Chemo-Photodynamic Therapy With Light-Triggered Disassembly of Theranostic Nanoplatform In Combination With Checkpoint Blockade For Immunotherapy of Hepatocellular Carcinoma

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Research Article

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Abstract

**Background:** Hepatocellular carcinoma (HCC) is a common malignant tumor with high rate of metastasis and recurrence. Although immune checkpoint blockade (ICB) has emerged as a promising type of immunotherapy in advanced HCC, treatment with ICB alone achieves an objective remission rate less than 20%. Thus, combination therapy strategies is needed to improve the treatment response rate and therapeutic effect.

**Methods:** A light-triggered disassembly of nanoplateform (TB/PTX@RTK) co-loaded an aggregation induced emission (AIE) photosensitizer (TB) and paclitaxel (PTX) was prepared for on-command drug release and synergistic chemo-photodynamic therapy (chemo-PDT). Nano-micells were characterized for drug loading content, hydrodynamic size, absorption and emission spectra, reactive oxygen species production, and PTX release from micells. The targeted fluorescence imaging of TB/PTX@RTK micells and the synergistic antitumor efficacy of TB/PTX@RTK micells-mediated chemo-PDT combined with anti-PD-L1 were assessed both in vitro and in vivo.

**Results:** The TB/PTX@RTK micells could specifically accumulate at the tumor site through cRGD-mediated active target and facilitate image-guided PDT for tumor ablation. Once irradiated by light, the AIEgens photosensitizer of TB could produce ROS for PDT, and the thioketal linker could be cleaved by ROS to precise release of PTX in tumor cells. Chemo-PDT could not only synergistically inhibit tumor growth, but also induce immunogenic cell death and elicit anti-tumor immune response. Meanwhile, chemo-PDT significantly upregulated the expression of PD-L1 on tumor cell surface which could efficiently synergize with anti-PD-L1 monoclonal antibodies to induce an abscopal effect, and establish long-term immunological memory to inhibit tumor relapse and metastasis.

**Conclusion:** Our results suggest that the combination of TB/PTX@RTK micell-mediated chemo-PDT therapy with anti-PD-L1 monoclonal antibodies can synergistically enhance systemic antitumor effects, and provide a novel insight into the development of new nanomedicine with precise controlled release and multimodal therapy to enhance the therapeutic efficacy of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death in the world [1]. Vast majority of patients are diagnosed at advanced stage that lose the chance of radical surgery, and the high probability of recurrence and metastasis leading to more than 90% of cancer-caused deaths is the major challenge in clinical treatment of HCC [2–4]. The current treatment strategies still cannot achieve satisfactory therapeutic effect, especially in unresectable HCC patients. Therefore, how to effectively eradicate the primary tumor and suppress recurrence and metastasis is the key to improve the efficacy of HCC. Recently, immune checkpoint blockade (ICB) therapy has emerged as a promising clinical treatment of advanced and metastatic malignant tumors [5, 6]. ICB therapies, especially programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) monoclonal antibodies (anti-PD-1/PD-L1), could
relieve the immune restriction and restore T lymphocyte function in the tumor microenvironment (TME) [7, 8]. Multiple types of anti-PD-1/PD-L1 monoclonal antibodies were recently approved by the FDA for the treatment of several types of malignancy, including first-line therapy for advanced HCC [9, 10]. However, the objective remission rate of ICB therapy in these patients was less than 20% [7, 11, 12], putatively because of the lack of sufficient release of tumor-associated antigen (TAA) and infiltration of cytotoxic T lymphocytes (CTLs) [13]. In addition, the lack of PD-L1 expression on tumor cells is one of the important reasons for the low response rate to ICB therapy [14, 15]. Hence, more efficient therapy strategy is urgently needed to synergistically improve the efficacy of ICB therapy.

Many other therapeutic methods, such as photodynamic therapy (PDT) and chemotherapy, have been exploited to elicit an antitumor immune response [2, 16–21]. PDT employs a combination of photosensitizer, light and oxygen to produce large amounts of reactive oxygen species (ROS) for inducing the immunogenic cell death (ICD) and releasing TAA [18–29]. However, limited by the tissue penetration and tumor hypoxia microenvironment, PDT only shows marginal to moderate therapeutic performance [2, 13]. Besides, although PDT can kill some tumor cells and generate certain levels of antitumor immune responses, PDT alone is usually insufficient to inhibit the growth of residual tumor cells in the body after PDT [2, 30, 31]. For chemotherapy, increasing evidences reveal that chemotherapy may increase tumor sensitivity toward immunotherapy [16, 22]. Some chemotherapeutic drugs (e.g., doxorubicin, oxaliplatin, paclitaxel, and so forth) induce ICD through diverse pathways [2, 16, 32, 33], including the concomitant release of neoplastic antigen, the translocation of tumor antigens to the dendritic cell surface, and the secretion of damage-associated molecular patterns (DAMPs) [34–37]. However, poor tumor targeting, multidrug resistance and severe side effects of chemotherapeutic agents have hurdled its application in triggering an immunogenic response in vivo [38, 39]. Combinational therapy that integrates different therapeutic modalities provides an opportunity to achieve better therapeutic efficacy and decreased side effects [16, 39–41]. Based on this concept, combination of PDT and chemotherapy may be a rational strategy for a more powerful activation of immune response and enhanced therapeutic effect with ICB therapy. Besides, PDT or chemotherapy related adaptive immune resistance caused by the up-expression of PD-L1 in tumor cells can be abolished by incorporating anti-PD-L1 monoclonal antibodies into the treatment strategy [13, 22, 23, 42, 43]. Notably, photosensitizer and chemotherapy drugs may have very different physicochemical properties and usually act on different targets, which make a great challenging for efficiently loading and delivering them to their specific targeting sites. Therefore, it is necessary to design an ideal drug delivery system that enable stable transportation of anti-tumor drugs in the circulation without leakage before reaching the tumor site, and precise release drugs in subcellular localization.

Self-assembly polymeric nanocarriers-based drug delivery systems (nano-DDSs) have received considerable attention for designing targeted and smart stimulus-responsive anticancer drug treatment strategies [13, 16, 24, 39, 44]. Nano-DDSs with high drug loading content can overcome the disadvantage of high hydrophobic drugs, elongate blood circulation time, and improve the bioavailability of drugs. By surface modification of specific ligands, nanomaterials can achieve specific tumor targeting ability [9, 13, 30, 31]. Moreover, the on-command drug release from stimuli-triggered disassembly of nanocarriers in
designated time and space could be achieved by some external stimulus, such as light [16, 24, 39]. For example, ROS-sensitive nanocarrier loaded with photosensitizers can not only play the role of PDT but also cleave ROS-sensitive groups to release drugs under light irradiation [16, 39]. Despite being promising, the design of versatile nano-DDSs with on-command drug delivery and drug release for highly efficient cancer therapy remains a formidable challenge.

Here, we designed a light-triggered disassembly of nanoplatform (TB/PTX@RTK) co-loaded an aggregation induced emission (AIE) photosensitizer (TB) and paclitaxel (PTX) for on-command drug release and synergistic chemo-PDT [22, 30, 31]. The nanocarrier originates from a ROS-sensitive thioketal (TK) linkage-bridged diblock copolymer of PEG with polylactic acid-glycolic acid (PLGA) (PEG-TK-PLGA). The cRGD peptide is coupled with NHS-PEG-TK-PLGA through an amidation reaction, and then through the self-assembly of cRGD-PEG-TK-PLGA and PEG-TK-PLGA, excellent tumor targeting can be obtained on the prepared micells of cRGD-modified PEG-TK-PLGA (RTK). As a photosensitizer, TB has stronger photosensitive properties and overcomes the aggregation-caused quenching effect [45, 46]. Under light irradiation, TB/PTX@RTK micells have a more powerful tumor killing and anti-tumor immunity activation effects. Meanwhile, chemo-PDT could significantly upregulate the expression of PD-L1 on tumor cells which could efficiently synergize with anti-PD-L1 antibody to induce an abscopal effect, and establish long-term immunological memory to inhibit the recurrence and metastasis of tumor (Scheme 1). This study might provide novel insight into the development of new nanomedicine with precise controlled release and multimodal therapy to enhance the therapeutic efficacy of HCC.

**Results And Discussion**

**Synthesis and characterization the ROS-sensitive micells with tumor targeting function**

The AIEgen photosensitizer of TPABDTO (TB) was synthesized referred to the previous report [45, 46]. The cRGD-PEG-TK-PLGA was obtained by amidation reaction between -NHS and -NH$_2$ from NHS-PEG-TK-PLGA and cRGD (RK-5) (Figure S1) [47]. The ROS-sensitive micells with tumor targeting function of RTK was prepared by dialysis method using cRGD-PEG-TK-PLGA and PEG-TK-PLGA. The hydrophobic AIE photosensitizer of TB and the chemotherapeutic drug of PTX loaded micells with targeting function were prepared by the same method, the PEG-TK-PLGA and cRGD-PEG-TK-PLGA were used as carriers material, that is TB@RTK, PTX@RTK and TB/PTX@RTK. The hydrodynamic sizes of RTK, TB@RTK, PTX@RTK and TB/PTX@RTK were 91.39 ± 0.2 nm (PDI = 0.182 ± 0.017), 100.4 ± 2.0 nm (PDI = 0.213 ± 0.009), 112.9 ± 4.5 nm ((PDI = 0.223 ± 0.019) and 121.2 ± 1.2 nm (PDI = 0.219 ± 0.015), respectively (Table S1 and Fig. 1A). The uniform spherical shape of TB@RTK, PTX@RTK and TB/PTX@RTK micells was observed by transmission electron microscopy (TEM) (Fig. 1B). It may be that the sample is in a dry state when testing the TEM, which causes the shrinkage of PEG, and the corresponding hydrodynamic size are therefore larger than their size of TEM. In addition, the drug loading content (DLC) of TB reached up to 7.43% and 7.83% for TB@RTK and TB/PTX@RTK micells, the DLC of PTX were 6.15% and 5.37% for PTX@RTK and TB/PTX@RTK micells, respectively. As shown in Fig. 1C, TB/PTX@RTK micells showed relatively strong emission peaking at approximately 684 nm in aqueous solution, which could be used for self-tracking.
Due to the structure of PEG-TK-PLGA containing the ROS-triggered TK group, the tumor-targeting micells of TB/PTX@RTK could show the expected ROS cleavage characteristic to realize the controlled release of the drug in tumor cells. In order to verify that the ROS-sensitive of TB/PTX@RTK, its size change in $\text{H}_2\text{O}_2$ (simulated oxidation environment) was determined by dynamic light scattering (DLS). After incubation in $\text{H}_2\text{O}_2$ for different time, the size of TB/PTX@RTK micells was changed from single peak to multiple peak for 2 h, and the PDI of TB/PTX@RTK micells was changed from 0.219 to 0.489 (Fig. 1D). Then, the ROS generation ability of AIE photosensitizers of TB loaded micells of TB@RTK and TB/PTX@RTK under light irradiation was evaluated using ABDA as indicator. As shown in Fig. 1E and 1F, the absorbance intensity of TB@RTK and TB/PTX@RTK micells was rapidly decreased during irradiation. These data clearly showed that the self-assembled amphiphilic micells could efficiently generate ROS under light irradiation for AIE photosensitizers loaded micells. Furthermore, the hydrodynamic size distribution of TB/PTX@RTK micells is more chaotic by DLS, which may be due to the stimulation of the TK structure of the carrier by ROS generated by light irradiation (Fig. 1G). This meant that the light administered during PDT treatment not only excited the AIE photosensitizer of TB to produce ROS, but also activates the release of PTX spatially. More importantly, drug controlled release experiments also demonstrated that the TB/PTX@RTK micells released the loaded PTX in a ROS-dependent manner. PTX released from TB/PTX@RTK micells was accelerated at the condition of $\text{H}_2\text{O}_2$ (10 mM), and about 75% of PTX released from TB/PTX@RTK micells for 48 h (Fig. 1H). As expected, it was found that light-triggered PTX release occurred after light irradiation (white light, 100 mW/cm$^2$, 10 min), and about 64% of PTX was released from TB/PTX@RTK micells under light irradiation for 48 h. In contrast, TB/PTX@RTK micells exhibited relatively slow PTX release under the condition without any treatment. These results indicated that the development of ROS-sensitive TB/PTX@RTK micells with AIE photosensitizers was an effective promising strategy to promote the release of PTX under light irradiation.

**Targeted imaging and subcellular localization of tumor cells by TB/PTX@RTK micells in vitro**

Our previous research has confirmed that integrin $\alpha_v\beta_3$, as cRGD receptor, was highly expressed in HCC cells [30]. To demonstrate the active targeted ability of cRGD modified micells to HCC cells, Hep 1–6 and Hep G2 cells were first treated with TB/PTX@RTK micells. Normal tissue cell lines L-O2 and HK-2 with low integrin $\alpha_v\beta_3$ expression were used as controls. After being co-incubated with the micells (5 µg/mL) for 4 h, cells were imaged using a confocal laser scanning microscopy (CLSM). As shown in Fig. 2A, B, the fluorescence intensity emitted by the AIE photosensitizer of TB in Hep G2 and Hep 1–6 cells was stronger than that in L-O2 and HK-2 cells, indicating that tumor cells exhibited more uptake of TB/PTX@RTK micells. Besides, when compared with TB/PTX@TK micells that without cRGD modification, Hep G2 cells exhibited more uptake of TB/PTX@RTK (Fig. 2C, D). To further evaluate the specificity of TB/PTX@RTK micells towards HCC cells, competitive binding test was performed. As anticipated, Hep G2 cells pretreated with cilengitide, an integrin $\alpha_v\beta_3$ inhibitor, prior to micells incubation exhibited a dramatically reduced uptake of TB/PTX@RTK micells (Fig. 2C, D). In addition, flow cytometry analysis was used to assess the specificity of TB/PTX@RTK micells targeting HCC cells, and the results were in accordance with the relative semi-quantitative fluorescence analysis based on CLSM imaging (Figure S2). To explore
the distribution of TB/PTX@RTK in tumor cells, LysoTracker Green was used to label the lysosomes. As shown in Fig. 2E, F, the subcellular localization of TB/PTX@RTK micells almost coincided with that of lysosomes, which suggested that micells mainly entered cells through endocytosis. These above results indicated that TB/PTX@RTK micells had the ability to target HCC cells, and mainly through ligand-receptor mediated active endocytosis.

**Fluorescence imaging, biodistribution analysis and pharmacokinetics of TB/PTX@RTK micells in vivo**

Prior to applications *in vivo*, hemolysis test *in vitro* was performed to assess the biocompatibility of TB/PTX@RTK micells. As shown in Figure S3A, no detectable hemolysis occurred in TB/PTX@RTK micells solutions under the studied time and concentration range. Furthermore, no obvious morphological change of RBCs was observed in TB/PTX@RTK micells as shown in Figure S3B. These results suggested that TB/PTX@RTK micells were hemocompatible and could be safely administered intravenously.

In order to demonstrate that TB/PTX@RTK micells could achieve targeted tumor accumulation *in vivo*, Hep 1–6 tumor-bearing Balb/c nude mice models were established to examine tumor accumulation and biodistribution of TB/PTX@RTK micells. Immediately following systemic administration, TB/PTX@RTK micells were distributed widely throughout the body and then accumulated in the major organs and tumor tissue (Fig. 3A, B). Selective high-level accumulation of TB/PTX@RTK micells in the tumor tissue at 12 h was confirmed by *ex vivo* fluorescence measurements (Fig. 3A, B). Next, in order to further evaluate the specificity of TB/PTX@RTK micells towards HCC cells, competitive binding test was performed *in vivo*. As anticipated, Hep 1–6 tumor-bearing Balb/c nude mice pretreated with cilengitide prior to micells injection exhibited a dramatically reduced uptake of TB/PTX@RTK micells (Fig. 3C). Meanwhile, to evaluate the biodistribution of TB/PTX@RTK micells *in vivo*, the mice were sacrificed 12 h after being injected with the micells and tumor tissues and major organs were harvested. The fluorescence intensity of the major organs and tumor tissues was measured by an In-Vivo FX PRO for semi-quantitative biodistribution analysis. As shown in Fig. 3D and Figure S4, the administration of cilengitide did not significantly influence the biodistribution of TB/PTX@RTK micells in the major organs. However, the fluorescence intensity of the tumor tissues in the non-blocking group was obviously higher than that in the blocking group. Together, these results demonstrated that cRGD modification significantly enhanced the targeting ability of TB/PTX@RTK micells *in vivo*.

It has been showed that the longer the nanomedicines circulate in the blood, the more likely they are to accumulate at the tumor site [48]. To verify the prolonged retention of TB/PTX@RTK micells in bloodstream, pharmacokinetic studies were performed. As shown in Fig. 3E, the clearance half-life ($t_{1/2}$) of TB/PTX@RTK micells (~ 12.5 h) in bloodstream was significantly longer than that of free TB (~ 4.0 h), possibly due to the reduced uptake of PEG-modified micells by the reticuloendothelial system, which provided sufficient time for TB/PTX@RTK micells to accumulate at the tumor site [30, 47].

**ROS generation, light-triggered PTX release and synergistic chemo-PDT in vitro**
According to our design, light triggered TB/PTX@RTK could produce high concentration of ROS in localized area, that on hand for PDT and the other hand for inducing the disassembly of micells and drug release. Based on this assumption, we thus firstly detected the ROS production of TB/PTX@RTK in HCC cells under light irradiation. We found that large amounts of ROS were generated in HCC cells treated with TB@RTK and TB/PTX@RTK micells under light irradiation, indicating that light could trigger efficient ROS generation by TB micells (Fig. 4A and S5). Release of PTX is the premise of TB/PTX@RTK micells to act chemotherapeutic effect. We thus investigated the PTX release from TB/PTX@RTK micells in Hep G2 cells under light irradiation. Previous studies have confirmed that PTX stabilized microtubules in their polymerized form leading to malignant tumor cell death [49]. Then, we assessed two markers of microtubule stability, acetylated α-tubulin and detyrosinated (Glu) α-tubulin to determine whether generating ROS would promote PTX release from micells [49]. Interestingly, in our research, the levels of acetylated α-tubulin and detyrosinated (Glu) α-tubulin dramatically increased in TB/PTX@RTK with light group, indicating that PTX was successfully released from the micells (Fig. 4B, S6-8). These results suggested that large amount of ROS produced by PDT was conducive to the release of PTX from TB/PTX@RTK micells.

We further explored the synergistic therapeutic effect of PTX chemotherapy and photosensitizer TB-mediated PDT. As shown in Fig. 4C, D and Figure S9, neither TB@RTK nor TB/PTX@RTK micells alone displayed obvious cytotoxic effects. However, in combination with light irradiation, TB/PTX@RTK micells displayed significantly stronger cell killing effect than TB@RTK micells. The difference suggested that the ROS generated by TB/PTX@RTK micells under light was sufficient to trigger rapid release of chemotherapeutics PTX, which effectively synergized with TB-mediated PDT to kill HCC cells (Fig. 4C, D). Viable and dead cells fluorescent staining using Calcein-AM and PI were performed to assess chemo-PDT-induced cell death. Cell death occurred only in areas where both TB/PTX@RTK micells and laser irradiation were present, and few cell death was detected in non-irradiated areas (Figure S10). These results further indicated that the cytotoxicity of TB/PTX@RTK micells was mainly controlled by light irradiation. As shown in Fig. 4E, with increasing concentration of TB/PTX@RTK micells or time of light irradiation, cell viability declined more rapidly, indicating that the therapeutic efficiency of TB/PTX@RTK-mediated chemo-PDT was concentration- and light irradiation time-dependent. In addition, flow cytometry analysis using the Annexin V-FITC/PI Apoptosis Assay Kit showed that apoptotic cells increased rapidly with increasing concentration of TB/PTX@RTK micells or time of light irradiation (Figure S11). These results indicated that the therapeutic efficiency of TB/PTX@RTK-mediated chemo-PDT changed with variations in laser irradiation exposure time and micell concentration.

**Effects of TB/PTX@RTK-mediated chemo-PDT in vivo**

C57BL/6 mice with subcutaneous Hep 1–6 tumors were used as the animal model to evaluate the therapeutic effect of TB/PTX@RTK-mediated chemo-PDT. Tumor-bearing C57BL/6 mice were divided into five groups (n = 18 per group) and treated with PBS (negative control), TB@RTK, TB@RTK + light, TB/PTX@RTK, or TB/PTX@RTK + light, respectively. The treatment efforts of different treatments were dynamically evaluated by measuring tumor volume and survival. In the PBS, TB@RTK, and TB/PTX@RTK
groups, tumor growth was not obviously inhibited (Fig. 5A, B). As shown in Fig. 5B, tumor growth in the TB@RTK + light group was inhibited in the early stages, but later the tumor appeared to recurrence, manifesting that PDT alone was not enough to completely inhibit tumor progression. Notably, the tumor suppressive effect of TB/PTX@RTK-mediated chemo-PDT was significantly better than that of PDT alone (Fig. 5B). On day 21 after different treatments, tumor tissues were harvested and weighed (n = 5 per group), and the representative tumor-bearing mice and their tumor tissues were photographed. As shown in Fig. 5C, a significant difference in tumor weight was observed between the TB/PTX@RTK + light group and the other four groups. To further assess the therapeutic efficacy of TB/PTX@RTK-mediated chemo-PDT, hematoxylin-eosin (H&E) staining, TUNEL assay and Ki-67 immunohistochemistry (IHC) were performed on tumor tissues at 3 day after different treatments (n = 5 per group). It was found that tumor cells in the PBS (negative control), TB@RTK and TB/PTX@RTK groups were dense (Fig. 5D). In contrast, tumor cells in TB/PTX@RTK + light groups showed the most nuclei absence, manifesting that a great deal of tumor cell was destroyed during the TB/PTX@RTK-mediated chemo-PDT process. In addition, the survival time of the tumor-bearing mice in the chemo-PDT group (n = 8 per group) was significantly prolonged (Fig. 5E). Together, these results suggested that TB/PTX@RTK-mediated chemo-PDT might serve as an effective strategy for anti-tumor therapy.

ICD induction, immune activation and adaptive immune resistance of TB/PTX@RTK-mediated chemo-PDT

Encouraged by the promising results of antitumor efficacy, we next explored the effect of TB/PTX@RTK mediated chemo-PDT on anti-tumor immunity. It was clarified that the immune profile of ICD pathways was defined by a cluster of molecules called DAMPs, such as calreticulin (CRT), heat shock protein 70 (HSP70), adenosine triphosphate (ATP), and high mobility group box 1 (HMGB1), and so on [20, 34, 35, 37]. Chemo-PDT stimulated the constellation of alterations in the dying tumor cells, such as HMGB1 efflux, HSP70 exposure and ATP secretion as “find me” signal, CRT exposure as an “eat me” signal to attract and activate antigen-presenting cells, leading to activation of adaptive anti-tumor immunity. Hence, we evaluated the ability of TB/PTX@RTK micells to efficiently induce two ICD-inducing modalities after light irradiation in vitro. We found that TB/PTX@RTK-mediated chemo-PDT did not change the total CRT content of HCC cells, but significantly increased the expression of CRT on the cell membrane surface more than PDT alone (Fig. 6A, S12). The reasonable explanation might be that ICD promoted the migration of CRT from the endoplasmic reticulum (ER) to the surface of the cell membrane [20]. As a “find me” signal, the extracellular secretion of HMGB and ATP, and the expression of HSP70 on the cell membrane were more efficiently induced by TB/PTX@RTK-mediated chemo-PDT to attract antigen-presenting cells into the tumor (Fig. 6B, C, S13-15). These results substantiated that TB/PTX@RTK-mediated chemo-PDT with two ICD-inducing modalities was more powerful to induce ICD than single therapy model.

DCs are the main antigen-presenting cells that present antigens to CD8+ CTLs and further activate CTLs [9, 19]. We then examined the effects of chemo-PDT-induced ICD on the maturation of bone marrow-derived DCs (BMDCs). Interestingly, we observed the significant maturation of BMDCs, as indicated by
increased CD11c+CD80+CD86+ cells in TB/PTX@RTK + light group (Fig. 6D, E). To further assess the maturation of DCs after chemo-PDT in vivo, the mice were euthanized 3 day after different treatments and ELISA was used to detect the serum IL-12 secreted by mature DCs upon TAA stimulation [19]. There was a significant upregulation of IL-12 levels in TB/PTX@RTK + light group compared with other groups (Figure S16), confirming that DC maturation was significantly induced by TB/PTX@RTK-mediated ICD.

Next, we explored whether TB/PTX@RTK-mediated chemo-PDT could effectively remodel the TME. Therefore, we further studied the antitumor CTL (CD45+CD3+CD8+) and immunosuppressive Treg cell (CD3+CD4+Foxp3+) populations of tumor after different treatments. Compared with PDT alone, TB/PTX@RTK-mediated chemo-PDT significantly increased the infiltration of CTLs and decreased the infiltration of Treg cells in the tumor (Fig. 8F-I). In addition, we studied CTLs activation by measuring the levels of IFN-γ produced by activated CTLs in tumor tissues [16, 23]. There was a significant upregulation of IFN-γ levels in TB/PTX@RTK + light group compared with the other four groups (Figure S17), confirming that CTLs were effectively activated by TB/PTX@RTK-mediated chemo-PDT. These results suggested that TB/PTX@RTK-mediated chemo-PDT enhanced anti-tumor immunity.

However, recent studies reported that PDT and multiple of chemotherapeutic agents could elevate the expression of PD-L1 on tumor cells. Membrane PD-L1 is able to bind with PD-1 on the surface of CTLs and inhibit its function. In addition, it was reported that the activated adaptive immune resistance in response to therapy was positively correlated with tumor recurrence and metastasis [13, 23, 50]. In this study, we found that the expression of PD-L1 on the cell membrane in TB/PTX@RTK + light group was increased compared with other groups (Fig. 6J, S18, S19). These results suggested that TB/PTX@RTK-mediated chemo-PDT could activate adaptive immune resistance. The increased expression of PD-L1 on tumor cells like a "double-edged sword". Although it inhibits the function of CTLs, the current researches show that the high expression of PD-L1 is positively correlated with tumor response to ICB therapy. Therefore, we assumed that the combination therapy with anti-PD-1/PD-L1 monoclonal antibody could effectively reverse adaptive immune tolerance and potentiate the antitumor efficacy of ICB.

The potential mechanism of ICD induced by TB@RTK micell-mediated PDT

As TB@RTK micell-mediated PDT has been proved as an effective treatment to incite tumor cell death and elicit the immunogenicity of dying tumor cells, there is growing interest in exploring the potential mechanisms of TB@RTK micells mediated ICD of tumor cells. Previous studies have reported that high production of ROS produced by photosensitizers located in lysosomes could damage lysosomes and further induce apoptosis through the mitochondria-mediated pathway [51]. Considering that the subcellular localization of TB@RTK micells almost coincided with that of lysosomes, we thus evaluated whether mitochondrial mediated apoptosis pathways were involved in the ICD induced by TB@RTK micell-mediated PDT.

ROS generation and lysosome disruption, leading to a release of large amounts of cathepsin from the lysosomes, serve as the primary event that induces mitochondria mediated apoptosis [51]. As shown in
Fig. 7A, micell-mediated PDT could disrupt the integrity of Hep 1–6 cell lysosomes, as observed from an increased lysosomal membrane permeability (Figure S20), and lysosomal deacidification (Fig. 7A, S21). These data suggested that TB@RTK micell-mediated PDT could trigger a rapid lysosome disruption. Previous studies elucidated that the release of cytochrome-c from mitochondria due to the disruption of membrane integrity induced by cathepsin is the second event characterizing the mitochondria-mediated apoptotic pathway [51]. We thus detected the mitochondrial membrane potential of Hep 1–6 cells after different treatments by a JC-1 fluorescent probe. As shown in Fig. 7B, the mitochondrial membrane potential was significantly damaged in the PDT group. In addition, the release of cytochrome-c from the mitochondria to cytosol was observed after the PDT process, which further confirmed the loss of integrity of mitochondrial (Fig. 7C, S22). Once the integrity of the mitochondria was lost, the ATP in the mitochondria was released and secreted outside the tumor cell (Fig. 6B). It has been elucidated that the activation of caspase-3, induced by cytochrome c released by mitochondria, is the third event resulting in the mitochondria mediated apoptosis [51]. GreenNuc™ Caspase-3 Assay Kit was performed to monitor the activation of caspase-3 in response to TB@RTK micell-mediated PDT using flow cytometry analysis. It was found that the caspase-3 activity in Hep 1–6 cells treated with TB@RTK micells plus light was elevated most significantly compared to the other groups (Fig. 7D). Taken together, these data verified our hypothesis that mitochondrial mediated apoptosis pathways were involved in the ICD induced by TB@RTK micell-mediated PDT in Hep 1–6 cells. Moreover, this finding was also confirmed in the Hep G2 cell (Figure S23). Based on these above studies, we speculated that TB@RTK mediated PDT produced ROS to disrupt lysosomes and release cathepsin, thus leading to mitochondrial damage. Injured mitochondria activate caspase 3 to induce apoptosis. In addition, it has been reported that PDT could cause ER stress to induce membrane translocation of CRT [20]. The translocation CRT induced by ER stress and the release of ATP induced by mitochondrial destruction may be the important causes of ICD induced by TB@RTK-mediated PDT.

**Effects of Chemo-PDT combined with anti-PD-L1 on tumor recurrence and metastasis and the immunological response**

Relapse and metastasis are the most challenges for HCC management. At present, there is no widely accepted therapeutic strategy to reduce these risks. Previous studies have showed that PTX or PDT therapy combined with ICIs could suppress tumor recurrence and metastasis [22, 23, 52]. Thus, we hope to verify the effect of TB/PTX@RTK mediated chemo-PDT combined with anti-PD-L1 antibody on recurrent and metastic tumors. As shown in Fig. 8A, we established a bilateral subcutaneous Hep 1–6 tumor model to mimic primary and distant tumors, in which light irradiation was given on one side (primary tumor) and no light irradiation was given on the other side (distant tumor). Tumor-bearing mice were divided into four groups (n = 5 per group): (1) PBS; (2) anti-PD-L1; (3) TB/PTX@RTK + light; and (4) TB/PTX@RTK + light + anti-PD-L1. The primary tumors in group 3 and group 4 were irradiated (540 nm, 100 mW/cm², 3 min) 12 h after injection of TB/PTX@RTK micells, and mouse anti-PD-L1 monoclonal antibodies were administered by i.v. injection on 1, 4, and 7 day at a dose of 2.0 mg/kg. The therapeutic effect of each group was dynamically monitored by measuring tumor volume. In addition, the bilateral
tumors were dissected and photographed on day 21 after different treatments. We found that treatment with anti-PD-L1 or TB/PTX@RTK + light showed moderate anticancer efficacy on primary tumors, while the combination of TB/PTX@RTK + light + anti-PD-L1 not only inhibited the growth of the primary tumor with no local recurrence during the observation period, but also significantly inhibited the growth of the distant tumors (Fig. 8B-E), suggesting that the combination of TB/PTX@RTK micell-mediated chemo-PDT therapy with anti-PD-L1 monoclonal antibodies could synergistically enhance systemic antitumor effects.

In order to explore the potential mechanism of the synergistic antitumor effects of TB/PTX@RTK + light and anti-PD-L1 monoclonal antibodies, the mice were sacrificed to harvest distant tumor tissues and the infiltrated immune cells were analyzed by flow cytometry. The percentages of CTLs (CD45+CD3+CD8+), immunosuppressive Treg cells (CD3+CD4+Foxp3+) and myeloid-derived suppressor cells (MDSCs, CD45+CD11b+Gr-1+) were shown in Fig. 8F-I and S24. It was found that the percentage of CTLs increased most significant in the TB/PTX@RTK + light + anti-PD-L1 group, while both Treg cells and MDSCs were significantly decreased when compared to the other groups (Fig. 8F-I and S24). Moreover, the levels of IFN-γ of distant tumor was determined post treatment. Compared with other groups, combination therapy induced the highest levels of IFN-γ in distant tumors (Figure S25), suggesting that combination therapy could relieve immune suppression and enhance the tumor-killing activity of CTLs, which then stimulate anti-tumor immunity and migrated to distant tumors to kill tumor cells.

Immunological memory enables the initiation of a more rapid and effective immune response, and effector memory T-lymphocyte (Tem) plays a significant role in antitumor immune memory[9, 16]. Therefore, we further examined whether the combination therapy strategy could inhibit tumor relapse. For these experiments, primary tumors were inoculated subcutaneously into the left flanks of C57BL/6 mice treated with TB/PTX@RTK + light or TB/PTX@RTK + light + anti-PD-L1. On day 28, 3×10⁶ Hep 1–6 cells were injected subcutaneously into the contralateral flanks of the treated C57BL/6 mice, and tumor volumes was closely monitored for the next 14 day (Figure S26). The secondary tumors in TB/PTX@RTK + light treatment group grew rapidly, while no tumors were detected in the combined treatment group (Figure S26). To examine the mechanism by which immunological memory is induced by the combined treatment, we detected Tem cells in the spleen on day 28 by flow cytometry. We found an obvious increase in Tem cells in mice treated with TB/PTX@RTK + light + anti-PD-L1, compared with TB/PTX@RTK + light (Figure S27). Taken together, these results indicated that TB/PTX@RTK-mediated chemo-PDT could significantly inhibit primary tumor growth and elicit a systemic antitumor immune response. In combination with anti-PD-L1 treatment, the antitumor immune effects of combination therapy were extended to inhibit the growth of metastatic and recurrent tumors.

**Toxicity evaluation of TB/PTX@RTK micells in vivo**

In clinic, the use of PTX has been limited to some extent because of its side effects. To assess the side effects of TB/PTX@RTK micells in mice, we monitored the body weight and multiple organ function of the treated mice 7 days after treatment. Double dose of TB/PTX@RTK micells used for chemo-PDT (10 mg/kg), was intravenously injected into healthy C57BL/6 mice. It was found that there was no significant
weight loss in the TB/PTX@RTK micells group compared with the saline (negative control) group (Fig. 9A). In contrast, intraperitoneal injection of free PTX led to weight loss in the mice. In addition, no significant toxicity or tissue damage of TB/PTX@RTK micells was observed in the major organs by the H&E staining (Fig. 9B). Except for free PTX group (same dose as TB/PTX@RTK group), all parameters of blood routine test of the other groups were not significantly abnormal (Fig. 9C). In contrast, free PTX led to a decline in white blood cells and platelets, indicating that free PTX had an inhibitory effect on the bone marrow (Fig. 9C). Then, the blood biochemistry test including serum creatine kinase-MB (CK-MB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and serum creatinine (sCr) was also performed (Fig. 9D). The blood biochemistry analysis showed that free PTX led to an increase in serum CK-MB, AST and ALT levels, which suggested that TB/PTX@RTK micells could avoid the toxic side effects of free PTX. Briefly, these studies demonstrated that TB/PTX@RTK micells exhibited good biocompatibility and could avoid the toxic side effects of free PTX in vivo.

Conclusion

In conclusion, we have prepared a targeted and ROS-sensitive TB/PTX@RTK micell to induce two ICD-inducing modalities for chemo-PDT. Under light irradiation, photosensitizer TB in micells uses oxygen to generate ROS that not only killed tumor cells during PDT, but also rapidly disintegrated micells to boost intracellular PTX release in tumor cells, conducing to effective tumor growth inhibition. Furthermore, TB/PTX@RTK micell-mediated chemo-PDT elicited an anti-tumor immune response and upregulated the expression of PD-L1 on tumor cell surface, which could effectively synergize with anti-PD-L1 monoclonal antibody to induce an abscopal effect, and establish long-term immunological memory to inhibit tumor relapse and metastasis. Taken together, the combination of TB/PTX@RTK micell-mediated chemo-PDT therapy with anti-PD-L1 monoclonal antibodies could synergistically enhance systemic antitumor effects.

Materials And Methods

PTX, PEG (5k)-TK-PLGA (2k), and NHS-PEG-TK-PLGA used in this study were synthesized by and purchased from Xi’an Ruixi Biological Technology Ltd. (Xi’an, China). Aminated cRGD peptide (RK-5, 187531) used in this study was synthesized by and bought from GL Biochem Ltd. (Shanghai, China). 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA) used in this study was bought from Sigma-Aldrich. The AIEgen photosensitizer of TB was synthesized as previously described [1-2]. 1H nuclear magnetic resonance (1H NMR) spectrum was measured by Bruker AM 400 apparatus. DLS (Nano-ZS ZEN3690 Malvern Instruments) was used to measure the average size of micells. TEM (JEM-2100 microscope) was used to observe the morphology of micells. The DLC of the hydrophobic AIE photosensitizer of TB and the chemotherapeutic drug of PTX was measured by high performance liquid chromatography (HPLC, waters 2695) and UV-vis spectrophotometer (UV-2600, SHIMADZU), respectively.

Synthesis of cRGD-PEG-TK-PLGA Block Copolymer
The cRGD peptide was coupled with NHS-PEG-TK-PLGA through an amidation reaction, and then through the self-assembly of cRGD-PEG-TK-PLGA and PEG-TK-PLGA, excellent tumor targeting could be obtained on the prepared micells of cRGD-modified PEG-TK-PLGA (RTK). In a nutshell, NHS-PEG-TK-PLGA in 5 mL of dimethyl formamide (DMF) was mixed into a solution of cRGD peptide (20 mg, 0.033 mmol) in DMF. After 24 h reaction at room temperature, the product of cRGD-PEG-TK-PLGA was purified by dialysis (MWCO 3500) in ultrapure water for 48 h and then lyophilized. Yield: 74.6%.

**Preparation of the Micells of RTK, TB@RTK, PTX@RTK, TB/PTX@TK and TB/PTX@RTK**

PEG-TK-PLGA (9 mg), RTK (1 mg), TB (1 mg) and PTX (1 mg) were dissolved in solvent of THF/CHCl$_3$ (v/v = 1:1, 1.5 mL) in the dark, ultrapure water (8.5 mL) was then slowly added to that solution. Afterwards, the solution was dialyzed against ultrapure water for 1 d to prepare TB/PTX@RTK micells with active tumor targeting. In the same way, PEG-TK-PLGA (10 mg), TB (1 mg) and PTX (1 mg) were mixed together to prepare TB/PTX@TK micells without active tumor targeting. The preparation methods of RTK, TB@RTK and PTX@RTK micells were similar to that of TB/PTX@RTK micells.

**In Vitro Drug Release**

The dialysis method was used to measure the release behavior of PTX from TB/PTX@RTK micells under different conditions. In a nutshell, 2 mL fresh prepared TB/PTX@RTK micells solution was transfer into a dialysis bag (MWCO 1000) and then 40 mL of release medium (PBS containing 0.1% Tween 80) was placed into the dialysis bag at 37 °C, accompanied by a steady gentle shake at 100 rpm. At a pre-set time, 2 mL of the release solution was taken out and an equal amount of fresh release medium was added at the same time. PTX concentrations were determined by HPLC. For investigating PTX release behavior, the release medium was contented 10 mM H$_2$O$_2$ or the micells were irradiated by light irradiation for 10 min at 100 mW/cm$^2$, respectively.

**Cell Lines**

The human HCC cell lines (Hep G2) and mouse HCC cell lines (Hep 1-6) used for testing the imaging and therapeutic efficacy of micells *in vitro* and *in vivo*, and human normal liver cell lines (L-O2) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human kidney proximal tubular cell lines (HK-2) were obtained from American Type Culture Collection. The cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin–streptomycin (Boster Biological Technology Co. Ltd., CA, USA) at 37 °C in a humidified atmosphere containing 5% CO$_2$ [3, 4].

**Experimental Animals**

Male 4~6-week-old Balb/c nude mice and C57BL/6 mice used in this study were provided by Beijing HFK Bioscience Co. Ltd. (Beijing, China) and the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All of the animal experiments and animal feeding were performed in accordance with the
guidelines of the Animal Care Committee at Tongji Medical College, HUST, Wuhan, China. This study was performed in compliance with the declaration of Helsinki [3].

Confocal Imaging

After being co-incubated with the micells (5 μg/mL) for 4 h, cells with or without receptor blocking (Cilengitide, 100 nM) were washed twice with PBS for 3 min and then fixed with 4% paraformaldehyde for 35 min. Commercial probe Actin-Tracker Green (Beyotime, Shanghai, China) was used to stain microfilament to show cell contours. Hoechst 33258 (Servicebio, Wuhan, China) was used to localize nuclei. After washing three times with PBS for 5 min, the cell culture slides were transferred to separate object slides and were subjected to confocal imaging analysis directly using the CLSM and relative semi-quantitative fluorescence analysis was performed by using the ImageJ software to assess cellular uptake of micells.

Quantitative Analysis of Micells Uptake by Flow Cytometry

After being co-incubated with the micells (5 μg/mL) for 4 h, the cells were washed twice with PBS for 3 min and harvested after digestion with trypsin into flow tubes in the dark. The flow cytometry analysis was performed for relative quantitative analysis of cellular uptake of micells and cells not co-incubated with micells were used as the blank control.

Co-localization between Micells and Lysosomes

After being co-incubated with the micells for 4 h, the cells were washed twice with PBS for 3 min and subsequently incubated with medium supplemented with 75 nM LysoTracker Green (Yeasen Biotech, Shanghai, China) for 2 h at 37 °C. Commercial probe LysoTracker Green was used to localize lysosome. Hoechst 33258 was used to localize nuclei. After washing three times with PBS for 5 min, the cell culture slides were transferred to separate object slides and were subjected to confocal imaging analysis directly using the CLSM and fluorescence co-localization analysis was performed by using the ImageJ software.

Hemolysis Tests

In order to perform *in vitro* hemolysis tests, fresh blood (0.5 mL) from orbital venous plexus of C57BL/6 mice was taken into anticoagulant tubes and then diluted with 2 mL PBS. The cell suspension was centrifuged (5000 rpm, 15 min) three times to separate the RBCs from the plasma and the RBCs were then diluted with 5 mL of PBS. 30 μL of the suspension of RBCs was mixed with 170 μL of distilled water (positive control), PBS (negative control), or a solution containing micells in different concentrations (5~80 μg/mL) into eppendorf tubes. After incubating at room temperature for different pre-set times, the suspension of each tube was centrifuged at 3000 rpm for 15 min. 100 μL of the supernatants of each eppendorf tube was added to a 96-well plate and the Multiskan GO microplate spectrophotometere was used to measure the OD values of supernatants at 570 nm. In addition, the precipitation of each eppendorf tube was used to make the cell smear to observe the morphological changes of erythrocyte [3].
**In Vivo Blood Safety Studies**

In order to perform *in vivo* blood safety studies of the micells, 4~6-week-old C57BL/6 mice were divided into six groups (n = 3 per group), and treated with (1) saline (negative control), (2) RTK, (3) TB@RTK, (4) free PTX, (5) PTX@RTK, (6) TB/PTX@RTK at an equal dose of 10 mg/kg via tail vein injection, respectively. We monitored the body weight and multiple organ function of the treated C57BL/6 mice 7 days after different treatment. On the 7th day, the whole blood of mice was collected for hematology analysis immediately [5]. In addition, fresh blood without heparinization from orbital venous plexus of C57BL/6 mice was taken into eppendorf tubes and then kept at room temperature for 30 min to clot and then centrifuged at 5000 rpm for 15 min to separate serum. The serum levels of biochemical markers of major organ function, including CK-MB, ALT, AST, BUN, and sCr, were measured to evaluate the function of major organs, moreover.

**In Vivo Fluorescence Imaging and Biodistribution Analysis**

HCC tumor models were established to evaluate active tumor targeting of micells and biodistribution analysis *in vivo*. In a nutshell, 5 × 10^6 Hep 1-6 cells were inoculated subcutaneously into the Balb/c nude mice to establish HCC tumor models. When the HCC tumor volume reached a mean size of approximately 300 mm^3, the Balb/c nude mice were intravenously injected with micells at a concentration of 5 mg/kg based on TB via the tail vein. For the receptor blocking test, mice were intra-peritonelly injected with cilengitide (250 μg) 2 h before injection of the TB/PTX@RTK micells. After the anaesthesia, the fluorescence imaging of each mouse at 620~720 nm was performed by using an In-Vivo FX PRO at different pre-set times. Subsequently, the Balb/c nude mice were sacrificed to harvest tumors and major organs in the dark, and the fluorescence intensity of tumors and major organs at 620~720 nm was measured by using the same equipment as above.

**Pharmacokinetics Studies of Micells**

For pharmacokinetics studies, the TB/PTX@RTK micells or free TB were intravenous injected with C57BL/6 mice via the tail vein. Fresh blood (100 μL) from orbital venous plexus of C57BL/6 mice was taken into anticoagulant tubes at different pre-set times and solubilized with lysis buffer (RIPA) for 30 min, and then subjected to ultrasound for 1 min to fully lyse the blood cells. The fluorescence intensity of the solution at 620~720 nm was measured by using a microplate reader and the exact fluorescence intensity of TB was calculated by deducting that of the blank control. Thereafter, the non-compartment elimination model was used to analyze the data of the relevant pharmacokinetic parameters [6]. The elimination half-life (t1/2) of the TB/PTX@RTK micells or free TB was calculated by using the WinNonlin v.5.1 software [3].

**Intracellular ROS Detection**

Commercial probe DCFH-DA (Beyotime, Shanghai, China) was used to evaluate intracellular ROS generation by TB-mediated PDT as previously described [3]. After being co-incubated with the micells for
4 h, the cells were washed three times with PBS for 3 min and then irradiated with a laser irradiation (540 nm, 100 mW/cm², 3 min). After washing twice with PBS for 3 min, Hep G2 and Hep 1-6 cells were subsequently incubated with 1 μM DCFH-DA at room temperature for 30 min in the dark and then captured by using a fluorescence microscope.

**Cytotoxicity Study**

The CCK-8 assays were used to measure Hep G2 and Hep 1-6 cells viability after different treatments as previously described [3]. Flow cytometry analysis was performed to quantify the proportion of apoptotic Hep G2 and Hep 1-6 cells after different treatments by using the Annexin V-FITC/PI Apoptosis Assay Kit (Boster Biological Technology Co. Ltd., CA, USA). Calcein-AM and PI stain were used to evaluate further TB/PTX@RTK-mediated chemo-PDT effects according to the protocol [4]. After being co-incubated with the micells for 4 h, Hep G2 and Hep 1-6 cells were washed three times with PBS for 3 min and then irradiated with a laser irradiation (540 nm, 100 mW/cm², 3 min) and subsequently incubated Calcein-AM (2 μM) and PI (5 μM) at 37 °C for 20 min, and images were captured with a fluorescence microscope.

**In Vitro Caspase-3 Assay**

Flow cytometry analysis was performed to quantify the caspase-3 activity of Hep G2 and Hep 1-6 cells after different treatments by using the GreenNuc™ Caspase-3 Activity Detection Kit for Live Cells (Beyotime, Shanghai, China) in accordance with the manufacturer’s protocol [5]. In a nutshell, after the indicated treatments, Hep G2 and Hep 1-6 cells were washed three times with PBS for 3 min and then harvested after digestion with trypsin into flow tubes in the dark. Subsequently, 200 μL of the suspension of HCC cells in the flow tube were incubated with 5 μM of GreenNuc™ Caspase-3 Substrate in the dark at room temperature for 30 min, and the cell samples were immediately analyzed by flow cytometry.

**Lysosomal Membrane Stability**

Lysosomal membrane stability was tested by using acridine orange (AO, Sigma-Aldrich) [7]. After being co-incubated with the micells for 4 h, Hep G2 and Hep 1-6 cells were washed three times with PBS for 3 min and then irradiated with a laser irradiation (540 nm, 100 mW/cm², 3 min) and subsequently incubated with complete medium containing AO (5 μg/mL) for 15 min at 37 °C, and images were captured with a fluorescence microscope.

**LysoTracker Green Staining**

After being co-incubated with the micells for 4 h, Hep G2 and Hep 1-6 cells were washed three times with PBS for 3 min and then irradiated with a laser irradiation (540 nm, 100 mW/cm², 3 min) and subsequently incubated with 75 nM LysoTracker Green (Yeasen Biotech, Shanghai, China) for 2 h at 37 °C. LysoTracker Green was used to localize lysosome and Hoechst 33258 was used to localize nuclei, and images were captured with a fluorescence microscope.

**Mitochondrial Membrane Potential Analysis**
The mitochondrial membrane potential ($\Delta \Psi_m$) of HCC cells after different treatments was detected by using the JC-1 $\Delta \Psi_m$ Assay Kit as previously described [3, 8]. Briefly, after being co-incubated with the micells for 4 h, Hep G2 and Hep 1-6 cells were washed three times with PBS for 3 min and then irradiated with a laser irradiation (540 nm, 100 mW/cm2, 3 min) and subsequently incubated with 500 μL of complete medium and 500 μL of JC-1 dyeing working fluid for 20 min at 37 °C and images were captured with a fluorescence microscope.

**Immunofluorescence Analysis**

Immunofluorescence analyses were performed as previously described [3]. Images were captured with a fluorescence microscope, and relative semi-quantitative fluorescence analysis and fluorescence co-localization analysis were performed using ImageJ software. To analyze the expression level of CRT, PD-L1, and HSP70 on the surface of cell, cells after different treatment were fixed with 4% paraformaldehyde. After washing three times with PBS, the cell samples were sealed with goat serum at room temperature for 30 min. After washing twice with PBS, cells were then incubated with the primary antibodies and FITC-conjugated secondary antibody successively in the dark [4]. Red fluorescent probe Dil (Beyotime, Shanghai, China) and Hoechst 33258 were used to label cell membranes and nuclei, respectively [8].

**Immunoblot Analysis**

Immunoblot analyses were performed as previously described to analyze the expression level of acetylated α-tubulin, Glu α-tubulin, cytochrome c, Bcl-2, and CRT in HCC cells after different treatment [3].

**ELISA Analysis**

The respective supernatant of cells, the cell extract, tumor tissues, and serum after different treatments were collected for measuring the levels of IFN-γ, IL-12, HMGB1, and APT by using the appropriate ELISA Assay Kits and following the manufactures’ instructions [4, 9]. ATP Assay Kit and Mouse HMGB1 Assay Kits were used to detect the extracellular release of ATP and HMGB1, respectively. IL-12 Assay Kits were used to test the serum levels of IL-12 of tumor-bearing C57BL/6 mice after different treatments and to indirectly evaluate the maturation of DCs in vivo [9]. IFN-γ Assay Kits were used to test the IFN-γ levels in tumor tissue lysates of tumor-bearing C57BL/6 mice after different treatments and to evaluate the activation of tumor-infiltrating CTLs in vivo.

**BMDC Isolation and BMDC Maturation Assays In Vitro**

To verify the effects of TB/PTX@RTK-mediated chemo-PDT on the maturation of DCs, we extracted bone marrow cells from the femoral bone marrow of C57BL/6 mice according to previous method and used GM-CSF and IL-4 to differentiate and cultured them into immature bone marrow DCs (BMDCs) [4, 10]. After incubation with the micells for 4 h, the Hep 1-6 cells were washed three times with PBS for 3 min and then laser irradiated with a laser irradiation (540 nm, 100 mW/cm2, 3 min). After incubation for a further 24 h, the medium supernatant of the tumor cells was collected and co-incubated with DCs for 24
h. Ater incubation with anti-CD11c-FITC, anti-CD86-APC, anti-CD80-PE fluorescent antibodies, the BMDCs were detected with a flow cytometer to evaluate the mature of BMDCs. Mature BMDCs were defined as CD11c⁺CD80⁺CD86⁺.

**Antitumor Efficacy Evaluation in the Hep 1-6 Tumor Models**

To evaluate the antitumor effects of TB/PTX@RTK-mediated chemo-PDT in the Hep 1-6 tumor models, Hep 1-6 tumor-bearing C57BL/6 mice were used. When the HCC tumor volume reached a mean size of approximately 200 mm³, tumor-bearing C57BL/6 mice were randomly divided into five groups (n = 18 per group): PBS, TB@RTK, TB@RTK + light, TB/PTX@RTK, and TB/PTX@RTK + light, respectively. Micells (5 mg/kg) were injected through the tail vein. The tumor sites of tumor-bearing C57BL/6 mice were laser irradiated with a laser irradiation (540 nm, 100 mW/cm², 3 min) 12 h after the injection of the micells. After different treatments, tumor volumes and weights were recorded everyday. The formula (tumor volume = tumor length × tumor width × tumor width × 0.5) was used to estimate tumor volume as previously described [4]. After 21 days of treatment, tumor-bearing C57BL/6 mice were photographed. To verify the therapeutic efficacy of the TB/PTX@RTK-mediated chemo-PDT, histological changes, proliferation and apoptosis levels of tumors were analyzed by using H&E staining, immunohistochemical analysis and TUNEL assay, respectively, on day 3 after different treatments (n = 5 per group). On day 21 after different treatment, tumor tissues were collected and weighed (n = 5 per group), and the representative tumor tissues were imaged. In addition, the survival time of the rest of tumor-bearing C57BL/6 mice (n = 8 per group) were recorded each day until the last mouse died.

To further evaluate the effects of the combined treatment on primary and metastatic tumors, the bilateral subcutaneous Hep 1-6 tumor models were established. When the HCC tumor volume reached a mean size of approximately 200 mm³, tumor-bearing C57BL/6 mice were randomly divided into four groups (n = 5 per group): (1) PBS; (2) anti-PD-L1; (3) TB/PTX@RTK + light; and (4) TB/PTX@RTK + light + anti-PD-L1, respectively. Light irradiation (540 nm, 100 mW/cm², 3 min) for group 3 and 4 was only given on one side (primary tumor) and no light irradiation was given on the other side (distant tumor) after 12 h postinjection, and anti-PD-L1 was administered by i.v. injection 1, 4, and 7 day at a dose of 2.0 mg/kg. The volume changes of bilateral tumors were detected, and bilateral tumors were dissected and photographed on day 21 after different treatment. In order to clarify the underlying mechanism of the synergistic antitumor effects of TB/PTX@RTK-mediated chemo-PDT and anti-PD-L1 treatment, tumor-bearing C57BL/6 mice were sacrificed to harvest the tumor tissues and the infiltrative immune cells in the distant tumors were analyzed by flow cytometry. The percentages of tumor-killing CTLs (CD45⁺CD3⁺CD8⁺), immunosuppressive Treg cells (CD3⁺CD4⁺Foxp3⁺) and MDSCs (CD45⁺CD11b⁺Gr-1⁺) were analyzed by flow cytometry.

To evaluate effects of combined treatment on tumor recurrence, we rechallenged mice previously treated with TB/PTX@RTK + light or TB/PTX@RTK + light + anti-PD-L1. The primary tumors was inoculated into the left flanks of the C57BL/6 mice as above. When the HCC tumor volume reached a mean size of approximately 200 mm³, tumor-bearing C57BL/6 mice were divided into two groups (n = 5 per group) and...
treated as above. On the 28th day after different treatments, secondary Hep 1-6 tumors were inoculated by injecting $3 \times 10^6$ cells into the right flanks of the mice. The percentage of second tumor-free C57BL/6 mice of the mice in each group ($n = 5$ per group) were recorded everyday. To investigate the effects of the combined therapy on antitumor immunological memory, splenic effector memory T cells (CD3$^+$CD8$^+$CD44$^+$CD62L$^-$) were analyzed by flow cytometry 28 d after treatment.

**In Vivo Tumor Immune Microenvironment Assays**

To study the effects of cascade chemo-PDT and anti-PD-L1 treatment on the tumor immune microenvironment in vivo, Hep 1-6 subcutaneous tumor model in C57BL/6 mice was used. Tumor tissues and spleen were collected after different treatments and digested to form a single-cell suspension. After lysing the red blood cells, the single-cell suspension was analyzed by flow cytometry. CTLs, Treg cells, MDSCs, and Tem cells were defined as above.

**H&E Staining**

H&E staining was performed to assess histological changes of tumors after different treatments as previously described and images were captured using optical microscopy [3].

**TUNEL Assay**

The TUNEL assay was performed to assess apoptosis levels of tumors after different treatments as previously described and images were captured by a fluorescence microscope [3].

**Immunohistochemical Analysis**

Immunohistochemical staining was performed as previously described to analyze the expression level of PD-L1 and Ki-67 in tumor tissue after different treatments and images were captured using optical microscopy [3].

**Statistical Analysis**

All of the measurement data were presented as mean ± standard deviation and were analyzed by utilizing Student's t-test or the Mann-Whitney U test wherever appropriate. Survival analyses for OS and tumor free survival were performed by utilizing the Kaplan–Meier method and log-rank test. Statistical differences were performed in Graphpad Prism 7.0. $P < 0.05$ was considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

**Abbreviations**

Hepatocellular carcinoma (HCC); immune checkpoint blockade (ICB); programmed cell death protein 1 (PD-1); programmed cell death ligand 1 (PD-L1); tumor-associated antigen (TAA); cytotoxic T lymphocytes (CTLs); photodynamic therapy (PDT); reactive oxygen species (ROS); immunogenic cell
death (ICD); damage-associated molecular patterns (DAMPs); nanocarriers-based drug delivery systems (nano-DDSs); aggregation induced emission (AIE); paclitaxel (PTX); polylactic acid-glycolic acid (PLGA); dendritic cells (DCs); transmission electron microscopy (TEM); drug loading content (DLC); dynamic light scattering (DLS); confocal laser scanning microscopy (CLSM); red blood cells (RBCs); hematoxylin-eosin (H&E); calreticulin (CRT); heat shock protein 70 (HSP70); adenosine triphosphate (ATP); high mobility group box 1 (HMGB1); endoplasmic reticulum (ER); bone marrow-derived DCs (BMDCs); enzyme-linked immunosorbent assay (ELISA); myeloid-derived suppressor cells (MDSCs); effector memory T-lymphocyte (Tem); creatine kinase-MB (CK-MB); alanine aminotransferase (ALT); aspartate aminotransferase (AST); blood urea nitrogen (BUN); serum creatinine (sCr).

Declarations

Ethics approval and consent to participate: All of the animal experiments were performed in accordance with the guidelines of the Animal Care Committee at Tongji Medical College, HUST, Wuhan, China.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare no competing financial interest.

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Authors' contributions

YG, XY, YX, JX, QZ, and XC supervised and conceptualized the study; JX, QZ, and XC performed most of the experiments and wrote the manuscript; SH, CZ, XZ, PS, WW, ZS, TZ, and ZS helped with project design and provided guidance on some experiments; all authors have read and approved the final manuscript.

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**Figures**
Preparation and characterization of RTK, TB@RTK, PTX@RTK and TB/PTX@RTK micells. (A) Hydrodynamic size distribution of RTK, TB@RTK, PTX@RTK and TB/PTX@RTK micells. (B) The TEM images of TB@RTK, PTX@RTK and TB/PTX@RTK micells. Scale bar: 100 nm. (C) FL spectra of TB/PTX@RTK micells in PBS (Ex = 530 nm). (D) Hydrodynamic size distribution of TB/PTX@RTK micells were treated with 10 mM H2O2 for 0 h, 2 h, 4 h and 6 h, respectively. (E, F) UV-vis absorption spectrum of TB@RTK (E) and TB/PTX@RTK (F) micells upon light irradiation (white light, 100 mW/cm2), respectively. (G) Hydrodynamic size distribution of TB/PTX@RTK micells upon light irradiation (white light, 100 mW/cm2, 10 min) after 2 h, 4 h and 6 h, respectively. (H) In vitro release of PTX from TB/PTX@RTK micells in PBS (pH 7.4, 0.1 M) containing 0.1% (w/v) Tween 80 at 37°C or H2O2 (10 mM) or light irradiation.
Figure 2

Targeted imaging and subcellular localization of tumor cells by TB/PTX@RTK micells in vitro. (A, B) CLSM images and relative semi-quantitative analysis of the fluorescence intensity of L-O2, HK-2, Hep G2 and Hep 1-6 cells after incubated with the micells (5 μg/mL) for 4 h. Red fluorescence is emitted by the micells. Green fluorescence and blue fluorescence label the cytoskeleton and the nuclei, respectively. Scale bar: 20 μm. (C, D) Confocal images and semi-quantitative analysis of Hep G2 after incubation with TB/PTX@TK or TB/PTX@RTK micells (5 μg/mL) for 4 h without or with pretreatment with cilengitide. Scale bars: 50 μm. (E, F) Confocal images and co-localization analysis of TB/PTX@RTK micells with lysosomes. Scale bar: 10 μm.
Figure 3

Fluorescence imaging, biodistribution analysis and pharmacokinetics of TB/PTX@RTK micells in vivo. (A) Fluorescent images of the Hep 1-6 tumor-bearing Balb/c nude mice at different time points after injection of TB/PTX@RTK micells (5 mg/kg). The white dotted ellipse indicates tumor sites. (B) Ex vivo fluorescence imaging of tumor tissues and major organs from tumor-bearing mice 12 h after intravenous injection of micells. (C) Biodistribution of TB/PTX@RTK micells in tumor-bearing mice 12 h after injection...
of TB/PTX@RTK micells (5 mg/kg) without or with pretreatment with cilengitide. The white dotted ellipse indicates tumor sites. (D) Quantitative analysis of organ distribution of TB/PTX@RTK micells in tumor-bearing mice with or without pretreatment with cilengitide. Data represent mean ± SD (n = 3). **p < 0.01. (E) Pharmacokinetics of free TB and TB/PTX@RTK micells in the blood. Data represent mean ± SD (n = 3).

Figure 4
ROS generation, intracellular PTX release and synergistic chemo-PDT in vitro. (A) Detection of the intracellular ROS generation induced by TB-mediated PDT in Hep 1-6 and Hep G2 cells. Scale bar: 20 μm. (B) Immunofluorescent staining of acetylated α-tubulin in Hep 1-6 and Hep G2 cells after treatment with PBS (negative control), TB@RTK, TB@RTK + light, TB/PTX@RTK, TB/PTX@RTK + light. Scale bar: 20 μm. (C) Flow cytometric analysis of cell apoptosis and necrosis in Hep 1-6 and Hep G2 cells after treatment with PBS (negative control), TB@RTK, TB@RTK + light, TB/PTX@RTK, TB/PTX@RTK + light. (D) Cell viability following treatment with PBS (negative control), TB@RTK, TB@RTK + light, TB/PTX@RTK, TB/PTX@RTK + light. (E) Cell viability after treatment with different concentration of TB/PTX@RTK micells or time of light irradiation.

**Figure 5**

Antitumor efficacy of TB/PTX@RTK micells in vivo. (A) Representative images of tumor-bearing mice and harvested tumors after various treatments. Scale bar: 1 cm. (B) Tumor volume at indicated time points after different treatments (n = 5 per group). Data represent mean ± SD. (C) Tumor weight at 21 days after different treatments. Data represent mean ± SD. (D) H&E staining for histological changes in Hep 1-6 tumor tissues after different treatments (upper row). Ki-67 expression detected by IHC in tumor tissues sections after different treatments (middle row). TUNEL assay for apoptosis levels in Hep 1-6 tumor tissues after different treatments (lower row). Scale bar: 20 μm. (E) Survival curves of Hep 1-6 tumorbearing C57BL/6 mice in different treatment groups (n = 8 per group). *P < 0.05, ***P < 0.001.
Figure 6

ICD induction, immune activation and adaptive immune resistance of TB/PTX@RTK-mediated chemo-PDT (G1: PBS; G2: TB@RTK; G3: TB@RTK + L; G4: TB/PTX@RTK; and G5: TB/PTX@RTK + L). (A) Immunofluorescence imaging of CRT expression on the membrane surface of Hep 1-6 cells after different treatments. Dil indicates the cytomembrane. Scale bars: 10 μm. (B, C) Extracellular ATP (B) and HMGB1 (C) levels after different treatments. *P < 0.05, **P < 0.01, ***P < 0.001. (D-I) Representative plots of flow...
cytometric analysis and statistical analysis of mature BMDCs (CD11c+CD80+CD86+; D and E), CTLs (CD45+CD3+CD8+; F and G) and Treg cells (CD3+CD4+Foxp3+; H and I) in the tumors. (J) Immunofluorescence imaging of membrane PD-L1 proteins on Hep 1-6 cells after different treatments. Dil indicates the cytomembrane. Scale bars: 10 μm.

Figure 7

Lysosome disruption, mitochondrial membrane potential depolarization, cytochrome-c release, as well as caspase-3 activation, following TB@RTK micell-mediated PDT in Hep 1-6 cells. (A) Lysosome disruption was assessed by LysoTracker Green staining after different treatments. In PDT group, the fluorescent intensity decreased. Scale bar: 50 μm. (B) Mitochondrial membrane potential (ΔΨm) in Hep 1-6 cells after the indicated treatments. Scale bar: 20 μm. (C) Immunofluorescence co-staining of cytochrome-c and Tom20. Tom20 indicates the mitochondria. Scale bar: 10 μm. (D) Caspase-3 activation in Hep 1-6 cells after different treatments.
Figure 8

Effects of chemo-PDT combined with anti-PD-L1 (G1, PBS; G2, Anti-PD-L1; G3, TB/PTX@RTK + L; and G4, TB/PTX@RTK + L + anti-PD-L1). (A) Treatment schedule for combination therapy strategy of TB/PTX@RTK micell-mediated Chemo-PDT and anti-PD-L1. (B, C) Primary tumor images and tumor growth curves in the different groups after the indicated treatments. (n = 5). Scale bar: 1 cm. *P < 0.05, **P < 0.01, ***P < 0.001. (D, E) Distant tumor images and tumor growth curves in the different groups.
after the indicated treatments. (n = 5). Scale bar: 1 cm. **P < 0.01, ***P < 0.001. (F-I) Representative plots of flow cytometric analysis and statistical analysis of the infiltration of CTLs (CD45+CD3+CD8+; F and G) and Treg cells (CD3+CD4+Foxp3+; H and I) in the distant tumors. (n = 5). Scale bar: 1 cm. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 9

Cytotoxicity of TB/PTX@RTK micells in vivo. (A) Body weight changes of C57BL/6 mice after injection of saline (negative control), free PTX, and TB/PTX@RTK micells, respectively. Data represent mean ± SD (n = 3). (B) H&E staining images of various organ slices from C57BL/6 mice 7 days after the indicated treatments. Scale bar: 50 μm. (C) Red blood cells (RBC), red blood cell distribution width (RDW), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), hemoglobin (HGB), weight blood cells (WBC), neutrophils (NE), platelet (PLT), and platelet distribution width (PDW) levels in C57BL/6 mice of different groups. (D) The serum levels of CK-MB, ALT, AST, BUN, and sCr in C57BL/6 mice of different groups.
Supplementary Files

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