Self-guiding Polymeric Prodrug Micelles with Two AIE Photosensitizers for Enhanced Chemo-Photodynamic Therapy

Xiaoqing Yi,†[a,b] Xiaoding Lou,*[a] Zujin Zhao,[e] Fan Xia,[a] and Ben Zhong Tang*†[d,e]

Abstract: Photodynamic therapy (PDT) has attracted extensive attention because of its properties of high spatial and temporal accuracy, controllability and non-invasive, which has been approved in clinic since the year of 1996. In the presence of photosensitizer and laser light, PDT induces the generation of reactive oxygen species (ROS) to inhibit the growth of target bacteria or cells. However, though delightful developments for PDT in recent years, its anti-tumor efficacy is still not as good as expected, which is closely related to the limited penetration of lasers in dense tumor sites. Fortunately, although ROS produced by photosensitizers in insufficient photodynamic energy have a reduced ability to directly kill tumor cells, they can improve the cytomembrane permeability and facilitate cellular uptake of anticancer drugs, which is called “photochemical internalization (PCI)”. In addition, PCI has been shown to effectively contribute to nanoparticle lysosomal escape by photochemically disrupted endocytic vesicles refuse with micelles-containing vesicles and subsequently facilitating intracellular drug release. Besides, based on the advantages of PDT and combination therapy, combining PDT with other different treatment modalities may have the opportunity to achieve better therapeutic efficacy and decreased side effects, such as combined chemo-photodynamic therapy. As a result, the dual-stage light irradiation strategy using PCI has been shown to improve the tumor suppressing efficacy of combined chemo-photodynamic therapy. Through a short-time light irradiation, a small amount of ROS produced by photosensitizer could induce lipid peroxidation and increase cell membrane permeability, leading to enhanced intracellular internalization of chemotherapeutic drugs and photosensitizer. After uptake by tumor cells, the released chemotherapeutic drugs and abundant ROS produced under the second light irradiation would play their respective roles in the cytoplasm and kill tumor cells subsequently.
Experimental Procedures

Materials. 2-Bromoethanol, 2,2’-bipyridine, 3,3’-dithiodipropionic acid, 3-bromopropyne, copper chloride, diethyl methylmalonate, NaH, LiAlH₄ and paclitaxel were used as received. Phosphate-buffered saline (PBS), Dulbecco’s Modified Eagle’s Medium (DMEM), bovine serum albumin (BSA), trypsin and penicillin-streptomycin were purchased from Gibco Invitrogen Corporation.

Cell culture. HeLa cell line was purchased from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) with 1% penicillin and 1% streptomycin at 37 °C in a 5% CO₂ atmosphere and 100% humidity.

Animals. BALB/c mice (4-6 weeks old, average body weight 16-18 g) were purchased from HFK Bioscience Co. (Beijing, China). HeLa cells (1 × 10⁶ per mouse) were injected subcutaneously into the right flank of BALB/c mice to generate the tumor mode. All animals received care in compliance with the guidelines set by the Animal Care Committee at Tongji Medical College. Tumor volumes were measured every other day using a caliper and calculated using the following formula: volume = ((tumor length) × (tumor width)²)/2.

Characterizations. ¹H Nuclear magnetic resonance (¹H-NMR) spectrum were measured by Bruker AM 400 apparatus. The solvent included CDCl₃ and DMSO-d₆, and the internal reference was tetra-methylsilane (TMS). The copolymer composition of PEG-b-PMPMC (PM) and PEG-b-PMPMC-g-PTX-g-PyTPE (PMPT) was calculated from the ¹H-NMR spectrum. The average size of micelles was measured by dynamic light scattering (DLS, Nano-ZS ZEN3690 Malvern Instruments). The morphology of micelles was observed by transmission electron microscopy (TEM, JEM-2100 microscope). High performance liquid chromatography (Agilent 1260) and liquid chromatograph mass spectrometry (LCMS, Thermo) were used to determine the drug release.

Synthesis of PEG-b-PMPMC-g-PTX-g-PyTPE (PMPT). PTX-SS-N₃, PyTPE, PM and 2,2’-bipyridine were dissolved in dry DMF in a Schlenk flask, CuBr was added after two freeze-pump-thaw cycles. The reaction was carried out for two days at 40 °C in an Ar atmosphere. The crude product solution was purified by silica gel chromatography and then precipitated three times in ether. Then, the product was purified by dialysis against DMSO for two days.

Determination of critical micelles concentration (CMC) of PMPT. PMPT solutions with or without pretreated with 2, 10 and 50 mM DTT for 1 h were added into sample bottles. The fluorescence emission intensity of PyTPE was analyzed as a function of the PMPT concentration (Ex = 400 nm, Em = 583 nm). When extrapolating the intensity of PMPT concentration region, the CMC values are determined as crossing points.

Preparation of PMPT and TB@PMPT micelles. TB@PMPT micelles were prepared by dialysis in the dark. PMPT (10 mg) and TB (1.2 mg) were dissolved in THF (2 mL), ultrapure water (9 mL) was then slowly added to that solution. The solution of PMPT and TB was transferred to the dialysis tube and dialyzed against ultrapure water for two days. The preparation method of PMPT micelles is similar to that of TB@PMPT micelles.

The drug loading content of PTX, PyTPE and TB. The drug loading contents (DLC) of PTX, TB and PyTPE was determined by HPLC. Briefly, lyophilized nanoparticles (2 mg) were dissolved in THF, the DLC of PTX, PyTPE and TB was determined by HPLC.

In vitro PTX release. PMPT and TB@PMPT micelles were continuously vibrated at 37 °C in the dark with or without 10 mM DTT. Samples from the solution (1 mL) were taken at different time points for HPLC analysis and replaced with 1 mL of fresh medium. The released PTX and PTX-SH from the micelles was evaluated by HPLC (CH₃CN/H₂O = 60/40, v/v; 1.0 mL min⁻¹).

Measurement of ROS generation in solution. ABDa was employed to measure ROS generation of micelles upon light irradiation. ABDa solution (15 μL, 4.5 mg mL⁻¹ in DMSO) was mixed to TB@PMP (30 μg mL⁻¹), PMPT (27 μg mL⁻¹) and TB@PMPT (30 μg mL⁻¹) micelles. Then, the mixed solution was irradiated under the with light (white light, 70 mW cm⁻²). The absorption of ABDa at 400 nm was recorded at various irradiation times to obtain the decay rate of photosensitizing process.

Measurement of ROS generation in living cells. PMPT (94 μg mL⁻¹) and TB@PMPT (105 μg mL⁻¹) micelles were pretreated with or without 10 mM DTT for 1 h in the dark at 37 °C. Subsequently, then the micelles incubated with HeLa cells for 4 h. DCFH-DA (final concentration 1 × 10⁻⁵ M) was added into the cells. After 20-min incubation, cells were washed three times with PBS, light irradiation (white light, 70 mW cm⁻², 6 min) was performed subsequently. The fluorescence images of cells were obtained using CLMS (Zeiss LSM 880). DCFH (green fluorescence, Ex: 488 nm, Em: 505-540 nm).

Detection of microtubules in living cells. PM and PMPT micelles were incubated with the cells in dark for 8 and 16 h, respectively, the concentration of micelles were 54 μg mL⁻¹. Then, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS and stained with anti-α-tubulin-FITC in the dark for 1 h at 37 °C. Anti-α-tubulin-FITC (green fluorescence, Ex: 488 nm, Em: 505-540 nm)

The reductive environment response of TB@PMPT micelles in tumor cells. PMPT (188 μg mL⁻¹) and TB@PMPT (210 μg mL⁻¹) were incubated with the cells for 2 h in dark, respectively. Then the cells were washed three time, further incubated for the predetermined
time intervals, the image of cells were observed by CLSM (Zeiss LSM 880). PyTPE (yellow fluorescence, Ex: 405 nm, Em: 500-560 nm), TB (red fluorescence, Ex: 488 nm, Em: 620-720 nm).

PTX release in cells. The PTX release in cells was studied as follows. After HeLa cells were seeded in 6-well plates and cultured for 24 h at 37 °C, then TB@PMPT micelles (210 μg mL⁻¹) were added. After incubation for 2 h at 37 °C in dark, the cells were replaced with PBS. At predetermined time intervals, cells were digested by trypsin, harvested by centrifugation, and resuspended in PBS. Cell suspension was freeze-thaw for three times under -20 °C, broken using cell disruptor. The broken cell suspension was centrifuged, and the supernatant was analyzed by HPLC (CH₃CN/H₂O =60/40, v/v; 1 mL min⁻¹). The cells treated with culture medium were used as a blank control.

Cellular uptake analysis. HeLa cells were seeded in petri dishes and treated with TB@PMP (94 μg mL⁻¹) and TB@PMPT (105 μg mL⁻¹) micelles for 1 h in dark. Then light irradiation (white light, 70 mW cm⁻²) was performed and incubation with cells continued for 3 h. LysoTracker Green was added and incubated with cells for 0.5 h to identify the intracellular drug location by CLSM.

CCK-8 assay. 96-well plate was used to culture HeLa cells of 1 × 10⁴ of cell suspension incubated for 24 h. Subsequently, micelles (200 μL) were added, and that cell were incubated for 1 h before the first light irradiation (L₁, 6 min, white light, 70 mW cm⁻²). After incubation with cells for 3 h, the cells were exposed to the second light irradiation (L₂, 18 min, white light, 70 mW cm⁻²). The micelles were removed and incubated for another 44 h before analysis. Cell viability was assessed by the CCK-8 assay.

Biodistribution in vivo. After the tumor volume reached to ~100 mm³, the TB@PMPT micelles were intravenously injected into the tumor-bearing BALB/c nude mice at the concentration of 2.0 mg mL⁻¹ in PBS through tail vein. Mice were anesthetized at predetermined time points and imaged by small animal imaging system in Institute of Virology (PerkinElmer). For tissue distribution studies, the mice were sacrificed at 24 h after injection. Heart, liver, spleen, lungs, kidneys and tumors were exfoliated and imaged.

Antitumor activity in vivo. The mice were randomly divided into four groups, and intravenously injected with PBS (-), TB@PMPT (without light irradiation, 2.0 mg mL⁻¹), TB@PMPT (LL, 24 min light irradiation for PDT, 2.0 mg mL⁻¹), and TB@PMPT (L₁L₂, 6 min light irradiation to enhance endocytosis and an 18 min light irradiation for PDT, 2.0 mg mL⁻¹) respectively on the first, fourth, seventh, and tenth days. The dose of TB@PMPT was 12.5 mg kg⁻¹ per mouse. The 6 min light irradiation was performed after injection for 6 h (L₁, 532 nm, 250 mW cm⁻²), the 18 min light irradiation (L₁) and the 24 min light irradiation (LL) were all performed after injection for 8 h (532 nm, 250 mW cm⁻²). Relative tumor volume and body weight was defined as V/V₀ and M/M₀, respectively (V₀ and M₀ was the tumor volume and body weight on the first day before treatment). After the mice were sacrificed, muscle, heart, liver, spleen, lung, kidney and tumor tissue were collected and stained with H&E staining.
Results and Discussion

Table S1. Properties of PMPT and TB@PMPT micelles

<table>
<thead>
<tr>
<th>Samples</th>
<th>DLC (wt%)</th>
<th>Mass ration (PyTPE: PTX: TB)</th>
<th>Size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMPT</td>
<td>7.35</td>
<td>18.5</td>
<td>f</td>
<td>116.8 ± 2.49</td>
</tr>
<tr>
<td>TB@PM</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>60.2 ± 1.50</td>
</tr>
<tr>
<td>TB@PMP</td>
<td>/</td>
<td>17.3</td>
<td>f</td>
<td>73.4 ± 2.30</td>
</tr>
<tr>
<td>TB@PMPT</td>
<td>6.79</td>
<td>17.1</td>
<td>7.57 f: 7.25</td>
<td>125.7 ± 3.26</td>
</tr>
<tr>
<td>TB@PMPT</td>
<td>6.65</td>
<td>16.7</td>
<td>9.57 f: 3.20</td>
<td>132.9 ± 1.63</td>
</tr>
<tr>
<td>TB@PMPT</td>
<td>6.55</td>
<td>16.5</td>
<td>10.8 1: 2.52 f: 1.65</td>
<td>148.5 ± 0.57</td>
</tr>
</tbody>
</table>

a No PyTPE was conjugated on backbone of amphiphilic polycarbonate.
b No PTX was encapsulated into PMPT micelles.
c No TB was encapsulated into PMPT micelles.
Table S2. The slope of decomposition curves of ABDA induced by ROS generation from TB@PMP, PMPT and TB@PMPT with or without 10 mM DTT were pre-incubated at 37 °C for 1 h.

<table>
<thead>
<tr>
<th>Samples</th>
<th>TB@PMP</th>
<th>PMPT</th>
<th>TB@PMPT</th>
<th>TB@PMP DTT, 1 h</th>
<th>TB@PMP DTT, 1 h</th>
<th>TB@PMPT DTT, 1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.07347</td>
<td>0.08902</td>
<td>0.1175</td>
<td>0.08153</td>
<td>0.04521</td>
<td>0.08811</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.0019</td>
<td>0.00198</td>
<td>0.00244</td>
<td>0.00236</td>
<td>0.00028</td>
<td>0.00237</td>
</tr>
</tbody>
</table>
Figure S1. Synthesis of reduction-sensitive amphiphilic polymeric prodrug PMPT.
Figure S2. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 5-methyl-5-propargyl-1,3-dioxan-2-one (MPMC).
Figure S3. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of PM.
Figure S4. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of PTX.
Figure S5. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of PTX-SS-N$_3$. 
Figure S6. $^1$H NMR spectrum (400 MHz, DMSO-$d_6$) of PyTPE.
Figure S7. Mass spectrum of PyTPE, calcd [M]^+ 553.2700.
Figure S8. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of PMPT.
Figure S9. Gel permeation chromatography traces of PMPT.
Figure S10. Transmission electron microscopy (TEM) images (A) PMPT and (B) TB@PMPT micelles with TB loading of 9.57% TEM images of (C) PMPT and (D) TB@PMP micelles with TB loading of 9.57% after stored at room temperature for 30 days. The scale bar is 100 nm.
Figure S11. (A) Hydrodynamic size distribution of PMPT and TB@PMPT micelles in PBS. (B) Stability assay of PMPT and TB@PMPT micelles in PBS during 30-day storage at room temperature.
Figure S12. UV-vis spectra of (A) PMPT and (B) TB@PMPT micelles.
Figure S13. Fluorescence spectra (Ex: 400 and 530 nm) of (A) PMPT and (B) TB@PMP micelles in aqueous solution.
Figure S14. UV-vis absorption spectra of the ROS indicator 9,10-anthracenediybis(methylene)dimalonic acid (ABDA) mixed with various micelles upon light irradiation (white light, 70 mW cm$^{-2}$). (A) TB@PMP; (B) PMPT; (C) TB@PMPT; (D) TB@PMP micelles with 10 mM DTT were pre-incubated at 37 °C for 1 h; (E) PMPT micelles with 10 mM DTT were pre-incubated at 37 °C for 1 h; (F) TB@PMPT micelles with 10 mM DTT were pre-incubated at 37 °C for 1 h.
**Figure S15.** Detection of intracellular ROS production by DCFH-DA in HeLa cells after incubation with TB@PMP (-), PMPT (-) and TB@PMPT (-) micelles, respectively. Scale bar: 20 μm.
Figure S16. Corresponding quantitative analysis of the fluorescence intensity in Figure 1B by the Zeiss LSM 880 software. Data was reported as mean ± SD and analysed through two-sided Student’s t-test. (**p < 0.01, *p < 0.05, ns means not significant)
Figure S17. Mass spectra of the released products from PTX-SS-N₃ with the treatment of 10 mM DTT. (A) PTX-SH: \([\text{M+H}]^+=942.3399\) (calcd 942.3365); (B) (PTX): \([\text{M+H}]^+=854.3415\) (calcd 854.3382); (C) 3-mercaptopropanoic acid: \([\text{M-H}]^+=105.0008\) (calcd 105.0005).
Figure S18. The release mechanism of free PTX from PTX-SH under the condition of 10 mM DTT.
Figure S19. The critical micelle concentration (CMC) value after PMPT micelles incubated with different concentrations of DTT for 1 h: (A) 0 mM DTT; (B) 2 mM DTT; (C) 10 mM DTT; (D) 50 mM DTT.
Figure S20. Mass spectra of the released products from TB@PMPT micelles with the treatment of 10 mM DTT. HRMS (A) PTX-SH: [M+H]+ 942.3387 (calcd 942.3365); (B) PTX: [M+H]+ 854.3381 (calcd 854.3382); (C) 3-mercaptopropanoic acid: [M–H]– 105.0007 (calcd 105.0005).
Figure S21. The size changes of (A) PMPT and (B) TB@PMPT micelles with TB loading of 9.57% in response to 10 mM DTT at pH 7.4 determined by DLS, respectively.
Figure S22 Mass spectra of the cell extract after incubation of TB@PMPT micelles with HeLa cells. HRMS (A) PTX-SH: [M+H]+ 942.3365 (calcd 942.3387); (B) PTX: [M+H]+ 854.3381 (calcd 854.3382); (C) 3-mercaptopropanoic acid: [M-H]- 105.0007 (calcd 105.0005); (D) TB: [M+H]+ 997.4232 (calcd 997.4279)
Figure S23. (A) Fluorescence (FL) spectra, (B) fluorescence intensity ratios $I_t/I_0$ and fluorescence intensity ratios of $I_0/I_t$ of PMPT micelles (100 mg L$^{-1}$) was incubated with 10 mM DTT for different times. Ex = 400 nm, Em = 583 nm.
Figure S24. (A) FL spectra, (B) fluorescence intensity ratios $I_t/I_0$ and fluorescence intensity ratios of $I_0/I_t$ of PMPT micelles (200 mg L$^{-1}$) was incubated with 10 mM DTT for different times.
Figure S25. FL spectra of TB@PMPT micelles with different concentrations and different TB loading content incubated with 10 mM DTT for different times. (PyTPE: Ex = 400 nm, Em = 583 nm; TB: Ex = 530 nm, Em = 684 nm). (A) C_{TB@PMPT}: 100 mg L^{-1}, DLC_{TB}: 7.57%; (B) C_{TB@PMPT}: 200 mg L^{-1}, DLC_{TB}: 7.57%; (C) C_{TB@PMPT}: 100 mg L^{-1}, DLC_{TB}: 9.57%; (D) C_{TB@PMPT}: 100 mg L^{-1}, DLC_{TB}: 10.8%.
Figure S26. The fluorescence signal of PyTPE was observed by CLSM after PMPT micelles were incubated with HeLa cells for 2 h. (A) HeLa cells without pretreatment, (B) pretreated with glutathione monoester (GSH-OEt) and (C) buthionine sulfoximine (BSO), respectively. (D) Their quantitative analysis of $I_0/I_h$ of PyTPE (n=3) for CLSM images. PyTPE: yellow signal, Ex = 405 nm. Scale bar: 20 μm.
Figure S27. (A) Fluorescence signal of PyTPE and TB was observed by CLSM after TB@PMPT micelles were incubated with HeLa cells for 2 h. HeLa cells were pretreated with GSH-OEt. (B) Quantitative analysis of the fluorescence intensity of PyTPE and TB (n=3). (C) Quantitative analysis of $I_{TB}/I_{PyTPE}$ (n=3) PyTPE: yellow signal, Ex = 405 nm; TB: red signal, Ex = 543 nm. Scale bar: 20 μm.
Figure S28. (A) Fluorescence signal of PyTPE and TB was observed by CLSM after TB@PMPT micelles were incubated with HeLa cells for 2 h. The HeLa cells were pretreated with BSO. (B) Quantitative analysis of the fluorescence intensity of PyTPE and TB (n=3). (C) Quantitative analysis of $I_{TB}/I_{PyTPE}$ (n=3) PyTPE: yellow signal, Ex = 405 nm; TB: red signal, Ex = 543 nm. Scale bar: 20 μm.
Figure S29. CCK-8 assay of blank HeLa cells (LL, L₁L₂) in HeLa cells after incubation for 48 h.
Figure S30. CCK-8 assay of free PTX (-), PMPT (-) and TB@PMPT (-) in HeLa cells after incubation for 48 h.
Figure S31. (A, B) The inhibition ratios of TB@PM (L₁-L₂), PMPT (L₁-L₂), and TB@PMPT (L₁-L₂) micelles treated cells upon light irradiation (white light, 70mW cm⁻²), respectively. The green bar denotes the additional cell inhibition ratio gained when TB@PMPT (L₁-L₂) are combined, compared with the sum of TB@PM (L₁-L₂), PMPT (L₁-L₂).
Figure S32. The inhibition ratios of TB@PM (LL), PMPT (LL), and TB@PMPT (LL) micelles treated cells upon light irradiation (white light, 70mW cm$^{-2}$), respectively. The green bar denotes the additional cell inhibition ratio gained when TB@PMPT (LL) are combined, compared with the sum of TB@PM, PMPT (LL).
Figure S33. Body weight in the mice after the intravenous of different samples: PBS (-), TB@PMPT (-), TB@PMPT (LL), and TB@PMPT (L₁L₂).
Figure S34. H&E staining images of the tumor and organs obtained from the mice after different treatments: intravenous injection of different samples: PBS (-), TB@PMPT (-), TB@PMPT (LL) and TB@PMPT (L₁L₂) micelles. Scale bar: 10 μm.