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Selective and effective killing of angiogenic vascular endothelial cells and cancer cells by targeting tissue factor using a factor VII-targeted photodynamic therapy for breast cancer

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Abstract

The cell surface receptor tissue factor (TF) is regarded as a common but specific target on angiogenic tumor vascular endothelial cells (VECs) and tumor cells in many types of cancer including breast cancer. The purpose of this study is to develop a selective and effective TF-targeting photodynamic therapy (PDT) by using its natural ligand factor VII (fVII)-conjugated Sn(IV) chlorin e6 (SnCe6) for the treatment of breast cancer. A cross linker EDC was used to covalently conjugate fVII protein to SnCe6, and the binding activity and phototoxicity was confirmed by ELISA and in vitro PDT. The efficacy of fVII-tPDT was assessed in vitro by crystal violet staining assay and in vivo by measuring tumor size in mice carrying murine or human breast cancer xenografts. We show that active site-mutated (K341A) fVII protein can be internalized into breast cancer cells and vascular endothelial growth factor (VEGF)-stimulated human umbilical vein endothelial cells (HUVECs) as angiogenic VECs. fVII-tPDT not only enhances 12-fold the in vitro efficacy but also selectively and effectively kills angiogenic HUVECs and breast cancer cells via specifically binding of fVII to TF and inducing apoptosis and necrosis as the underlying mechanism. Furthermore, fVII-tPDT can significantly inhibit the tumor growth of murine and human breast cancer without obvious toxicities in mice. We conclude that fVII-tPDT using fVII-SnCe6 conjugate can selectively and effectively kill angiogenic VECs and breast cancer cells in vitro and significantly inhibit the tumor growth of murine and human breast cancer in mice.

Keywords

Targeting tumor neovasculature and tumor cells; Targeted photodynamic therapy; Coagulation factor VII; Tissue factor; Breast cancer

Introduction

Accumulating evidence suggests that the receptor tissue factor (TF) is expressed on endothelial cells of pathological capillary blood vessels associated with solid tumors [9, 18–20, 38], wet macular degeneration (wMD) [6, 40], and endometriosis [23]; however, this receptor is not expressed on endothelial cells of normal blood vessels, including the smallest capillary vessels and larger blood vessels such as veins and arteries [9, 12, 13, 20]. TF thus serves as a common but specific therapeutic target for treatment of these diseases. As TF is overexpressed on many cancer cells in solid tumors [7, 9, 18–20, 35] and leukemia [2, 3, 15], TF-targeting therapies could also be used to eradicate tumor cells. TF is a normal cell surface receptor and is expressed on extravascular cells of several normal tissues and in the adventitial layer of large blood vessel walls [12, 42]; however, it is anatomically sequestered from its natural ligand coagulation factor VII (fVII) and from TF-targeting therapeutic agents circulating in blood by the intact semipermeable endothelial layer of normal blood vessels [26]. In contrast, newly formed tumor capillary blood vessels are leaky because they lack tight junctions between endothelial cells, and their walls may even lack endothelial cells entirely [8, 26]. Systemically or intratumorally administered TF-targeting therapeutic agents can thus access TF on angiogenic tumor vascular endothelial cells (VECs) and tumor cells via the leaky tumor neovasculature, but cannot access TF on normal tissue cells in the adventitial layer and outside the vessel wall.

To target TF for eradication of tumor neovasculature and tumor cells, Hu and Garen [18–20] have constructed an antibody-like fVII/IgG1 Fc immunoconjugate (called an Icon) for cancer immunotherapy. TF was targeted using fVII instead of an antibody because fVII binds its cognate receptor TF with exceptionally high specificity and affinity [41]. The dissociation constant (K_d) for fVII binding to TF is up to 10^{-12} M [41], while the K_d s of anti-TF antibodies are 10^{-8} – 10^{-9} M [22, 31]. Furthermore, the dimeric Icons (fVII/IgG1Fc) used for immunotherapy and monomeric fVII proteins in this study are produced by recombinant DNA technology, allowing mouse Icon (mouse fVII/human IgG1 Fc) and mfVII proteins to be made for tests in preclinical animal models and human Icon (human fVII/human IgG1 Fc) and hfVII proteins to be made for future clinical trials without need for a humanization process. Delivery of the Icon cDNA by an adenoviral vector or injection of the Icon protein resulted in dramatic eradication of the pathological neovasculature in animal models of cancer [18–20, 38], wMD [6, 40], and human endometriosis [23] without side effects on fertility [23].

Here we report another TF-targeting therapeutic, photodynamic therapy (PDT). PDT can be used as a treatment for cancer and wMD. It involves three components, a photosensitizer, a laser light, and tissue oxygen. Unlike immunotherapy, PDT primarily kills cells by induction of apoptosis and/or necrosis and can thus be used as a standalone modality or in combination with Icon immunotherapy, surgery, radiotherapy, and chemotherapy. Due to the limited selectivity of photosensitizers, conventional non-targeted PDT (ntPDT) may cause side effects in normal tissues [33]. To improve the safety and efficacy of PDT, antibodies or ligands have been conjugated directly or indirectly to photosensitizers [25, 34]. This targeted PDT (tPDT) allows selective targeting of the photosensitizer to the diseased cells that express the cognate antigen or receptor. As TF is a common but specific receptor on tumor cells and angiogenic tumor VECs [7, 9, 18–20, 35, 38], we hypothesized that targeting a

photosensitizer to TF by using its ligand fVII could overcome the poor selectivity and improve the therapeutic effects of ntPDT. We recently developed an effective fVII-targeted verteporfin (VP) PDT for treatment of wMD [24] and cancer [43]; however, VP is very costly and must be extracted from commercial Visudyne, a liposomal formula of VP. Here we report the development of another fVII-tPDT by covalently conjugating fVII protein to another potent but much less expensive photosensitizer, Sn(IV) chlorin e6 (SnCe6). To the best of our knowledge, this is the first report of targeting the angiogenic receptor TF using an fVII–SnCe6 conjugate for ligand-tPDT for simultaneous targeting of both pathological neovasculature and tumor cells.

Materials and methods

Construction of a plasmid containing the fVII cDNAs

Construction of a plasmid containing fVII cDNA was similar to our previous Icon plasmid vectors [18–20]. Briefly, the mouse factor VII cDNA containing a K341A mutation was derived from a previously constructed Icon plasmid vector pcDNA3.1(+)/mfVII(K341A)/hIgG1 Fc [18–20]. The new mfVII cDNA is composed of the coding sequence of mfVII, a BamHI site, and a sequence encoding the first 15 amino acid residues of ribonuclease S-peptide with a D14N mutation [21] followed by eight histidines (His tag). S-peptide and His tag were designed for detection and purification of fVII proteins.

Production and purification of the mfVII protein

As similarly described [18–20], fVII plasmid vector was stably transfected into CHO-K1 cells and fVII protein was purified from the collected serum-free medium from the transfected CHO-K1 cells using Ni-NTA affinity resins for His tag (Qiagen).

Conjugation of fVII protein to SnCe6

The following conjugation procedure has been used to conjugate another –COOH group-containing photosensitizer (VP) to fVII protein [24]. Sn(IV) Ce6 dye (MW 850.21, Fig. 1a) (Frontier Scientific, Logan, UT) was completely dissolved in one volume of 1× DPBS (PBS), and then nine volumes of DMF (dimethylformamide, Sigma) to a final concentration of 10 mg/ml. SnCe6 was activated by EDC in a reaction tube containing 6 µl DMF, 2 µl of 10 mg/ml SnCe6, and 2 µl of freshly prepared 25 mg/ml EDC (*N*'-3-dimethylaminopropyl-*N*-ethylcarbodiimide hydrochloride, Sigma) in DMF. The mixture was incubated at room temperature (RT) for 30 min, and then 80 µl of 1 mg/ml mfVII protein in 1× DPBS or 1× DPBS buffer only (as a buffer control) were added to the 10 µl of activated dye and incubated at RT for 1 h. The 90 µl of mfVII–SnCe6 or PBS–SnCe6 conjugation mixture was loaded onto Sephadex G50 spin columns (Roche), which were washed three times with 1× DPBS prior to loading of conjugation mixture, in order to remove the unconjugated dye, following the manufacturer's instructions. After separation by spin columns, the collected mfVII–SnCe6 conjugate (green) and PBS–SnCe6 control (colorless) were characterized by spectral scanning (200–800 nm) and by reading absorbance at 635 nm (A_{635nm}) using a spectrophotometer (Beckman). The concentration of protein was determined by Protein Assay Reagent (Bio-Rad) using bovine serum albumin (BSA, Pierce) as standard protein and the concentration of SnCe6 was calculated based on A_{635nm} using an SnCe6 standard curve [$y = 0.0007x - 0.0014$, $R^2 = 0.9999$, in which y is A_{635nm} and x is the concentration of unconjugated SnCe6 in a linear range of 0–2,000 µg/ml diluted 1:50 in ddH₂O]. The molar ratio of dye to protein was determined based on their molar concentrations (µM).

Similarly, bovine serum albumin (BSA, Sigma) was conjugated to SnCe6 as an irrelevant protein control.

Binding activity of fVII and its SnCe6 conjugate to cancer cells was determined by cancer cell ELISA

MDA-MB-231 cancer cells were seeded and grown overnight in 96-well plates at a density of 20,000 cells per well. The next day, the cells were washed once with pre-warmed TBS-Ca-T (10 mM Tris-HCl pH 7.4, 140 mM NaCl, 10 mM CaCl₂, 0.1% Tween 20), fixed with 1% paraformaldehyde (J.T. Baker) and then blocked with 2% BSA. The fixed breast cancer cells were incubated with 0.01, 0.1, 1, and 10 µg/ml mfVII or mfVII-SnCe6 conjugate diluted in TBS-Ca-T at 37°C for 1 h. After three washes, 2 µg/ml goat anti-mfVII (R&D Systems) was added to detect mfVII bound to the cancer cells. At this point, 0.01, 0.1, 1, and 10 µg/ml goat anti-HTF (American Diagnostica) was added to separate duplicate wells as the positive control and incubated at 37°C for 1 h. After three washes, 5 µg/ml anti-goat IgG Fc HRP (Vector) was added and incubated at 37°C for 1 h, and OPD substrate solution (Sigma) was added. After 30 min at 37°C, OPD color development was stopped by 4.5 N H₂SO₄, and A490nm was read using a microplate reader (Gemini). Binding activity was presented as A490nm after being subtracted from average A490nm of secondary antibody HRP control wells.

Detection of TF expression on breast cancer cells and 293 cells by flow cytometry using monoclonal anti-HTF

This flow cytometry was done using 20 µg/ml goat anti-HTF (American Diagnostica) followed by 20 µg/ml secondary anti-goat IgG Fc FITC (Vector Laboratories), similarly as described [18–20].

Endocytosis of fVII by TF-expressing VECs and breast cancer cells

TF expression was induced on HUVECs using 1 nM VEGF (BD Biosciences) as previously described [44]. The detailed procedure is described in supporting information.

In vitro PDT in tissue culture plates

The in vitro PDT tests were done in 96-well plates containing $1-2 \times 10^4$ cells in 100 µl growth medium per well or in 48-well plates containing 9×10^4 cancer cells in 200 µl per well. The detailed procedure is described in Supporting Information. Briefly, fVII-tPDT or ntPDT was carried out by incubating the cells with fVII-SnCe6 conjugate or unconjugated SnCe6 at 37°C for 90 min, then washing the cells once and adding the growth medium. The cells were irradiated with a 635-nm fiber-coupled diode laser (BWF2-635-0.1–100-0.22, B&W Tek, Inc.) for various time durations at 100 mW/cm². After treatment with PDT, the cells were incubated at 37°C and 5% CO₂ until they reached 95% confluence and were ready for the crystal violet staining assay or clonogenic assay described below.

Crystal violet staining and clonogenic assays for determining the in vitro efficacy of SnCe6 PDT

Non-clonogenic crystal violet staining was performed as described previously [27] with a minor modification to the more convenient reading of absorbance at 595 nm (A595nm). The in vitro efficacy was presented as the percent of surviving cells based on A595nm readings by the crystal violet staining assay or colony numbers by clonogenic assay (Supporting Information).

Assessing apoptosis and necrosis as mechanism of action of SnCe6 PDT

Human MDA-MB-231 breast cancer cells were treated in 96-well plates with fVII-tPDT or ntPDT (2 µM, 72 J/cm²) as described above. At various time points after PDT treatment, the plates were separately assayed for necrosis (cytotoxicity) using the CytoTox 96 Non-

Radioactive Cytotoxicity Assay (Promega) and apoptosis using the fluorescence-based Apo-ONE Homogeneous Caspase-3/7 assay (Promega) (Supporting Information).

In vivo fVII-tPDT treatment of mouse and human breast cancer in mouse subcutaneous tumor xenograft models

The animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Yale University. Mouse breast cancer EMT6 xenografts were generated by subcutaneously (s.c.) injecting 1×10^6 EMT6 cancer cells in 100 μ l PBS into the right flanks of 4–6 weeks old female Balb/c mice (Taconic Farms). Human MDA-MB-231 breast cancer xenografts were generated in 4–6-week-old female CB-17/SCID mice (Taconic Farms) by s.c. injection of 1×10^6 human MDA-MB-231 breast cancer cells in 100 μ l of PBS: Basement membrane Matrigel (BD Biosciences) into the right flank, as previously described [18–20]. After the tumors reached 100–200 mm in size, fVII-SnCe6 or unconjugated SnCe6 were intravenously (i.v.) injected into mouse tail veins at a final concentration of 2 or 4 μ M SnCe6 using an estimate of blood volume based on body weight (35 ml blood/kg of mouse body weight). Ninety minutes after injection of fVII-SnCe6 or SnCe6, the tumors were irradiated with 72 J/cm² using a 635-nm laser under anesthesia by intraperitoneal (i.p.) injection of 100 mg ketamine/kg + 10 mg xylazine/kg. The date for the first treatment was designated as day 0, and the same treatment procedure (i.e., i.v. injection of drug and 90 min later irradiation of tumor by laser light) was repeated once on day 7 for EMT6 tumors or on days 7, 14, 21, and 28 for MDA-MB-231 tumors. The control mice were i.v. injected with saline buffer but were not irradiated with laser light.

The efficacy of SnCe6 was determined by measuring tumor width and length with calipers and calculating tumor volume (mm³) using the formula (width)² $\times$ length/2 as previously described [18–20]. At the end of the experiments, the mice were examined morphologically and histologically, and blood samples from the mice with EMT6 tumors were examined by complete blood counts with differential analysis by Antech Diagnostics (Lake Success, NY) to assess the safety of this treatment.

Statistical analyses

In vitro studies were carried out in duplicate wells in each group, and in vivo efficacy in mouse tumor models were assessed in groups of five mice. These data are presented as mean $\pm$ standard deviation (s.d.). The statistical significance of differences between the treated group(s) and the control group were analyzed using paired or unpaired *t* tests, ANOVA single factor, one-way ANOVA with Tukey's Multiple Comparison Test, or two-way ANOVA tests using Prism 5.0c (GraphPad Software Inc). *P* values less than 0.05 were considered to be statistically significant. The half maximal effective concentrations (EC₅₀) of fVII-tPDT and ntPDT were calculated using the best-fit semilog or one phase decay equations using Prism 5.0c.

Results

The fVII protein can be covalently conjugated to SnCe6 without affecting the ability of fVII to bind breast cancer cells or the absorption spectrum of SnCe6

We used the cross linker EDC to covalently conjugate mfVII protein (MW, 52,800 daltons) to SnCe6 (MW: 850 Da; Fig. 1a). The absorption spectrum of free SnCe6 in PBS had two separate peaks at 635 nm (Q band) and 409 nm (Soret band), and size exclusion Sephadex G50 spin columns separated free dye from protein-conjugated dye by holding it in the resin so the absorption spectrum of the collected solution from the PBS-SnCe6 conjugation reaction did not have any peaks (Fig. 1b). In contrast, the absorption spectrum of the fVII-SnCe6 conjugate had peaks at 635 and 409 nm along with a protein peak at 280 nm after

column separation (fVII–SnCe6 in Fig. 1b), indicating that SnCe6 was successfully and covalently conjugated to fVII protein. In contrast to a previous report that conjugation of a monoclonal antibody to chlorin e6 resulted in a shift in the Q band absorption peak of chlorin e6 [10], we found that the peak wavelengths of fVII-conjugated SnCe6 were the same as those of free SnCe6 (Fig. 1b).

Using a cell-based ELISA, we showed that the ability of SnCe6-conjugated mfVII to bind MDA-MB-231 cancer cells was similar to that of unconjugated mfVII and of a positive control (goat anti-HTF antibody) (Fig. 1c) ($P > 0.05$ by one-way ANOVA). This result indicates that the conjugation procedure did not affect the binding activity of fVII (Fig. 1c). The molar ratios of SnCe6 to mfVII were stable at $4.21 \pm 0.95:1$ (mean $\pm$ SD, $n = 11$) in the mfVII–SnCe6 conjugates.

The fVII protein is internalized by TF-expressing breast tumor cells and vascular endothelial growth factor (VEGF)-stimulated human umbilical vein endothelial cells (HUVECs)

It has been shown that wild-type human fVII protein can be endocytosed by TF-transfected baby hamster kidney (BHK) cells [17]; however, the ability of TF-expressing cancer cells and angiogenic tumor VECs to endocytose murine fVII protein with a coagulation active site mutation (K341A), which is key for successful development of tPDT, remains unknown. To address this question, we used an mfVII(K341A)/human IgG1 Fc Icon immunoconjugate [18–20], which contained an mfVII protein containing a K341A mutation [19, 20] conjugated to the Fc portion of an IgG1 antibody, to detect co-localization of fVII with the intracellular F-actin cytoskeleton by immunofluorescence staining. We observed co-localization of fVII/Fc (green) with F-actin (red) in VEGF-stimulated HUVECs and MDA-MB-231 cells (Fig. 2), demonstrating that the mfVII(K341A) protein could be internalized by both the cancer cells and angiogenic VECs.

fVII-targeting not only enhances the efficacy but also improves the selectivity of SnCe6 PDT for killing breast cancer cells

Using the optimized incubation time (90 min) and crystal violet staining to assess the efficacy of fVII–SnCe6 for PDT in vitro, we found that the half maximal effective concentration (EC_{50}) of fVII–SnCe6 was 13.9-fold lower than that of SnCe6 used for ntPDT (1.43 vs. 19.84 μ M) when laser fluence was 18 J/cm² (Fig. 3a, b). When the laser fluence was increased to 36 J/cm², the EC_{50} of fVII–SnCe6 decreased to 0.58 μ M (Fig. 3c) and was still 12-fold less than that of SnCe6 used for ntPDT (7.00 μ M) (Fig. 3d). We thus conclude that fVII-targeting enhances the ability of SnCe6 PDT to kill breast cancer cells in vitro by 12- to 13-fold.

We next tested the effect of TF expression levels on the efficacy of fVII-tPDT. Using 2 μ M fVII–SnCe6, we found that the EC_{50} of laser fluence was fourfold greater for MCF-7 cells, which express low levels of TF (Fig. 4b), than for MDA-MB-231 cells, which express higher levels of TF (Fig. 4a) (70.6 and 17.3 J/cm², respectively). In contrast, fVII-tPDT did not kill the 293 cells, which do not express TF (Fig. 4c). Taken together, these data demonstrate that fVII-tPDT can selectively kill cancer cells without side effects in normal tissue cells.

fVII-tPDT can selectively kill angiogenic VECs but has no side effects in normal quiescent VECs

We next asked whether fVII-tPDT could selectively kill TF-expressing angiogenic tumor VECs. To answer this question, we first induced TF expression in HUVECs by treating with 1.1 nM VEGF as a model of angiogenic VECs (Fig. 2a). Control HUVECs, which were also

starved but were not stimulated with VEGF and thus did not express TF [44], were used as a model of normal VECs. We found that fVII-tPDT (2 μ M SnCe6, 36 J/cm²) selectively killed the angiogenic HUVECs but did not kill the unstimulated normal HUVECs ($P < 0.0001$ by twotailed t test) (Fig. 4d), whereas ntPDT did not kill either type of HUVEC ($P = 0.846$ by two-tailed t test) (Fig. 4d). These findings show that fVII-tPDT can selectively and significantly eradicate angiogenic VECs.

Induction of apoptosis and necrosis is the underlying mechanism of action of fVII-targeted SnCe6 PDT

MDA-MB-231 breast cancer cells released more cyto-plasmic LDH after 1 h of treatment than at later time points (4 and 12 h), indicating that necrosis occurred early during fVII-SnCe6 treatment (Fig. 5a). In contrast, a higher level of caspase 3/7 activity, which indicates apoptosis, was detected after 12 h of PDT treatment than at 1, 4, 16.5 and 18 h time points (Fig. 5b). Consistent with the therapeutic effect of fVII-tPDT observed in vitro ($P < 0.01$ or 0.05 vs. fVII-SnCe6 alone, ntPDT, SnCe6 or laser alone by one-way ANOVA with Tukey's Multiple Comparison Test) (Fig. 5c), the frequencies of cells undergoing necrosis or apoptosis were significantly higher when cells were treated with fVII-SnCe6 than when cells were treated with ntPDT ($P < 0.0001$ for fVII-tPDT vs. ntPDT by one-way ANOVA with Tukey's Multiple Comparison Test) (Fig. 5a, b). Treatment with either the drug or laser light alone did not induce necrosis or apoptosis (Fig. 4a, b) or result in therapeutic efficacy (Fig. 4c).

The therapeutic effect of fVII-tPDT is specifically mediated by fVII protein

We showed that at a molar ratio of 1:1 unconjugated fVII:fVII-SnCe6, 50% of the effect of fVII-tPDT (2 μ M SnCe6, 36 J/cm²) was inhibited by addition of unconjugated mfVII protein ($P < 0.0001$ vs. untreated control and vs. fVII-tPDT with a 0:1 molar ratio by one-way ANOVA with Tukey's Multiple Comparison Test) (Fig. 5d). Further increasing the ratio to 10:1 completely abolished the effect of fVII-tPDT ($P > 0.365$ vs. untreated control and $P < 0.0001$ vs. fVII-tPDT with 1:1 and 0:1 molar ratios, respectively), whereas fVII-tPDT killed about 80% of breast cancer MDA-MB-231 cells ($P < 0.0001$ vs. untreated control) without inhibition (0:1) (Fig. 5d). These results indicate that the therapeutic effect of fVII-tPDT was mediated by fVII protein.

To further rule out the possibility that any protein could mediate the PDT effect, we conjugated bovine serum albumin (BSA), an irrelevant protein, to SnCe6. BSA-SnCe6 PDT (2 μ M SnCe6) did not kill breast cancer cells even at a laser fluence of 72 J/cm² ($P > 0.05$ vs. 0 J/cm² and other fluences by one-way ANOVA with Tukey's Multiple Comparison Test) (Fig. 5e), while fVII-tPDT killed almost all of the MDA-MB-231 cancer cells at this laser fluence (Fig. 4a). We thus conclude that the effect of fVII-tPDT is specifically mediated by fVII protein but not by other irrelevant proteins.

fVII-tPDT can significantly inhibit mouse and human breast cancer growth in vivo in mouse models

Using an EMT6 tumor model in Balb/c mice, we found that fVII-tPDT with 2 μ M SnCe6 significantly inhibited tumor growth ($P = 0.0004$ by ANOVA single factor; All P values in Fig. 6 were analyzed by ANOVA single factor), and increasing the SnCe6 concentration to 4 μ M further enhanced this effect ($P = 0.0028$ vs. the control); however, the difference between the two fVII-tPDT groups (2 and 4 μ M SnCe6) was not statistically significant ($P = 0.0778$) (Fig. 6a). Tumors from fVII-tPDT-treated mice weighed significantly less than that those from control mice (2.72 ± 1.23 g) and ntPDT-treated mice (2.65 ± 0.55 g) (Fig. 6b), and tumors from fVII-tPDT-treated mice with 4 IM SnCe6 weighed significantly less than

those of mice treated with 2 μ M SnCe6 (0.90 ± 0.34 and 0.27 ± 0.12 g, respectively) ($P = 0.0004$) (Fig. 6b).

Similar results were obtained using an MDA-MB-231 tumor model. Treatment with fVII-tPDT significantly inhibited human breast tumor growth ($P = 0.010$ and 0.0015 vs. the control and ntPDT by ANOVA single factor, respectively) (Fig. 6c), whereas treatment with ntPDT under the same conditions had no effect ($P = 0.466$ vs. the control) (Fig. 6c). The average weight of tumors from the fVII-tPDT-treated mice (0.32 g) was only half that of tumors from ntPDT-treated mice (0.69 g) and about one-third of that of tumors from control mice (0.86 g) (Fig. 6d); however, these differences in tumor weight were not statistically significant ($P > 0.05$ in Fig. 6d), possibly due to the limitations of measuring tumors using calipers, which measure both width and length but not depth of a tumor. In both experiments, there were no differences in mouse body weight, complete blood counts and differential analyses of leukocytes between the control mice and PDT-treated mice, and none of the mice had any signs of toxicity during or at the end of the experiments. We thus conclude that fVII-tPDT is effective and safe for treatment of human and murine breast tumors in mice.

Discussion

In this study, we developed an effective and selective ligand-targeted SnCe6 PDT for breast cancer therapy that simultaneously targets and eradicates angiogenic tumor VECs and tumor cells. To the best of our knowledge, there are no published articles reporting the successful development of targeted PDT that can simultaneously target both the tumor neovasculature and tumor cells for cancer therapy. Successful development of targeted PDT requires identification of a target molecule that is selectively expressed on the diseased cells, identification of a targeting vehicle that can specifically bind the target molecule and be internalized by the diseased cells, and the ability to covalently conjugate this vehicle to a photosensitizer without affecting its binding activity or the photoactivity of the photosensitizer. Macromolecules such as antibodies or ligands are frequently used as targeting vehicles [25, 34]. In this regard, we chose the receptor TF as the target molecule and its ligand fVII as the targeting vehicle for the development of ligand-targeted SnCe6 PDT in this study. We show that fVII protein can be covalently conjugated to SnCe6 without affecting the binding activity or absorption spectrum of the fVII-SnCe6 conjugate. In addition, we show that TF is overexpressed on both human and mouse breast cancer cell lines and angiogenic VECs but not on normal VECs. Due to the difficulty of maintaining the angiogenic features of tumor VECs in tissue culture, control and VEGF-stimulated HUVECs were used as models of normal and angiogenic VECs, respectively, in this study. Selective expression of TF on cancer cells and angiogenic VECs provides the basis and rationale for the selective therapeutic effect of fVII-targeted therapies on tumor VECs and tumor cells observed in this study and the efficacy of cancer, wMD, and endometriosis in our previous studies [6, 18–20, 23, 24, 38, 40].

In the course of the development of TF-targeting therapeutics, Hu and Garen [20] reported for the first time in 1999 antibody-like fVII-targeted Icon (fVII/IgG1 Fc) that has been tested for immunotherapy; the Icon immunotherapy targets TF for eradication of pathological neovasculature in tumors [18–20, 38] and non-cancerous diseases such as wMD [6, 40] and human endometriosis [23]. Similar to the studies in which Hu and Garen [20] introduced a mutation (K341A) in the fVII protein in order to reduce its coagulation activity [18–20], Shoji and coworkers [36] recently reported the use of an active site-inactivated recombinant human fVIIa (FFRck-fVIIa) as a carrier for the targeted delivery of a potent synthetic curcumin analog (EF24) to TF-expressing tumor-associated VECs and tumors. This study by Shoji et al. provides independent evidence in support of our

previous findings regarding the efficacy of TF-targeting therapeutics [18–20]. Here we report the development of another TF-targeting therapeutic, fVII-targeted SnCe6 PDT, which can induce apoptosis and necrosis and is effective, selective, and safe for in vitro and in vivo treatment of mouse and human breast cancer in mouse models. Our results suggest that fVII-tPDT can be used as a stand-alone modality, and potentially in combination with Icon immunotherapy, surgery, and other modalities for treatment of breast cancer and potentially of other laser light-accessible tumors on skin surfaces or in the internal cavities.

The choice of SnCe6 is based on the following considerations. (1) Like VP, SnCe6 is a potent photosensitizer and has been used in ntPDT and tPDT; SnCe6 has been targeted to tumor cells by insulin [1], cell surface receptor ligands (e.g., epidermal growth factor, EGF) [14, 37], and antibodies [10, 11, 16, 28, 29, 32]. In previous studies, targeting vehicles such as insulin, EGF, or antibodies also significantly reduced the IC₅₀ or EC₅₀ of SnCe6 for PDT in vitro; of which two articles reported the in vivo efficacy (survival) study on targeting cancer cells by antibody-tPDT [11, 28]. In this regard, we extend the previous studies in the field not only by choosing the receptor (TF) as a targeting molecule expressed selectively on both tumor VECs and tumor cells but also by carrying out and demonstrating better selectivity and stronger efficacy of fVII-tPDT than ntPDT in eradication of breast cancer in vitro and in vivo. (2) Like VP, SnCe6 contains carboxyl groups that can be covalently conjugated to the amino groups of proteins via cross linkers such as EDC and NHS. We modified a previously reported procedure [5] by using EDC alone. This modified procedure was sufficient to conjugate fVII proteins to SnCe6, as reported in this article, and to VP, as reported in our recent study [24], while the binding activity of fVII proteins retained. This conjugation/separation procedure can theoretically be used to conjugate other carboxyl group-containing photosensitizers to fVII or other targeting protein molecules for tPDT. (3) SnCe6 is much cheaper than VP (1 mg SnCe6 costs \$1, while 1 mg Visudyne costs ~ \$100), and SnCe6 in PBS solution is stable at least for 1 year without any loss of its phototoxicity to cancer cells when stored at 4°C. The reduced dosage, the consequent reduced toxicity and cost, and the stable shelf life of SnCe6 could significantly reduce the cost of future clinical trials and the eventual clinical application of fVII-targeted SnCe6 PDT.

However, cautions should be taken for the design and enrollment of patients in future clinical trials using TF-targeting therapeutics. Numerous articles have reported that inflammatory cytokines such as IL-1, TNF α , and products from pathogens such as endotoxin (bacterial lipopolysaccharide, LPS) can induce TF expression on vascular endothelial in the diseased tissues [4], such as at sites of inflammation in endotoxemia [30], or in atherosclerosis [39]. Nevertheless, we show in Fig. 5 that fVII-SnCe6 conjugate or SnCe6 drug alone without laser irradiation does not have killing effect, indicating that fVII-tPDT as a localized modality should not have side effects on those inflammatory diseases as long as laser light is limited to the target tumor. In addition, fVII-tPDT should have dual selectivity due to control of laser light delivery and to selective targeting of photosensitizer to tumor neovasculature and/or tumor cells and, therefore, could minimize any side effects in those inflammatory conditions.

In conclusion, we report for the first time an effective and selective fVII-targeted SnCe6 PDT that can selectively and effectively kill angiogenic VECs and tumor cells in vitro and significantly inhibit the tumor growth of murine and human breast cancer in vivo. As TF is a common but specific target molecule on many types of tumor cells, including other solid tumors [7, 9, 18–20, 35] and leukemia [2, 3, 15] and angiogenic VECs in pathological neovasculature [6, 7, 9, 18–20, 23, 35, 38, 40], fVII-tPDT could have broad therapeutic potential for breast tumors and other tumors as well as for other pathological neovasculature-associated diseases [8] in which the diseased lesions express TF and can be

accessed by laser light, for instance, delivered by optical fibers through endoscopic, interstitial, or intracavitary techniques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TF	Tissue factor
fVII	Coagulation factor VII
VEC	Vascular endothelial cells
VEGF	Vascular endothelial growth factor
PDT	Photodynamic therapy
tPDT	Targeted photodynamic therapy
ntPDT	Nontargeted photodynamic therapy
wMD	Wet macular degeneration
SnCe6	Sn(IV) chlorin e6
VP	Verteporfin
Kd	Dissociation constant
SCID mouse	Severe combined immunodeficient mouse

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Fig. 1.

The fVII-SnCe6 conjugate retains the same absorption spectrum as free SnCe6 and the same binding activity as unconjugated fVII protein. **a** Chemical structure of SnCe6. **b** Absorption spectra of SnCe6 and fVII-SnCe6. The samples in all three panels were diluted 1:50 in ddH₂O to a concentration of 0.6 μM SnCe6. **c** Binding activity of fVII-SnCe6 and fVII to MDA-MB-231 as determined by cell ELISA. Results in **b** and **c** are representative of eleven and two experiments, respectively

Fig. 2. fVII is internalized by TF-expressing VEGF-stimulated angiogenic HUVECs and MDA-MB-231 breast cancer cells. fVII protein primarily bound to the cell plasma membrane (green, arrows in **a** and **d**) of the fixed cells (0 min), while endocytosis of fVII, shown as co-localization intracellularly with Alexa Fluor 633-phalloidin-stained cytoskeletal F-actin (red, arrows in **b** and **e**), was observed at 37°C for 30 min after incubation with VEGF-stimulated HUVECs (**b**) and human breast cancer MDA-MB-231 cells (**e**). **c** and **f** Secondary antibody FITC controls for non-specific FITC background. Scale bars, 20 μm

Fig. 3.

fVII-targeting enhances the ability of SnCe6 PDT to kill human MD-MBA-231 breast cancer cells in vitro more than 12-fold. The half maximal effective doses (EC_{50} , represented by *dashed line*) of SnCe6 for MDA-MB-231 cancer cells were shown (**a, c** for fVII-tPDT; **b, d** for ntPDT) using crystal violet staining. Equations and R^2 values were generated using semilog line for **a–c** and one phase decay for **d** with Prism 5.0c software.

a–d Representative of two experiments

Fig. 4. fVII-tPDT selectively kills breast cancer cells (**a**, **b**) and angiogenic HUVECs (**d**) but has no side effects in normal cells such as 293 cells (**c**) and unstimulated HUVECs (**d**). **a–c** TF expression and selective effect of fVII-tPDT on human breast cancer MDA-MB-231 (**a**) and MCF-7 (**b**) cells and normal 293 cells (**c**). The level of TF expression was determined by flow cytometry using goat anti-HTF antibody (solid line). The control (dashed line) was incubated only with the secondary antibody FITC. The fVII-tPDT (2 μ M SnCe6) treatment of these cell lines was performed with various laser fluence values, and efficacy was determined by crystal violet staining. **d** fVII-tPDT and ntPDT (2 μ M SnCe6) treatment of

angiogenic (VEGF-stimulated) and quiescent (unstimulated) HUVECs. **a–d** Representative of two experiments

Fig. 5.

The mechanism of action of fVII-tPDT is to induce necrosis and apoptosis, and the effect of fVII-tPDT is specifically mediated by fVII protein. MDA-MB-231 cancer cells were treated separately with fVII-tPDT, ntPDT (2 μ M, 36 J/cm^2), photosensitizer alone (2 μ M) or laser light alone (36 J/cm^2). After treatment, the cells were separately assessed for necrosis (**a**) and for apoptosis (**b**). After these assays, the MDA-MB-231 cancer cells were stained with crystal violet for the therapeutic effect (**c**). **d** 2 μ M fVII-SnCe6 was pre-mixed with mfVII protein in various molar ratios prior to incubation with MD-MB-231 cells, and the cells were then treated with fVII-tPDT (36 J/cm^2). **e** MDA-MB-231 cells were treated with BSA-targeted SnCe6 PDT (2 μ M, 36 J/cm^2)

Fig. 6. fVII-targeted SnCe6 PDT is efficacious for treatment of mouse (**a, b**) and human (**c, d**) breast cancer in mouse models. **a** Mouse EMT6 tumors in Balb/c mice were treated by fVII-tPDT (2 or 4 μM SnCe6, 72 J/cm²) or ntPDT (4 μM SnCe6, 72 J/cm²) on days 0 and 7. **b** Mouse EMT6 tumor weights from the Balb/c mice (**a**) on day 12. **c** Human MDA-MB-231 tumors in SCID mice were treated with fVII-tPDT or ntPDT (2 μM SnCe6, 72 J/cm²) on days 0, 7, 14, 21, and 28. **d** MDA-MB-231 tumor weights from the SCID mice on day 45. Each group contained five mice, and the data are presented as mean ± S.D. in all panels and analyzed using ANOVA single factor