Reactive Oxygen Species-Responsive Nanoparticles Based on PEGlated Prodrug for Targeted Treatment of Oral Tongue Squamous Cell Carcinoma by Combining Photodynamic Therapy and Chemotherapy

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ABSTRACT: In this study, a reactive oxygen species (ROS)-responsive nanoparticle system was designed for combining photodynamic therapy (PDT) and chemotherapy for oral tongue squamous cell carcinoma (OTSCC)-targeted treatment. A PEGlated prodrug (RPTD) of doxorubicin (DOX) via thiokeletal linkage and cRGD peptide modification was synthesized and then used to prepare nanoparticles for encapsulating photosensitizer hematoporphyrin (HP). Thus, the obtained HP-loaded RPTD (RPTD/HP) nanoparticles had a regular spherical shape and small size, approximately 180 nm. The RPTD/HP nanoparticles showed a remarkable PDT efficiency and successfully induced ROS generation upon laser irradiation both in vitro and in vivo. DOX exhibited significant ROS-responsive release property from RPTD/HP nanoparticles because of the rupture of the thiokeletal linker. In OTSCC cells, RPTD/HP nanoparticles were efficiently internalized and showed potent effects on cell growth inhibition and apoptosis induction after laser irradiation. In OTSCC tumor-bearing mice, RPTD/HP nanoparticles displayed excellent tumor-targeting ability and notably suppressed tumor growth through multiple mechanisms after local laser irradiation. Taken together, we supplied a novel therapeutic nanosystem for OTSCC treatment through combining PDT and chemotherapy.

KEYWORDS: ROS-responsive, nanoparticle, photodynamic therapy, chemotherapy, oral tongue squamous cell carcinoma

INTRODUCTION

Oral tongue squamous cell carcinoma (OTSCC) is the commonest oral cancer. Even with combined treatment of surgical operation, chemotherapy, and clinical radiation, the global 5-year survival rate of OTSCC patients is less than 60% because of local-regional recurrence and lymph node metastasis.¹,² Many OTSCC patients are diagnosed definitely at the middle or late stage, and thus unfortunately lose the chance of surgery. Furthermore, ablative surgery is often unacceptable to OTSCC patients because it will severely impair the oral structure and functions, for example, speech, mastication, and swallowing. Other traditional treatments such as chemotherapy and radiotherapy also cannot suppress OTSCC deterioration effectively because of their lack of tumor-targeting ability and/or their significant toxicities.³,⁴ Besides, drug resistance induced by long-term chemotherapy will also reduce therapeutic efficacy and further lead to treatment failure.⁵ In view of these facts, it is increasingly necessary to explore effective therapeutic strategies for improving the survival and life quality of OTSCC patients.

Photodynamic therapy (PDT) is an advanced noninvasive method and has been extensively applied for treating various cancers and nonmalignant diseases. PDT involves three key comments, light, photosensitizer, and molecular oxygen.⁶ Photosensitizers can undergo photochemical reaction upon activation with laser irradiation at a specific wavelength and subsequently induce the generation of reactive oxygen species (ROS),⁷ which can directly kill cancer cells by inducing oxidative damages to lipids, proteins, and nucleic acids.⁸,⁹ A large amount of ROS can also destroy tumor vasculature¹⁰ and activate antitumor immunity of the host,¹¹,¹² thus exerting indirect anticancer effects. Some investigations have also shown that PDT can distinctly enhance chemotherapy efficacy in drug-resistant cancers.¹³ It can be seen that PDT has many advantages for OTSCC treatment and is favorable for preserving the oral structure and functions.

However, there are limitations in the clinical applications of photosensitizers by their poor water-solubility, short circulation half-life, and low tumor-targeting ability.¹⁴ The application of nanocarrier technology can overcome these limitations. Nanocarriers have many advantages for carrying anticancer drugs such as good solubilization to indissolvable

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drugs, tumor-targeted drug delivery, drug delayed and controlled release, and high bioavailability. As is known to all, nanocarriers can deliver drugs targeting tumors via the enhanced permeability and retention (EPR) effect, and furthermore can enhance drug accumulations in the tumor site through surface-modification of antibodies or ligands for the receptors overexpressed by cancer cells. The RGD (arginine–glycine–aspartic acid) peptide is a well-known ligand of integrin αvβ3, which is often overexpressed in malignant tissues such as OTSCC. Hence, many RGD peptide-modified nanomaterials have been prepared for targeted delivery of photosensitizers. Additionally, nanocarriers can simultaneously encapsulate photosensitizers and other therapeutic agents (chemotherapeutic drug, gene or immune regulator), and thus obtain synergistic anticancer effects by combining PDT with other treatment methods. Recently, smart nanocarriers that can respond to stimulus outside such as enzyme, pH, temperature, ROS, and ultrasound wave are attracting more and more attention because of their controlled and on-demand drug release properties. In consideration of the induction effect of PDT on ROS generation, ROS-responsive nanocarriers have unique advantages on combining PDT with other treatments. For example, the thioketal bond can be rapidly cleaved upon ROS, and currently it has been used as a chemical linker to design ROS-responsive nanocarrier for codelivery of a photosensitizer and a chemotherapeutic drug. The ROS generation triggered by photochemical reaction can destabilize these nanocarriers, thus releasing a chemotherapeutic drug and obtain synergistic anticancer effects.

In view of the facts described above, we designed a ROS-responsive nanoparticle system for combining PDT and chemotherapy for OTSCC-targeted treatment. As shown in Scheme 1, a PEGlated prodrug of doxorubicin (DOX) is synthesized through thioketal linkage and cRGD (cyclo-arginine–glycine–aspartic acid–D-phenylalanine–cysteine)
peptide modification. This prodrug, named as RPTD, has an amphiphilic property and can form nanoparticles in aqueous media through self-assembly. Photosensitizer hematoporphyrin (HP) has a large ring π conjugate structure and can be efficiently encapsulated into RPTD nanoparticles via π−π stacking interaction according to our previous report,1,3,4, thus obtaining HP-loaded RPTD (RPTD/HP) nanoparticles. Scheme 1B illustrates the function mechanisms of RPTD/HP nanoparticles on OTSCC by combining PDT and chemotherapy. RPTD/HP nanoparticles can reach and be accumulated in the tumor through the EPR effect and specific binding of the cRGD peptide with integrin αvβ3 overexpressed on OTSCC cells. After cell internalization, HP loaded by RPTD/HP nanoparticles can induce the intracellular ROS generation upon laser irradiation, thereby leading to the mitochondria-mediated cell apoptosis. Meanwhile, the thiolketal linker between DOX and PEG will rupture by the ROS, and subsequently DOX can be released from RPTD/HP nanoparticles to exert its antitumor effects. All these show that RPTD/HP nanoparticles will facilitate OTSCC treatment through combining PDT and chemotherapy. In this study, the synergistic efficacy of RPTD/HP nanoparticles against OTSCC was also investigated both in vitro and in vivo.

### EXPERIMENTAL SECTION

#### Materials

Diacectoxy thiolkeletal was synthesized by us according to the lab according to our previous report.33 Maleimide-PEG-amine (MAL-PEG-NH2, mPEG MW = 2000) was obtained from Yare Biotech Inc. (Shanghai, China). DOX-HCl, HP, N-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethyl amino-propyl)carbodiimide hydrochloride (EDC) were purchased from J&K Scientific Ltd. (Beijing, China). The cRGD peptide with a purity of 96.8% was obtained from Synpeptide Co., Ltd. (Nanjing, China). Cyanine 5.5 (Cy5.5) and singlet oxygen sensor green (SOSG) were obtained from Thermo Fisher Scientific (Waltham, USA). Rhodamine 123 (Rh123), 4′,6-diamidino-2-phenylindole (DAPI), and 2′,7′-dichlorofluorescein in diacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, USA).

The human OTSCC cell line, CAL-27, was obtained from American Type Culture Collection (Manassas, VA, USA). The human oral epithelial cell line (HOEC) was obtained from BeNa Culture Collection (Beijing, China). Both CAL-27 and HOEC cells were cultured in L120-Dulbecco’s modified Eagle’s medium ( Gibco, Life Technologies, USA) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. The cells were incubated at 37 °C under an atmosphere of 5% CO2. For PDT treatment, CAL-27 cells were irradiated by a 633 nm laser for 10 min at an intensity of 100 mW/cm² according to the method we previously reported.24

Female BALB/c nude mice with a body weight of 20 ± 2 g were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were raised in a specific pathogen-free environment and fed with normal diets. The animal model for OTSCC was established by subcutaneously injecting CAL-27 cells into the flanks of mice at 3.0 × 10⁷ cells/mouse. For PDT treatment, the tumors in the mice were irradiated by a 633 nm laser for 10 min at an intensity of 100 mW/cm² according to the method we previously reported.4 All the procedures mentioned above were approved by the Tianjin Medical University Animal Care and Use Committee and executed according to the China Physiological Society “Guiding Principles in the Care and Use of Animals”.

#### Synthesis and Characterization of RPTD

mPEG-thiolkeletal-DOX conjugate (PTD) was first synthesized by connecting mPEG and DOX, respectively, to the two end carboxyl groups of diacetoxyl thiolkeletal through amide bonds. Briefly, 22 mg of diacetoxyl thiolkeletal (0.1 mmol), 12 mg of NHS (0.1 mmol), and 20 mg of EDC (0.1 mmol) were mixed in 4 mL of anhydrous dimethyl sulfoxide (DMSO) and stirred for 2 h at room temperature to activate one end carboxyl group of diacetoxyl thiolkeletal. mPEG (200 mg, 0.1 mmol) was dissolved in 10 mL of DMSO and then added dropwise into the above mixture. After stirring for 36 h, the reactant solution was transferred into a dialysis bag with 1 kDa molecular weight cut-off (MWCO), purified by dialysis in deionized water, and further freeze-dried to obtain mPEG-thiolkeletal carbodiimide acid (PTD). Next, PT was mixed with EDC/NHS in DMSO at an equimolar ratio for 2 h. DOX-HCl was desalted overnight in triethylamine solution and then mixed with EDC/NHS-activated PT. After 48 h of reaction at room temperature, the reactant solution was purified by dialysis as mentioned above and finally freeze-dried to obtain PTD. It should be noted that all steps were done in the dark.

The cRGD peptide was next conjugated to PTD through the Michael addition reaction between the sulfhydryl group in the cRGD peptide and maleimide moiety in mPEG. Briefly, PTD was dissolved in DMSO and diluted with deionized water to obtain a final concentration of 10 mg/mL, thus forming a micellar dispersion. The cRGD peptide was dissolved in deionized water at a concentration of 1 mg/mL and subsequently added into the above solution at a cRGD peptide/PTD weight ratio of 1/5. Next, the mixed solution was stirred under nitrogen atmosphere for 2 h at room temperature in the dark, and then purified by dialysis to remove unreacted cRGD peptide. Finally, PTD was obtained by freeze-drying.

The chemical structures of mPEG, cRGD peptide, PT, PTD, and RPTD nanoparticles were confirmed by Fourier transform infrared spectroscopy and proton nuclear magnetic resonance spectroscopy (1H NMR) using a NEXUS 470 IR spectrometer (Nicoleot, USA) and AVANCE III NMR spectrometer (400 MHz, Bruker, Germany). The DOX contents in PTD and RPTD were detected using an ultraviolet–visible (UV–vis) spectrophotometer (U-3310, Hitachi, Japan) at 488 nm.

#### Preparation and Characterization of RPTD/HP Nanoparticles

RPTD/HP nanoparticles containing HP and DOX were prepared by self-assembly using the dialysis method. In detail, HP was first dissolved in methanol at a concentration of 1.0 mg/mL and stirred overnight at room temperature. After being dissolved in DMSO at a concentration of 10 mg/mL, RPTD was added dropwise into the above solution of HP (DOX/HP molar ratio was 1/4). Next, the mixture was placed into a dialysis bag with 2 kDa MWCO after 12 h of stirring in the dark, and dialyzed in deionized water for 12 h with water change every 3 h, finally obtaining RPTD/HP nanoparticles. For comparison, HP-loaded PTD (PTD/HP) nanoparticles (without modification of the cRGD peptide) were prepared by the same method, and RPTD and PTD nanoparticles without loading of HP were also prepared meanwhile.

The morphologies of nanoparticles prepared above were characterized using a HT7700 transmission electron microscope (TEM, Tokyo, Japan). The size and size distributions of nanoparticles were determined using a Zeta sizer Nano ZS detector (Malvern Instruments, Worcestershire, UK). The in vitro stabilities of PTD/HP and RPTD/HP nanoparticles were investigated through monitoring the changes of their size and size distribution during 5 day storage in deionized water and 10% FBS solution. Furthermore, we also evaluated the π−π stacking interaction between HP and RPTD by monitoring the intermolecular fluorescence quenching. RPTD was mixed with HP in methanol at a DOX/HP molar ratio of 1/4, and then the fluorescence emission spectrum of this mixture was recorded on a RF-5301 fluorescence spectrophotometer (Shimadzu, Japan) at an emission wavelength of 395 nm. The fluorescence emission spectra of free HP and free DOX were also scanned at the same HP and DOX concentrations.

#### Evaluation of ROS-Responsivity of RPTD/HP Nanoparticles

First, we evaluated the ROS-responsive cleavage ability of the thiolkeletal linker in PTD using the NMR method as reported previously.29 In detail, PTD was incubated in DMSO solution containing H₂O₂ (400 mM) and CuCl₂ (3.2 μM) for 48 h at 37 °C. Then, the solution was placed into a dialysis bag with 1 kDa MWCO and dialyzed in deionized water to fully remove DMSO, and further freeze-dried to acquire the sample. Finally, the 1H NMR spectrum of
this sample was detected using an AVANCE III NMR spectrometer and compared with that of PTD.

Next, the ROS generations triggered by RPTD/HP nanoparticles upon different times of laser irradiation at 633 nm at 100 mW/cm² were detected using a fluorescent probe of SOSG.35 For comparison, the ROS generations in solutions of phosphate buffered saline (PBS), free DOX, free HP, and PTD/HP nanoparticles were also detected after laser irradiation. The concentrations of HP and DOX used in the above experiments were 4.0 and 1.0 μg/mL, respectively.

Finally, we evaluated the ROS-responsive release of DOX from RPTD/HP nanoparticles. RPTD/HP nanoparticles were irradiated by a 633 nm laser for 10 min at 100 mW/cm². Then, these nanoparticles were placed into dialysis bags with 1 kDa MWCO and dialyzed separately in PBS solutions with pH values of 7.4 and 6.5 at 37 ± 2 °C under shaking at 100 rpm. At the scheduled times, 0.5 mL of released media were collected and 0.5 mL of fresh media were added at the same time. Finally, the DOX concentrations in the released media were measured by a UV−vis spectrophotometer at 488 nm. For comparison, the DOX releases from RPTD/HP nanoparticles without laser irradiation were also evaluated in PBS solutions at pH 7.4 and 6.5.

Cellular Uptakes of PTD/HP and RPTD/HP Nanoparticles. Cellular uptakes and intracellular distributions of PTD/HP and RPTD/HP nanoparticles in CAL-27 and HOEC cells were first visualized by confocal microscopy according to our previous report.36 The cells were incubated with PTD/HP and RPTD/HP nanoparticles for 6 h at the HP and DOX concentrations of 4.0 and 1.0 μg/mL, respectively. After staining with DAPI, the cells were observed under an FV-1000 confocal microscope (Olympus, Tokyo, Japan). Moreover, we also observed the intracellular locations of PTD/HP nanoparticles in CAL-27 cells at 2, 6, and 12 h after laser irradiation. Additionally, the uptakes of PTD/HP and RPTD/HP nanoparticles by CAL-27 and HOEC cells were quantitatively detected using flow cytometry. After 6 h of incubation with PTD/HP and RPTD/HP nanoparticles, the cells were digested, collected, and washed sequentially, and then resuspended in PBS. Afterwards, the cellular fluorescence intensities were detected using flow cytometry (Beckman Coulter, California, USA).

Detection of Intracellular ROS Generation. The ROS generations in CAL-27 cells triggered by RPTD/HP nanoparticles with laser irradiation were assessed by flow cytometry and confocal microscopy according to the method we previously reported.32 For flow cytometric detection, the cells were incubated with free DOX, free HP, PTD/HP, and RPTD/HP nanoparticles for 2 h at the HP and/or DOX concentrations of 4.0 and 1.0 μg/mL, respectively. Some of these cells were irradiated by laser, and then all the cells were further incubated for 12 h. After that, the cells were processed with DCFH-DA, and then the intracellular fluorescence intensities were detected using a flow cytometer. For confocal microscopic observation, the cells were treated by the same method as mentioned above, stained with DAPI, and finally observed under a confocal microscope.

Cytotoxicity Assessment. Cytotoxicities of RPTD/HP nanoparticles alone and combined with laser irradiation in CAL-27 cells were determined using the MTT assay. Briefly, the cells were incubated with various concentrations of free DOX, free HP, PTD/HP, and RPTD/HP nanoparticles for 2 h. Some of these cells were processed with laser irradiation, and then all the cells were further incubated for 48 h. Next, the cells were treated with the MTT reagent and then detected using an ELX800 absorbance microplate reader (Bio-Tek Epoch, Winooski, USA). According to the previous report,37 we further calculated the combination index (CI) values of PTD/HP and RPTD/HP nanoparticle-mediated combination treatments of PDT and chemotherapy. The calculation formula was shown as follows, and the CI value >1, =1, and <1 represented antagonism, additivity, and synergism, respectively.

\[
\text{CI} = \frac{IC_{50} \text{(HP in nanoparticles with laser irradiation)}}{IC_{50} \text{(HP in nanoparticles)}} + \frac{IC_{50} \text{(DOX in nanoparticles with laser irradiation)}}{IC_{50} \text{(DOX in nanoparticles)}}
\]

We also used the LIVE/DEAD Cell Staining Kit to visualize the cytotoxicity of RPTD/HP nanoparticles combined with laser irradiation in CAL-27 cells. The cells received the same treatments as described above for the MTT assay, and were then stained with calcein-AM and ethidium bromodimer-1. After that, the cells were imaged using an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Cell Apoptosis Assay. CAL-27 cells were seeded into 12-well plates at a density of 1 × 10⁴ cells/well. After preincubation of 24 h, the cells were treated with free DOX, free HP, PTD/HP, and RPTD/HP nanoparticles for 2 h at the HP and DOX concentrations of 4.0 and 1.0 μg/mL, respectively. Some of these cells were processed with laser irradiation, and then all the cells were further incubated for 24 h. Next, the cells were treated with the Annexin V-APC Apoptosis Detection Kit (BD Biosciences, USA) and finally analyzed using flow cytometry.

Evaluations of Mitochondrial Membrane Damage and Subcellular Locations of Cytochrome c. The same treatments were carried out in CAL-27 cells as described above for the cell apoptosis assay. To detect the damage of the mitochondrial membrane, the treated cells were stained sequentially with Rh123 and Hoechst 33342 (Invitrogen, Carlsbad, USA), and finally imaged by confocal microscopy. To observe the subcellular locations of cytochrome c (Cyt c), the treated cells were stained with MitoTracker Green (M7514, Eugene, USA), fixed with 4% paraformaldehyde (PFA), and blocked with 1% BSA. After that, the cells were processed sequentially with anti-Cyt c monoclonal antibody (Abcam, Cambridge, UK, 1:1000 of dilution) and Alexa-647-conjugated goat antirabbit antibody (Life Technologies, 1:300 of dilution). Finally, the Cyt c subcellular locations were observed under a confocal microscope.

Tissue Distribution and Intratumoral Accumulation of RPTD/HP Nanoparticles. Cy5.5, a near-infrared fluorescent dye, was first encapsulated into PTD/HP and RPTD/HP nanoparticles to prepare PTD/HP/Cy5.5 and RPTD/HP/Cy5.5 nanoparticles. Briefly, Cy5.5 and HP were mixed in methanol at a weight ratio 1/10 and stirred for 12 h. Next, this mixture was processed using the method described above for the preparation of PTD/HP and RPTD/HP nanoparticles, and thus obtained PTD/HP/Cy5.5 and RPTD/HP/Cy5.5 nanoparticles.

Next, we evaluated biodistributions and intratumor accumulations of PTD/HP/Cy5.5 and RPTD/HP/Cy5.5 nanoparticles in OTSCC mice using the living fluorescent imaging technique. In detail, CAL-27 tumor-bearing mice were randomly divided into four groups with 3 mice/group and then intravenously injected with sterile physiological saline (the control), free Cy5.5, PTD/HP/Cy5.5, and RPTD/HP/Cy5.5 nanoparticles, respectively. At the scheduled times, these mice were imaged using a vis in vivo imaging system (PerkinElmer, Waltham, USA). Afterwards, all the mice were sacrificed, and their major organs (heart, liver, kidney, spleen, and lung) and tumors were collected for observing the fluorescence distributions under an in vivo imaging system.

Evaluation of in Vivo Antitumor Activities of RPTD/HP Nanoparticles. CAL-27 tumor-bearing mice were randomly divided into 10 groups with 5 mice/group and treated separately with sterile physiological saline (the control), free DOX, free HP, PTD/HP, and RPTD/HP nanoparticles, in combination with and without laser irradiation. All treatments underwent intravenous injection once every 3 d for four consecutive times. The doses of HP and DOX were, respectively, 12 and 3 mg/kg. At 24 h after administration, the tumors in the mice were processed with laser irradiation. During the whole treatment period, the tumor sizes and mouse body weights were monitored continuously. After treatments, all the mice were sacrificed.
and their major organs and tumors were removed for further experiments.

The above tissue and tumor samples were fixed with 4% PFA, embedded with paraffin, and then cut into sections with 5 μm thickness. For histopathological analysis, these sections were stained with hematoxylin & eosin (H&E) and then observed under a microscope. For further evaluation of tumor angiogenesis, the tumor sections were stained immunohistochemically with rabbit polyclonal antibody against CD31 (Abcam, Cambridge, UK) according to the method we previously reported.32 After staining with hematoxylin, these sections were finally observed using a fluorescence microscope.

Statistical Analysis. All detections underwent at least three isolated experiments and all data were demonstrated using means ± SD deviation. Student’s t-test was used for statistical analysis and a P value of less than 0.05 denoted statistical significance.

■ RESULTS AND DISCUSSION

Synthesis and Characterization of RPTD. RPTD, a ROS-responsive PEGlated prodrg of DOX, was synthesized and then used to prepare nanoparticles for carrying a photosensitizer in this study. The synthesis route of RPTD is shown in Scheme 1A. First, PDT was synthesized by connecting mPEG and DOX separately to the two end carboxyl groups of diacetoxyl thioketal through amide bonds and characterized by the IR and NMR methods. Figure 1A shows the IR spectra of diacetoxyl thioketal, mPEG, PTD, and assignments of characteristic peaks. In the IR spectrum of PDT, the stretching vibrations of the benzene skeleton in DOX and ether bond in the PEG backbone were obviously visible at 1500–1600 and 1100 cm⁻¹, respectively. Figure 1B shows the ¹H NMR spectra and signal assignments of diacetoxyl thioketal in deuterated DMSO, mPEG, and PTD. Figure 1C shows the ¹H NMR spectra of RPTD, PTD, and cRGD.
responsive cleavage ability of the thioketal linker in PTD using the NMR method previously reported.26 After incubation in DMSO solution containing H2O2 and CuCl2, mimicking a ROS environment, the proton signal of methyl groups in thioketal at 1.5 ppm was notably weakened (Figure 1C). This indicated that the thioketal bond in PTD was partially cleaved by ROS.

The cRGD peptide is highly specific for integrin ανβ3 that is often overexpressed by OTSCC cells.17,18 Hence, we used the cRGD peptide to prepare a novel OTSCC-targeted drug carrier in this study. As shown in Scheme 1A, the cRGD peptide was conjugated with PTD to synthesize RPTD through the Michael addition reaction between the sulfydryl group in the cRGD peptide and maleimide moiety in mPEG. The chemical structure of RPTD was characterized using the NMR method in deuterated DMSO. The proton signals of the benzene ring (7.2–7.5 ppm) and the amino groups (8.0–8.5 ppm) in the cRGD peptide were clearly visible, and meanwhile the proton signal of the maleimide group at 6.7 ppm almost completely disappeared in the 1H NMR spectrum of RPTD (Figure 1D). Thus, it can be seen that RPTD was successfully synthesized through a highly efficient coupling reaction between the cRGD peptide and PTD. The DOX contents in PTD and RPTD were detected using UV−vis spectrophotometry and their values were approximately 17.8 and 13.5%, respectively.

Characterization and ROS-Responsive Drug Release Behavior of RPTD/HP Nanoparticles. Because of the existence of both hydrophobic DOX and hydrophilic PEG, RPTD had a significant amphiphilic property. Therefore, it could self-assemble to form nanoparticles through hydrophobic interaction in aqueous media according to our previous report.27 By using a simple dialysis method, RPTD nanoparticles were prepared and exhibited a spherical shape with a small size of approximately 160 nm. The photosensitizer HP has a large ring π conjugate structure in a molecule, and therefore it could be efficiently encapsulated into RPTD nanoparticles through π−π stacking. First, we evaluated the π−π stacking interaction between HP and RPTD using a fluorescence quenching method. As shown in Figure 2A, the mixture of HP and RPTD displayed significantly reduced fluorescence signals as compared to both free HP and RPTD at the same concentrations, confirming a strong π−π stacking interaction between these two molecules. RPTD/HP nanoparticles prepared at a DOX/HP molar ratio of 1/4 had a spherical shape (Figure 2B) and a small size of about 180 nm with a relatively narrow distribution (Figure 2C). During 5 days of storage in deionized water and 10% FBS, RPTD/HP nanoparticles maintained almost unchanged sizes (Figure S1A) and polydispersity indexes (Figure S1B), demonstrating their excellent in vitro stability.

In view of the ROS-responsive cleavage of the thioketal linker between DOX and PEG in RPTD, we believed DOX could be controllably released from RPTD/HP nanoparticles through the HP-induced ROS generation upon laser irradiation. To evaluate this ROS-responsive drug release behavior, we first monitored the ROS generations induced by RPTD/HP nanoparticles after different times of laser irradiation by using a fluorescence probe SOSG. The detection results are shown in Figure 2D. Compared to PBS and free DOX, free HP, PTD/HP, and RPTD/HP nanoparticles notably enhanced the SOSG fluorescence intensities during the 10 min laser irradiation period, indicating that large amounts of ROS were produced in these systems. At the same time.
HP concentrations, the ROS productions induced by PTD/HP and RPTD/HP nanoparticles were almost equivalent to those induced by free HP. This meant that HP maintained a high PDT efficiency even after encapsulation into these nanoparticles. Next, we detected the in vitro DOX releases from RPTD/HP nanoparticles at different pH values in the case of laser irradiation or not. The DOX release profiles are shown in Figure 2E. The DOX release was notably accelerated after laser irradiation (100 mW/cm², 10 min) at both pH 7.4 and pH 6.5, indicating that the thioketal linkage between DOX and PEG was successfully cleaved by the generated ROS. Additionally, DOX had an accelerated release rate at pH 6.5 from RPTD/HP nanoparticles whether with or without laser irradiation. This was perhaps because DOX has an enhanced solubility in weak acid solution than in neutral and alkaline ones.

**Evaluation of Specific Cellular Uptake and Subcellular Distribution.** It is well known that modification with targeting peptide can provide specific interactions between nanoparticles and cancer cells, and therefore can influence tumor-accumulation, cellular internalization, and intracellular location of nanoparticles. Integrin αvβ3, a common cell adhesion molecule overexpressed in tumor vasculature and some cancer cells, plays key roles in tumor neovascularization and cancer cell migration, invasion, and metastasis. The cRGD peptide is a potent antagonist for integrin αvβ3 and has been widely used as an effective cancer-targeting ligand. Because OTSCC cells often overexpress integrin αvβ3, we prepared RPTD/HP nanoparticles with surface modification of the cRGD peptide, and further investigated their specific cellular uptake and subcellular distribution in OTSCC cells using the intrinsic fluorescence of DOX. First, we compared the cellular uptakes of PTD/HP and RPTD/HP nanoparticles in integrin αvβ3 highly expressed OTSCC CAL-27 cells and low-expressed normal HOEC cells. As compared with PTD/HP nanoparticles, RPTD/HP nanoparticles exhibited significantly stronger fluorescence signals in CAL-27 cells after 6 h of incubation (Figure 3A,B). However, this difference was not visible in HOEC cells (Figure 3C,D). These results suggested that RPTD/HP nanoparticles could be specifically internalized into CAL-27 cells through the mediation of the cRGD peptide. Free DOX could rapidly diffuse into CAL-27 cells and was mostly located in the cell nucleus (Figure S2), whereas DOX loaded by RPTD/HP nanoparticles was mainly distributed in the cytoplasm (Figure 3A). After laser irradiation, DOX was obviously released from RPTD/HP nanoparticles and entered the nuclei of CAL-27 cells (Figure S3). All these results suggested that DOX can be controllably released from RPTD/HP nanoparticles through the cleavage of the thioketal bond triggered by the PDT-induced ROS generation.

**Intracellular ROS Generation Triggered by RPTD/HP Nanoparticles with Laser Irradiation.** As the effect or molecules of PDT, ROS can cause oxidative stress in cancer cells, thus leading to cell apoptosis or necrosis. To evaluate the PDT efficiency, we detected the ROS generation in CAL-27 cells after 6 h of incubation with RPTD/HP nanoparticles and 10 min of laser irradiation. DCFH-DA, a cell-permeable nonfluorescent precursor, can be cleaved by the intracellular esterases and then transformed into the fluorescent dichlorofluorescein (DCF) upon reaction with intracellular ROS. After treatment of RPTD/HP nanoparticles with laser irradiation, CAL-27 cells displayed intensive green fluorescence (Figure S4A) and the fluorescence intensity reached a high level at 12 h (Figure S4B). This demonstrated that RPTD/HP nanoparticles could efficiently induce the intracellular ROS generation. The fluorescence signals of DCF in cells treated with free HP, PTD/HP, and RPTD/HP nanoparticles were significantly enhanced at 12 h after laser irradiation (Figure 4A), confirming the strong PDT efficiencies of these treatments. We further quantitatively compared the intracellular ROS levels induced by different treatments using flow cytometry. As compared to both free HP and PTD/HP nanoparticles, RPTD/HP nanoparticles showed an obviously higher induction effect on the ROS generation after laser irradiation (Figure 4B,C), which was because more amount of HP entered CAL-27 cells through the delivery of RPTD/HP nanoparticles because of the mediation of the cRGD peptide.

**Synergistic Effects of RPTD/HP Nanoparticles with Laser Irradiation against OTSCC Cells.** Synergistic effects of PDT/chemotherapy combination treatment mediated by RPTD/HP nanoparticles were investigated in OTSCC cells. First, cytotoxicities of different treatments were detected by MTT assay and the results are shown in Figure 5A. Free HP only slightly inhibited the growth of CAL-27 cells when its concentration increased; on the contrary, PTD/HP and RPTD/HP nanoparticles exhibited evidently enhanced cytotoxicities at the same HP concentrations. This demonstrated that DOX loaded by these nanoparticles was released partially and then exerted its cytotoxicity. After 10 min of laser irradiation, the cytotoxicities of free HP, PTD/HP, and RPTD/HP nanoparticles all increased notably, confirming their strong PDT efficiencies. Furthermore, PTD/HP and RPTD/HP nanoparticles showed more potent cytotoxicities than free HP. This meant that most of the DOX was released from these nanoparticles through ROS-triggered cleavage of the thioketal linker between DOX and PEG. However, laser irradiation did not influence the cytotoxicity of free DOX in...
CAL-27 cells (Figure S5). The synergistic effects of PDT and chemotherapy combined treatments mediated by PTD/HP and RPTD/HP nanoparticles were also evaluated, and their CI values were approximately 0.89 and 0.68 (<1). These results indicated that these treatments had synergistic cytotoxicity in CAL-27 cells. Moreover, RPTD/HP nanoparticles displayed slightly higher cytotoxicity than PTD/HP nanoparticles whether with or without laser irradiation, which was because RPTD/HP nanoparticles delivered more amounts of HP and DOX into CAL-27 cells to exert synergistic effects.

Next, we used the LIVE/DEAD Cell Staining Kit to further visualize the synergistic cytotoxicity mentioned above. As shown in Figure S5B, the live and dead CAL-27 cells after various treatments emitted intensive green and red fluorescence, respectively. The cells treated with laser irradiation were almost fully alive, which indicated that laser irradiation alone could not cause the cell damage. At 24 h after laser irradiation, free HP (4.0 μg/mL), PTD/HP, and RPTD/HP nanoparticles (4.0 μg/mL HP and 1.0 μg/mL DOX) obviously induced the cell death; but by contrast, PTD/HP and RPTD/HP nanoparticles showed more potent induction efficacy, further confirming the synergistic cytotoxicity of PDT and chemotherapy. Similar to the results of the MTT assay, RPTD/HP nanoparticles also exhibited a much higher cytotoxicity than PTD/HP nanoparticles perhaps because of their specific uptake by CAL-27 cells.

Subsequently, we evaluated the apoptosis of CAL-27 cells with different treatments, and the flow cytometry data are shown in Figure S5C. Without laser irradiation, free HP exhibited no significant induction effect on the cell apoptosis, whereas PTD/HP and RPTD/HP nanoparticles both induced the cell apoptosis to some extent, indicating that DOX was partially released from these nanoparticles. Laser irradiation alone only caused the cell apoptosis slightly, but increased notably the apoptosis rates of CAL-27 cells treated with free HP, PTD/HP, and RPTD/HP nanoparticles, which confirmed the strong PDT efficiencies of these treatments. However, RPTD/HP nanoparticles with laser irradiation displayed the highest induction potency on the cell apoptosis, for example, the rate of normal cells was only 37.4%. Additionally, free DOX whether with laser irradiation or not both contributed to the cell apoptosis, but exhibited no significant difference in the rates of apoptotic cells (Figure S6). Accordingly, we deduced that RPTD/HP nanoparticles can induce the apoptosis of OTSCC cells through combining PDT with chemotherapy.

**Cell Apoptotic Mechanism Induced by RPTD/HP Nanoparticles with Laser Irradiation.** Many investigations have reported that the mitochondria-mediated apoptosis plays key roles in antitumor effects of PDT. The ROS generation induced by PDT can cause the depolarization of the mitochondrial membrane and further mediate the release of Cyt c from the mitochondria into the cytoplasm to initiate the cell apoptosis.30 Here, we monitored the depolarization of the mitochondrial membrane in CAL-27 cells with different treatments using Rh123 as a fluorescence probe. When the mitochondrial membrane was depolarized, the change of the mitochondrial membrane potential will excite Rh123 to emit intensive green fluorescence, and thereby we can detect it using confocal microscopy and flow cytometry. Compared to the control, all treated cells exhibited visible green fluorescence, indicating the polarization of the mitochondrial membrane in these cells (Figure 6A). Figure 6B shows the flow cytometry results. CAL-27 cells exhibited similar fluorescence intensities after treatments of free DOX with and without laser irradiation, which should be attributed to the DOX-induced mitochondrial membrane damage as reported previously.31 By comparison, the intracellular fluorescence intensities induced by free HP, PTD/HP, and RPTD/HP nanoparticles were notably enhanced after laser irradiation, indicating their strong PDT efficiencies on the polarization of the mitochondrial membrane. PTD/HP and RPTD/HP nanoparticles, whether with or without laser irradiation, showed markedly higher potencies on the polarization of mitochondrial membrane as compared to free HP. This meant that DOX loaded by these nanoparticles exerted its cytotoxic effects. After laser irradiation, RPTD/HP nanoparticles induced the mitochondrial membrane polarization at a much higher level than that of

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**Figure 4.** Intracellular ROS generations in CAL-27 cells using DCFH-DA as an indicator. (A) Confocal microscopic images of CAL-27 cells treated with free DOX, free HP, PTD/HP, and RPTD/HP nanoparticles. Flow cytometry analysis (B) and comparison of intracellular DCF fluorescence intensities (C) in CAL-27 cells after various treatments. The concentrations of HP and DOX were 4.0 and 1.0 μg/mL, respectively. **P < 0.01 for the comparison between two treatment groups.**
induced by PTD/HP nanoparticles because of their higher cellular uptake.

Cyt c is a small heme-protein in the mitochondrial intermembrane space and its release from the mitochondrial to cytoplasm is believed to be an early indicator of cell apoptosis.33 Hence, we further observed subcellular distributions of Cyt c in CAL-27 cells after various treatments by immunofluorescence staining. The confocal images are shown in Figure 6C. The blue, green, and red fluorescence present the cell nuclear, mitochondria, and Cyt c, respectively. Laser irradiation effectively promoted the release of Cyt c from the mitochondria to the cytoplasm in CAL-27 cells treated with free HP, PTD/HP, and RPTD/HP nanoparticles. Meanwhile, the nuclear translocation of Cyt c was also observed in these treated cells. We believed this was because large amounts of intracellular ROS resulted in the damage of the nuclear membrane, and thus increased its permeability. The results above indicated that RPTD/HP nanoparticles had a powerful PDT efficiency and successfully activated the mitochondrial apoptosis pathway in CAL-27 cells after laser irradiation.

Tissue Distribution and Tumor Accumulation of RPTD/HP Nanoparticles in the OTSCC Model Mice. To evaluate the OTSCC-targeting ability of RPTD/HP nanoparticles, we established a mouse xenograph model by subcutaneously implanting CAL-27 cells into BALB/c nude mice. Cy5.5 was used as a near-infrared fluorescent dye to prepare PTD/HP/Cy5.5 and RPTD/HP/Cy5.5 nanoparticles. The mice bearing the CAL-27 tumor were injected with these nanoparticles via the tail vein, and then their tissue distributions and tumor accumulations were assessed using the in vivo imaging technique. Compared to free Cy5.5, PTD/HP/Cy5.5 and RPTD/HP/Cy5.5 nanoparticles displayed significantly slowed elimination rates and increased tumor distributions (Figure 7A). They began to appear in the tumors...
at 4 h and showed visible tumor-accumulations at 8 and 24 h. Here, the EPR effect played an important role in the tumor-accumulations of PTDH/Cy5.5 and RPTDH/Cy5.5 nanoparticles because of their nanoscaled sizes. At 24 h post administration, the main organs and tumors were removed from the mice and then analyzed using a fluorescence imaging system. Compared with PTD/HP/Cy5.5 nanoparticles, RPTD/HP/Cy5.5 nanoparticles exhibited notably increased tumor-accumulation (Figure 7B), which should result from the specific binding of the cRGD peptide with integrin αvβ3 overexpressed on the CAL-27 cells. The above results meant that RPTD/HP nanoparticles had excellent OTSCC-targeting

Figure 6. Activation effects of RPTD/HP nanoparticles on the mitochondrial apoptosis pathway. (A) Confocal microscopic images of CAL-27 cells treated with free DOX, free HP, PTD/HP, and RPTD/HP nanoparticles alone and with laser irradiation. Rh123 was used as a probe for detecting the mitochondrial membrane potentials with green fluorescence. (B) Intracellular mean fluorescence intensities determined by flow cytometry after various treatments. (C) Subcellular locations of Cyt c in CAL-27 cells after various treatments. The mitochondria were stained with M7514 and emitted green fluorescence. Cyt c was immunofluorescence stained with anti-Cyt c antibody and emitted red fluorescence. The concentrations of HP and DOX were 4.0 and 1.0 μg/mL, respectively. *P < 0.05 and **P < 0.01 for the comparison between two treatment groups.

Figure 7. Tissue distributions and intratumor accumulations of free Cy5.5, PTD/HP/Cy5.5 and RPTD/HP/Cy5.5 nanoparticles in CAL-27 tumor-bearing mice. (A) In vivo fluorescence images of mice at different times post administration. (B) Fluorescence images of hearts, livers, kidneys, spleens, lungs, and tumors removed from the mice at 24 h post administration.
ability and could realize the tumor-targeted delivery of HP and DOX.

**In Vivo Antitumor Activities of RPTD/HP Nanoparticles with Laser Irradiation.** Encouraged by the synergistic effects of PDT/chemotherapy combination treatment based on RPTD/HP nanoparticles in vitro and their remarkable tumor-targeting property in vivo, we further investigated the antitumor efficiency of RPTD/HP nanoparticles combined with laser irradiation in CAL-27 tumor-bearing mice. All treatments including normal saline (the control), free DOX, free HP, PTD/HP, and RPTD/HP nanoparticles with and without laser irradiation were carried out once every 3 d consecutively four times. The tumor sizes and body weights of the treated mice were monitored continuously during the treatment period. Figure 8A shows the curves of tumor growth. All treatments remarkably inhibited the tumor growth as compared to the control. Furthermore, PTD/HP and RPTD/HP nanoparticles showed much more significant inhibitory effects on tumor growth than both free DOX and free HP with laser irradiation, which resulted from their efficient tumor-accumulations mediated by the EPR effect. After laser irradiation, PTD/HP and RPTD/HP nanoparticles further notably slowed the growth speed of tumors, confirming their strong synergistic effects of PDT and chemotherapy. Additionally, the tumors of mice treated with RPTD/HP nanoparticles and laser irradiation were the smallest in size (Figure 8B), which was because HP and DOX delivered by RPTD/HP nanoparticles were more efficiently accumulated in the tumor site through specific binding of the cRGD peptide with integrin αvβ3. The changes of mouse body weights are shown in Figure 8C. Because of the toxic and side effects, free DOX with a dose of 3.0 mg/kg decreased the body weights of mice. However, the mice receiving other treatments showed no significant changes in

![Figure 8](image-url)
their body weights, demonstrating that these treatments are safe for in vivo applications.

After treatments, the main organs and tumors were removed from the mice and then were made into histopathological sections for further analysis. From the images of H&E-stained tissue sections, no histopathological changes and injuries appeared in the main organs of all the treated mice (Figure S7). However, the inflammatory infiltration, cytoplasmic vacuolation, and even cell necrosis were evidently observed in the tumors of mice with treatments of PTD/HP and RPTD/HP nanoparticles with laser irradiation (Figure 8D). This meant that these treatments had remarkable synergistic effects on tumor growth suppression through combination of PDT with chemotherapy. It is well known that the damage of tumor vasculature is another main mechanism of PDT fighting against cancers. Therefore, we further detected tumor angiogenesis through immunohistochemical staining of CD31 after various treatments. As shown in Figure 8E, the large vessels were visible in the tumors of the control mice, but almost fully disappeared in the tumors in other treatment groups. Figure 8F shows the average microvessel densities (MVD) in tumor tissues. PTD/HP and RPTD/HP nanoparticles notably decreased the intratumor MVD in CAL-27 tumor-bearing mice after laser irradiation. It probably contributed to their strong antiangiogenic efficacies by combining PDT and chemotherapy. In addition, RPTD/HP nanoparticles with laser irradiation also displayed a significantly higher inhibitory effect on tumor angiogenesis than PTD/HP nanoparticles with laser irradiation because of their more efficient tumor accumulation. These results suggested that RPTD/HP nanoparticles could efficiently combine PDT and chemotherapy to obtain synergistic effects against OTSCC.

■ CONCLUSIONS

In this study, we synthesized a PEGlated prodrg of DOX via thioketal linkage and cRGD peptide modification and prepared its nanoparticles for encapsulation with photosensitizer HP. These nanoparticles with coloading of HP and DOX, named as RPTD/HP, showed significant ROS-responsive ability and successfully realized the controlled DOX release through the rupture of the thioketal linker upon laser irradiation. At the cellular level, RPTD/HP nanoparticles were specifically uptaken by CAL-27 cells through the cRGD peptide-mediated cell internalization and showed a strong PDT efficiency. Furthermore, RPTD/HP nanoparticles with laser irradiation showed significant synergistic effects on inhibiting tumor cell proliferation and inducing cell apoptosis. At the animal level, RPTD/HP nanoparticles exhibited brilliant tumor-targeting property and almost completely suppressed the growth of tumor in CAL-27 tumor-bearing mice. Therefore, our results demonstrated that RPTD/HP nanoparticles could be used as a novel nanoplatform for OTSCC treatment through combining PDT with chemotherapy.

■ ASSOCIATED CONTENT

$\bigcirc$ Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b08269.

In vitro stability of RPTD/HP nanoparticles, confocal images of CAL-27 cells after incubation with free DOX and RPTD/HP nanoparticles post laser irradiation, intracellular ROS generation of RPTD/HP nanoparticles at different times after laser irradiation, viabilities of CAL-27 cells with free DOX treatment with and without laser irradiation, apoptosis analyzed by flow cytometry in CAL-27 cells after treatment with free DOX with and without laser irradiation, and microscopic images of tissue sections with H&E staining after various treatments (PDF)

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Notes

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