

Enhanced chemo-photodynamic therapy of an enzyme-responsive prodrug in bladder cancer patient-derived xenograft models

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Abstract: Patient-derived xenograft (PDX) models are powerful tools for understanding cancer biology and drug discovery. In this study, a polymeric nano-sized drug delivery system poly(OEGMA)-PTX@Ce6 (NPs@Ce6) composed of a photosensitizer chlorin e6 (Ce6) and a cathepsin B-sensitive polymer-paclitaxel (PTX) prodrug was constructed. The photochemical internalization (PCI) effect and enhanced chemo-photodynamic therapy (PDT) were achieved via a two-stage light irradiation strategy. The results showed that the NPs@Ce6 had great tumor targeting and rapid cellular uptake induced by PCI, thereby producing excellent anti-tumor effects on human bladder cancer PDX models with tumor growth inhibition greater than 98%. Bioinformatics analysis revealed that the combination of PTX chemotherapy and PDT up-regulated oxidative phosphorylation and ROS generation, blocked cell cycle and proliferation, and down-regulated the pathways related to tumor progression, invasion and metastasis, including hypoxia, TGF- β signaling and TNF- α signaling pathways. Western blots analysis confirmed that proteins promoting apoptosis (Bax, cleaved caspase-3, cleaved PARP) and DNA damage (γ H2A.X) were up-regulated, while those inhibiting apoptosis (Bcl-2) and mitosis (pan-actin and α/β -tubulin) were down-regulated after chemo-PDT treatment. Therefore, this stimulative responsive polymer-PTX prodrug-based nanomedicine with combinational chemotherapy and PDT evaluated in the PDX models could be a potential candidate for bladder cancer therapy.

Key words: Polymer-paclitaxel prodrug; Chemo-photodynamic therapy; Photo-chemical internalization; PDX models; Bladder cancer; Bioinformatics analysis

1. Introduction

Nanomedicines produced by the incorporation of therapeutic agents in a nanoscale polymer drug delivery system to treat malignant tumors have made considerable progress, however, their therapeutic effects are still unsatisfactory due to the complexity of the macroenvironment of the human body and the tumor microenvironment.[1, 2] The nanomedicine needs to overcome multiple physiological barriers before arriving at the tumor site after intravenous administration, including evading elimination by the mononuclear phagocyte system, avoiding premature release of therapeutic agents encapsulated in the nanocarrier into the blood system, and achieving deep penetration into the tumor. In addition, after overcoming these barriers, the nanomedicine must be able to be effectively engulfed by tumor cells, and the encapsulated drugs must be released specifically and rapidly inside tumor cells.[3-6] All these steps play a critical role in maximizing therapeutic effects of nanomedicines. Therefore, tumor microenvironment-responsive polymer-based nanomedicines have been designed to specifically overcome these biological barriers with improved delivery efficiencies to tumor tissues, resulting in enhanced therapeutic indexes as well as reduced adverse effects.[7, 8]

To prepare such polymeric prodrug-based nanomedicines with high stability during circulation and rapid drug release at the tumor site, the properties of polymeric carriers need to be finely tuned, including their chemical composition, chemical structure and molecular weight.[9] An outer layer of polyethylene glycol (PEG) on nanoparticles can significantly reduce non-specific interactions between nanoparticles and cells and decrease adsorption of serum proteins during the circulation, therefore, PEGylation of nanoparticles has become a commonly used method to overcome the elimination of nanoparticles by the mononuclear phagocyte system.[10, 11] Recently, poly(oligo (ethylene glycol) methacrylate) (poly(OEGMA)) has been reported as an efficient drug delivery carrier due to the synergistic effect of PEGylation, its high molecular weight and flexibility for chemical modification.[12-15] The density of PEG or oligo (ethylene glycol) (OEG) has a great impact on the uptake of

nanoparticles by cells.[16, 17] Therefore, a balance of maintaining the function of PEGylation and enhancing cellular uptake of nanoparticles should be carefully considered for poly(OEGMA)-based or PEGylated nanomedicines.

Various methods have been used to enhance the cellular uptake of nanoparticles including grafting of targeting moieties on nanoparticles and tuning the morphology and/or size of nanoparticles.[18-20] A new strategy of photochemical internalization (PCI) has been developed by encapsulating a photosensitizer (PS) in nanoparticles. In such systems, the permeability of cell membranes is increased to facilitate the cellular uptake of nanoparticles due to the effect of reactive oxygen species (ROS) generated by PS under transitory low-energy-density irradiation.[21-23]

In addition, the nanomedicine incorporating a PS could be used for a combinational therapy since the PS is also an essential component for photodynamic therapy (PDT).[24, 25] It has been demonstrated that a single chemotherapeutic drug in a nanomedicine can be ineffective in complete elimination of tumors and it often induces chemo-resistance from tumors during the treatment.[26] Combining chemotherapy and PDT has become a viable option to improve the effectiveness of cancer treatment.[27] PDT relies on cytotoxic ROS, especially singlet oxygen, produced by a PS under laser irradiation at a specific wavelength to impair biological activity of tumor cells and ultimately induce cell death.[28, 29] Therefore, PDT has been considered to be an ideal, flexible and non-invasive method for precise treatment of tumors. .

Although many polymeric nanomedicines show encouraging therapeutic effects in animal models, their clinical performances are often far below the initial expectations. Failure to translate animal study results into clinical outcomes is often associated with the limitations of cell lines and cell line-derived xenografts in preclinical animal studies due to the lack of their clinical relevance and heterogeneity.[30] Recently, patient-derived tumor xenografts (PDXs) models have been established to mimic human tumors and they are considered as a more feasible tool for translational research.[31] Importantly, PDXs can maintain the cellular and

histopathological structure of their parental tumors, and genomic and proteomic profiles between PDXs and their parental tumors are preserved.[32, 33] It is worth noting that the sensitivity to anticancer drugs in PDXs is well correlated with the response of patients to these drugs in clinical treatment.[34] PDXs are a promising model in predicting the efficacy of conventional and novel anti-cancer therapies.

Therefore, in this study, we constructed a stimuli-responsive, amphiphilic, block poly(OEGMA)-PTX prodrug loaded with Ce6, and studied its potential as an efficient nanomedicine in the bladder cancer PDX model (**Scheme 1**). The hydrophilic fragment of the polymer prodrug was prepared by reversible addition fragmentation chain transfer (RAFT) polymerization of OEGMA, and an anti-tumor drug PTX was covalently linked to the polymer backbone through a cathepsin B-responsive tetrapeptide Gly-Phe-Leu-Gly (GFLG) (Scheme S1). The self-assembled nanostructure could efficiently encapsulate Ce6 for PDT and PCI. The therapeutic effect of the multifunctional nanomedicines (poly(OEGMA)-PTX prodrug incorporating Ce6) was evaluated in a bladder cancer PDX model, including the examination of the underlying anticancer mechanisms at the genomic and proteomic level. With synergistic action between the PS and the carrier, the delivery vehicles could overcome multiple physiological barriers in the delivery process, and effectively suppress tumor growth through the combination of chemotherapy and PDT. The anticancer efficacy of the nanomedicine in the bladder cancer PDX model indicates that combinational chemotherapy and nanomedicine-facilitated PDT have a great potential in anti-cancer treatment.

2. Materials and experimental procedures

2.1 Materials and methods

The information about materials, measurements and characterizations of poly(OEGMA)-PTX@Ce6 (NPs@Ce6), including preparation, size characterization, stability, drug release, and additional anticancer mechanism studies were provided in Supporting Information.

2.2 Tumor cells and tumor spheroids culture

Human T24 bladder cancer cells were acquired from Shanghai Institute for Biological Sciences (China) and cultured by following the vendor's instructions. Briefly, cells were maintained in the PRMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin) in a 5% CO₂, humidified incubator at 37 °C. To culture T24 multicellular tumor spheroids (MTSs), T24 cells were seeded in 96-well U-bottom plates at a density of 2×10^4 cells per well. After 1 week, tumor spheroids were formed with a diameter of approximately 200 μm.

2.3 Human tumor samples and PDX mice model

The study protocol and subsequent amendments were approved by the Institutional Review Board of the West China Hospital, Sichuan University, Chengdu, China (2020 Review, No. 330). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of West China Hospital, Sichuan University and approved by the Animal Ethics Committee of China. Human tumor samples were obtained with written consent from patients. All patients had been pathologically diagnosed with muscle invasive bladder cancer and underwent radical cystectomy at West China Hospital, Sichuan University. Their tumor tissues were collected immediately after surgery and minced into pieces and injected to the NSG mouse (NOD SCID gamma mouse) via subcutaneous transplantation. When the tumors in mice grew to an approximate volume of 500 mm³, they were harvested and subcutaneously transplanted into BALB/c nude mice. After multiple passages (> 3), the PDX tumors could be used for *in vivo* experiments. The tumor volume (mm³) was calculated as $(L \times W^2)/2$, where L and W were the length and width of tumors, respectively.

2.4 PCI-induced internalization

T24 cells were seeded in glass bottom dishes and cultured for 24 h. The medium was replaced with fresh one containing Ce6 or NPs@Ce6 at 0.5 μg/mL of Ce6. Cells were incubated for 1 h

and treated with 660 nm NIR irradiation (0.2 J/cm^2). At different incubation durations (2 h, 4 h, 6 h), cells were incubated in fresh medium containing $1\times$ Hoechst 33342 for nuclei staining and observed under a confocal laser scanning microscope (CLSM). PCI-induced internalization of NPs@Ce6 was quantified by ImageJ software. In terms of PCI-induced internalization in T24 MTS, MTSs were incubated with fresh medium containing $1\times$ Hoechst 33342 overnight, and then treated with NPs@Ce6 at $0.5 \mu\text{g/mL}$ of Ce6 for 1 h and subjected to 660 nm NIR irradiation (0.5 J/cm^2). At different incubation time points (2 h, 4 h, 12 h), the MTSs were collected and analyzed under a microscope.

2.5 In vitro antitumor treatment

To evaluate the anticancer effect of the nanoparticles, T24 cells were seeded in 96-well plates at a density of 3,000 cells per well and incubated for 24 h. Cells were treated with different concentrations of free Ce6, PTX, NPs, and NPs@Ce6 with or without 660 nm NIR irradiation (0.6 J/cm^2), respectively. For NPs@Ce6+SL group (short-long-term irradiation), cells were subjected to short-term irradiation (0.2 J/cm^2) after 1 h incubation for PCI-induced internalization, and 0.4 J/cm^2 irradiation after 6 h incubation. In the NPs@Ce6+L group (long-term irradiation), 0.6 J/cm^2 irradiation was given after 6 h incubation. Unless specified otherwise, the other experiments were performed using the same irradiation protocol. After PDT treatment, cells were further incubated for 24 h. $10 \mu\text{L}$ of the CCK8 reagent was added into each well and incubated for 2 h. The absorbance at 450 nm was recorded via a Varioscan Flash microplate reader (Thermo Fisher Scientific, USA). The combination index (CI) of PTX and Ce6 was calculated by CompuSyn software.[35]

2.6 Antitumor effect studies in bladder cancer PDX models

When the tumor volumes reached around 100 mm^3 , the mice were divided into seven groups ($n = 5$ for each group): Ce6+L, PTX, NPs, NPs@Ce6, NPs@Ce6+SL, NPs@Ce6+L and saline. The nanoparticles were injected at an equivalent PTX dose of 7 mg/kg and a Ce6 dose of 5

mg/kg. The tumors in the NPs@Ce6+SL-treated group were subjected to NIR irradiation (660 nm, $3 \text{ J/cm}^2 = 2 \text{ min} \times 25 \text{ mW/cm}^2$) for PCI-induced internalization after 1 h injection, and mice in this group received second NIR irradiation ($6 \text{ J/cm}^2 = 4 \text{ min} \times 25 \text{ mW/cm}^2$) at 6 h after injection. The tumors in both Ce6+L and NPs@Ce6+L groups received NIR irradiation (9 J/cm^2) after 6 h injection, while other groups were kept in dark. The above anticancer treatment procedure was performed every 4 days and four times in total. The tumor sizes and body weights were recorded every two to four days. All mice were sacrificed at day 19, tumors and main organs of each group were harvested. The tumors in each group were weighed. The tumors and main organs were treated with Hematoxylin and Eosin (H&E) and immunohistochemistry (IHC) staining. In addition, fresh tumor tissues in the control, Ce6+L, NPs and NPs@Ce6+L groups were used for bulk RNA-seq to unveil the anticancer mechanisms.

2.7 Western blots

T24 Cells were treated in the same way as in the apoptosis assay. The treated cells were harvested, and the cell lysate was immediately prepared in a RIPA buffer containing protease inhibitors and phosphatase inhibitors. Electrophoresis was performed according to a standard protocol using 10% SDS-polyacrylamide gel and transferred to Immobilon-P (Millipore). Primary antibodies were applied at 1:500-1:2000 dilution in 5% BSA in TBST for incubation overnight at 4 °C. Membranes were incubated with HRP-conjugated secondary antibodies (1:10000) for 1 h at room temperature. The proteins were visualized using NcmECL Ultra Reagent (NCM biotech). The Immobilon® Western Chemiluminescent HRP Substrate (WBKLS0500, MILIPORE) was used for detection of proteins.

2.8 RNA-seq analysis

2.8.1 Library Construction

According to a standardized Illumina protocol, tumor tissues from the PDX mouse model at the end of treatment (day 19) were used for the preparation and construction of transcriptome

libraries. Subsequent transcriptome sequencing analysis was performed on the Illumina NovaSeq 6000 platform.

2.8.2 Pre-processing raw data

After 150 bp paired-end sequencing raw data was obtained, adapter, ploy-N, and low-quality reads were removed to obtain high-quality clean data. Clean data was checked against the criteria of $Q20 > 90$ and $Q30 > 85$. Clean data was used for subsequent analysis of alignment, differential genes, and pathway enrichment.

2.8.3 Sequence alignment and differential expression analysis

Sequence alignment was executed with STAR (v 2.6.0)[36] using the reference human genome (GRCh38). Deseq2()[37] was performed to generate normalized expression data with the option of “counts(normalized=TRUE)”. The significance score and log2fold-change were calculated.

2.8.4 Drawing heatmap and volcano map

The heat map matrix was composed of different genes with a p-value < 0.05 by comparing genes from the treatment group and those from the control group, and the data normalization was performed by DESeq2. The function adapted from Seurat[38, 39] DoHeatmap() was used for visualization and gene labeling. The volcano map matrix included log2Foldchange and $-\log_{10}(\text{p_value})$ which were obtained by comparing NPs@Ce6+L versus Ce6+L by DESeq2. Red plots were drawn for genes up-regulated in the NPs@Ce6+L-treated group (p-value < 0.05 and log2foldchange > 0.3), and blue plots for genes down-regulated in the NPs@Ce6+L-treated group (p_value < 0.05 and log2foldchange < -0.3). Data visualization and gene labeling were performed by ggplot2 (<https://ggplot2.tidyverse.org>) and ggrepel.

2.8.5 Pathway enrichment analysis

Differentially expressed genes (p_value < 0.05 and $|\log_2\text{fc}| > 0.3$) which were identified by comparing samples between NPs versus Control, NPs@Ce6+L versus Control, NPs@Ce6+L

versus Ce6+L, and NPs@Ce6+L versus NPs were used in Gene Ontolog by clusterProfiler (v3.10.1)[40] and gene set enrichment analysis (GSEA).[41, 42]

2.9 Statistical analysis

Student's t test was applied for statistical analysis of experimental data in comparison with the controls. All data were presented as mean \pm standard deviation (SD). Statistical significance was indicated by $P < 0.05$ and highly statistical significance by $P < 0.01$.

3. Results and discussion

3.1 Preparation and characterization of NPs@Ce6

Ce6-loaded nanoparticles (NPs@Ce6) were prepared by encapsulating Ce6 with an enzyme-responsive diblock poly(OEGMA)-PTX prodrug. To obtain the prodrug, a cathepsin B-responsive monomer (MA-GFLG-PTX) was first synthesized according to a previously reported method.[43] Subsequently, a hydrophilic macromolecular chain transfer agent, poly(OEGMA)-CTA, was prepared by RAFT polymerization as sketched in Scheme S1 (Supporting Information). The GPC results showed that the poly(OEGMA)-CTA had a high molecular weight and a narrow molecular weight distribution ($M_n = 25.8$ kDa, $PDI = 1.13$). Compared with many PEG-modified polymers, this poly(OEGMA)-based hydrophilic fragment has a significantly higher molecular weight, so it was expected to have a better assembly performance and encapsulation properties.

The proportion of hydrophilic and hydrophobic segments in polymer prodrugs has an important impact on its performance. In this study, the content of PTX in the polymer can be adjusted by controlling the reaction conditions. To obtain polymeric prodrugs with optimized physicochemical properties, we first studied the self-assembly properties of polymer prodrugs obtained after copolymerization of the poly(OEGMA)-CTA and MA-GFLG-PTX monomer through dissipative particle dynamics (DPD) simulation. As shown in Figure S1 (Supporting Information), we constructed the structural models of polymer prodrugs polymerized with 2 to

5 MA-GFLG- PTX respectively and studied their self-assembly processes and structures through DPD simulation (Figure S2 and S3, Supporting Information). The DPD simulation results showed that the poly(OEGMA)-PTX prodrug with different contents of PTX (the theoretical range is 6.0% to 13.2 wt%) could self-assemble to form nanoparticles with a hydrophobic inner core constituted with PTX and GFLG, and a hydrophilic shell from poly(OEGMA) (**Figure 1a**). However, as the PTX drug content in the prodrug increased, the outer poly(OEGMA) segment could not completely cover the inner PTX molecules, and the morphology of the formed nanoparticles gradually changed from a complete core-shell structure to a semi-packaged structure. Although a low content of PTX was more conducive to self-assembly of the prodrug, such an amount of PTX resulted in a lower drug loading into the drug delivery system. To balance a high drug content for a desired therapeutic index and successful self-assembly for a complete core-shell structure, the polymer structure containing three GFLG-PTX repeating units may be an ideal polymeric prodrug structure. Therefore, we used the results of DPD simulation as a guide and optimized the reaction conditions to copolymerize MA-GFLG-PTX and poly(OEGMA)-CTA to prepare an amphiphilic block polymer prodrug poly(OEGMA)-PTX.

Compared with the ^1H NMR spectrum of poly(OEGMA)-CTA (Figure S4, Supporting Information), a characteristic peak of PTX (7.25-8.00 ppm) was observed in the ^1H NMR spectrum of poly(OEGMA)-PTX (Figure S5, Supporting Information), which indicated that PTX was successfully conjugated to the polymer through a tetrapeptide GFLG. The results of amino acid analysis showed that the percent weight of the amino Gly, Phe, and Leu were 1.19%, 1.42%, and 1.21%, respectively, further indicating the presence of GFLG in the polymer (Table S1, Supporting Information). GPC confirmed a molecular weight of 28.3 kDa for poly(OEGMA)-PTX and a PDI of 1.28. The cleavage of GFLG by papain that has a similar activity to cathepsin B could specifically release covalently linked PTX. The content of PTX in

poly(OEGMA)-PTX was determined to be 8.3% by HPLC analysis, indicating that on average, each polymer prodrug contained 2.7 PTX repeating units.

The self-assembly structure was further examined from its ^1H NMR spectrum. As shown in Figure S6 (Supporting Information), all peaks of the polymer prodrug were observed in $(\text{CD}_3)_2\text{SO}$, while only the peak for the hydrophilic OEGMA chain was seen in D_2O , indicating that it had a stable core-shell structure in an aqueous solution. The critical micelle concentration (CMC) value of poly(OEGMA)-PTX was measured to be $20.0 \mu\text{g/mL}$ using pyrene as a fluorescence probe (Figure 1b). The hydrodynamic diameter of the nanoparticles formed by the polymer prodrug was $131.96 \pm 8.16 \text{ nm}$ by DLS (Figure 1c), which was close to the result of TEM. These results indicated that the amphiphilic polymer prodrug could form stable micelles while encapsulating hydrophobic therapeutic agents.

To obtain a better anti-tumor effect, a Ce6-encapsulated polymer nano drug delivery system (NPs@Ce6) was prepared by a thin-film hydration method, so that it could be used for chemical-photodynamic synergistic therapy. As shown in Figure 1d, the prepared NPs@Ce6 has a uniform spherical structure, and the hydrodynamic particle size was about $168.20 \pm 1.12 \text{ nm}$. In addition, NPs@Ce6 showed excellent colloidal stability in phosphate buffered saline (PBS) containing 10% fetal bovine serum, and cell culture media (Figure 1e). The results of photostability (Figure 1f) and ROS generation (Figure 1g) confirmed that the encapsulated Ce6 had similar photostability/photoactivity to free Ce6, and it could rapidly produce cytotoxic ROS under 660 nm laser irradiation, indicating NPs@Ce6 could exert their PDT effect against tumors.

To establish the release profiles of PTX and Ce6 triggered by cathepsin B in tumor cells, NPs@Ce6 were incubated in a simulated tumor microenvironment (McIlvaine's buffer at pH 5.4 with papain for mimicking cathepsin B) in comparison to a normal physiological environment (McIlvaine's buffer without papain at pH 7.4). HPLC detection results showed that the absorption peak for free PTX was detected after the NPs@Ce6 were incubated with

papain (Figure 1 h and Figure S7, Supporting Information), indicating that the polymer prodrug could release the original PTX drug through enzymatic cleavage. As shown in Figure 1i, PTX was released rapidly in McIlvaine's buffer containing papain at pH 5.4, and the release amount reached 80% within 8 h. In contrast, in PBS (pH 7.4 or 5.4) without papain, the release of PTX was less than 20% in 24 h. The release profile of Ce6 in different buffer solutions was similar to PTX, and rapid release of Ce6 from NPs@Ce6 occurred in the presence of papain (Figure 1j). These results indicated that NPs@Ce6 were responsive to cathepsin B, and responsive degradation of GFLG unpacked the core-shell nanostructure, thereby quickly releasing the contained drugs.

3.2 Uptake of NPs by cells and tumor spheroids

The uptake of free Ce6 and NPs@Ce6 by T24 cells was studied via flow cytometry and CLSM. As shown in Figure S8 (Supporting Information), the fluorescence intensity of Ce6 (red) in the cytoplasm was very weak in T24 cells post incubation with free Ce6 from 2 to 6 h. The fluorescence intensity of Ce6 was significantly higher in T24 cells after they were treated with NPs@Ce6 compared to those treated with free Ce6. As shown in **Figure 2a** and **2b**, the cellular fluorescence intensity of Ce6 gradually increased in T24 cells treated with NPs@Ce6, suggesting that the internalization of NPs@Ce6 by T24 cells was time dependent. A relatively low dose of light (0.2 J/cm^2) was used to evaluate the PCI effect on the cellular uptake of NPs@Ce6. A notably enhanced fluorescence signal of Ce6 was observed at 2 h, 4 h, and 6 h in cells exposed to a low dose of NIR light irradiation (Figure 2a). Semi-quantitative analysis confirmed that NIR light irradiation induced statistically significant cellular uptake of NPs@Ce6 (Figure 2b).

As the incubation time increased, the fluorescence intensity in the spheroids significantly increased, indicating time-dependent penetration of NPs@Ce6 (Figure S9, Supporting Information). In addition, the fluorescence intensity in T24 spheroids at the incubation time

ranging from 2 to 12 h was enhanced after the spheroids received NIR light irradiation (0.5 J/cm²), indicating the penetration of NPs@Ce6 was enhanced by the PCI effect (Figure 2c, 2d and Figure S9, Supporting Information).

Flow cytometry analysis also revealed that free Ce6 was not readily uptaken by T24 cells and supported time-dependent internalization of NPs@Ce6 by T24 cells (Figure 2e and 2f). Importantly, flow cytometry analysis also demonstrated that the low dose irradiation increased the uptake of nanoparticles through the PCI effect. Thus, these results suggested that photodynamic treatment at a low dose could promote cellular uptake of NPs@Ce6 and enhance their spheroid penetration. In addition, live cell images revealed red fluorescence of NPs@Ce6 was largely observed in regions that was stained with LysoTracker green, implying intracellular distribution of NPs@Ce6 was in the lysosomes of T24 cells (Figure S10, Supporting Information).

3.3 Imaging of ROS generation

The intracellular ROS level after PDT treatment was measured via DCFH-DA in T24 cells and spheroids. As shown in **Figure 3a** and Figure S11 (Supporting Information), the ROS level significantly increased in cells treated with NPs@Ce6 and PDT treatment compared with those that received the free Ce6 and PDT treatment (abbreviated as Ce6+L). Moreover, the production of ROS in cells or tumor spheroids was enhanced in the NPs@Ce6+SL group in comparison with the NPs@Ce6+L group, which may be contributed to the PCI effect induced by low-dose light irradiation. The cellular ROS generation was also noticed to be enhanced after cells were treated by PTX, NPs, NPs@Ce6, which was consistent with previous reports that PTX could induce ROS generation.[44, 45]

3.4 *In vitro* anticancer treatment

The synergistic antitumor effect of PTX and PDT was evaluated by staining live and dead cells with Calcein AM and PI in T24 spheroids. The fluorescence images confirmed that

treatment with NPs@Ce6 plus PDT induced more dead cells (red) in comparison with chemotherapy or PDT treatment alone (Figure 3b). In addition, more dead cells were observed in the NPs@Ce6+SL group than those in the NPs@Ce6+L group. Moreover, the cell counting kit-8 (CCK8) was employed to evaluate the cytotoxicity against T24 cells induced by NPs@Ce6 plus PDT treatment. As shown in Figure 3c, treatment with free Ce6 did not impact the viability of T24 cells even when the concentration of free Ce6 was increased up to around 7 $\mu\text{g/mL}$, but the cell viability decreased when the cells were treated with free Ce6 at a concentration of more than 1 $\mu\text{g/mL}$ and subjected to NIR light irradiation (1.0 J/cm^2). In addition, there was no statistically significant difference in the cell viability between the Ce6+SL and Ce6+L group, indicating free Ce6 under NIR irradiation was unable to exert a PCI effect on cells. Along with an increase in the PTX concentration, the cell viability gradually decreased after treatment with PTX, NPs or NPs@Ce6. The IC_{50} (PTX) values of NPs (11.2 $\mu\text{g/mL}$) and NPs@Ce6 (5.7 $\mu\text{g/mL}$) were higher than that of PTX (3.5 $\mu\text{g/mL}$), which may be attributed to a more complex process of internalization, trafficking, and drug release of NPs and NPs@Ce6 than free PTX. Notably, the IC_{50} (PTX, Ce6) value of NPs@Ce6+SL (0.16 $\mu\text{g/mL}$) was lower than that of NPs@Ce6+L (0.38 $\mu\text{g/mL}$). In addition, the combination indexes (CIs) of PTX and Ce6 were less than one, indicating a synergistic antitumor effect between them (Figure S12, Supporting Information). Treatment with PTX, NPs, NPs@Ce6 with or without NIR light irradiation also significantly inhibited the cloning formation of T24 cells (Figure S13, Supporting Information). These results may be attributed to a better penetration ability and a higher cellular uptake efficiency of NPs@Ce6 induced by the PCI effect.

Microtubules, one of the intracellular cytoskeletal filaments, play a critical role in mitosis and intracellular vesicular trafficking. PTX has been demonstrated to be able to stabilize microtubules and suppress their dynamicity, thereby blocking mitosis and inducing cell death. To evaluate the impact of PTX from NPs@Ce6 on the microtubules of T24 cells, the morphology of microtubules in cells after treatment with different formulations was observed

under a CLSM. As shown in Figure 3d, the microtubules congregated around the cellular cortex in cells treated with PTX, NPs and NPs@Ce6, while microtubules were evenly distributed throughout the cytoplasm in the control and Ce6-treated groups. In addition, the cells in the control and Ce6-treated groups maintained an integrated and intact nuclear structure, while multiple disintegrated nuclei structures were observed in the cells treated with PTX, NPs and NPs@Ce6, indicating PTX released from NPs and NPs@Ce6 exerted a similar effect on disruption of mitosis and damage of DNA to free PTX.

Furthermore, the impact of PTX released from NPs@Ce6 on cell cycle progression of T24 cells was assessed by PI staining and detected by flow cytometry. As shown in Figure 3e and 3f, the cells in the control and Ce6+L groups were mainly in the G0/G1 phase (~66%) and the S phase (~25%), while only ~7% cells were in the G2/M phase. After T24 cells were treated with PTX, NPs, or NPs@Ce6, the proportion of cells in the G2/M phase significantly increased to 85.83%, 83.45%, and 83.52%, respectively; and the percentages of cells in the G0/G1 and S phase were significantly decreased to around 1% and 15%, respectively. These results suggested that PTX released from NPs or NPs@Ce6 could induce cell cycle arrest in the G2/M phase of mitosis in a similar manner as free PTX, which led to cancer cell death.

Apoptosis of T24 cells induced by the combinational PTX and PDT treatment was also evaluated by flow cytometry (Figure 3g and 3h). The results revealed that the treatment with Ce6 and NIR irradiation did not increase cell apoptosis, which may be due to extremely poor cellular uptake of free Ce6 by T24 cells. However, exposure to free PTX and PTX from NPs or NPs@Ce6 could significantly induce apoptosis, and apoptotic cells accounted for 27.87 % of the PTX-treated group, 38.37% of the NPs-treated group and 49.37% of the NPs@Ce6-treated group. Moreover, a combinational PDT and PTX treatment further increased the percentage of apoptotic cells; and more apoptotic cells were found in the NPs@Ce6+SL group compared with the NPs@Ce6+L group (83.4% vs 70.17%, $p < 0.0001$), which may be attributed to the PCI

effect due to photo-responses of Ce6 under NIR illumination to enhance penetration of NPs@Ce6 in tumor spheroids and cellular uptake of NPs@Ce6 to improve their toxicity to tumor cells.

3.5 *In vivo* anticancer treatment

To assess the biodistribution of NPs@Ce6 after systemic administration and their *in vivo* therapeutic efficacy, a human bladder cancer PDX model was established for imaging and anticancer treatment. Briefly, the bladder cancer tissue was extracted from a patient who was diagnosed with muscle invasive bladder cancer in the West China Hospital and underwent radical cystectomy. H&E staining of the cancer tissue confirmed the diagnosis of bladder cancer in this patient (**Figure 4a**). To establish a bladder cancer PDX model, the fresh tumor tissue was minced into pieces at $\sim 1 \text{ mm}^3$ and subcutaneously implanted into NSG mice. H&E staining of the xenografts from mice after several *in vivo* passages showed that the PDX model inherited the histopathological features of the parental tumor. Thus, the established PDX model mimicking the human bladder cancer could be used for evaluating the therapeutic effect of drugs *in vivo*. The bladder cancer PDX model at the fifth passage was employed for assessment of *in vivo* biodistribution and therapeutic outcomes of NPs@Ce6.

3.5.1 Pharmacokinetics and biodistribution

Ce6 is a fluorescence probe, which exhibits deep tissue penetration and low autofluorescence.[46] Thus, through detection of Ce6 fluorescence, it was feasible to reveal dynamic distribution of NPs@Ce6 in a specific organ or tissue and their excretion pathways. *In vivo* and *ex vivo* bioimaging was performed in PDX mice after intravenous injection of NPs@Ce6 or free Ce6. Quantitative fluorescent signals of Ce6 from NPs@Ce6 and free Ce6 at different time points were shown in Figure 4b and Figure S14 (Supporting Information), respectively. Noticeably enhanced accumulation of NPs@Ce6 at the tumor site was observed with an increase in the post-injection time compared with free Ce6, indicating NPs@Ce6 could significantly increase Ce6 accumulation at tumor sites. After *in vivo* imaging at 48 h, main

organs and tumors of mice were harvested and imaged. The fluorescent signal was strongest in tumors compared with other organs. In addition, the tumor tissues were harvested at 12 h after injection and imaged under a CLSM. The images revealed the fluorescence intensity of Ce6 in tumors treated with NPs@Ce6 was more intense compared to that in tumors treated with free Ce6 (Figure 4c). Moreover, *in vivo* pharmacokinetic analysis showed the concentration of Ce6 in the blood of mice treated with NPs@Ce6 reduced at a relatively slower rate, with a half-life of 447.5 min, than that in the mice treated with free Ce6 with a half-life of 79.1 min (Figure 4d and Table S2, Supporting Information). This may be attributed to a high MW of NPs@Ce6, a stable linker in the chemical structure, a slightly negatively charged surface, and a branched structure with excellent chain flexibility and deformability. The prolonged circulation time could contribute to the passive accumulation of NPs@Ce6 at the tumor site and boost their therapeutic effects.

3.5.2 Antitumor therapy

The *in vivo* synergistic anticancer efficacy was evaluated in bladder cancer PDX models. Mice bearing bladder tumors were divided into 7 groups (n = 5 for each group). The control group was intravenously injected with PBS. For PCI-induced internalization, the tumors first received NIR light irradiation at 20 mW cm⁻² for 2 min at 1 h after injection of NPs@Ce6. A total of 4 doses were administrated to mice during the entire treatment procedure. Changes in the tumor volume were monitored every 2-4 d and mice were photographed after treatment on day 19 (Figure 4e and 4f). During the 19-day treatment, the tumors rapidly grew with a 13.8-fold increase in the volume in the mice that received PBS, while PDT treatment with free Ce6 slightly inhibited tumor growth with a 9.8-fold increase in the tumor volume, which may be due to the relatively weak enhanced permeability and retention (EPR) effect and poor cellular uptake of free Ce6 by tumor cells (Figure 4g-i), which were consistent with the *in vitro* effect of free Ce6. In addition, free PTX, NPs and NPs@Ce6 without PDT treatment showed a similar

inhibition efficacy with a 6-fold, 4.8-fold and 5.3-fold increase in the tumor size, respectively. However, the combinational treatment (NPs@Ce6+L and NPs@Ce6+SL) displayed a significantly improved inhibitory effect on tumor growth compared with chemotherapy via free PTX, NPs and NPs@Ce6. Notably, the tumors in the NPs@Ce6+SL treatment group were significantly smaller with a 5-fold reduction in the original tumor volume compared with those that were subjected to NPs@Ce6+L treatment with a 3-fold increase in the tumor volume. The inhibition score of tumors in the NPs@Ce6+SL group reached 98.5%. These results were consistent with tumor growth kinetics (Figure S15, Supporting Information), average tumor weights (Figure 4h) and morphologies of tumors upon harvest on day 19 (Figure 4i). The tumors in two mice in the NPs@Ce6+SL treatment group were completely eradicated (Figure 4i), which may be ascribed to passive accumulation and PCI-induced penetration of NPs@Ce6 at the tumor sites and PCI-enhanced cellular uptake of NPs@Ce6 to exert a synergistic therapeutic effect after rapid release of PTX and Ce6 under NIR irradiation. Moreover, slight fluctuations in the body weight were observed in mice from all groups, but there was no significant difference in the body weight change among all groups (Figure S16, Supporting Information). In addition, no obvious pathological changes were seen in major organs after treatment by NPs@Ce6+SL, NPs@Ce6+L, NPs@Ce6, NPs, PTX, Ce6+L, or saline on day 19, which indicated that NPs@Ce6 had great biocompatibility (Figure S17, Supporting Information).

3.5.3 Mechanisms of the combined PDT and PTX treatment

All mice were euthanized after the treatment and the tumors were extracted for mechanistic analysis. H&E staining of tumor tissues revealed that there was a large area of necrosis and fragmented nuclei were observed in the NPS@Ce6+SL-treated group (Figure 4j and Figure S18, Supporting Information). IHC staining of cleaved caspase-3 and the TUNEL assay in tumor tissues indicated the combinational treatment with PTX and PDT significantly induced more apoptotic cells (Figure 4j and Figure S18, Supporting Information). Moreover, more apoptotic

cells were produced in the NPs@Ce6+SL group compared with the NPs@Ce6+L group, which may be due to the PCI effect (Figure 4j). In addition, Ki-67 staining showed that proliferation of tumor cells was significantly inhibited after treatment by NPs@Ce6+SL and NPs@Ce6+L (Figure S19, Supporting Information). These results demonstrated that the combinational treatment of chemotherapy and PDT effectively inhibited proliferation of tumor cells and induced their apoptosis.

To further unveil the underlying molecular mechanisms of the anticancer effect from the combinational approach with NPs@Ce6 under NIR illustration, bulk RNA-sequencing was performed to compare the transcriptomics of tumor samples extracted from mice after different treatments. The heatmap showed differentially expressed genes (DEGs) in tumors subjected to the combinational treatment with PTX and PDT (**Figure 5a**). The DEGs included SOD2, BID, H2AX, COA3, NDUFAB2, PIM1, and TACC3. Among them, TACC3 was involved with spindle, H2AX with DNA damage, and SOD2, COA3 and NDUFAB2 with reactive oxygen species (ROS) generation pathways. The volcano plots showed the oncogenes, such as KLF9, STC1, IGF2, ABCC1 and CAPN6, were down-regulated, while the tumor suppressor genes including CDH11 and SMOC2 were up-regulated in the NPs@Ce6+L group compared with the Ce6+L group (Figure 5b). Gene Ontolog (GO) term enrichment analysis revealed that up-regulated genes after treatment with NPs@Ce6+L compared with Ce6+L were mainly involved in ROS generation, mitochondrial respiratory pathways, DNA damage and mitosis-associated pathways, including oxidative phosphorylation, activation of the mitochondrial respiratory chain, impedance of the repair function for broken double-strand DNA and prevention of disassembly of spindle microtubules (Figure 5c). Meanwhile, down-regulated genes associated with mitosis-related pathways were significantly enriched in the NPs group compared with the control group (Figure 5d).

Gene set enrichment analysis (GSEA) indicated that the gene sets associated with oxidative phosphorylation (OXPHOS, NES=1.75, FDR=0.00), DNA repair (NES=1.38, FDR=4.84E-3)

and mitochondria gene module (NES=1.83, FDR=0.00) had significantly positive enrichment scores in the tumors treated with NPs@Ce6+L compared with those with Ce6+L treatment, while gene sets associated with TGF- β signaling (NES= -1.39, FDR=0.03), hypoxia_UP (NES= -1.73, FDR=0.00) and TNF- α signaling via NF- κ B (NES= -1.46, FDR=0.03) had significantly negative enrichment scores (Figure 5e), which confirmed the results from GO term analysis. Meanwhile, gene sets related to proliferation, division, cell cycle and mitosis were negatively enriched in tumors treated with NPs, such as Myc target_V1, cell division, G2M checkpoint and proliferation, compared with those treated with Saline (Figure S20, Supporting Information). When gene sets were compared between tumors treated with NPs and those with NPs@Ce6+L, gene sets for oxidative phosphorylation (NES=1.56, FDR=0.008) had a positive enrichment score, while TGF- β signaling (NES= -1.66, FDR=0.003) and TNF- α signaling via NF- κ B (NES= -1.25, FDR=0.19) had negative enrichment scores (Figure S20, Supporting Information).

Mitochondrial ROS are generated mainly on the inner mitochondrial membrane during the process of oxidative phosphorylation. The level of oxidative phosphorylation is universally downregulated in cancer due to glycolysis.[47] Down-regulated OXPHOS is often associated with poor clinical outcomes across all cancer types and closely correlated with invasion and metastasis of tumors.[48, 49] However, a high level of mitochondrial ROS generated by the upregulated OXPHOS pathway could activate apoptosis pathways that is capable of inducing cell death.[50] Our data supported that OXPHOS was up-regulated by the NPs@Ce6+L treatment for production of mitochondrial ROS to induce cell apoptosis.

It has been suggested that TGF- β could promote cancer progression by increasing invasion and metastasis of tumor cells, and the TGF- β activity is negatively correlated with clinical outcomes.[51] Meanwhile, TNF- α could also promote tumor invasion and metastasis via the NF- κ B pathway.[52] In particular, it has been revealed that hypoxia in tumors contributes to tumor progression, angiogenesis, metastasis and resistance to therapy.[53] The NPs@Ce6+L

treatment resulted in down-regulation of genes related to TGF- β , TNF- α and hypoxia for preventing tumor progression and metastasis.

Besides bioinformatics analysis, proteins related to apoptosis, DNA damage, and cytoskeleton aggregation in bladder cancer T24 cells were also analyzed at the proteomics level (Figure 5f-g, and Figure S21, Supporting Information). The protein expression analysis suggested that apoptosis-related genes including cleaved caspase-3, cleaved PARP, and Bax were upregulated after treatment with free PTX, NPs@Ce6, or NPs@Ce6+L, while the apoptosis-inhibiting gene Bcl-2 was down-regulated, which suggested the number of apoptotic cells significantly increased in these groups (Figure 5f and 5g). Meanwhile, cells treated with NPs@Ce6+L and NPs@Ce6+SL had a higher expression level of cleaved caspase-3 compared with those treated with PTX, NPs or NPs@Ce6 (Figure 5f and 5g). However, cells treated with Ce6 and Ce6+L did not show any difference in the expression of apoptotic proteins compared with those without any treatment, which may be due to the poor cellular uptake of Ce6 by bladder cancer cells. In addition, protein expression of the DNA damage-associated gene γ H2A.X was significantly decreased in cells after PTX treatment alone or combined PTX and PDT treatment (Figure 5f and 5g). Interestingly, expression of microfilament and microtubules-associated proteins, α/β -tubulin and pan-actin, were reduced after treatment with PTX, but PDT treatment did not alter the expression of these proteins.

In summary, we have revealed the bladder tumor-killing mechanisms of the combined PTX and PDT treatment via NPs@Ce6 at both cellular and molecular levels (Figure 5h). Our results suggested that chemotherapy by PTX from NPs@Ce6 could block cell division and proliferation, which resulted in cancer cells death; while a combination treatment with PTX and PDT via NPs@Ce6 could significantly inhibit the genes and pathways related to tumor progression, invasion, and metastasis, and up-regulate OXPHOS for ROS generation to induce DNA damage and cell death.

4. Conclusions

We established bladder cancer PDX models for evaluating the therapeutic efficacy of an enzyme-responsive polymeric nano-drug delivery system (NPs@Ce6) that efficiently delivered PTX and Ce6 into tumor cells. The drug delivery system had great biocompatibility with an extended circulation time for passive accumulation at tumor tissues. The PCI effect induced by short-term irradiation contributed to cellular uptake and tissue penetration of NPs@Ce6. In the bladder cancer PDX models, treatment with NPs@Ce6 with short-term and long-term irradiation had the best performance in inhibiting bladder tumors. Bioinformatics analysis suggested that the combination of PTX chemotherapy and PDT via NPs@Ce6 could down-regulate the hypoxia pathway, TGF- β and TNF- α signaling pathways which were involved in tumor progression, invasion and metastasis, and could arrest the cell cycle, suppress proliferation, and promote oxidative phosphorylation and ROS generation. Meanwhile, western blots analysis revealed that proteins related to promoting apoptosis (Bax, cleaved caspase-3, cleaved PARP) and DNA damage (γ H2A.X) were up-regulated, and those related to inhibiting apoptosis (Bcl-2) and mitosis (pan-actin and α/β -tubulin) were down-regulated. This encouraging therapeutic outcome in the PDX models could be achievable via NPs@Ce6 with two-stage irradiation against bladder cancer.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (52073193, 82002692, 51873120, 81621003), 1·3·5 project for disciplines of excellence, West China Hospital, Sichuan University (ZYJC21013), Department of Science and Technology of Sichuan Province (2018JY0574), Post-Doctor Research Project of Sichuan University (2021SCU12016), and Post-Doctor Research Project, West China Hospital, Sichuan University (2018JY0574, 2019HXBH057, 2020HXBH094). M. Kopytynski was supported by the NIHR Imperial Biomedical Research Centre (BRC). We thank Ping Liao (Laboratory of Anesthesia and

Critical Care Medicine, Translational Neuroscience Center, Department of Anesthesiology of West China Hospital, Sichuan University, Chengdu, Sichuan, China) for imaging analysis. We thank Guang Yang, Yang Yang, Xiaoting Chen and Guangneng Liao from the Animal Experimental Center of West China Hospital of Sichuan University for technical assistance in animal experiments. We are also grateful to Sisi Wu, Xuemei Chen, Zhiqian Li, Guonian Zhu, Yanjing Zhang, Hongying Chen, Yan Wang, Yu Ding, Fengfeng Chen, Li Zhou, Jinkui Pi, Bo Su, Lei Wu, Jie Zhang, Shiqing Fu and Yi Zhang (Core Facility of West China Hospital of Sichuan University) for their technical assistance in *in vitro* and *in vivo* studies.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure Legends

Scheme 1. Schematic illustration of (a) constructing a bladder cancer PDX model; (b) degradation of the poly(OEGMA)-based polymer prodrug upon treatment with cathepsin B to release PTX; and (c) self assembly of the amphiphilic polymer poly(OEGMA)-PTX into nanoparticles NPs@Ce6 encapsulated with the hydrophobic Ce6. (d) NPs@Ce6 can produce ROS and exert the PCI effect under light irradiation for a short time and enhance the cellular uptake of NPs@Ce6. The PTX and Ce6 are released from NPs@Ce6 via Cathepsin B-responsive degradation in a tumor cell. Under long-time light irradiation, Ce6 can produce a large amount of ROS, which has a synergistic anti-tumor effect with PTX-mediated chemotherapy, and induce cell death by blocking cell mitosis, promoting cell apoptosis and damaging DNA.

Figure 1. Characterization of NPs and NPs@Ce6. **a)** Structures of nanoparticles formed by polymer prodrugs with different PTX drug contents obtained by DPD simulation. **b)** Determination of CMC of the poly(OEGMA)-PTX prodrug using pyrene as a fluorescence probe. Hydrodynamic size distributions and TEM images of **c)** NPs and **d)** NPs@Ce6. **e)** Colloidal stability of NPs@Ce6 in different solutions. **f)** Photostability of NPs@Ce6 and free Ce6 dissolved in PBS or DMSO. **g)** Time-dependent recovery of SOSG fluorescence from free Ce6, NPs@Ce6 or NPs under light exposure (660 nm, 2 mW/cm²). **h)** HPLC chromatograms of poly(OEGMA)-PTX incubated with papain for 0 h (middle) and 12 h (down), with free PTX used as a control (up). **i)** *In vitro* PTX release profiles from NPs@Ce6 in different conditions. **j)** *In vitro* Ce6 release profiles from NPs@Ce6 in different conditions.

Figure 2. PCI-induced internalization and penetration. **a)** CLSM images for intracellular localization of NPs@Ce6 (0.5 µg/mL of Ce6) at different time-points with or without NIR light irradiation at 0.2 J/cm². Hoechst 33342 (blue) for nuclei, and fluorescence of Ce6 (red) for NPs@Ce6. Scale bar: 20 µm. **b)** Semi-quantitative analysis of integrated fluorescence density of intracellular Ce6 of Figure 2a. * P < 0.05, *** P < 0.001, **** P < 0.0001 (n = 16). **c)** CLSM images of T24 spheroids after treatment with NPs@Ce6 (0.5 µg/mL of Ce6) for 12 h with or without NIR light irradiation at 0.5 J/cm². Purple for the fluorescence of Ce6. Scale bar: 100 µm. **d)** Fluorescence distribution of Ce6 in T24 spheroids along the cross-section of the spheroid marked in Figure 2c. **e)** Flow cytometry histograms and **f)** mean fluorescence intensity (MFI) of Ce6 in T24 cells after treatment with NPs@Ce6 for different durations with or without NIR light irradiation at 0.2 J/cm². *** P < 0.001 (n = 3).

Figure 3. *In vitro* anticancer treatment. **a)** ROS generation in T24 tumor spheroids treated with Ce6, PTX, NPs and NPs@Ce6 with or without NIR light irradiation. NPs@Ce6+SL (0.5 J/cm² for PCI-induced internalization at 1 h of incubation, and 1.0 J/cm² at 6 h of incubation). Scale bar: 100 µm. **b)** CLSM images of T24 tumor spheroids after treatment with Ce6, PTX, NPs and NPs@Ce6 with or without NIR light irradiation. Live cells were stained with Calcein AM (green) and dead cells with PI (red). Scale bar: 100 µm. **c)** Cytotoxicity of Ce6, PTX, NPs and NPs@Ce6 with or without NIR light irradiation towards T24 cells (n = 3). NPs@Ce6+SL (0.2 J/cm² for PCI-induced internalization at 1 h of incubation, and 0.4 J/cm² at 6 h of incubation). **d)** Microtubule aggregation in T24 cells after treatment with Ce6, PTX, NPs and NPs@Ce6 with or without NIR light irradiation for 12 h. Red fluorescence for tubulin with Tubulin-Tracker Red and blue for nuclear with DAPI. Scale bar: 20 µm. **e, f)** flow cytometry analysis of cell cycle distribution in T24 cells after treatment with Ce6, PTX, NPs and NPs@Ce6. Mean ± SD (n = 3). * P < 0.05, **** P < 0.0001. **g, h)** flow cytometry analysis of apoptosis in T24 cells after treatment with Ce6, PTX, NPs and NPs@Ce6 with or without NIR light irradiation. Mean ± SD (n = 3), **** P < 0.0001.

Figure 4. *In vivo* anticancer treatment. **a)** Establishment of a human bladder cancer PDX model in BALB/C nude mice for evaluation of the antitumor effect with representative H&E staining images for the parental tumor and xenografts of the first (F1), third (F3) and fifth generation (F5). Xenografts (F5) were used for *in vivo* imaging and drug treatment. **b)** *In vivo* fluorescent images of mice bearing PDX tumors treated with NPs@Ce6 and free Ce6 (5 µg/mL) at different time-points, and ex vivo fluorescent images for harvested tumors and major organs at the end of *in vivo* imaging (48 h). **c)** Representative CLSM images of tumor tissues treated with NPs@Ce6 or Ce6 for 12 h. Red fluorescence denotes to Ce6, Green fluorescence denotes to CD31 for blood vessels, and DAPI fluorescence denotes to nuclei. Scale bar: 100 µm. **d)** *In vivo* pharmacokinetic curves of the Ce6 concentration after intravenous injection of NPs and Ce6, respectively (Mean ± SD, n = 5). **e)** Experiment procedure for injection of different formulations with 4 doses and light irradiation. **f)** Representative whole-animal photographs of mice bearing PDX tumors after treatment. Red circles refer to the region with PDX tumors. **g)** Changes in tumor volume in mice treated with saline, Ce6, PTX, NPs and NPs@Ce6 with or without NIR light irradiation (660 nm, 6 min at 250 mW/cm²), * P < 0.05, ** P < 0.01 and ns = not significant (n = 5). PCI-induced internalization and penetration were performed under 660 nm NIR light irradiation for 2 min at 250 mW/cm². **h)** Tumor weights from bladder cancer PDX mice after treatment (Mean ± SD, n = 5, ** P < 0.01). **i)** Photographs of tumors harvested from mice on day 19 after different treatments. Red circles refer to eliminated tumors. **j)** Images of H&E staining, IHC staining for Cleaved Caspase-3, and TUNEL assays for bladder cancer PDX tumors. In the IHC staining, brown for apoptotic cells. In TUNEL assays, green for apoptotic cells. Scale bar: 100 µm.

Figure 5. Mechanisms for combining PDT with PTX for anticancer treatment. **a)** Heatmap of differentially expressed genes (DEGs) in tumors from bladder cancer PDX models after treatment, measured by RNA-seq. Red for upregulation and blue for downregulation. P adjusted value < 0.05. Three biologically independent animals per group (n = 3). Key DEGs were listed on the right. **b)** Volcano plots of DEGs in tumors after NPs@Ce6+L and Ce6+L treatment. **c)** Gene Ontolog (GO) term enrichment analysis of up-regulated DEGs after NPs@Ce6+L treatment compared with Ce6+L treatment. **d)** GO term enrichment analysis of down-regulated DEGs after NPs treatment compared with the Control; **e)** Gene signatures with positive enrichment scores from oxidative phosphorylation, DNA repair, and mitochondria gene module and with negative enrichment scores for TGF-beta signaling, TNFα signaling via NF-κB, and hypoxia from Gene Set Enrichment Analysis (GSEA) in tumors treated NPs@Ce6+L, comparing to those treated with Ce6+L. **f)** Western blotting plots of proteins for apoptosis (PARP, cleaved PARP, Caspase-3, Cleaved Caspase-3, Bcl-2, and Bax), DNA damage (γH2A.X), and cytoskeletal filaments (α/β-tubulin and pan-actin) in T24 cells after treatment with blank, Ce6, PTX, NPs, NPs@Ce6 with or without NIR light irradiation (660 nm, 0.6 J/cm²). **g)** Semiquantitative analysis of protein levels in different mice groups from Figure 5e. **** P < 0.0001. **h)** Schematic mechanisms of the anticancer effect of the combinational PTX and PDT treatment against bladder cancer.











