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Tumor Mitochondria-targeted Photodynamic Therapy with a Translocator Protein (TSPO)-specific Photosensitizer

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Abstract

Photodynamic therapy (PDT) has been proven to be a minimally invasive and effective therapeutic strategy for cancer treatment. It can be used alone or as a complement to conventional cancer treatments, such as surgical debulking and chemotherapy. The mitochondrion is an attractive target for developing novel PDT agents, as it produces energy for cells and regulates apoptosis. Current strategy of mitochondria targeting is mainly focused on utilizing cationic photosensitizers that bind to the negatively charged mitochondria membrane. However, such an approach is lack of selectivity of tumor cells. To minimize the damage on healthy tissues and improve therapeutic efficacy, an alternative targeting strategy with high tumor specificity is in critical need. Herein, we report a tumor mitochondria-specific PDT agent, IR700DX-6T, which targets the 18 kDa mitochondrial translocator protein (TSPO). IR700DX-6T induced apoptotic cell death in TSPO-positive breast cancer cells (MDA-MB-231) but not TSPO-negative breast cancer cells (MCF-7). *In vivo* PDT study suggested that IR700DX-6T-mediated PDT significantly inhibited the growth of MDA-MB-231 tumors in a target-specific manner. These combined data suggest that this new TSPO-targeted photosensitizer has great potential in cancer treatment.

Graphical abstract

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Conflict of interest

No potential conflicts of interest relevant to this article are reported.

Keywords

Photodynamic therapy; Photosensitizer; Translocator protein; TSPO; Mitochondria; Apoptosis

1. Introduction

Photodynamic therapy (PDT) is a clinically approved, minimally invasive, and highly controllable therapeutic procedure, which has become popular as an alternative or additional approach to conventional cancer treatments, such as chemotherapy and surgery [1, 2]. A regime of PDT requires three key components: photosensitizer (PS), light irradiation, and oxygen [3]. In the presence of oxygen, a PS is activated by irradiation at a specific wavelength to produce reactive oxygen species (ROS), such as singlet oxygen and free radicals, which consequently lead to cell death [4]. Undoubtedly, development of effective PSs is essential to the advance of PDT. In recent years, a number of PSs have been developed for research as well as clinical use, such as porphyrin derivatives, chlorins, and phthalocyanines [5, 6].

The efficacy of PDT largely depends on the tumor-selectivity and subcellular localization of PSs. Upon administration, different PSs may locate to distinct cell organelles, such as mitochondria, lysosomes, and plasma membranes, depending on their physicochemical and binding properties, such as lipophilicity, charge, and chemical structure [7]. In fact, the subcellular distribution of PSs often correlates with specific type of cell death [8]. For example, antibody-PS conjugates used in photoimmunotherapy bind to plasma membrane and often lead to necrotic cell death [9].

Among the various subcellular targets, the mitochondrion holds particularly great promise as a PDT target, as it plays an essential role in supplying energy for cells and regulating cell apoptosis [10-12]. For this reason, great effort has been focused on developing new mitochondria-specific PDT agents [13, 14]. Current mitochondria-targeting PSs are mostly based on cationic molecules, because the negative charges on mitochondria membranes allow for ionic interaction with these PSs. However, given that mitochondrion is a universal cell organelle, such PSs can also damage mitochondria in healthy cells, leading to unwanted side effects. To address this limitation, we aimed to develop a PS that specifically binds to tumor mitochondria.

In the present study, we chose the 18 kDa translocator protein (TSPO), previously termed the peripheral benzodiazepine receptor (PBR), as the target for PDT. TSPO is a protein mainly found on the outer mitochondrial membrane and associated with a number of cellular

processes, such as cholesterol transport, steroidogenesis, cell proliferation, porphyrin transport and apoptosis [15]. Although normal tissues and organs express TSPO at various levels, significantly increased expression level of TSPO has been found in multiple cancers including breast [16, 17], colorectal [18], prostate [19], and brain cancer [20]. In addition, higher TSPO expression levels correlate with increased tumor aggressiveness and metastasis as well as with a poorer prognosis [18]. Furthermore, deregulation of TSPO expression or function has been reported to contribute to cell apoptosis. Consequently, TSPO is a promising target for improved cancer treatment efficacy.

Since TSPO regulates porphyrin transport, much effort has been invested into TSPO-targeted PDT using endogenous and exogenous porphyrin molecules, as summarized in a recent review [21]. Unfortunately, most of these TSPO-PDT studies resulted in limited efficacy and selectivity. To improve the TSPO targeting effect, Chen *et al.* recently developed a TSPO targeted PS for bi-functional positron emission tomography (PET) imaging and PDT by conjugating ^{124}I -labeled TSPO ligand, PK11195, with a well known porphyrin derivative, HPPH, as the PS [22]. The resulting agent showed significantly improved PDT efficacy over the HPPH PS in a breast cancer mouse model, although lengthy synthetic process and skin damage was involved.

In this study, we report a new TSPO-targeted PS, IR700DX-6T, which consists of a phthalocyanine PS (IR700DX), a six-carbon linker, and a TSPO ligand (DAA1106) with a high binding affinity to TSPO. This construct allows for effective PDT with only low-power LED light irradiation, thus avoiding potential skin phototoxicity issues. The photo therapeutic effect of IR700DX-6T was examined using both TSPO-positive and TSPO-negative human breast cancer cells. Furthermore, we evaluated the *in vivo* PDT efficacy using a breast cancer xenograft animal model.

2. Materials and methods

2.1. General

The solvents used are of ACS grade or HPLC grade. The PS, IR700DX-NHS ester, was purchased from LICOR Bioscience (Lincoln, NE). ^1H NMR spectra were recorded on a Bruker Avance III 400 MHz system. Mass spectra were recorded on a Waters LCT Premier mass spectrometer. UV/Vis absorption spectra were recorded on a Cary 100 Bio UV-vis Spectrophotometer (Agilent Technologies, Santa Clara, CA). Fluorescence emission spectra were recorded on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA). MDA-MB-231 and MCF-7 human breast cancer cells were gifts from Dr. Carolyn J. Anderson's lab (University of Pittsburgh, Pittsburgh, PA). The following instruments, supplies and assay kits were used: SynergyTM H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT), Zeiss Axio Observer fluorescent microscopy system (Zeiss, Jena, Germany), 96-well optical black plates (Fisher Scientific, Pittsburgh, PA), CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI), ApopTag[®] In Situ Apoptosis Detection Kits (EMD Millipore, Billerica, MA), and IVIS Lumina XR *in vivo* imaging system (PerkinElmer, Waltham, MA).

2.2. Synthesis of IR700DX-6T

The DAA1106 analog with a six-carbon linker, 6-TSPOMBb732, was prepared using the previously reported procedure [23]. IR700DX-6T was then synthesized by coupling IR700DX-NHS with 6-TSPOMBb732. Briefly, IR700DX-NHS and 6-TSPOMBb732 were dissolved in DMSO under argon and stirred in the dark for 2 days at room temperature. The resulting mixture was purified using dialysis tubing with a molecular weight cutoff of 500 Dalton in water. The remaining solution in the dialysis tubing was then lyophilized to dryness to give IR700DX-6T as a green powder. ^1H NMR (CD_3OD): δ = 9.77 (m, 5H), 9.61 (m, 1H), 9.42 (d, 1H, J = 5.7 Hz), 8.54 (s, 1H), 8.50 (m, 5H), 8.45 (m, 1H), 8.05 (d, 1H, J = 6.1 Hz), 7.28 (td, 2H, J = 7.0, 2.1 Hz), 7.12 (tt, 1H, J = 7.4, 1.0 Hz), 7.07 (dddd, 2H, J = 15.8, 11.0, 7.8, 3.1 Hz), 6.77 (dd, 1H, J = 9.0, 5.1 Hz), 6.73 (dd, 1H, J = 8.8, 3.0 Hz), 6.61 (dq, 2H, J = 8.4, 1.6 Hz), 6.43 (d, 1H, J = 8.9 Hz), 6.23 (d, 1H, J = 3.0 Hz), 4.59 (m, 23H), 3.57 (m, 7H), 3.15 (m, 4H), 2.72-2.81 (m, 18H), 2.21 (t, 2H, J = 7.5 Hz), 2.01 (t, 2H, J = 8.1 Hz), 1.97 (s, 3H), 1.61-1.78 (m, 12H), 1.52 (m, 4H), 1.27-1.42 (m, 6H), -0.96 (m, 4H), -2.11 (q, 4H, J = 5.2 Hz), -2.77 (d, 12H, J = 2.0 Hz). MS (ESI): m/z ($M + 6\text{H}$) $^{2+}$ calcd for $\text{C}_{98}\text{H}_{131}\text{FN}_{14}\text{O}_{27}\text{S}_6\text{Si}_3^{2+}$, 1115.847; found, 1115.849.

2.3. Cell culture

MDA-MB-231 and MCF-7 human breast cancer cells were used as the TSPO-positive [24] and TSPO-negative [25] cells, respectively. Both MDA-MB-231 and MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS, Fisher Scientific, Pittsburgh, PA), and 1% Penicillin-Streptomycin-Glutamine (Life Technology, Carlsbad, CA). Cells were incubated in a water jacketed incubator (37 °C, 5% CO_2).

2.4 Stability of IR700DX-6T in cell culture medium

A solution of IR700DX-6T or Indocyanine green (ICG, Sigma-Aldrich, St. Louis, MO) in cell culture medium was placed inside a capped cuvette. The initial absorption spectrum was measured before the cuvette was placed inside an incubator. Additional absorption spectra were recorded after 24 and 48 h incubation.

2.5. Determination of binding affinity using surface plasmon resonance (SPR)

To evaluate the binding affinity of IR700DX-6T to TSPO, SPR was performed using a Biacore X100 instrument (GE Healthcare, Little Chalfont, UK) based on the established methods [26]. Briefly, 25 $\mu\text{g/mL}$ of human TSPO full-length recombinant protein (Abnova Corporation, Taiwan) was immobilized via amine coupling (~1500 response units, RU) on a CM5 sensor chip (GE Healthcare, Little Chalfont, UK). Serial dilutions of IR700DX-6T (10, 5, 2.5, 1.25, 0.62, 0.31, 0.15 μM) in HBS-EP buffer (GE Healthcare, Little Chalfont, UK) were flowed for 180 sec at a rate of 20 $\mu\text{L/min}$ followed by dissociation with HBS-EP buffer flowed for 600 sec at a rate of 20 $\mu\text{L/min}$. After each sample injection, the surface was regenerated with 0.75 mM NaOH solution for 30 sec at a rate of 10 $\mu\text{L/min}$. Rabbit monoclonal TSPO antibody (Abcam, Cambridge, UK) was used to monitor the stability of the immobilized TSPO protein with serial dilutions (1, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} nM) in HBS-EP buffer. The consistently strong binding of the antibody to TSPO indicated

that TSPO was stable during the regeneration steps (data not shown). All sensorgrams were double referenced by subtracting the surface effect from the control flow cell and the buffer effect from the blank buffer. The association rate constant (k_{on}) and dissociation rate constant (k_{off}) were obtained using Biacore X100 Evaluation Software (GE Healthcare, Little Chalfont, UK) assuming the Langmuir 1:1 binding model. The binding constant (K_D) was calculated using $K_D = k_{off} / k_{on}$. SPR sensorgrams were plotted using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA).

2.6. Co-localization study of IR700DX-6T and a TSPO antibody

MDA-MB-231 cells were seeded into 8-well chamber slide (Fisher Scientific, Pittsburgh, PA) and incubated for 24 h. Cells were grouped into three treatments: (1) 5 μ M of IR700DX-6T for 16 h; (2) 10 μ M of DAA1106 (blocking agent) for 1 h followed by 5 μ M of IR700DX-6T for 16 h; and (3) 5 μ M of IR700DX for 16 h. Subsequently, cells were fixed with 4% paraformaldehyde in PBS for 15 min at 25 °C, and permeabilized with 0.1% Triton X-100 in PBS (Sigma-Aldrich, St. Louis, MO) for 15 min at 25 °C. Immunofluorescence staining of TSPO was performed by treating the cells with a monoclonal anti-TSPO antibody (Abcam, Cambridge, UK) for 1 h at 25 °C (1 : 100 in 0.2% BSA, 0.05% Tween20 in PBS) as the primary antibody, followed by treatment with anti-rabbit Alexa Fluor 488 (Invitrogen, Grand Island, NY) for 1 h at 25 °C (1 : 500 in 0.2% BSA, 0.05% Tween20 in PBS) as the fluorochrome-conjugated secondary antibody. Cell nuclei were stained with 10 μ g/mL DAPI (Life Technology, Carlsbad, CA) for 1 h at 25 °C. Cells were washed with PBS for three times before and after the secondary antibody treatment. Fluorescence microscope was used to capture fluorescence images. IR700DX-6T or IR700DX was visualized with a Cy5 filter (excitation/emission: 625-655/665-715 nm). Immunofluorescence staining of the TSPO antibody was visualized with a green fluorescent protein (GFP) filter (excitation/emission: 450-490/500-550 nm). Nuclear staining was imaged with a DAPI filter (excitation/emission: 335-383/420-470 nm).

2.7. In vitro PDT study

Cells were seeded into 96-well plates and incubated for 24 h prior to treatment. *In vitro* IR700DX-6T-PDT was performed as follows: cells were incubated with 5 μ M of IR700DX-6T at 37 °C for 16 h. Cells were then washed once to remove the unbound photosensitizer with cell culture medium. Next, cells were placed directly underneath the LED light (L690-66-60, Marubeni America Co., New York, NY), aligned to the center of the irradiation region. The distance from the tip of the LED light lens to the bottom of the plate was maintained at 3 cm. The cells were irradiated with LED light (wavelengths of 670-710 nm, peak at 690 nm) for 30 min (54 J/cm²). The irradiated area was 19.63 cm². Details regarding the experimental setup (Fig. S1) and irradiation energy can be found in the supplementary information. After the light irradiation, cells were incubated for an additional 90 min (unless otherwise indicated).

Cell viability was determined using the CellTiter-Glo assay per the manufacturer's instructions. The luminescent intensity was directly proportional to the amount of remaining viable cells. Cell death rates were determined by one minus recorded luminescent intensity in each group over that of the vehicle group times one hundred percent.

In comparison with IR700DX-6T-PDT treatment, MDA-MB-231 cells were also treated with (1) vehicle; (2) light irradiation; (3) IR700DX-6T; and (4) IR700DX-PDT. Additionally, MCF-7 cells were also treated with IR700DX-6T-PDT. Cell death rates were compared among these groups. To determine the blocking effect, MDA-MB-231 cells were pretreated with 10 μ M of DAA1106 or vehicle for 24 h, and then *in vitro* IR700DX-6T-PDT was carried out. Cell death rates were compared between the blocking and non-blocking treatments.

2.8. Cytotoxicity under the dark (non-illuminated) condition

To measure the dark cytotoxicity of IR700DX-6T, MDA-MB-231 cells were seeded into 96-well plates and incubated for 24 h. Subsequently, cells were treated with IR700DX-6T (2.5, 5, 10, 20 μ M) without light irradiation for 48 h. ICG was used as the negative (non-toxic) control. Cisplatin (COSH Healthcare Ltd., Tucker, GA), a commonly used chemotherapy drug, was used as the positive (toxic) control. Cell viability was determined as mentioned above.

2.9. Apoptosis study

To visualize the morphological changes induced by IR700DX-6T-PDT, we captured the real-time cell images during PDT and processed them into videos by ImageJ. MDA-MB-231 cells were seeded into 35 mm MatTek dishes (MatTek Corporation, Ashland, MA), treated with 5 μ M of IR700DX-6T for 16 h, washed once and then exposed to continuous light illumination (15 mW/cm²) at room temperature for 1 h, during which time frame-by-frame images were captured every 10 seconds through a trans light differential interference contrast (DIC) filter, using a fluorescence microscope.

Furthermore, we monitored morphological changes in the mitochondria after IR700DX-6T-PDT treatment. MDA-MB-231 cells were seeded into 35 mm MatTek dishes (MatTek Corporation, Ashland, MA) and incubated for 24 h prior to treatment. Cells were treated with IR700DX-6T-PDT or IR700DX-6T only (without irradiation). Mitochondria were labeled with 200 nM of Mito-Tracker[®] Green (Life Technology, Carlsbad, CA) for 30 min at 37 °C. Cell nuclei were stained with 10 μ g/mL of DAPI for 30 min at 37 °C. Fluorescence images were captured using our Zeiss Axio Observer fluorescent microscopy equipped with an Apotome.2 optical sectioning system. Mito-Tracker[®] Green and nuclear images were obtained with a GFP and DAPI filter set, respectively.

Lastly, we used ApopTag[®] kit to verify apoptotic death caused by IR700DX-6T-PDT. This kit distinguishes cell apoptosis from necrosis based on detecting terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). MDA-MB-231 cells were seeded into 8-well chamber and incubated for 24 h. Cells were then treated with IR700DX-6T-PDT or IR700DX-6T only, fixed in 1% formaldehyde (Sigma-Aldrich, St. Louis, MO) for 10 min, and labeled by ApopTag[®] staining according to the manufacturer's instructions.

2.10. In vivo PDT study

The animal studies have been approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Female athymic nude mice at 6 to 8 weeks old were

purchased from the Jackson Laboratory (Bar Harbor, ME). 5×10^6 MDA-MB-231 cells were injected subcutaneously into the left flank of the mice. Mice were anesthetized with a 2.5% isoflurane/oxygen gas mixture during treatments. The mice were euthanized with cervical dissection under anesthesia once the tumor diameter reached 15 mm at any direction.

To determine the optimal drug-light interval (time between the PS administration and light irradiation), tumor-bearing mice were i.v. injected with 10 nmol of IR700DX-6T or IR700DX via the tail vein. *In vivo* optical imaging was performed with an IVIS *in vivo* imaging system using the following parameters: excitation filter, 605 nm; emission filter, 700 nm; exposure time, 1 sec; binning, small; field of view, 12; f/stop, 2; open filter. The images were captured at pre-injection, 1 min, 2 h, 3 h, 9 h, 24 h, and 48 h post-injection. Images were analyzed by using Living Image 4.4 software (Caliper Life Sciences, Hopkinton, MA). Region-of-interest (ROI) in the tumor area was drawn. Fluorescent intensity within ROI was then measured, as represented by Radiant Efficiency ([photons/sec/cm²/sr]/[μW/cm²]). The time point with the highest fluorescent intensity was used as the drug-light interval for each drug treatment, respectively.

In vivo PDT experiments were carried out at approximately 7 days after cell injection. Mice with tumor sizes of 90–120 mm³ were selected. The LED light was placed directly above the tumor area of the target animal. The distance from the tip of LED lens to the tumor was approximately 1.5 cm. The tumor area was completely covered by the irradiation region. The irradiated area was 12.56 cm². During the PDT treatment, light irradiation at the tumor area was given by an LED light at a power density of 50 mW/cm² for 15 min (45 J/cm²). The light dose for *in vivo* study was comparable with *in vitro* study, and this amount of light dose has been proved to be effective for *in vivo* PDT from our previous type 2 cannabinoid receptor (CB₂R)-targeted PDT study [27]. Tumor-bearing mice were randomized into 6 groups (n = 4 per group) with the following treatments: (1) Untreated; (2) IR700DX-6T-PDT: 10 nmol IR700DX-6T i.v. injection, followed by light irradiation after 2 h and 24 h; (3) Blocked IR700DX-6T-PDT: 100 nmol DAA1106 i.v. injection; after 1 h, 10 nmol IR700DX-6T i.v. injection, followed by light irradiation after 2 h and 24 h; (4) IR700DX-6T: 10 nmol IR700DX-6T without light irradiation; (5) Light irradiation: light irradiation at 0 h and 24 h; (6) IR700DX-PDT: 10 nmol IR700DX i.v. injection, followed by light irradiation after 1 min and 24 h. The above treatments were repeated every 6 days. The tumor sizes were measured daily by a caliper and the volume was calculated as (tumor length) × (tumor width)²/2.

2.11. Ex vivo imaging and biodistribution

MDA-MB-231 tumor bearing mice (n = 3) were i.v. injected with 10 nmol IR700DX-6T through the tail vein. At 2 h post-injection, mice were euthanized. Tissues and organs (blood, heart, lung, liver, spleen, pancreas, kidneys, tumor, muscle of the left leg, and brain) were excised and imaged under IVIS Lumina XR system. The fluorescent intensity of IR700DX-6T was evaluated by drawing ROI along the excised tissues and organs. The quantitative imaging contrast profiles were obtained by dividing the fluorescence intensity from the target tissues/organs by that from the muscle of the left leg.

2.12. Data Processing and Statistical analysis

All of the data given in this study are the mean $\pm$ SEM (standard error of the mean) of n independent measurements ($n = 3$ for *in vitro* study, $n = 4$ for *in vivo* study, and $n = 3$ for *ex vivo* study). Statistical analyses were performed using Student's t test or one-way ANOVA method, with p values < 0.05 considered statistically significant. The analyses were performed using SPSS11.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Synthesis, spectroscopic, stability, and binding properties of IR700DX-6T

In our previous study, we developed a DAA1106 analog with a six-carbon linker and terminal amino group, 6-TSPOMBb732 (Scheme 1), which was subsequently conjugated to fluorescent dyes for TSPO-targeted imaging [23, 28]. Building upon this success, we attached this functional DAA1106 analog to a phthalocyanine dye, IR700DX-NHS, via a simple acylation reaction (Scheme 1). The resulting IR700DX-6T exhibits maximum absorption and emission at 690 nm and 699 nm in water, respectively (Fig. 1A).

The stability of IR700DX-6T in cell culture medium was accessed using UV/Vis absorption spectra (Fig. S2). After 48 h incubation, only a small decrease in IR700DX-6T absorption was observed, whereas the absorption intensity of ICG dropped dramatically during the same time period.

The binding kinetics of IR700DX-6T to human TSPO recombinant protein was measured by SPR. Serial dilutions of IR700DX-6T in HBS-EP buffer were injected over a TSPO-immobilized sensor chip followed by injection of HBS-EP buffer for dissociation. The resulting SPR sensorgrams were fitted using the Langmuir 1:1 binding model. The association rate constant $k_{on} = (2.2 \pm 0.7) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and dissociation rate constant $k_{off} = (4.3 \pm 0.3) \times 10^{-3} \text{ s}^{-1}$ (Fig. 1B, Table 1). The binding constant $K_D = (1.9 \pm 0.4) \times 10^{-6} \text{ M}$. Results are based on average of triplicate data.

3.2. Fluorescence microscopy

IR700DX-6T was shown to primarily localize in the cytoplasm (Fig. 2). There is co-localization between IR700DX-6T fluorescence and TSPO immunofluorescence staining, although some signals did not overlap. Pretreatment of DAA1106 significantly reduced the IR700DX-6T signal and non-targeted PS IR700DX showed a minimal fluorescent signal in cells. These data suggested that IR700DX-6T specifically bound to intracellular TSPO and alternative binding sites might exist, as discussed below.

3.3. In vitro PDT study

We evaluated the therapeutic effect of IR700DX-6T-PDT treatment using TSPO-positive MDA-MB-231 and TSPO-negative MCF-7 cells. As shown in Fig. 3A, immediately after treatment with IR700DX-6T-PDT, only $23.4\% \pm 0.9\%$ MDA-MB-231 cell death was observed. However, when the cells were returned to the incubator for an extended period of time (up to 90 min), increased cell death was observed in a time-dependent manner (cell death: $42.1\% \pm 4.0\%$ at 30 min, $51.5\% \pm 1.2\%$ at 60 min, and $70.6\% \pm 1.4\%$ at 90 min).

In Fig. 3B, no significant cell death were associated with light irradiation alone (cell death: $-3.6\% \pm 0.8\%$), or IR700DX-6T alone (cell death: $1.2\% \pm 2.4\%$). IR700DX-PDT (non-targeted) caused significantly less cell death than IR700DX-6T-PDT (cell death: $9.7\% \pm 2.1\%$ after IR700DX-PDT vs $71.9\% \pm 3.0\%$ after IR700DX-6T-PDT, $p < 0.001$). Additionally, significant difference of cell death was observed between MDA-MB-231 and MCF-7 cells treated with IR700DX-6T-PDT (cell death: $71.9\% \pm 3.0\%$ in MDA-MB-231 vs $13.7\% \pm 2.7\%$ in MCF-7 cells, $p < 0.001$). As shown in Fig. 3C, the blocking agent DAA1106 significantly reduced IR700DX-6T-PDT effect (cell death: $60.4\% \pm 2.9\%$ in the blocking group vs $82.5\% \pm 2.3\%$ in the non-blocking group, $p < 0.05$). These data suggested that IR700DX-6T-PDT caused effective cell death in a target-specific manner.

We also evaluated the influence of PS incubation time and LED light dose on PDT effect. As shown in Fig. S3A, the cell death rate, induced by IR700DX-6T after equal amount of light irradiation, was dependent on the incubation time. Specifically, cells with an incubation time of 16 h showed a significantly higher cell death rate ($75.0\% \pm 0.4\%$) than cells incubated for 4 h ($31.8\% \pm 3.5\%$). When the incubation time was reduced to only 30 min, no significant PDT effect was observed ($2.4\% \pm 2.4\%$). It is likely that the bulky structure of IR700DX-6T requires a prolonged incubation time for cellular uptake, and the PDT therapeutic effect is associated with the amount of PS inside cells. The light irradiation dose is another important factor in PDT efficacy. In Fig. S3B, significant amount of cell death ($79.2\% \pm 1.2\%$) was observed after 30 min of LED light irradiation (54 J/cm^2). When the light irradiation time was reduced to 15 or 5 min (27 or 9 J/cm^2 , respectively), much lower cell death rates were observed ($10.2\% \pm 1.3\%$ and $8.1\% \pm 1.3\%$, respectively). It is possible that the amount of ROS generated under low dosage of light irradiation was insufficient to cause significant cell death. Additionally, to demonstrate that IR700DX-6T PDT is safe to non-cancerous cells, the effect of IR700DX-6T PDT on non-cancerous human embryonic kidney HEK293 cells was evaluated. As shown in Fig. 3C, no significant cell death was observed in HEK293 cells as compared to MDA-MB-231 cells (cell death: $6.7\% \pm 1.2\%$ in HEK293 vs $75.0\% \pm 0.4\%$ in MDA-MB-231).

3.4. Cytotoxicity under dark (non-illuminated) condition

We first studied the dark cytotoxicity of IR700DX-6T in MDA-MB-231 cells. Similar to the FDA-approved ICG dye, IR700DX-6T caused negligible cell death in the tested concentration range ($2.5\text{--}20 \mu\text{M}$) (Fig. 4) in the absence of irradiation, whereas the positive control (Cisplatin, a chemotherapy drug) led to greatly elevated cell death under the same condition.

3.5. Apoptosis study

To identify the classification of MDA-MB-231 cell death induced by IR700DX-6T-PDT, real-time videos of the IR700DX-6T-treated cells were recorded during PDT (Video 1A). A combined treatment of IR700DX-6T and light irradiation yielded morphological changes typically seen in apoptotic cell death, such as cell shrinkage, membrane blebbing without loss of cell integrity, and apoptotic bodies formation [29]. In contrast, cells treated with IR700DX-6T without light irradiation exhibited normal morphology (Video 1B). It is noteworthy that in Video 1A, cells were exposed to both LED and white light irradiation,

whereas cells shown in Video 1B were only exposed to white light. The exposure time in Video 1A was therefore reduced to show similar overall brightness of cells as that in Video 1B, accounting for the variation of “bright particles” shown in the videos.

We also studied the morphological changes in the mitochondria caused by IR700DX-6T-PDT treatment using Mito-Tracker[®] Green staining, which labels mitochondria (Fig. 5A). Without PDT treatment, mitochondria showed a thread-like shape. However, upon IR700DX-6T-PDT treatment, mitochondria turned into swelling and circle-like shape, indicating mitochondrial damages. Furthermore, we used ApopTag[®] kit to verify the apoptotic cell death induced by the IR700DX-6T-PDT treatment. Green fluorescence signal indicates that DNA damage occurred during apoptosis. In Fig. 5B, green fluorescence signals were found in the cell nuclei of IR700DX-6T-PDT-treated cells, but cells treated with only IR700DX-6T were lack of green fluorescence signals. The combined data indicated that IR700DX-6T-PDT resulted in apoptosis in MDA-MB-231 cells, accompanied by significant mitochondrial damage.

3.6. In vivo TSPO-targeted PDT

We first determined the optimal drug-light interval for IR700DX-6T and IR700DX by *in vivo* imaging using MDA-MB-231 tumor-bearing mice (Fig. 6). After injection, the uptake of IR700DX-6T in tumors increased and reached the maximum at 2 h post injection. After that, IR700DX-6T was gradually cleared from the tumor area. *Ex vivo* imaging and biodistribution study showed that the tumor/muscle ratio reached 2.3 ± 0.2 at 2 h post-injection. IR700DX-6T uptake was also detected in the kidney, liver and lung (Fig. S4). At 24 h post-injection, IR700DX-6T still showed relatively high tumor uptake (62.4% of uptake at 2 h post-injection, Fig. 6). Therefore, for IR700DX-6T-PDT treated mice, two light irradiation treatments, at 2 h and 24 h post-injection, respectively, were deployed after each drug administration. The uptake of non-targeted IR700DX showed an apex of accumulation right after administration and decreased sharply over time. To correspond with this clearance profile and minimize the influence of light irradiation treatment, two light irradiation treatments, at 1 min and 24 h post-injection respectively, were deployed after IR700DX administration. The above treatments were repeated every 6 days.

The *in vivo* PDT results are summarized in Fig. 7. At six days post-injection, the treatment of IR700DX-6TPDT significantly reduced tumor growth as indicated by the tumor volume compared with that of the untreated mice (average tumor volume 164.5 mm^3 vs 845.7 mm^3 , $p < 0.001$). In contrast, mice treated with light irradiation showed a rapid tumor growth (average tumor volume: 1332.9 mm^3 , $p < 0.01$). Additionally, IR700DX-6T treatment without irradiation (average tumor volume: 321.1 mm^3 , $p < 0.01$) and IR700DX-PDT (average tumor volume: 273.7 mm^3 , $p < 0.01$) also exhibited tumor inhibition effect, but the therapeutic effect was inferior in comparison with IR700DX-6T-PDT. Lastly, blocking agent DAA1106 effectively suppressed the therapeutic effect induced by IR700DX-6T-PDT (average tumor volume: 459.3 mm^3 in blocking group vs 164.5 mm^3 in IR700DX-6T-PDT group, $p < 0.05$).

Untreated mice were sacrificed after 7 days when the tumor size reached 15 mm in diameter, as required by the IACUC protocol. In contrast, the tumor volume of the IR700DX-6T-PDT

group, after five treatment cycles, did not reach the mandatory termination point until day 30. The tumor volumes of the other groups reached the termination point on day 6 (light irradiation), 11 (IR700DX-6T), 12 (IR700DX-PDT) and 10 (blocking), respectively.

4. Discussion

We see considerable opportunity in targeting the TSPO for PDT treatment. In addition to the elevated expression levels in various types of cancers, TSPO plays an important role in the regulation of apoptosis. Specifically, through interaction with several proteins in the outer and inner mitochondrial membrane, TSPO regulates the mitochondrial permeability transition pore (MPTP), which controls mitochondrial membrane integrity by maintaining mitochondrial transmembrane potential [30, 31]. TSPO-targeted therapy may cause prolonged opening of the MPTP, which leads to the release of apoptotic factors (such as cytochrome c) from the mitochondria into the cytosol and trigger the mitochondrial apoptosis cascade [15]. In particular, high levels of ROS have been proven effective in disrupting the MPTP, resulting in immediate dissipation of mitochondrial transmembrane potential and osmotic swelling of the mitochondrial matrix [31]. The matrix swelling leads to the rupture of the outer mitochondria membrane and subsequently apoptotic cell death [32, 33]. As such, TSPO-targeted PDT agents appear to be effective therapeutic tools to directly introduce ROS to the MPTP and initiate the apoptosis cascade.

The present study was focused on developing a new TSPO-targeted PDT agent, IR700DX-6T, and evaluate the efficacy in cells and a xenograft mouse breast cancer model. This PS exhibits low dark toxicity and high target-specific phototoxicity. Specifically, under irradiation with low-power LED light, IR700DX-6T was lethal to TSPO-positive MDA-MB-231 cells while the TSPO-negative MCF-7 cells remained intact. In addition, this PS effectively induced apoptotic cell death in TSPO-positive MDA-MB-231 cells, accompanied with mitochondria matrix swelling. *In vivo* PDT study suggested that IR700DX-6T-PDT significantly inhibited the growth of MDA-MB-231 tumor in a TSPO-specific manner.

DAA1106 partially blocked the intracellular TSPO uptake of IR700DX-6T (Fig. 1B, 2), as well as the *in vitro* IR700DX-6T-PDT effect (Fig. 3C). Although the specific targeting of IR700DX-6T has been demonstrated, we also noticed non-specific binding of the PS, which correlates with the partial blocking effects. This is not surprising, because attachment of a bulky dye molecule to a small targeting ligand often impede the proper interaction with the target receptor, leading to non-specific binding and lower binding affinity [23, 34-37]. Additionally, the IR700DX phthalocyanine dye may lead to binding with alternative proteins, such as Bcl-2. It has been reported that phthalocyanine molecules could bind to anti-apoptotic Bcl-2 protein [38]. Interestingly, Bcl-2 is also an important regulator of apoptosis and photodamage to Bcl-2 is pertinent to apoptotic cell death caused by PDT treatment [33, 38]. Therefore, it is possible that IR700DX-6T binds to both TSPO and alternative apoptosis-regulatory proteins, and the cell death caused by IR700DX-6T-PDT is due to photodamage at both binding sites.

Another interesting finding during our study is that the cell death determined 90 min post IR700DX-6T-PDT treatment was significantly higher than the instantaneous measurement.

Because the CellTiter-Glo Luminescent Cell Viability Assay kit used to determine cell viability is based on ATP measurement, our observation indicates that a gradual ATP depletion occurred over time after IR700DX-6T-PDT. This is in agreement with TSPO-targeted therapy as photodamage of TSPO is expected to collapse the mitochondrial membrane potential over time, which is the driving force of ATP synthesis [39]. It is noteworthy that apoptosis triggered by photodamage of mitochondria takes shorter time than regular apoptosis, which usually progresses through lengthy signal-transduction pathways. Mechanistic studies of PDT-induced apoptosis indicate that mitochondria photodamage may cause loss of the mitochondrial membrane potential, leading to rapid release of cytochrome c [40, 41]. Release of cytochrome c from mitochondria is the key trigger to apoptotic cell death [42]. Therefore, unlike other triggers of apoptosis, mitochondria stress evoked by photodamage can bypass intermediate apoptotic pathways and lead to rapid apoptosis and efficient cell death.

Because TSPO regulates apoptosis, TSPO-targeted PDT is expected to cause apoptotic cell death. In our study, typical morphological changes corresponding to apoptosis, such as cell shrinkage and cell fragmentation into small blebs, were observed during the IR700DX-6T-PDT (Video 1A). At the subcellular organelle level, mitochondrial matrix swelling was found only in IR700DX-6T-PDT-treated cells (Fig. 5A). In conjunction of these morphological criteria of apoptosis, we deployed ApopTag[®] kit post IR700DX-6T-PDT to validate the type of cell death caused by the treatment. This kit labels DNA fragment from apoptosis with green fluorescence through the TUNEL method [43]. Green signals were detected in nuclei after MDA-MB-231 cells were treated with IR700DX-6T-PDT (Fig. 5B). These data confirm the apoptosis-like cell death induced by IR700DX-6T-PDT, and are in sharp contrast to those from our previous PDT study using a CB₂R-targeted PS IR700DX-mbc94 [37, 44], which shares the same phthalocyanine dye with IR700DX-6T. CB₂R belongs to the G-protein coupled receptor family and is up-regulated in various types of cancers [45]. The most distinct outcomes caused by IR700DX-mbc94-PDT include: (1) cell death was observed instantly after completion of PDT and (2) morphological changes associated with necrotic cell death, such as cell swelling and cell membrane rupture, were recorded. It appears that the subcellular location of PS plays a critical role in the type of cell death. Further studies will involve exploring the mechanisms of different cell death phenotypes mediated by these molecular targets.

IR700DX-6T-PDT treatment using the LED irradiation significantly suppressed the growth of MDA-MB-231 tumors. In fact, it took over 30 days for the tumor diameter in this group to reach the 15 mm mandatory termination point. This time period is 3 to 5 times longer than the other control treatment groups. We also found that the tumor growth was even accelerated in the light irradiation only group. These observations are consistent with the *in vitro* PDT results and our previous *in vivo* PDT study targeting the CB₂R [45]. It is possible that, despite the low energy output, the LED light irradiation could still have induced thermal effect, which caused the tumor microenvironment (*i.e.* angiogenesis, oxygenation, immune response, *etc.*) to be more favorable for tumor growth [46]. However, investigation of the tumor microenvironment changes is beyond the scope of this study.

Although PDT is considered a minimally invasive treatment process, one of the main issues of clinical PDT is skin damage [21]. To address this limitation, we adopted low-power LED light as the irradiation source. During the *in vivo* IR700DX-6T-PDT treatment, we closely monitored the skin damage. Mild temporary swelling around the tumor area was noticed during 48 h to 72 h post-injection of PS. It is possible that this swelling is an inflammatory response associated with PDT-caused cell death. In our *in vitro* study, we found that IR700DX-6T-PDT caused apoptotic cell death. Apoptosis has been reported to induce minimal inflammation reaction compared to necrosis, as cell contents are not released during apoptosis and apoptotic bodies can be scavenged by phagocytes [4]. However, the concept that apoptosis is non-inflammatory is not absolute, because in some cases, apoptotic cells undergo a process known as secondary necrosis, which may induce inflammation response [47]. It is noteworthy that this temporary local swelling was recovered quickly and no visible side effects were observed, such as skin scarring and infection, weight loss or any life threatening complications.

For *in vitro* study, two human breast cancer cell lines, MDA-MB-231 (TSPO-positive) and MCF-7 (TSPO-negative) were used to determine the target specificity of IR700DX-6T-PDT. While using MCF-7 as a negative control for the subsequent *in vivo* study seems to be a rational expansion from the *in vitro* study, it was not utilized because, in xenograft model, the MCF-7 tumor expresses high level of murine TSPO due to inflammation. As such, the MCF-7 xenograft model is not qualified as a TSPO-negative tumor model [25].

5. Conclusions

In conclusion, we developed a novel TSPO-targeted PS, IR700DX-6T. *In vitro* study indicated that IR700DX-6T induced apoptotic cell death in TSPO-positive but not TSPO-negative breast cancer cells. Remarkably, *in vivo* PDT study suggested that IR700DX-6T-mediated PDT significantly inhibited the growth of MDA-MB-231 tumors in a target-specific manner. These results suggest that IR700DX-6T-PDT may have great potential in treating TSPO-positive tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Spectroscopic and binding properties of IR700DX-6T. (A) Normalized UV/Vis absorption (green) and emission (red) spectra of IR700DX-6T in water at the concentration of 1×10^{-6} M ($\lambda_{\text{ex}} = 660$ nm). (B) Representative SPR sensorgrams with global fitting curves of IR700DX-6T to immobilized sensor chip.

Fig. 2.

Co-localization study of IR700DX-6T and TSPO immunostaining. MDA-MB-231 cells were grouped into three treatments: (1) 5 μ M of IR700DX-6T for 16 h; (2) 10 μ M of DAA1106 (blocking agent) for 1 h followed by 5 μ M of IR700DX-6T for 16 h; (3) 5 μ M of IR700DX for 16 h. Cells were fixed and immunofluorescence stained with anti-TSPO. Nuclei were stained with DAPI. IR700DX-6T or IR700DX (red), TSPO (green), nuclei (blue). Scale bar: 10 μ m.

Fig. 3.

In vitro PDT effect of IR700DX-6T. (A) MDA-MB-231 cells were treated with IR700DX-6T-PDT, and incubated for the indicated time period. (B) MDA-MB-231 cells were divided into 5 groups and given the following treatments: (1) vehicle; (2) light irradiation; (3) IR700DX-6T; (4) IR700DX-PDT; (5) IR700DX-6T-PDT. In addition, MCF-7 cells treated with IR700DX-6T-PDT were used as a TSPO-negative cell control. (C) The effect of IR700DX-6T PDT on non-cancerous HEK293 cells as compared to MDA-MB-231 cells. (D) MDA-MB-231 cells were treated with or without blocking agent before IR700DX-6T-PDT treatment.

Fig. 4.

Cytotoxicity of IR700DX-6T under dark condition. MDA-MB-231 cells were treated with IR700DX-6T (2.5, 5, 10, 20 μM) without light irradiation for 48 h. Cell death was determined. ICG was used as a negative (non-toxic) control. Cisplatin was used as a positive (toxic) control.

Fig. 5. IR700DX-6T-PDT induced mitochondria damage and apoptotic cell death. MDA-MB-231 cells were treated with IR700DX-6T with or without light irradiation. (A) Morphological changes of mitochondria were studied using Mito-Tracker[®] Green as a mitochondria marker. Upon IR700DX-6T-PDT treatment, mitochondria turned into swelling and circle-like shape. (B) Apoptosis-like cell death was identified using the ApopTag[®] apoptosis detection kit. Green fluorescence signals indicating apoptosis were found in the cell nuclei of IR700DX-6T-PDT treated cells. Scale bar: 20 μ m.

Fig. 6.

Bio-clearance of IR700DX-6T and IR700DX. MDA-MB-231 tumor-bearing mice were i.v. injected with 10 nmol of IR700DX-6T or IR700DX. *In vivo* optical imaging was performed with IVIS Lumina XR. The images were captured at pre-injection, 1 min, 2 h, 3 h, 9 h, 24 h, and 48 h post-injection. Fluorescence intensity in tumor areas was measured and plotted.

Fig. 7.

In vivo PDT effect of IR700DX-6T. MDA-MB-231 tumor-bearing mice were divided into the following groups: (1) untreated; (2) IR700DX-6T-PDT; (3) Blocked IR700DX-6T-PDT; (4) IR700DX-6T; (5) Light irradiation; (6) IR700DX-PDT. (A) White light image showing tumor inhibition effect among the above groups before (day 0) and after (day 6) the first treatment. Red dotted circles represented the tumor area. (B) Quantitative analysis of tumor growth.

Scheme 1.
Synthesis of IR700DX-6T.

Table 1

Association rate constant (k_{on}), dissociation rate constant (k_{off}), and equilibrium constant (K_{D}) of IR700DX-6T to human TSPO recombinant protein.

	k_{on} ($\text{M}^{-1}\text{s}^{-1}$)	k_{off} (s^{-1})	K_{D} (M)
Mean $\pm$ SEM	$(2.2 \pm 0.7) \times 10^3$	$(4.3 \pm 0.3) \times 10^{-3}$	$(1.9 \pm 0.4) \times 10^{-6}$