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Multilayer Photodynamic Therapy for Highly Effective and Safe Cancer Treatment

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Abstract

Recent efforts to develop tumor-targeted photodynamic therapy (PDT) photosensitizers (PSs) have greatly advanced the potential of PDT in cancer therapy, although complete eradication of tumor cells by PDT alone remains challenging. As a way to improve PDT efficacy, we report a new combinatory PDT therapy technique that specifically targets multilayers of cells. Simply mixing different PDT PSs, even those that target distinct receptors (this may still lead to similar cell-killing pathways), may not achieve ideal therapeutic outcomes. Instead, significantly improved outcomes likely require synergistic therapies that target various cellular pathways. In this study, we target two proteins upregulated in cancers: the cannabinoid CB₂ receptor (CB₂R, a G-protein coupled receptor) and translocator protein (TSPO, a mitochondria membrane receptor). We found that the CB₂R-targeted PS, IR700DX-mbc94, triggered necrotic cell death upon light irradiation, whereas PDT with the TSPO-targeted IR700DX-6T agent led to apoptotic cell death. Both PSs significantly inhibited tumor growth *in vivo* in a target-specific manner. As expected, the combined CB₂R- and TSPO-PDT resulted in enhanced cell killing efficacy and tumor inhibition with lower drug dose. The median survival time of animals with multilayer PDT treatment was

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Conflict of interest

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

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extended by as much as 2.8-fold over single PDT treatment. Overall, multilayer PDT provides new opportunities to treat cancers with high efficacy and low side effects.

Graphical Abstract

Photodynamic therapy (PDT) is increasingly used as a minimally invasive, controllable and effective therapeutic procedure for cancer treatment. However, complete eradication of tumor cells by PDT alone remains challenging. In this study, we investigate the potential of multilayer PDT in cancer treatment with high efficacy and low side effects. Through PDT targeting two cancer biomarkers located at distinct subcellular localizations, remarkable synergistic effects in cancer cell killing and tumor inhibition were observed in both *in vitro* and *in vivo* experiments. This strategy may be widely applied to treat various cancer types by using strategically designed PDT photosensitizers that target corresponding upregulated receptors at tactical subcellular localization.

Keywords

Photodynamic therapy; CB₂ receptor; TSPO; synergistic; combination therapy

1. Introduction

Photodynamic therapy (PDT) offers a minimally invasive, effective and highly controllable therapeutic strategy, and has become popular as an alternative or additional approach to conventional cancer treatments, such as chemotherapy and surgery [1, 2]. During the process of PDT, a light-sensitive photosensitizer (PS) is activated by light irradiation at a specific wavelength to produce reactive oxygen species (ROS), such as singlet oxygen and free radical, which consequently lead to cell death [3]. PDT has been clinically approved to treat several types of cancers, such as esophageal and non-small cell lung cancer, as well as precancerous changes of Barrett's esophagus and skin (actinic keratosis). Moreover, many clinical trials are currently under way to study the potential of PDT in the treatment of various other types of cancers [4]. To date, the FDA has approved several PDT PSs to treat cancer, including Photofrin, Levulan, Metvix and Foscan [5].

Despite the promise of PDT, most available PDT PSs can also cause phototoxicity to normal tissues in the irradiated region due to the lack of tumor specificity. To overcome this limitation, much effort has been invested in developing tumor-targeted PSs [6]. Many tumor associated antibodies [7] and peptides [8, 9] have been used in PDT as targeting molecules. For example, Taratula *et al.* attached luteinizing hormone-releasing hormone (LHRH) peptide to a phthalocyanine-encapsulated dendrimer for targeted PDT treatment of ovarian

cancer that overexpresses the LHRH receptor [10]. In a similar way, Master and co-workers coupled GE-11 peptide to the surface of phthalocyanine-incorporated micelles and evaluated the efficacy of the resulting PDT nanomedicine in a xenograft mouse head & neck tumor model [11]. Despite these and other recent efforts to develop tumor-targeted PDT PSs, complete eradication of tumor cells by PDT alone remains challenging, mainly due to the limited efficacy [12].

Recently, combination therapy that involves PDT and other treatments, particularly chemotherapy, has become a promising strategy to further improve therapeutic efficacy [13, 14]. The combination of chemotherapy and PDT can promote synergism between different cell killing pathways, and therefore greatly enhance the anticancer efficacy [15, 16]. For example, combined treatment of PDT and methotrexate, a chemotherapy drug, caused a synergistic cytotoxic effect in epithelial squamous carcinoma models both *in vitro* and *in vivo* [17]. More recently, He and co-workers developed core-shell nanoparticles for combined chemotherapy and PDT of resistant head and neck cancers. This combination therapy approach shows superior efficacy against tumor progression (83% reduction of tumor volume) by introducing apoptosis and necrosis simultaneously [18]. The results from these combination therapy studies are encouraging, although most of the PDT PSs used in combination therapy lack tumor targeting capability and the systemic toxicity caused by chemotherapy is of concern. In an effort to achieve outstanding therapeutic efficacy, with high tumor-targeting capability and low side effects, here we report a new combinatory PDT approach through targeting multiple cellular layers.

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 [19]. For example, researchers have attached PSs to cancer-related antibodies, which bind to the plasma membrane and lead to effective cancer cell death [20, 21]. Another promising subcellular target for PDT is the mitochondria, which plays an essential role in supplying energy for cells and regulating cell apoptosis [22–24]. Great effort has been focused on developing new mitochondria-specific PDT agents [25, 26], although most mitochondria-targeting PSs are based on cationic molecules, which lack specificity for tumor mitochondria. The subcellular distribution of PSs often correlates with specific type of cell death [27]. For example, antibody-PS conjugates bind to plasma membrane and often lead to necrotic cell death [28], whereas mitochondria-targeted PDT typically causes apoptotic cell death [24]. Since combination therapies can promote therapeutic synergy through multi-target mechanisms [29], we expected that specifically targeting multiple cellular layers associated with different cancer cell-killing mechanisms by combined PDT treatment would offer stronger and more sustained responses for durable tumor destruction with the potential of lower toxicity.

In our recent studies, we developed two cancer-specific PDT PSs, IR700DX-*mbc94* and IR700DX-6T, which target cannabinoid CB₂ receptor (CB₂R) and translocator protein (TSPO), respectively [30–32]. CB₂R belongs to the G protein-coupled receptors (GPCRs, plasma membrane receptors) family and is predominantly a peripheral receptor abundantly expressed by immune cells [33, 34]. Many types of cancers, including prostate, skin, liver,

brain, thyroid, lymphoma, lung, colon, ovarian and breast cancers, up-regulate CB₂R expression [35]. An association between CB₂R levels and tumor aggressiveness has also been identified [36]. The 18 kDa translocator protein (TSPO), previously termed the peripheral benzodiazepine receptor (PBR), is a protein mainly found on the outer mitochondrial membrane with overexpression in multiple cancers, including ovarian [37–39], breast [40, 41], colorectal [42], prostate [43], and brain cancers [44]. Similar to CB₂R, higher TSPO expression levels correlate with increased tumor aggressiveness and metastasis as well as with a poorer prognosis [42]. Furthermore, deregulation of TSPO expression or function has been reported to contribute to cell apoptosis. PDT treatment using IR700DX-mbc94 or IR700DX-6T showed significant therapeutic effect in cancer cells and mouse tumor models in a target-specific manner. In addition, CB₂R-PDT and TSPO-PDT caused predominantly necrotic and apoptotic cell death, respectively.

In the present study, we set out to investigate the potential of multilayer PDT in cancer treatment through combined targeting of CB₂R and TSPO. As expected, combination of these two treatments led to a remarkable synergistic effect. Although simultaneous administration of two PSs for PDT of cancer was recently reported, these PSs lack active targeting of cancer-associated receptors [45]. To our best knowledge, this study represents the first multilayer PDT approach by specifically targeting cancer-related receptors, with the promise of enhanced efficacy and minimal side effects.

2. Materials and methods

2.1. General

The solvents used are of ACS or HPLC grade. The photosensitizer, IR700DX-NHS ester, was purchased from LI-COR Bioscience (Lincoln, NE). The following instruments, supplies and assay kits were used for *in vitro* and *in vivo* studies: 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), apoptosis/necrosis cell detection kit (PromoKine, Heidelberg, Germany), IVIS Lumina XR *in vivo* imaging system (PerkinElmer, Waltham, MA).

2.2. Synthesis of IR700DX-6T and IR700DX-mbc94

IR700DX-6T and IR700DX-mbc94 were synthesized by coupling IR700DX with a conjugable TSPO ligand, 6-TSPOMBb732 and CB₂R ligand, mbc94, respectively, using the previously reported method [31].

2.3. Cell culture

MDA-MB-231 human breast cancer 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% CO₂).

2.4. In vitro PDT study

MDA-MB-231 cells were seeded into 96-well plates and incubated for 24 h prior to treatment. *In vitro* PDT was performed as follows: cells were incubated with indicated concentration of IR700DX-6T (TSPO-PDT), IR700DX-mbc94 (CB₂R-PDT) or IR700DX-6T + IR700DX-mbc94 (combined PDT) at 37 °C for 16 h. To evaluate single PDT treatment effect, IR700DX-6T (0.125, 0.1875, 0.25, 0.3125, 0.375, 0.5, 1, 2, 4, and 8 μM) or IR700DX-mbc94 (0.5, 0.75, 1, 1.25, 1.5, 2, and 4 μM) was used. To evaluate synergistic effect of multilayer PDT treatment (IR700DX-6T/IR700DX-mbc94=1:4), IR700DX-6T (0.125, 0.1875, 0.25, 0.3125, and 0.375 μM), IR700DX-mbc94 (0.5, 0.75, 1, 1.25, 1.5 μM), or combined IR700DX-6T (0.125, 0.1875, 0.25, 0.3125, and 0.375 μM) and IR700DX-mbc94 (0.5, 0.75, 1, 1.25, and 1.5 μM) were used. We also evaluated the synergistic effect of multilayer PDT treatment using two other ratios (IR700DX-6T/IR700DX-mbc94=1:1 and 4:1) and the same method was used.

Cells were then washed once to remove the unbound probe with cell culture medium. Cells were irradiated with LED light (L690-66-60, Marubeni America Co., New York, NY) at wavelengths of 670–710 nm and a power density of 30 mW/cm² for 30 min (54 J/cm²). After the treatment of light irradiation, cells were returned to incubator for an additional 24 h.

Cell viability was determined by 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.

2.5 Combination index calculations

The median-effect analysis of Chou-Talalay method [46] was used to determine additive, synergistic or antagonistic effect of combined TSPO-PDT and CB₂R-PDT treatment. *In vitro* therapeutic effect data were used to calculate combination index (CI) via CompuSyn (Combosyn, Inc., Paramus, NJ) software.

$$CI = \frac{C_{A,x}}{IC_{x,A}} + \frac{C_{B,x}}{IC_{x,B}}$$

$C_{A,x}$ and $C_{B,x}$ are the concentrations of drug A and drug B used in combination to achieve x % drug effect. $IC_{x,A}$ and $IC_{x,B}$ are the concentrations for single agents to achieve the same effect [47]. When CI value is <1, the combination is synergistic; when CI value is =1 the combination is additive, and when CI value is >1 the combination is considered antagonistic [48]. Chou-Talalay plot was generated to provide visual illustration. Chou-Talalay plot is a plot of CI on y-axis as a function of effect level (fa) on the x-axis. Below the CI = 1 horizontal line indicates synergism, above the CI = 1 horizontal line indicates antagonism, and on the CI = 1 horizontal line indicates additive effect.

2.6 Apoptosis/necrosis assay

MDA-MB-231 cells were seeded into 35 mm MatTek dishes (MatTek Corporation, Ashland, MA). Cells were treated with 0.25 μM of IR700DX-6T, 1 μM of IR700DX-mbc94 or combined IR700DX-6T (0.25 μM) and IR700DX-mbc94 (1 μM) for 16 h. *In vitro* PDT was performed. Cell death was detected using apoptosis/necrosis cell detection kit by staining cells with Annexin V and Ethidium Homodimer III (EthD-III) labeling. An early indicator of apoptosis is the rapid translocation and accumulation of the phospholipid phosphatidylserine from the inner to the outer portion of the plasma [49]. This disruption of membrane asymmetry can be detected using a Ca^{2+} -dependent phospholipid-binding protein Annexin V that has a high affinity to phosphatidylserine. EthD-III is a positively charged nucleic acid probe and not permeant to live cells. It can be used for identifying necrotic and late apoptotic cells. MDA-MB-231 cells were seeded into 35 mm petri dishes. After treatment of TSPO-PDT, CB₂R-PDT, or combined-PDT, the cells were stained with apoptosis/necrosis cell detection kit as per the manufacturer's instructions and observed under fluorescent microscopy. Live cells did not show fluorescent signals. Early apoptotic cells were labeled as positive green fluorescent plasma membrane staining, while necrotic cells were identified by red fluorescent nuclear staining. Late apoptotic cells may show both red and green staining.

2.7. In vivo phototherapy

The animal studies have been approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Female athymic nude mice at 6–8 weeks old were purchased from the Jackson Laboratory (Bar Harbor, ME). 5×10^6 MDA-MB-231 cells were injected subcutaneously into the right 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.

In vivo PDT experiments were carried out approximately 7 days after cell injection. Mice with tumor sizes of 50–60 mm³ were selected. Tumor-bearing mice were randomized into 4 groups ($n = 4$ per group) with the following treatments: (1) Untreated, (2) TSPO-PDT: 2.5 nmol IR700DX-6T i.v. injection, followed by light irradiation at 2 h post injection, (3) CB₂R-PDT: 10 nmol IR700DX-mbc94 i.v. injection, followed by light irradiation at 2 h post injection, and (4) Combined PDT therapy: (10 nmol IR700DX-mbc94 + 2.5 nmol IR700DX-6T) i.v. injection, followed by light irradiation at 2 h post injection. 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 above treatments were repeated every 6 days. The tumor sizes were measured daily by a caliper until termination point and the volume was calculated as $(\text{tumor length}) \times (\text{tumor width})^2 \div 2$.

2.8. Data Processing and Statistical analysis

All data given in this study are the mean $\pm$ SEM (standard error of the mean) of n independent measurements ($n = 4$ for *in vitro* study and *in vivo* PDT study, $n = 3$ for bio-distribution study). Graphs were plotted using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Statistical analyses were performed using the one-way

ANOVA method, with p values <0.05 considered statistically significant. The analyses were performed using SPSS11.0 (SPSS Inc., Chicago, IL). Kaplan Meier Survival analysis was performed using GraphPad Prism software.

3. Results

3.1 Combined treatment with TSPO-PDT and CB₂R-PDT induces synergistic cytotoxicity effect in MDA-MB-231 cells

Triple negative MDA-MB-231 human breast cancer cells express CB₂R and TSPO at a low and high level respectively [40, 50], and was used to evaluate the combined therapeutic effect. TSPO-targeted PS, IR700DX-6T [32], and CB₂R-targeted PS, IR700DX-mbc94 [31] (Figure 1), which share the same PS (IR700DX), were synthesized using previously reported methods. We first evaluated ROS production from IR700DX-mbc94 and IR700DX-6T upon light irradiation using singlet oxygen sensor green (SOSG). After 30 min of light irradiation, 0.25 μ M of IR700DX-6T (RFU SOSG: 3664.6 ± 78.3), 1 μ M of IR700DX-mbc94 (RFU SOSG: 10948.5 ± 236.8) and combined 0.25 μ M of IR700DX-6T + 1 μ M of IR700DX-mbc94 (RFU SOSG: 11492.5 ± 80.7) induced significant ROS production, which was successfully quenched when singlet oxygen scavenger NaN₃ was added into the solution (Supplementary Figure 1). We then investigated effect of single treatment of TSPO-PDT or CB₂R-PDT in MDA-MB-231 cells. As shown in Figure 2, after treatment with TSPO-PDT or CB₂R-PDT alone, cell death increased in a concentration-dependent manner. TSPO-PDT effect was less potent than CB₂R-PDT, with an IC₅₀ of 2.04 μ M (TSPO-PDT) and 1.45 μ M (CB₂R-PDT), respectively.

To evaluate multilayer PDT effect, MDA-MB-231 cells were divided into 3 treatment groups: (1) TSPO-PDT; (2) CB₂R-PDT; and (3) combined PDT (TSPO-PDT + CB₂R-PDT). PDT effect was subsequently evaluated. As shown in Figure 3A, a remarkable increase in cell death was observed with combined treatment. For example, PDT with 1 μ M of CB₂R-PDT and 0.25 μ M of TSPO-PDT alone induced only $5.2\% \pm 4.8\%$ and $22.2\% \pm 3.4\%$ cell death, respectively. However, combined therapy caused as high as $57.8\% \pm 1.9\%$ cell death.

To quantify the synergistic effect of multilayer PDT treatment with CB₂R-PDT and TSPO-PDT, CI value was calculated based on the Chou and Talalay's median-effect equation [1]. As shown in Table 1, the CIs in all combined treatments were less than 1, indicating that the combination of TSPO-PDT and CB₂R-PDT had a synergistic cytotoxic effect on MDA-MB-231 cells at the indicated concentration. As shown in Figure 3B, the simulated curve of Fa-CI plot (Chou-Talalay plot) for multilayer PDT treatment was below the CI = 1 horizontal line, indicating a synergism effect.

We also evaluated the stability of IR700DX-mbc94 and IR700DX-6T in biological conditions using high performance liquid chromatography (HPLC). We found that IR700DX (Supplementary Figure 2), IR700DX-mbc94 (Supplementary Figure 3) and IR700DX-6T (Supplementary Figure 4) are all stable in cell medium, even after 24 h incubation (99% IR700DX, 86% IR700DX-mbc94 and 80% IR700DX-6T remained un-decomposed, Supplementary Table 1). In cell medium with MDA-MB-231 cells, 99% IR700DX, 78% IR700DX-mbc94 and 73% IR700DX-6T remained un-decomposed after 2 h incubation.

After 24 h incubation, the amount of IR700DX remained the same, although un-decomposed IR700DX-mbc94 and IR700DX-6T decreased to 63% and 30%, respectively. Overall, IR700DX-mbc94 showed higher stability than IR700DX-6T in the tested biological conditions.

3.2 Combined TSPO-PDT and CB₂R-PDT induces apoptosis and/or necrosis in MDA-MB-231 cells

We evaluated cell death using apoptosis/necrosis cell detection kit. Most of the untreated cells were viable. No significant cell death was found in single PDT (TSPO-PDT or CB₂R-PDT) treated cells under the indicated condition. In contrast, the cells undergoing late apoptosis and/or necrosis were significantly found in combined PDT treatment group compared to the single treatment group (Figure 4). The above data suggested that multilayer PDT induced higher degree of late apoptosis and/or necrosis than single PDT treatment.

3.3 In vivo multilayer PDT inhibits tumor growth in a murine xenograft model

We first studied tumor uptake and biodistribution of IR700DX-mbc94 and IR700DX-6T using *in vivo* and *ex vivo* fluorescence imaging at 4 different time points (2 h, 12 h, 24 h and 48 h post-injection). Both IR700DX-6T (Supplementary Figure 5 and 6) and IR700DX-mbc94 (Supplementary Figure 7 and 8) showed high tumor uptake, especially within 24 hours post-injection. The only other organs with high PS uptake are kidneys and liver, which are the major organs for drug clearance. Both IR700DX-6T and IR700DX-mbc94 cleared slowly in kidneys, but relatively quickly in liver.

In vivo tumor inhibition effect was investigated in subcutaneous MDA-MB-231 xenograft tumor-bearing mice (Figure 5A–B). Animals were randomized into 4 groups with the following treatments: (1) Untreated, (2) TSPO-PDT, (3) CB₂R-PDT, and (4) Combined TSPO-PDT and CB₂R-PDT. After 8 days post-treatment, TSPO-PDT effectively inhibited tumor growth compared with that of the untreated mice (average tumor volume $288.9 \pm 42.3 \text{ mm}^3$ vs $786.9 \pm 27.7 \text{ mm}^3$, $p < 0.001$). Additionally, CB₂R-PDT also significantly reduced tumor growth ($354.0 \pm 61.4 \text{ mm}^3$, $p < 0.001$). Combined TSPO- and CB₂R-PDT treatment showed greater therapeutic effect than TSPO-PDT ($147.4 \pm 27.0 \text{ mm}^3$ vs $288.9 \pm 42.3 \text{ mm}^3$, $p < 0.05$) or CB₂R-PDT ($147.4 \pm 27.0 \text{ mm}^3$ vs $354.0 \pm 61.4 \text{ mm}^3$, $p < 0.01$).

We also compared the long-term survival of the tumor-bearing mice (Figure 5C) among the 4 groups. Untreated mice quickly reached the termination point owing to aggressive tumor growth (median survival time 9 days). Single treatment of CB₂R-PDT did not significantly prolong the survival time (median survival time 10 days). In contrast, single treatment of TSPO-PDT prolonged the survival time almost 2 times compared with the untreated group (median survival time 16 days). Mice received combined treatment of CB₂R-PDT and TSPO-PDT exhibited the longest survival time (median survival time 28 days). These data suggest that multilayer PDT treatment offers the greatest therapeutic benefit.

Discussion

The balance between cell death and cell survival, which is essential to normal homeostasis, is severely disrupted in cancer. Cancer cells often acquire the ability to aberrantly proliferate

by inactivating normal cell death pathways [51]. Such impairment of cell death function also allows cancer cells to escape chemotherapy, immune attack, and radiation. The resulting resistance accounts for many deaths of cancer patients. It is therefore urgent to develop strategies that can address the disrupted cell death function of cancer cells. Despite the stronghold of cancer cells, it is highly unlikely that they are resistant to all death pathways [52]. Recent studies suggest that it is feasible to bypass resistance by simultaneously activating multiple cell death pathways [53]. A promising approach is strategic combination of two or more drugs to promote therapeutic synergy through multi-target mechanisms [29]. Encouraged by these findings, we speculated that multilayer PDT treatment would offer high efficacy by simultaneously triggering different cancer cell-killing mechanisms.

Multilayer PDT provides a new combinatory approach with multiple advantages: (1) Through specifically targeting distinct cellular layers, this new approach offers the unique opportunity to achieve desired synergistic effects. Simply mixing different PDT PSs, even those that target distinct receptors (this may still lead to similar cell-killing pathways), may not achieve ideal therapeutic outcomes. Instead, significantly improved outcomes likely require synergistic therapies that target various cellular pathways, which can be attained by introducing phototoxicity to multiple cellular layers; (2) Due to the multiple targeting effects (localized light irradiation and multiple receptor-targeting effects), multilayer PDT may offer minimal side effects. Off-target binding of receptor-targeted molecular agents is a common issue. Compared to single receptor-targeted PDT, multiple receptor-targeted PDT requires a much lower dose to reach high efficacy; therefore, the amount of PSs non-specifically bound to normal tissue is greatly reduced. Similarly, lower light dose can be used, which will reduce skin damage. In our *in vivo* studies, we did not observe any side effects caused by PDT treatments (Supplementary Figure 9); (3) The intrinsic fluorescence in PSs will allow for optical imaging and surgical guidance. The multiple receptor-targeting effects will greatly enhance imaging accuracy; and (4) Multilayer PDT has the promise of positively and widely impacting cancer treatment. Here CB₂R and TSPO were used as examples. Both CB₂R and TSPO are overexpressed in many cancers, such as breast [41, 54] [55, 56], bladder [57] prostate [43, 58], colorectal [42, 43] cancer. In addition, the higher expression level of CB₂R and TSPO correlate with tumor aggressive and poorer prognosis, and both are considered to be the target of cancer therapy [59–61]. For cancers that do not up-regulate CB₂R and TSPO, we can target other cancer-related receptors, making this concept widely applicable.

We chose triple-negative breast cancer (TNBC) cell, MDA-MB-231, to evaluate multilayer PDT effect *in vitro* and *in vivo* for the following reasons: (1) TNBC is defined by the absence of expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [62, 63]. TNBC accounts for 15% of all breast cancers, which characterized by aggressive biology, poor prognosis and lacking of targeted proteins for chemohormonal treatment (e.g. HER2-targeted Trastuzumab) [64, 65]. Compared with other types of breast cancer, those with triple negative breast cancer (TNBC) have a poorer survival rate. Therefore, treatment of TNBC represents a great clinical challenge; (2) In our recent study, we found that TSPO-targeted PDT was effective in treating MDA-MB-231 cells and tumors in a target-specific manner [32]. In this study, we were interested in investigating whether adding CB₂R-PDT to TSPO-PDT would further

improve the therapeutic effect; (3) Although MDA-MB-231 cells express CB₂R at a relatively low level, the binding affinity of IR700DX-mbc94 is much higher than IR700DX-6T ($K_D = 42$ nM for IR700DX-mbc94 vs 1.9 μ M for IR700DX-6T) [31, 32]. Therefore, we expected that CB₂R-PDT would have significant therapeutic effect on MDA-MB-231 cells. Interestingly, we found that CB₂R-PDT is even more effective than TSPO-PDT in killing MDA-MB-231 cells ($IC_{50} = 1.45$ μ M for CB₂R-PDT vs 2.04 μ M for TSPO-PDT, Figure 2).

Our study suggests that multilayer PDT may address the challenge of TNBC treatment with high therapeutic efficacy and safety. To allow for treatment of tumors that are relatively large in size and deep in tissue, a near infrared (NIR) PS, IR700DX, was incorporated into the targeted PDT agents design. NIR window (650–900 nm) is desired for *in vivo* PDT treatment and fluorescence imaging due to deep tissue penetration. Several NIR photosensitizing nanosystems have recently been reported for *in vivo* PDT treatment [66–68].

The main purpose of this study is to evaluate the synergistic effect caused by multilayer PDT. We didn't conduct comprehensive *in vitro* and *in vivo* experiments to evaluate the targeting specificity of IR700DX-mbc94 and IR700DX-6T, respectively, because such studies have been reported in our recent papers [30–32]. We evaluated synergistic effect of combined IR700DX-mbc94 and IR700DX-6T PDT treatment using three ratios, including IR700DX-6T/IR700DX-mbc94=1:4 (Figure 3), 4:1 (Supplementary Figure 10) and 1:1 (Supplementary Figure 11). Although synergistic effect was observed in all conditions, multilayer PDT treatment using IR700DX-6T/IR700DX-mbc94=1:4 produced the most consistent synergy at all concentrations. It is possible that the crosstalk between necrosis and apoptosis plays an important role in the synergy [69]; however, the exact mechanism may be rather complicated [27] and investigation of such mechanism is beyond the scope of this study. We therefore selected IR700DX-6T/IR700DX-mbc94=1:4 for *in vivo* studies. All therapeutic results were compared with those from single dose PDT and untreated group. Our results showed that the combination of TSPO-PDT with CB₂R-PDT led to significant synergistic inhibition of MDA-MB-231 cell and tumor growth, particularly at lower concentrations. At higher concentrations, PDT with single dose caused dramatic cell death; therefore, less significant synergy was observed.

To explore the mechanism of synergistic effects caused by multilayer PDT, we assessed cell death phenotype. Apoptosis/necrosis study showed that the cells underwent late apoptosis or necrosis synergistically increased in the combined treatment group as compared to single PDT treatment groups (Figure 4).

It is interesting to note that the *in vitro* studies showed TSPO-PDT is less potent than CB₂R-PDT. However, *in vivo* results revealed a better tumor inhibition effect in TSPO-PDT than that in CB₂R-PDT. Despite the consistent molar ratio of IR700DX-6T and IR700DX-mbc94 (1:4) for both *in vitro* and *in vivo* studies, the final quantity of the corresponding PDT agent in animal tumor and subcellular locations might be different. This information is inaccessible as the actual *in vivo* biodistribution of each agent in the combined therapy group cannot be distinguished. More importantly, the cell death mechanism associated with

each PDT agents could influence the *in vivo* tumor inhibition performance. As we previously reported [31], CB₂R-PDT caused significant necrotic cell death, while TSPO-PDT induced apoptotic cell death [32]. The distinct *in vivo* tumor responses to the two types of cell death can lead to differences in post treatment tumor growth pattern. Therefore, TSPO-PDT might lead to a higher extent of tumor inhibition response than CB₂R-PDT in animal studies. It's noteworthy that we did not conduct immunohistochemical analysis on tumor tissues because all currently available CB₂R antibodies have significant non-specific binding issues, leading to unreliable results [70, 71].

Conclusions

In the present study, we investigate the potential of multilayer PDT in cancer treatment. Through PDT targeting TSPO and CB₂R, two tactically critical cellular receptors associated with cancers, remarkable synergistic effects in cancer cell killing and tumor inhibition were observed in both *in vitro* and *in vivo* experiments. The median survival time of tumor-bearing animals was also largely extended. Our results suggest that multilayer PDT has great promise in enhancing cancer therapeutic efficacy and reducing adverse effect by utilizing multiple tumor targets located at distinct cellular layers. Further, this strategy may be widely applied to treat various cancer types by using strategically designed PSs that target corresponding upregulated receptors at tactical subcellular localization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2. Therapeutic effect of TSPO- or CB₂R-PDT on MDA-MB-231 cells
 (A) MDA-MB-231 cells were treated with TSPO-PDT using IR700DX-6T. The IC₅₀ of TSPO-PDT is 2.04 μM. (B) MDA-MB-231 cells were treated with CB₂R-PDT using IR700DX-mbc94. The IC₅₀ of CB₂R-PDT is 1.45 μM.

Figure 3. Synergistic effect of multilayer PDT treatment using IR700DX-6T and IR700DX-mbc94 on MDA-MB-231 cells

(A) The combined therapeutic effect of TSPO-PDT and CB₂R-PDT on MDA-MB-231 cells.

(B) Chou-Talalay plot to determine the synergistic effect of combined TSPO-PDT and CB₂R-PDT.

Figure 4. Combination of TSPO-PDT and CB₂R-PDT induces MDA-MB-231 cells apoptosis
Apoptosis/necrosis staining of MDA-MB-231 cells after TSPO-PDT, CB₂R-PDT, or combined PDT treatment. Annexin V and EthD-III negative cells represented viable cells. Cells treated with combined TSPO-PDT and CB₂R-PDT showed both Annexin V positive and EthD-III positive staining, which indicated late apoptotic and/or necrotic cells. Scale bar: 20 μ m.

Figure 5. Combination of TSPO-PDT and CB₂R-PDT inhibited tumor growth in MDA-MB-231 tumor bearing mice

Tumor growth was compared among the following groups: (1) untreated, (2) TSPO-PDT, (3) CB₂R-PDT, (4) Combined CB₂R-PDT and TSPO-PDT. (A) White light images of representative mice in each group at 8 days post-PDT treatment. (B) Tumor growth was compared among groups at 8 days post-PDT treatment. (C) Survival curves compared among groups.

Table 1

Synergistic effect of combined treatment of TSPO-PDT and CB₂R-PDT on MDA-MB-231 cells.

IR700DX-6T (μM)	IR700DX-mbc94 (μM)	CI
0.125	0.5	0.73
0.1875	0.75	0.80
0.25	1	0.75
0.3125	1.25	0.86
0.375	1.5	0.92