferating vascular endothelial cells[21, 22] and peritumoral veins also express this receptor[19], and there exist a large number of somatostatin analogs, some of which are in clinical use to treat or image neuroendocrine tumors[23, 24]. We decided to use one of these, octreotate, to target SST2 receptors. This peptide, derives from the binding domain of native somatostatin, is a cyclic peptide containing two d-amino acids to improve its serum stability, and has a COOH terminal to support cell internalization[25]. Octreotate would also be expected to have angiostatic properties by itself[24].

Our goal in this project is to evaluate the effects of two different PDT targeting agents with respect to their abilities to induce tumor regressions and cures. To do this, the same porphyrin based 2-photon activatable sensitizer was coupled to either octreotate[25] or a phage display derived EGF targeting peptide, GE11[26]. Results from these targeted PDT experiments were also compared to results from PDT using the same sensitizer with no targeting moiety.

2. Materials and methods

2.1. Cell lines and culture conditions

The NCI-H69 human small cell, and the human A 549 non-small cell, lung cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). While the NCI-H69 line overexpresses SST2 receptors[27], the A 549 cell line does not express SST2 receptors[28]. The human FaDu cell line, which originated from a hypopharyngeal tumor, was also obtained from ATCC[29]. We conducted microarray expression profile experiments using mRNA isolated from FaDu cells grown in tissue culture and from subcutaneous FaDu tumors induced in *SCID* mice. RNA integrity was assessed using an Agilent 2100 bioanalyzer, and all RNAs scored at least 8/10. The Affymetrix GeneChip human genome array HG-U133A 2.0 was used to evaluate the expression profile using 10µg labeled and fragmented cRNA per chip. On analyzing the results, we found no evidence for expression of SST2 receptors by FaDu cells. The T-47D human ductal breast carcinoma was obtained from ATCC[30], and shown by us, using immunostaining, to express SST2 receptors (this manuscript). The murine lewis lung carcinoma[31] was also obtained from ATCC, and subcutaneous tumors were induced in C57/BL6 mice from cells harvested from near confluent tissue cultures of these cells. Bovine aortic endothelial (BAE) cells [32] were from frozen stock generously provided by Dr Stephen M. Schwartz, University of Washington, Seattle.

With the exception of the BAE cells, all cell lines were propagated in tissue culture in 50%: 50% DMEM/F12 medium (Hyclone), supplemented with 10% fetal bovine serum (Hyclone), insulin, transferrin and selenium supplement (ITS, Mediatech) and cover antibiotics. BAE cells were propagated in high glucose DMEM (Gibco Life Sciences),

supplemented with 10% fetal bovine serum, 100 µg/ml porcine intestine mucosal heparin (Sigma), 5µg/ml endothelial cell growth supplement from bovine neural tissue (Sigma) and cover antibiotics. Standard tissue culture plastic flasks (Techno Plastic Products) were used, and cells were subcultured using a wash of Ca²⁺ - and Mg²⁺ -free Tyrode's balanced saline followed by a minimal exposure to the same saline solution containing 0.05% trypsin and 0.02% EDTA. Cells were harvested in complete serum containing medium to inactivate any remaining trypsin.

2.2. Preparation of tumor tissue for immunostaining

Tumors were allowed to grow subcutaneously in *SCID* mice until they were at least 1 cm³. Mice were sacrificed and the tumor was dissected out. The tumor tissue was trimmed to only keep areas that showed no signs of necrosis. The tissue was then placed in a cryomold (Tissue Tek®), embedded in OCT compound matrix (Tissue Tek®), and the mold was set to float within a plastic petri dish in liquid nitrogen until frozen. Frozen tissue was stored at -80°C. Tissues were sectioned into 10 µm sections using a cryostat set at -20°C and picked up by melting onto a Superfrost Plus Gold slide. Prepared slides were stored at -80°C until needed for staining.

2.3. Immunohistochemical staining

Prepared slides were dried overnight at room temperature. To begin staining, sections were fixed in 75% cold acetone and 25% water for 10 minutes at 4°C then air dried at room temperature for 20 minutes. Sections were washed three times in PBS buffer and twice in PBS containing 0.05% Tween 20 (PBS-Tween). The sections were incubated in the primary antibody for one hour at room temperature in a hydration chamber, followed by three rinses in PBS-Tween. The primary antibody was detected by incubation with fluorescent Protein A for one hour at room temperature in a hydration chamber. Sections were washed three times in PBS-Tween, twice in PBS and once in sterile deionized water. Coverslips were mounted on tissue sections using Prolong Gold Antifade (Molecular Probes) and dried. Sections were examined using a Leica® TCS-NT laser scanning confocal microscope equipped with a 63× 0.9 NA oil immersion objective, and either 488 nm or 568 nm laser excitation sources. Fluorescence filter combinations used for fluorescein dyes were TD 488/568/633, RSP 580, BP 525/50. For SST2 receptor staining, the primary antibody used was a rabbit polyclonal anti-somatostatin 2 receptor (SST2r) (Advanced Targeting Systems) diluted 1:250 in PBS-Tween. The probe for primary antibody detection was Protein A-FITC (Pierce) diluted 1:50 in PBS-Tween. The anti-SST2r antibody was developed in a rabbit using a peptide corresponding to the extracellular domain of SST2r, therefore, membrane antibody binding is expected in non-permeable cells expressing this receptor. To ensure tumor vasculature was being stained using the anti-SST2r antibody, tissue sections were also stained independently with rabbit polyclonal anti-human vonWillebrand Factor (vWF) (Factor VIII) (Dako), an established endothelial cell marker. The anti-vWF antibody was diluted 1:100 in PBS-Tween. Negative control slides omitted the primary antibody.

To demonstrate SST2 receptor expression on BAE cells, we used octreotate, conjugated to biotin. Octreotate was biotinylated with EZ-Link® Amine-PEGn-Biotin (Pierce) using the procedure recommended by Pierce. After conjugation over 2 hours, any unreacted biotinylation reagent and EDC by-products were removed by passing the mixture through a Bio-Gel P-GDG desalting column. The biotinylation was confirmed using binding to streptavidin conjugated to Alexa Fluor 488 (Pierce), and the presence of octreotate by spectrophotometer readings at λ=214 for the peptide bond. Based on the readings for the fractions separated on the desalting column, the fractions were pooled into three samples. One of these, having the highest biotin and peptide readings, was considered to contain successful product. This sample was dialyzed using 1000 kD cut off pore size dialysis tubing

(Spectrum Labs) using three changes of 1/30 diluted Dulbecco's PBS. The dialyzed product was lyophilized and reconstituted in 2 ml sterile deionized water.

2.4. Competitive binding of antibody and octreotate to SST2 receptors in vitro

T-47D cells were grown on glass coverslips, then fixed and stained as described above. One group of coverslips was first washed gently with PBS then incubated in a 1mg/ml solution of octreotate for 4 hours at room temperature. Following several washes with PBS to remove unbound octreotate, the cells were stained for the SST2 receptor using the anti-SST2r antibody as described earlier. The control coverslips omitted the octreotate incubation.

2.5. Vascular effects of free octreotate blockade on SST2 receptor targeted PDT

SCID mice, bearing subcutaneous NCI-H69 xenograft tumors in the flank, were used for this experiment. The tumors were induced from freshly harvested tissue cultures of NCI-H69 cells by injecting 5×10^6 cells in 0.1 ml Tyrode's CMF subcutaneously, and were used when 8–10 mm in diameter. As a blood flow tracer, FITC conjugated dextran 150K was purchased from Sigma and was made up at 10 mg per ml in CMF. 2 mg in 0.2 ml CMF were injected slowly intravenously in each mouse, and fluorescent *in vivo* scans were carried out 40 minutes later. These scans were performed using a 465 narrow band pass excitation and a 535 high band pass emission filter for FITC on a Kodak 2000MM scanner. Baseline *in vivo* fluorescence scans were taken for each tumor 48 hours before PDT treatment, and the effects of PDT treatment were monitored by fluorescence scanning 6 hours after laser PDT activation. In one group of 4 mice, octreotate, 100 μ g, was injected intravenously two minutes before PDT sensitizer infiltration to evaluate the role of endothelial SST2 receptors in any vascular shutdown. The control group of 4 mice received an equivalent amount of CMF intravenously in place of the octreotate injection. Six hours after the PDT treatment, all mice were again intravenously injected with FITC conjugated dextran 150K, and were scanned once more at 40 minutes after that injection.

2.6. Synthesis of targeted porphyrin sensitizers and two-photon PDT conditions used

The basic synthesis of these porphyrin sensitizers, including the linkage to the SST2r targeting peptide, octreotate, is described in [7]. The EGF receptor targeting peptide, GE11, was originally identified from phage display experiments [26], and was shown to be internalized by cells after binding to the EGF receptor. For the experiments described herein, we purchased aliquots of targeted and untargeted sensitizers from SensoPath Technologies (Bozeman, MT).

2.7 PDT treatment of tumor bearing mice

This is described in detail in [7]. Briefly, tumors were sensitized by local infiltration of the tumor with the sensitizer dissolved in HS-15 (BASF) excipient and diluted in serum free DMEM/F12 medium. After 4 hours to allow for the tumor to take up the sensitizer, the mice were anesthetized using isoflurane in oxygen and the tumor laser irradiated as described in [7]. Hair over the tumor and in the region where the laser would impact the skin of the animal (if different) was removed under anesthesia the day before PDT treatment. The narrow laser beam was rastered through the area of the tumor by having the mouse supported on a mechanical microscope stage and rastering the stage via its computer control software [7]. Mice received buprenorphine subcutaneously at the start of laser irradiation, as well as 1 ml CMF subcutaneously at the start and the end of laser irradiation. The subcutaneous CMF ameliorated the effects of fluid shifts that can occur during PDT treatment.

2.8 Laser pulse requirements for two-photon PDT depth efficacy

Light from three different light sources was used to investigate how the pulse parameters influence the activation of the sensitizer: (a) Output from 1 kHz repetition rate, 1.2 W average power regenerative Ti:sapphire amplifier (Coherent Legend HE[®]); (b) Output from 75 MHz repetition rate, 600 mW average power Ti:sapphire femtosecond oscillator (Coherent Mira 900[®]); (c) continuous wave (CW) 2 W average power diode laser. An optical power meter with a thermoelectric probe (Coherent, LabMaster) was used to measure the average power at the mouse. The peak wavelength of the Ti:sapphire laser pulses and of the diode laser output were measured with diffraction grating spectrometer (Ocean Optics USB 4000), and constituted $\lambda = 795$ nm and $\lambda = 805$ nm, for the Ti:sapphire and the CW diode laser, respectively. The pulse duration (FWHM) of the Ti:sapphire oscillator as well as of the optimally compressed Ti:sapphire regenerative amplifier output was 120 fs. In addition, the pulse duration at the output of the regenerative amplifier was stretched to ~3 ps by moving the corner reflector prism in the pulse compressor. The stretched pulse had the same wavelength and the same energy as the optimally compressed 120 fs pulse. The duration of the pulses was measured with a second harmonic generation autocorrelator (Clark MXR AC 100). A schematic of the experimental setup for PDT treatment of tumor bearing mice is shown in Fig. 1. The same light delivery system consisting of mirrors, an electronically operated shutter (Uniblitz[®]) and focusing lens ($f=350$ mm) was used for all illumination modes.

The mouse was anesthetized with Isoflurane[®] in oxygen, and positioned on an x-y motorized translation stage (Prior[®]) controlled via a PC computer interface. A clear plastic plate was used as a support for the anesthetized mouse, which allowed the transmitted light intensity to be measured, and also the transmitted scattered light to be imaged with a digital camera. The laser beam spot size was adjusted by moving the focusing lens in a vertical z-direction. During the irradiation, a PC-controlled routine was used to move the mouse, and therefore, the tumor, in an x-y raster pattern relative to the laser beam spot. Typical irradiation areas were 15×15 mm² and the distance between neighboring spots was 1 mm, whereas the diameter of the laser spot on the proximal surface of the animal was not less than 2–3 mm. The irradiation time at each spot was 5 sec and the total irradiation times were 20–30 min. Thermal damage to the skin was avoided by (a) the rastering of the laser spot and (b) by using a watch glass laid on top of the mouse with glycerin contact fluid between the mouse skin and the watch glass. The average power of the laser at the skin level was 700–900 mW for the regenerative amplifier, 400–500 mW for the Ti:sapphire oscillator and 1.3–1.5 W for the diode laser. The average irradiance was 10–25 W cm⁻², which corresponds to the pulse energy from the regenerative amplifier, 0.6–0.8 mJ. The corresponding peak irradiance for the optimally compressed pulses was 10–100 GW cm⁻² and for the stretched pulses was 1–10 GW cm⁻². Under all illumination conditions, about 10% of the average incident power was transmitted through the mouse, and was measured with the same optical power meter positioned under the support plate. This indicated that most of the light was not absorbed by the mouse (tissue thickness 1–2 cm) but, passed through with about ~10–20% of photons scattered close to the forward direction.

2.9 Quantitation of tumor growth/regression

With the exception of the experiment to evaluate the vascular effects of free octreotate competition in octreotate targeted PDT, experimental groups of PDT treated mice consisted of 8–10 animals. The tumors were measured in three dimensions the day before PDT treatment, then every second day until the end of the experiment. Tumor volumes were calculated assuming a spheroid. The resultant data was analyzed using Graph Pad Prism and, with the exception of the comparative data from the targeted and untargeted sensitizers, means and linear regression curves plotted for each group. Means and error bars (SEM)

were plotted for all data sets. In the case of 3LL tumors treated directly with pulse elongated PDT, a spline fit was used for the regression curve to accommodate the two distinct phases of the curve.

2.10 Comparison of therapeutic efficacies for untargeted and for EGF receptor and SST2 receptor targeted PDT

Four groups of 8–10 mice were used for this experiment. Each group consisted of 8–10 *SCID* mice bearing subcutaneous FaDu xenografts in the flank area. As indicated earlier, our microarray expression profiles failed to show SST2 receptor expression for these tumors. 5×10^6 FaDu cells in 0.1ml CMF were injected subcutaneously to induce the tumors which were used when they reached ~8mm diameter. PDT was carried out as described earlier. One group of mice received no PDT treatment, the second received PDT treatment with SST2r targeted sensitizer only, the third received PDT treatment using EGFr targeted sensitizer only and the fourth received PDT treatment using a 50:50 mixture of SST2r and EGFr targeted sensitizers. All tumors were measured and tumor volumes calculated as described earlier. The means and SEMs were plotted. In a separate experiment, one group of mice was not treated and the second was treated with PDT using untargeted sensitizer.

3. Results

3.1 Validation of two-photon activation conditions

While we have demonstrated effective two-photon PDT of tumors laser irradiated through the body of the host mouse [7], tissue scattering of the laser light at this depth (2cm) raises some questions as to the possibility of efficient multi-photon uptake by the PDT sensitizer. We could find no previous information available that would shed light on this question. We, therefore, decided to evaluate the laser pulse requirements as they affect our deep tissue PDT treatment. Three experiments were carried out using SST2 receptor targeted PDT. In each case the tumor in one group of mice was laser irradiated directly, and in the other through the body of the host mouse. Figure 2 shows the results of these experiments. Panel A shows the results for PDT treatment of FaDu xenografts using optimally compressed femtosecond laser pulses, from the regenerative amplifier, to activate the sensitizer. The tumor regressed in both groups of mice, and was still regressing 10 days after treatment. For the animals treated through the depth of the body of the mouse, single photon activation (say, from the red tail of the laser spectrum) would be exceedingly unlikely. Therefore, the PDT sensitizer appeared to be activated by two photon mechanisms in both groups of mice. Panel B shows the results of a repeat experiment using FaDu xenografts where the only change was a tenfold elongation of the laser pulse. The pulse stretching was achieved by slightly misaligning the optics in the pulse compressor. The tumors in the mice, that were treated directly, stopped growing but did not regress. Degradation of PDT efficacy was even more severe for the tumors treated through the body of the mouse. Here, the tumors continued to grow with a rate similar to untreated FaDu tumors (see Figure 6.). Panel B shows no evidence for PDT depth efficacy. Panel C shows the effect of pulse elongation on PDT treatment of a different tumor, the Lewis lung carcinoma. Again no PDT effect was seen for tumors treated through the body of the mouse. Tumors treated directly started to regress, then grew at a rate only slightly slower than that of tumors treated through the body of the mouse. While we cannot yet model how two-photon uptake takes place in sensitizers at tissue depth, we can conclude that it only takes place only if the pulses are sufficiently short and have sufficiently high peak power.

Similar experiments were carried out also with the Ti:sapphire oscillator and with the CW diode laser. In both cases no PDT effect was observed (data not shown). In the case of the diode laser the average power was about factor of two higher than with the regenerative

amplifier. We conclude that because high laser pulse peak intensity (a situation satisfying the requirement for a high photon density in the irradiation laser beam) appears to be critical, the two-photon absorption process must be responsible for achieving efficacy of the treatment.

3.2 Staining patterns for SST2 receptors in vitro and in vivo

Figure 3, panels A and B demonstrate staining for the SST2 receptor after (panel A) and before (panel B) tissue fixation. The punctate membrane staining pattern is easy to see in panel B, but is overwhelmed by intracellular staining, including nuclear staining (N), in panel A. Nuclear staining has been reported before for SST2r [33, 34] which might imply that the receptors could be co-localized there. Punctate regions of perinuclear and surface membrane staining can be discerned. The fibrillar appearance of the rest of the cytoplasmic staining is likely a precipitation/aggregation artifact from the acetone fixation. Panel C shows the effective blocking of staining induced by the presence of excess octreotate. Since we were concerned not only with SST2 receptors on tumor cells but also with these receptors on endothelial cells, we also stained cultured bovine aortic endothelial cells (panel D). However, for greater relevance to our targeting studies, in this case, we used biotinylated octreotate in place of the primary antibody. Good staining was seen using this alternative protocol, and these cells did not show nuclear staining.

Figure 4 shows staining patterns for tumor xenografts in *SCID* mice. Each panel is a reconstructed z stack of confocal images. Panels A and B are images of NCI-H69 SST2 receptor positive lung cancer tumors. Panel A shows clear positive staining of tumor vessels with the endothelial marker, anti-factor 8 associated antigen. Panel B, stained for SST2 receptors, shows a very similar pattern of staining for the tumor vessels. In addition, faint punctate staining of the overall tumor tissue is apparent. Interestingly, staining of the tumor vasculature is more intense than that of the tumor cells themselves. Panel C shows the staining pattern seen in a xenograft of the SST2 receptor negative A549 lung tumor. Staining of the tumor vasculature is apparent, although smaller vessels do not appear to be stained. No staining of the tumor cells can be discerned in panel C.

3.3 Octreotate blockade ablates the vascular shutdown characteristic of SST2 receptor targeted PDT

Figure 5 shows the tumor vascular shutdown characteristic of our SST2 receptor targeted PDT. Panel A shows the fluorescence from high molecular weight FITC conjugated dextran that has leaked from blood vessels in the NCI-69 tumor. This *in vivo* scan was taken at 40 minutes after the intravenous injection of fluorescent dextran and 48 hours before PDT treatment. 40 minutes post tracer injection is the time at which maximum fluorescence is found in the tumor as shown in panel F. Panel B shows a similar *in vivo* scan taken 6 hours after PDT treatment. Almost no fluorescence is seen in the tumor in panel B indicating that vascular flow has been interrupted. Panel E shows the tumor bearing mouse that was scanned in panels A and B. Panels C and D show the result of injecting excess octreotate 2 minutes before PDT sensitizer infiltration. Panel C shows the characteristic fluorescence pattern from high molecular weight FITC conjugated dextran that has leaked from the tumor vessels. Panel D, where PDT sensitizer infiltration was carried out under octreotate blockade, shows the same pattern as seen for panel C, indicating that blood flow through the tumor is still intact.

3.4 Comparison of the efficacies of untargeted, SST2r targeted and EGFr targeted PDT

In Table 1 and Figure 6, we show the PDT efficacy of targeting to two different receptors and of using only untargeted PDT. The tumor xenograft used is the human hypopharyngeal carcinoma, FaDu that we have shown to lack expression of the SST2 receptor using

microarray experiments. These same expression profiling experiments demonstrated good expression of the EGF class of receptors. Tumors treated with EGF receptor targeted PDT sensitizer, and our standard laser activation protocol stopped growing, but did not regress (Figure 6, panel A). Tumor regression was seen for tumors treated with SST2 receptor targeted PDT sensitizer and our standard laser protocol, and for tumors treated with a 50:50 equimolar mix of these two differently targeted PDT sensitizers and our standard laser protocol (Figure 6, panel A). Long term “cures” (no tumor recurrence in 3 months) were seen in both of the last two groups, but in neither case did they exceed 25% of the test group. For the mice treated with EGF receptor targeted PDT sensitizer alone, all tumors eventually regrew, and no long term “cures” were seen. Overall, the least effective treatment was PDT using untargeted sensitizer (Figure 6, panel A). The tumors did regress for a few days, but the regression started later and was not maintained. All treated tumors regrew, and two mice died on the last day of the experiment. Two separate control tumor growth curves (Figure 6, panel B) are shown, since one relates to the targeted sensitizers experiment, and the other to the untargeted sensitizer experiment which was conducted at a different time. In conclusion, these experiments strongly support the importance of targeting PDT to tumor vasculature. It also demonstrates that mixed targeting to both tumor and vasculature is effective, but more work would be needed to define optimal targeting ratios.

4. Discussion and conclusions

4.1. Targeting PDT to the SST2 receptor

In this study, we show that tumor vasculature for both SST2r positive and SST2r negative human lung cancer cell lines stains for SST2 receptors. Even for xenograft tumors grown from SST2r positive cell lines, the intensity of SST2r staining is greater for the vasculature than for the tumor cells themselves (Figure 4). Expression of this receptor has been previously shown to occur on peritumoral veins [19] and proliferating endothelium [21, 22]. Thus, endothelial cell expression of SST2 receptors, like tissue factor expression [35–39], is likely part of the switch of tumors to angiogenic growth [39]. Targeting this receptor is facilitated by the large number of well-studied somatostatin analogues whose chemistry and clinical activity are well known [23].

Another approach used to target immunotherapy or PDT has been to use factor VII to bind to tissue factor expressed by tumor cells and tumor vasculature [17, 38, 40–44]. Like SST2r, tissue factor is often overexpressed in malignant tumor cells, particularly those of epithelial origin [35, 45, 46], and is expressed on the endothelial cells of proliferating, but not quiescent, blood vessels [42]. The strong association of tissue factor and factor VII/factor VIIa constitutes the initial step in blood coagulation [47], and this could have safety implications were widespread intravascular coagulation to occur as a result of the use of factor VII for targeting. To reduce this concern, the active site of factor VII protein has been mutated to inhibit procoagulant activity while not affecting its affinity for tissue factor, and the modified protein was shown to function well as a targeting moiety [44]. Factor VII/factor VIIa-tissue factor complex formation on the cell surface leads to more tissue factor mobilization to the cell surface from the golgi [48], and such a local upregulation could lead to further unwanted coagulation. Overall, while SST2r and tissue factor present similar expression profiles for therapeutic targeting of malignant cancer, targeting SST2r is likely to be safer for the patient. It is difficult to directly compare the results of SST2r targeting for PDT with tissue factor targeting for PDT since our SST2r targeted studies use a single two-photon PDT treatment and the tissue factor targeting studies use multiple single photon PDT treatments. Nonetheless, our studies suggest that SST2r targeting of tumor vasculature is highly effective because we get good PDT results with the FaDu xenografts even though the tumor cells themselves lack expression of SST2r. As expected, appropriately targeted PDT performed better than untargeted PDT.

Our *in vivo* imaging study on PDT induced shutdown of tumor blood flow, Figure 5, further substantiates the importance of vascular targeting for effective PDT. Here, free intravenous octreotate completely prevents shutdown of tumor blood flow, presumably by blocking the binding of targeted sensitizer to endothelial SST2 receptors. While tumor vessels are sometimes incomplete allowing some tumor parenchymal cells to directly contact the blood contents [49, 50], it seems very unlikely that enough tumor cells would interact with free intravascular octreotate to dominate the response. Thus, this vascular shutdown is most probably due to sensitizer targeting the vascular endothelial cells, with subsequent PDT damage to tumor blood vessels.

4.2 Sensitizer activation conditions

Many experimental factors can modify the efficiency of how tumors are killed by PDT treatment. It is well known that altering the light fluence rates can change the balance between direct singlet oxygen killing and inflammatory killing [51]. Whether the sensitizer localizes in the mitochondria or the lysosomes can have significant effects on killing efficiency [52]. Lysosomal locating sensitizers are 4–6 times more efficient at tumor cell killing [52]), and we have determined that our octreotate targeted sensitizer is located in the lysosomal system (data not shown). Concurrent tumor vascular damage is important to PDT success (e.g. this manuscript), and the type of cell death mechanism(s) used by the cancer cells also effects killing efficiency [53].

In order to better understand the dependence of the efficiency of PDT treatment on the illumination conditions, we estimated the dose of singlet oxygen generated in the tumor cells for four different light sources: (a) continuous wave (cw) diode laser; (b) Ti:Sapphire mode-locked fs oscillator; (c) Ti:Sapphire regenerative amplifier with stretched output pulse; (d) Ti:Sapphire regenerative amplifier with compressed output pulse. Our calculations of maximum number of singlet oxygen molecules produced per cell (see Supplementary information) is in all four cases below the limiting value, 2×10^8 molecules, that was found recently to be the minimum dose required to reduce the surviving fraction of MatLyLu cells by $1/e$ [54]. This leaves the question, of what mechanism(s) may be facilitating our deep tissue PDT effect, open for future investigations.

4.3 Comparison of the efficacy of SST2r targeted with EGFr targeted PDT

Our results show that PDT targeted to SST2 receptors is more effective than PDT targeted to EGF receptors (Figure 6, Table 1). Untargeted PDT was found to be the least effective, likely due to poorer retention of the sensitizer in tumor tissue (Figure 6, Panel A). We have previously used the SST2r targeting peptide, octreotate, in successful targeted PDT [7]. The targeting peptide, GE11, used for EGFr targeting, has previously been used successfully by others for therapeutic targeting [55]. Like octreotate, it has been shown to be internalized after binding to its receptor [26]. Our microarray data suggests that FaDu cells would express sufficient EGF receptors for targeting when compared to our data for SST2r microarray expression data for other tumor cell lines (data not shown). While tumor vascular endothelial cells are well known to respond to the effects of EGF signaling in the tumor cells themselves [56,57], it is less clear that the endothelial cells are significantly affected by EGF directly. However, it has been shown that in a xenograft system where the tumor cells lacked EGFr, targeting only the vascular cells to block EGF signaling was sufficient to inhibit tumor growth [58]. Relatively few cell surface EGF receptors could be sufficient to produce this result. Histopathology from our studies shows less early hemorrhage in EGFr targeted PDT treated tumors than in SST2r targeted PDT treated tumors (data not shown). Therefore, we conclude that, in our experiments with targeted PDT, EGFr targeting alone fails to produce an adequate vascular response. When FaDu tumors were treated with PDT using a 50:50 molar mixture of EGFr and SST2r targeted sensitizer, the result was very similar to

using SST2r targeted sensitizer alone (Figure 6). This underscores the importance of inducing vascular shutdown in this therapeutic modality. Further studies would be needed to evaluate how small a percentage of vascular targeted sensitizer is needed to achieve adequate vascular shutdown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PDT	photodynamic therapy
NIR	near infrared
UCNP	upconversion nanoparticle
VEGF	vascular endothelial growth factor
ATCC	American type culture collection
SST2r	somatostatin type 2 receptor
EGF	epidermal growth factor
BAE	bovine aortic endothelial
DMEM	Dulbecco's minimal essential medium
EDTA	Ethylenediaminetetraacetic acid
SCID	severe combined immunodeficient
FITC	Fluorescein isothiocyanate
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
PBS	phosphate buffered saline
CMF	calcium and magnesium free saline
GE11 peptide	YHWYGYTPQNV

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Highlights

- Targeting PDT to SST2 receptors is more efficient than to EGF receptors.
- Using the same sensitizer, untargeted PDT was least effective.
- SST2r targeting led to a number of cures whereas EGFr targeting did not.
- SST2r targeting of PDT led to tumor vascular shutdown.
- For 2-photon activation, laser pulse parameters were critical for depth efficacy.

Fig. 1. SCID mouse receiving NIR laser activation. The watch glass on the side of the mouse along with added drops of glycerin reduced scattering on the optical interface between the laser beam and the mouse, and also aided heat dissipation at the skin surface.

Fig. 2.

Comparison of the regression of tumors using our normal laser parameters for deep PDT treatment with pulse lengthened (10 fold) laser parameters. Treated through the mouse = $\blacksquare$ treated directly = $\blacktriangle$ Panel A = FaDu xenograft subcutaneous tumors induced as described in materials and methods, treated using our normal femtosecond laser pulse protocol for PDT. Panel B = FaDu xenograft subcutaneous tumors induced as described in materials and methods, treated using 10 fold pulse elongation of the laser beam. Panel C = 3LL subcutaneous tumors induced as described in materials and methods, treated using 10 fold pulse elongation of the laser beam. Note that the last day possible for tumor

measurement for the group treated through the mouse was day +8 after laser treatment. This was due to aggressive growth of the tumor in this group.

Fig. 3. SST2 receptor expression *in vitro*. T47D human breast carcinoma cells (panels A, B, and C) and bovine aortic endothelial cells (D) were propagated on glass coverslips as described in materials and methods. Panels A) and B) were stained for SST2 receptors as described in materials and methods. Panel A) was fixed with acetone then stained with the primary antibody while panel B) was first stained with the primary antibody and then fixed with acetone. Panel C) was stained with the primary antibody in the presence of competing excess octreotate peptide. Panel D) was stained with biotinylated octreotate, then developed with neutravidin secondary reagents as described in materials and methods. Bar = 20 μm. N = nucleus.

Fig. 4. Staining of blood vessels in tumor tissues. Panels A) and B) are samples from human SST2 receptor positive NCI H69 human xenograft tissue in *SCID* mice. Panel C) is from the SST2 receptor negative xenograft A549 human lung tumor in *SCID* mice. Panel A) is stained for factor 8 associated antigen, while panels B) and C) are stained for SST2 receptors as described in materials and methods. Bar = 750 μm .

Fig. 5.
In vivo imaging scans of tumor blood flow in NCI H69 xenograft tumors in *SCID* mice. 150K molecular weight FITC dextran was used to monitor tumor blood flow as described in materials and methods. NCI H69 xenograft subcutaneous tumors were used. Panel A) shows the extravasation of the dextran tracer from leaky vessels in the tumor tissue 40 minutes after intravenous tracer injection via the tail vein. Panel B) shows the effect on blood flow 6 hours after PDT treatment. Panel C) shows the blood flow pattern 48 hours before PDT treatment. Panel D) shows the blood flow pattern 6 hours after PDT treatment when the sensitizer was injected under conditions of octreotate blockade as described in materials and methods. Panel E) shows a picture of the tumor bearing mouse imaged in panels A) and B). The red circle outlines the tumor. Panel F) shows the pattern of fluorescence seen in a control tumor at increasing times after intravenous injection of FITC conjugated dextran 150K. Bar = 1cm. Red star = area of FITC fluorescence.

Fig. 6.

Comparison of the effectiveness of PDT treatments using different targeting peptides or untargeted PDT. All groups of mice carried subcutaneous FADU xenografts in the flank region. Neither the FaDu cell line nor the xenografts induced by this line in *SCID* mice were shown to express the SST2 receptor as described in materials and methods. Panel A) tumors received treatment with untargeted sensitizer = ○, tumors received PDT using sensitizer targeted to the EGF receptor = ▲, tumors received PDT using sensitizer targeted to the SST2 receptor = △, tumors received PDT using a 50:50 mixture of sensitizer targeted to the EGF receptor and sensitizer targeted to the SST2 receptor = ★. Panel B) tumors received no treatment (control group for groups treated with targeted sensitizers in panel A) = ●, tumors received no treatment (control group for the group treated using untargeted sensitizer in panel A) = ○.

Table 1

Number and average size of regressing tumors, 10 days after a single PDT treatment, compared to the peptide used to target the photosensitizer. Our standard two photon PDT laser conditions, $\lambda = 790$ and 900mW power at the mouse, were used.

	Targeting peptide used		
	EGRr binding	SST2r binding	50:50 mix of EGRr binding and SST2r binding
Number of tumors still regressing at +10 days	56%	75%	100%
Average size (% of original volume) of regressing tumors at +10 days	37 ± 23	27 ± 12	14 ± 11