Supporting Information

Single-molecule Förster Resonance Energy Transfer-Based Photosensitizer for Synergistic Photodynamic/Photothermal Therapy

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EXPERIMENTAL PROCEDURES

Materials and Instrumentation

All general chemicals for fluorescence detection and organic synthesis including 1,3-diphenylisobenzofuran (DPBF), acridine orange (AO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Energy Chemical Co. and all of the solvents were analytically pure. LysoTracker Green DND 26, Hoechst 33342 and MitoTracker Green FM were purchased from Life Technologies Co. (USA). Calcein AM/propidium iodide (PI) Detection Kit was purchased from Beyotime Biotechnology Co. (China). Annexin V-FITC apoptosis detection kit and Reactive Oxygen Species Assay Kit (DCFH-DA) were obtained from Beyotime biotechnology Co.. Ltd. Human breast cancer MCF-7 cells, Human cervical cancer Hela cells, Mouse melanoma B16 cells and Mouse breast cancer 4T1 cells were purchased from Institute
of Basic Medical Sciences (IBMS) of the Chinese Academy of Medical Sciences. $^1$H-
NMR and $^{13}$C-NMR spectra of all compounds were performed with Bruker Avance III
500 spectrometer. Mass spectrometric (MS) data were carried out using LTQ Orbitrap
XL instruments. Absorption and emission spectra for CR, BDP and BDP-CR were
performed with a Lambda 35 UV-visible spectrophotometer (PerkinElmer) and a
VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018),
respectively. Fluorescence quantum yield was obtained with the HAMAMATSU
absolute fluorescence quantum yield spectrometer (Serial No. C11347). Femtosecond
time resolved transient absorption spectra were obtained from the ultrafast pump-probe
transient absorption spectroscopy (Spectra Physics, Helios). Confocal laser scanning
microscope (CLSM) images were performed on Olympus FV3000 confocal laser
scanning microscope. Small animals’ fluorescence imaging was carried out by
NightOWL II LB983 living imaging system. All cell experiments were performed in
accordance with guidelines approved by the ethics committee of Dalian Medical
University.

**Synthesis of BDP-CR, BDP and CR**

**Synthesis of compound 1**

2-Iodothiophene (4.2 g, 20 mmol), copper powder (254 mg, 20 mol%) and potassium
phosphate tribasic (9.2 g, 40 mmol) were suspended in 2-(ethylamino)ethanol (21.4 g
240 mmol) and heated at 70 °C for 24 h under N$_2$ protection. After this time, the reaction
mixture was allowed to cool to room temperature and water (80 mL) added. The
solution was extracted with diethyl ether (3*100 mL), and the combined organic
fractions washed with brine (1*60 mL), and then the organic layer was dried over anhydrous MgSO₄ and evaporated under reduced pressure. Purification was undertaken by neutral silica gel column chromatography (hexane: ethyl acetate = 2: 1, v/v) to elute the product as an orange oil (2.52 g, 80%). ESI-HRMS, calcd for (C₈H₁₃NOS) m/z: [M+H]⁺:172.0791, found: 172.0790; [2M+H]⁺:343.1508, found: 343.1509.

**Synthesis of compound 2**

Compound 1 (0.45 g, 2.6 mmol) and propargyl bromide (0.74 mL, 9.36 mmol) in toluene (18 mL) was added a solution of tetrabutylammonium bisulfate (100 mg) in 50% sodium hydroxide solution (10 mL) and the reaction mixture was stirred at room temperature for 3 days. The organic layer was separated and the solvent removed in vacuo. The resulting residue was dissolved in CHCl₃ (50 mL), washed with water (2*25 mL), dried over MgSO₄ and the solvent removed in vacuo. Purification was undertaken by silica gel column chromatography (hexane: ethyl acetate = 100: 3, v/v) to elute the product as a yellow oil (250 mg, 84%). ESI-HRMS, calcd for (C₁₁H₁₅NOS) m/z: [M+H]⁺: 210.0947, found: 210.0944.

**Synthesis of compound CR**

Croconic acid (85.24 mg, 0.6 mmol) and compound 2 (250 mg, 1.2 mmol) were dissolved in 1:1 anhydrous toluene/1-butanol (30 mL) and heated at reflux for 3 h under N₂ protection. After this time, the solution was allowed to cool to room temperature and the solvent removed in vacuo. The crude product was purified by neutral silica gel column chromatography (CH₂Cl₂: CH₃OH = 75: 1, v/v), giving the CR as a dark brown solid (161 mg, 55%). ¹H NMR (500 MHz, CDCl₃) δ 8.89 – 8.66 (m, 2H), 6.50 (s, 2H),
4.17 (t, $J = 2.0$ Hz, 4H), 3.90 – 3.81 (m, 4H), 3.76 (d, $J = 24.8$ Hz, 4H), 3.49 (s, 5H), 2.46 – 2.45 (m, 1H), 1.36 (dd, $J = 9.5$ Hz, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 186.24, 186.10, 185.57, 185.42, 183.06, 173.66, 173.13, 172.97, 141.89, 141.83, 141.10, 140.76, 136.67, 136.47, 136.42, 136.34, 129.93, 123.99, 123.88, 123.72, 113.17, 113.09, 112.99, 78.86, 75.31, 67.06, 58.65, 53.83, 50.83, 50.71, 29.71, 12.37. ESI-HRMS, calcd for (C$_{27}$H$_{28}$N$_2$O$_5$S$_2$) m/z: [M+H]$^+$: 525.1512, found: 525.1502.

**Synthesis of compound 3**

4-hydroxybenzaldehyde (0.50 g, 4.1 mmol) and 2,4-dimethylpyrrole (0.92 mL, 8.94 mmol) were kept in a 500 ml round-bottomed flask with THF (120 mL) under N$_2$ protection at room temperature. After being stirred for 15 min, trifluoroacetic acid (0.1 mL, 1.3 mmol) was added to the mixture. After 12 hours, a solution of DDQ (0.93 g, 4.1 mmol) in THF (160 mL) was added to the round-bottomed flask. After 4 hours, the round-bottomed flask was cooled on the ice bath, then Et$_3$N (25 mL) was added dropwise. After 15 minutes, BF$_3$·Et$_2$O (25 mL) was added dropwise to the solution. The ice bath was removed, and the mixture was kept stirring at room temperature for 16 hours, then filtered by celite. The celite was washed by CH$_2$Cl$_2$ (3*100 mL), and the filtrate was evaporated. The residue was dissolved in CH$_2$Cl$_2$ (200 mL), then the solution was washed with NaHCO$_3$ solution, deionized water and brine. The organic layer was dried over anhydrous MgSO$_4$. The solvent was removed under reduced pressure and the crude product was purified by neutral silica gel column chromatography (hexane: CH$_2$Cl$_2$ = 1: 1, v/v). The desired compound as a red-orange solid (0.68g, 48%). ESI-MS, calced for (C$_{19}$H$_{19}$BF$_2$N$_2$O) m/z: [M-H]$^-$: 339.15, found:
Synthesis of compound 4

Compound 3 (1 g, 2.94 mmol) and 1-azido-2-bromo-ethane (0.72 g, 4.4 mmol) were dissolved in acetone (50 mL). K$_2$CO$_3$ (1.5 g, 44 mmol) were added. The reaction was refluxed for 12 h under N$_2$ protection. The solvent was evaporated in vacuo, extracted with water and CH$_2$Cl$_2$. Organic layer was dried with MgSO$_4$ and evaporated under reduced pressure. The product was purified by silica gel column chromatography using (hexane: CH$_2$Cl$_2$ = 2: 1, v/v). Fraction containing compound 5 was collected then the solvent was removed under reduced pressure (2.85 g, 89%). ESI-MS, calcd for (C$_{21}$H$_{22}$BF$_2$N$_5$O) m/z: [M+H]$^+$:410.20, found: 410.33; [M+Na]$^+$:432.18, found: 432.32.

Synthesis of compound 5

Compound 4 (81.8 mg, 0.2 mmol) and NIS (85.4 mg, 0.48 mmol) in HFIP (2 mL) was stirred at room temperature for 5 min. After reaction, the solvent was removed and recovered by distillation. The residue was extracted with CH$_2$Cl$_2$, washed with H$_2$O, dried over Na$_2$SO$_4$, and concentrated under reduced pressure. The crude product was further purified using column chromatography (hexane: CH$_2$Cl$_2$=10: 3, v/v) to afford the products. ESI-MS, calcd for (C$_{21}$H$_{20}$BF$_2$I$_2$N$_5$O) m/z: [M+H]$^+$:661.99, found: 662.01.

Synthesis of compound BDP

Compound 5 (200 mg, 0.3 mmol) and triethylene glycol monomethyl ether benzaldehyde (465.9 mg, 1.75 mmol) were dissolved in toluene (40 mL). Glacial acetic acid (0.3 mL) and piperidine (0.3 mL) were added into the above solution. Then, the mixture was refluxed using Dean-Stark apparatus under a nitrogen atmosphere. The
residue was extracted with CH$_2$Cl$_2$, washed with H$_2$O, dried over Na$_2$SO$_4$, and concentrated under reduced pressure. The crude product was further purified using column chromatography (CH$_2$Cl$_2$/CH$_3$OH=25: 1, v/v). Finally, a dark green solid BDP was obtained (130.8 mg, 37%). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.13 (d, $J = 16.6$ Hz, 2H), 7.63 – 7.55 (m, 6H), 7.19 (d, $J = 8.4$ Hz, 2H), 7.06 (d, $J = 8.4$ Hz, 2H), 6.96 (d, $J = 8.6$ Hz, 4H), 4.22 (t, $J = 4.8$ Hz, 2H), 4.20 – 4.17 (m, 4H), 3.91 – 3.87 (m, 4H), 3.78 – 3.74 (m, 4H), 3.71 – 3.69 (m, 4H), 3.68 – 3.66 (m, 6H), 3.57 – 3.54 (m, 4H), 3.39 (s, 6H), 1.50 (s, 6H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 159.97, 159.16, 150.46, 145.66, 139.09, 138.23, 133.20, 129.80, 129.24, 128.04, 116.77, 115.41, 114.99, 82.71, 71.95, 70.89, 70.68, 70.60, 69.69, 67.56, 67.03, 59.06, 50.21, 17.73. HRMS (MALDI) ([C$_{49}$H$_{56}$BF$_2$I$_2$N$_5$O$_9$]): calcd m/z: 1161.2229, found: 1161.2254.

**Synthesis of compound BDP-CR**

Compound BDP (90 mg, 0.078 mmol), Compound CR (52 mg, 0.1 mmol), CuSO$_4$·5H$_2$O (37.5 mg, 0.15 mmol), and sodium ascorbate (59 mg, 0.3 mmol) were dissolved in the mixed solvent (14 mL, CHCl$_3$:EtOH:H$_2$O = 12: 1: 1, v/v/v), then the mixture was stirred at r.t. for 24 h under N$_2$ protection. When the reaction was finished, the resulting mixture was extracted with DCM and water, and the organic layer was further dried over anhydrous Na$_2$SO$_4$. After the solvent was removed, the crude product was purified by neutral silica gel column chromatography (DCM/CH$_3$OH = 20: 1, v/v), giving the BDP-CR as a dark green solid (60 mg, 55%). $^1$H NMR (500 MHz, DMSO-d$_6$) δ 8.50 (dd, $J = 67.3$, 28.6 Hz, 4H), 8.06 (d, $J = 16.5$ Hz, 8H), 7.57 (d, $J = 8.4$ Hz, 8H), 7.42 (d, $J = 16.7$ Hz, 4H), 7.26 (d, $J = 7.8$ Hz, 4H), 7.05 (t, $J = 14.0$ Hz, 16H), 4.77
(s, 4H), 4.57 (s, 4H), 4.41 (s, 6H), 4.19 – 4.13 (m, 8H), 3.79 – 3.75 (m, 12H), 3.61 – 3.59 (m, 8H), 3.55 (d, J = 4.7 Hz, 8H), 3.54 – 3.51 (m, 12H), 3.45 – 3.42 (m, 8H), 3.34 (s, 8H), 1.34 (s, 12H), 1.23 (s, 12H). 

$^{13}$C NMR (126 MHz, DMSO-d$_6$) δ 174.45, 174.23, 172.75, 159.85, 158.63, 149.59, 145.43, 143.92, 138.93, 138.44, 132.77, 129.71, 129.61, 128.86, 128.64, 126.68, 116.09, 115.26, 105.39, 91.28, 87.87, 84.14, 74.77, 73.24, 71.25, 69.95, 69.77, 69.58, 68.82, 67.37, 66.68, 66.22, 58.02, 54.88, 48.98, 48.57, 35.09, 33.63, 31.25, 29.05, 29.00, 28.95, 28.80, 28.71, 28.66, 28.55, 26.52, 25.08, 24.46, 22.06, 17.21, 13.91. HRMS (MALDI), calcd for (C$_{125}$H$_{140}$B$_2$F$_4$I$_4$N$_{12}$O$_{23}$S$_2$): m/z [M+H]$^+$: 2848.6003; found: 2848.6013.

**UV-vis Spectra and Fluorescent Emission Spectra**

The UV-vis spectrum of CR, BDP and BDP-CR was obtained using UV-visible spectrophotometer and fluorescent emission spectrum of CR, BDP and BDP-CR in PBS buffer (0.01 M PBS, PBS/DMSO = 2:1 v/v, pH = 7.4) was acquired with fluorescence spectrometer (FLS-980, Edinburgh, UK) under 660 nm excitation.

**Femtosecond Transient Absorption Spectra**

The femtosecond transient absorption spectra of compound BDP-CR and BDP were measured by a home-made ultrafast pump–probe setup. The pulse duration was 30 fs. The wavelength of the pump beam was chosen to be 470 nm. The used concentration of samples was 5 μM for both BDP-CR and BDP in DMSO at room temperature. Measurements were performed in 1 mm quartz cuvettes.

**Fluorescence Quantum Yield**

The absolute fluorescence quantum yield apparatus was used to measure
fluorescence quantum yield of CR, BDP and BDP-CR in PBS buffer (0.01 M, PBS/DMSO = 2:1 v/v, pH = 7.4) at the concentration of 5 μM, respectively. The excitation wavelength was 671 nm.

Singlet Oxygen Detection

The $^1$O$_2$ quantum yield (FD) was detected according to the literature method. The singlet oxygen ($^1$O$_2$) generation efficiency of CR, BDP and BDP-CR were evaluated by singlet oxygen capture agent, 1, 3-diphenlisobenzofuran (DPBF), respectively. Briefly, the absorbance of DPBF at 415 nm was adjusted to about 1.0, and then BDP, BDP-CR or MB was added to the cuvette and the maximum absorption was adjusted to 0.3, respectively. The relative quantum yields were calculated with reference to Methylene Blue (MB) in DMSO for which the quantum yield is 0.52. The mixture was then placed in a cuvette and irradiated with a 671 nm light source (2 mW/cm$^2$) for different time (15, 30, 45, 60, 75, 90, 105, 120 s), and the corresponding absorption spectra was measured immediately. The slopes of absorbance of DPBF at 415 nm versus irradiation time were measured and used to compare the $^1$O$_2$ generation ability. The emission maxima of DPBF with different irradiation times were obtained, and the singlet oxygen quantum yields were determined using the following equation:

$$\Phi_{\Delta \text{sam}} = \Phi_{\Delta \text{std}} \left( \frac{m_{\text{sam}}}{m_{\text{std}}} \right) \left( \frac{F_{\text{std}}}{F_{\text{sam}}} \right)$$

Where “sam” and “std” designate the “PSs” and “MB”, respectively. “m” is the slope of absorbance attenuation curve of DPBF at 415 nm, and “F” is the absorbance correction factor, which is obtained by $F = 1-10^{-O.D}$. (O.D. is the absorbance of the solution at 671 nm). The superscripts indicated the reference.
Photothermal Performance Detection

CR, BDP and BDP-CR was irradiated by 671 nm light at different power densities (0 mW/cm², 25 mW/cm², 50 mW/cm², 100 mW/cm² and 200 mW/cm²) to discuss the effect of power density on conversion efficiency, respectively. Next, different concentrations of CR, BDP and BDP-CR were prepared including 0 μM, 5 μM, 10 μM, 20 μM and 40 μM and then irradiated by 671 nm light (100 mW/cm²), respectively. A thermometer submerged in the solution was used to monitor the temperature during 1200s. Besides, BDP-CR solutions (100 mW/cm²) were imaged by infrared thermal camera. To measure the conversion efficiency, 40 μM BDP-CR was exposed to 200 mW/cm² irradiation (671 nm) for 6 min, and then the irradiation was removed for cooling down to room temperature. The temperature of the solution was recorded at an interval of 30s during this process. The photothermal conversion efficiencies (\( \eta \)) were measured according to a previously described method:\(^3\)

\[
\eta = \frac{hs(T_{\text{Max}}-T_{\text{Sur}})-Q_{\text{Dis}}}{I(1-10^{-A_{671}})}
\] ----equation (S1)

\( h \) is the heat transfer coefficient, \( s \) is the surface area of the container, and the value of \( hs \) is determined from the equation (S2). \( Q_{\text{Dis}} \) represents heat dissipated from the light mediated by the solvent and container. \( I \) is the light power and \( A \) is the absorbance at 671 nm.

\[
hs = \frac{mC}{\tau_s}
\] ----equation (S2)

\( m \) is the mass of the solution containing the photoactive material, \( C \) is the specific heat capacity of the solution, and \( \tau_s \) is the associated time constant, which can be determined from equation (S3).
\[
t = -\tau_2 \ln(\theta)
\]  
-----equation (S3)

\(\theta\) is a dimensionless parameter, known as the driving force temperature, as calculated using equation (S4).

\[
\theta = \frac{T - T_{\text{Surr}}}{T_{\text{Max}} - T_{\text{Surr}}}
\]  
-----equation (S4)

\(T_{\text{max}}\) and \(T_{\text{Surr}}\) are the maximum steady state temperature and the environmental temperature, respectively. Moreover, the solvent (PBS/DMSO = 2:1, v/v, 0.01 M, pH = 7.4) heat capacity is 2.805 J/(g•°C).

**Cell Culture Conditions and Cellular Uptake**

Human breast cancer MCF-7 cells, Human cervical cancer Hela cells and Mouse melanoma B16 cells were maintained in DMEM medium. Mouse breast cancer 4T1 cells were maintained in RPMI 1640 medium. All of them were supplemented with 1% penicillin-streptomycin and 15% FBS, and the atmosphere was of 5% CO\(_2\) and 95% air at 37 °C. In order to mimic hypoxic tumor environment, cells were cultured in an incubator chamber (MIC-101, Billups-rothenberg) at 37 °C in a humidified, 2% O\(_2\) and 5% CO\(_2\) atmosphere and using an oxygen detector (Nuvair, O\(_2\) QuicKstick) to monitor the O\(_2\) content in the chamber. Cancer cells were incubated with 2 \(\mu\)M BDP and BDP-CR at 37°C for 0.5, 1, 2, 4 h, respectively. After cells were washed with PBS for twice, the confocal fluorescence imaging was performed and images were collected (excited at 640 nm, monitored at 660-760 nm).

**Intracellular Singlet Oxygen Imaging**

DCFH-DA (2,7-dichlorofluorescein diacetate) Detection Kit was used to validate the generation of singlet oxygen in living 4T1 cells. The cells were incubated onto 35 mm
confocal dishes for 24 h. 4T1 cells were divided into eight groups: 1) irradiated with 671 nm light (0.1 W/cm², 5 min); 2) incubated with 10 μM BDP for 4 h and irradiated with 671 nm light (0.1 W/cm², 5 min); 3) incubated with 5 μM BDP-CR for 4 h and irradiated with 671 nm light (0.1 W/cm², 5 min); 4) incubated with 5 μM BDP-CR and 10 mM NAC for 4 h and irradiated with 671 nm light (0.1 W/cm², 5 min); 5) untreated; 6) incubated with 10 μM BDP for 4 h; 7) incubated with 5 μM BDP-CR for 4 h; 8) incubated with 5 μM BDP-CR and 10 mM NAC for 4 h. DCFH-DA Detection Kit according to the manufacture instruction. Then, confocal fluorescence imaging was used to observe the intracellular $^1$O₂ level (excited at 488 nm, monitored at 490-520 nm).

Subcellular Colocalization Assay

4T1 cells were planted onto 35 mm confocal dishes and incubated for 24 h. After incubated with 2 μM BDP-CR for 4 h at 37°C under 5% CO₂, the cells were further stained by LysoTracker Green DND 26 (100 nM), MitoTracker Green FM (100 nM), or Hoechst 33342 (2 mg/mL). Cells were then visualized with laser confocal microscopy. BDP-CR was excited with 640 nm wavelength, while the excitation wavelength for LysoTracker Green DND 26 and MitoTracker Green FM were 488 nm, and Hoechst 33342 was 405 nm. The emission wavelength was collected from 660 to 760 nm for BDP-CR, 500 to 540 nm for LysoTracker Green and Mito Tracker Green, and 440 to 480 nm for Hoechst 33342.

Lysosomes Disruption Assay

4T1 cells were seeded and cultured in 35 mm confocal dishes for 24 h. Then the cells
were exposed to the different following treatments: group 1, untreated (Control); group 2, incubated with 5 μM BDP-CR for 4 h at 37°C (BDP-CR); group 3, irradiated with 671 nm light (0.1 W/cm²) for 5 min (Light); group 4, incubated with 5 μM BDP-CR for 4 h at 37°C and irradiated with 671 nm light (0.1 W/cm²) for 5 min (BDP-CR + light). Before imaging, all cells in different treatment groups were stained with AO (5 μM) for 0.5 h. Then the fluorescence signals of AO within 4T1 cells were detected using confocal microscopy with the excitation wavelength of 488 nm, capture emission region from 515 nm to 545 nm for green channel and from 610 nm to 640 nm for red channel.

**Dead/Live Cell Co-Staining**

Firstly, 4T1 cells were incubated 1640 medium for 24 h under normoxic or hypoxic conditions, then exposed to different following treatments: group 1, untreated (Control); group 2, irradiated with 671 nm light (0.1 W/cm²) for 5 min; group 3, incubated with 20 μM BDP for 4 h at 37°C; group 4, incubated with 10 μM BDP-CR for 4 h at 37°C; group 5, incubated with 20 μM BDP for 4 h at 37°C and irradiated with 671 nm light (0.1 W/cm²) for 5 min; group 6, incubated with 10 μM BDP-CR for 4 h at 37°C and irradiated with 671 nm light (0.1 W/cm²) for 5 min. The other six groups were treated under hypoxic conditions in the same way as normoxia. After different treatments, Calcein AM and Propidium Iodide co-staining was performed. The excitation wavelength of Calcein AM and Propidium Iodide were 488 nm, and emission signals were collected from 505 to 545 nm with green fluorescence and from 600 to 700 nm with red fluorescence, respectively. The same method was applied to other three kinds
of cells treated with **BDP-CR** to compare the difference killing effect on each group.

**Confocal Imaging of Photon-induced Cell Death**

Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit was used to analysis the **BDP-CR** mediated photon-induced cell death. Briefly, 4T1 cells were seeded onto 35 mm confocal dishes and incubated for 24 h, then the cells were divided into five groups on different treatments: group 1, irradiated with 671 nm light (0.1 W/cm²) for 5 min (Light); group 2, incubated with 10 μM **BDP** at 37 °C for 4 h (**BDP**); group 3, incubated with 5 μM **BDP-CR** at 37 °C for 4 h (**BDP-CR**); group 4, incubated with 10 μM **BDP** at 37 °C for 4 h and irradiated with 671 nm light (0.1 W/cm²) for 5 min (**BDP + Light**); group 5, incubated with 5 μM **BDP-CR** at 37 °C for 4 h and irradiated with 671 nm light (0.1 W/cm²) for 5 min (**BDP-CR + Light**). After different treatments, cells were stained with Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit according to the manual. The cell apoptosis was visualized by fluorescence microscopy with excitation wavelength of 488 nm. The emission wavelength was collected from 505 to 545 nm for Annexin V-FITC green channel, and from 600 to 680 nm for PI red channel.

**Flow Cytometric Assay**

For apoptosis analysis, 4T1 cells (1×10⁶ cells per well) were seeded in 6-well plates and cultured overnight. Then cells were incubated with different conditions and divided into six groups: group 1, untreated (Control); group 2, irradiated with 671 nm light (0.1 W/cm²) for 5 min (Control + light); group 3, incubated with 10 μM **CR** at 37 °C for 4 h (**CR**); group 4, incubated with 10 μM **BDP-CR** for 4 h at 37°C (**BDP-CR**); group 5,
incubated with 20 μM BDP for 4 h at 37°C and irradiated with 671 nm light (0.1 W/cm²) for 5 min (BDP + light); group 6, incubated with 10 μM BDP-CR for 4 h at 37°C and irradiated with 671 nm light (0.1 W/cm²) for 5 min (BDP-CR + light). After incubation for another 24 h, the cells were centrifuged at 1300 rpm for 5 min and resuspended in Annexin V binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂). Then cells were stained with Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit according to the manual. Finally, cells were analyzed on a flow cytometer.

**In Vitro Cytotoxicity Studies**

4T1, MCF-7, B16 and Hela cells were seeded onto 96-well plates at 10000 cells per well and incubated at 37 °C for 24 h under normoxic or hypoxic conditions. Different concentrations of BDP-CR, BDP + CR, BDP and CR from 0 to 32 μM in 1640 medium were added to the wells, respectively. The concentration of BDP was twice that of BDP-CR. And BDP + CR was the mixture of BDP (twice the concentration of BDP-CR) and CR (the same concentration as BDP-CR). Then, the cells were further incubated for 4 h and were subjected with 0.1 W/cm² (671 nm light) for 5 min under normoxic or hypoxic conditions, respectively. After 24 h, MTT solution (5 mg/mL) was added in 1640 medium to each well. The solution in each well was removed and then added 100 μL DMSO to each well after incubating the cells for 4 h. The absorbance at 490 nm was measured with a Bio-Rad microplate reader. The cell viability was obtained by the following equation:

\[
\text{cell viability (\%)} = \left( OD_{ps} - \frac{OD_{black\ control}}{OD_{control}} - OD_{black\ control}\right) \times 100\%
\]
For dark toxicity of **BDP-CR, BDP + CR, BDP and CR**, no light irradiation was applied to this experiment under normoxic or hypoxic conditions, and all other steps were the same. The same method was used to analysis the cell cytotoxicity on MCF-7, Hela and B16 cells treated with **BDP-CR, BDP and CR** under normoxic condition, respectively. The effects of PDT and PTT with the **BDP-CR** on cell viability were investigated separately. 4T1 cells were cultured in a medium containing 32 µM **BDP-CR** for 4 h. For the PDT experiments, cells were irradiated with 671 nm light on ice (5 min) to ensure that the temperature of the wells never exceeded 10°C. For PTT experiments, cells were pretreated with NAC (10 µL, 20 µM) for 4 h and were then irradiated with an NIR light for 5 min. For combined PDT and PTT therapy studies, cells were irradiated with a 671 nm light at room temperature and without NAC pretreatment.

**In Vivo Fluorescence Imaging**

All animal operations were in accordance with institutional animal use and care regulations were approved by the Model Animal Research Center of Dalian Medical University (MARC). Specific female Balb/c mice, 6-7 weeks of age, were obtained from Laboratory Animal Center of Dalian Medical University. Then, 1×10^6 4T1 cells were injected subcutaneously into the selected armpit positions to establish the breast cancer tumor model of Balb/c mice. After about 7 days, tumors grew to about 160 mm³ in volume before used for *in vivo* imaging and phototherapy. For *in vivo* tumor imaging, **BDP-CR** (20 nM, 100 µL) or **BDP** (40 nM, 100 µL) was injected intratumorally. The fluorescence signals were monitored at different post-injection time (0.5h, 1h, 2h, 4h
and 6h). The excitation wavelength was 665 nm, and the collected emission wavelength was 690-710 nm.

**In Vivo Photothermal Imaging**

For *in vivo* photothermal imaging (IR) of the 4T1 tumor-bearing mice, the tumor sites were irradiated with 0.3 W/cm² (671 nm light) for 10 min at 4 h postinjection of the BDP-CR, BDP or PBS, respectively. Temperature changes of the tumor site were recorded by IR thermal camera every 60 s.

**In Vivo Tumor Therapy**

The breast cancer tumor model of 4T1 tumor-bearing mice were randomly divided into eight groups (n = 5 per group), including group 1, “PBS”; group 2, “PBS + 671 nm light”; group 3, “CR”; group 4, “BDP”; group 5, “BDP-CR”; group 6, “BDP + 671 nm light”; group 7, “BDP-CR + 808 nm light” and group 8, “BDP-CR + 671 nm light”, respectively. On day 0, PBS (100 μL) were injected intratumorally in group “PBS” and “PBS + 671 nm light”. At the same time, mice of group “CR”, “BDP-CR”, “BDP-CR + 808 nm light” and “BDP-CR + 671 nm light” were injected with each of the corresponding drugs (20 nM, 100 μL) in the same way, respectively. The mice of group “BDP” and “BDP + 671 nm light” were injected with BDP (40 nM, 100 μL). After injection for 4 h, group “PBS + 671 nm light”, “BDP + 671 nm light”, “BDP-CR + 808 nm light” and “BDP-CR + 671 nm light” were continuously irradiated with 671 nm or 808 nm light (0.3 W/cm²) for 10 min and group “PBS”, “CR”, “BDP” and “BDP-CR” without subsequent light irradiation. In the following 21 days, the tumor volume of all mice was measured every day using a vernier caliper. Then, the greatest longitudinal
diameter (length) and the greatest transverse diameter (width) were used to calculate the tumor volume, as follows: tumor volume = width × width × length/2. Relative tumor volume was calculated as V/V₀ (V₀ was the initial tumor volume).

**Hemolysis assay**

Hemolytic activity of our photosensitizers were evaluated as the reported method.² Erythrocytes were isolated from sterile defibrinated sheep blood, and washed three times with saline solution and then suspended in saline at a 2% of hematocrit. Different concentrations of BDP-CR, BDP and CR solutions were incubated with properly diluted red blood cells (RBCs) at 37 °C for 4 h and then cooled using an ice bath to stop hemolysis, respectively. After centrifugation at 1500 rpm for 8 min, the supernatant was analyzed for hemoglobin at 540 nm using UV-spectrophotometer. The percent of hemolysis was determined as follows:

Hemolysis (%) = \((Abs-Abs₀)/ (Abs₁₀₀-Abs₀)\) × 100

Abs is the absorbance of the sample, Abs₁₀₀ absorbance of 100% lysed sample treated with deionized distilled water, Abs₀ absorbance of unlysed sample treated with saline.

**Evaluation of Intratumoral Oxidative Stress**

The mice were divided randomly into 8 groups: 1) PBS; 2) PBS + 671 nm light; 3) CR; 4) BDP; 5) BDP-CR; 6) BDP + 671 nm light; 7) BDP-CR + 808 nm light and 8) BDP-CR + 671 nm light. 1×10⁶ 4T1 cells suspended in 100 μL PBS were subcutaneously injected into each mouse to establish the tumor models. 7 days after the tumor cells were implanted (the tumor volume is about 160 mm³ at this point), corresponding drugs (20nM, 100 μL) were injected intratumorally into mice in group 3
to 8 while the mice in group 1 and 2 were injected with the same volume of pure PBS. Then the fluorescent dye, DCFH-DA (10 μmol/L, 50 μL) was injected intratumorally 4 h after injection in all groups. Next, laser (671 nm, 0.3W/cm², 10 min) was performed in group 2, 6, 7 and 8. Subsequently, tumors from each group were dissected. The cryosections were observed by a confocal laser scanning microscope.

**In Vivo Biosafety Assay**

The *in vivo* biosafety was evaluated by the body weight of all mice and H&E slice histological analysis major organs. After 21 days post-treatment, the mice were euthanized, and main organs including heart, liver, spleen, lung, kidneys and tumor were harvested for histological analysis by means of hematoxylin-eosin (H&E) staining.

**Statistical Analysis**

Data were expressed as mean ± standard deviation. Student's t test was used to evaluate the statistical significance. P values < 0.05 were regarded statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

**Note**: no unexpected or unusually high safety hazards were encountered during the experiment.
Scheme S1. Synthesis routes of CR, BDP and BDP-CR.

Figure S1. (a) Photo-degradation curves of DPBF in the presence of (a) BDP or (b) CR under 671 nm light irradiation in DMSO.
**Figure S2.** Temperature change of CR in the PBS buffer solutions at various concentrations (0 μM, 5 μM, 10 μM, 20 μM and 40 μM) under 100 mW/cm² irradiation (671 nm).

**Figure S3.** Temperature change of (a) BDP or (b) CR (20 μM) in the PBS buffer solutions at different power densities (0 mW/cm², 25 mW/cm², 50 mW/cm², 100 mW/cm² and 200 mW/cm²) under 671 nm irradiation.
Figure S4. (a) Photothermal effects observed upon irradiating BDP-CR with 671 nm light (200 mW/cm$^2$) for 360 s, and then the light was switched off. (b) Time versus-$\ln(\theta)$ plot (with $\theta$ being the driving force temperature, cf. eq S4) obtained using the data recorded during the cooling period of the experiment outlined in (a).

Figure S5. Absorption spectra of BDP-CR pre- and post-808 nm light irradiation (200 mW/cm$^2$, 10 min). Insets are photographs of BDP-CR dispersion pre- (left) and post- (right) light irradiation.
Figure S6. Temperature evolutions of BDP-CR under light irradiation (200 mW/cm²) during five circles of heating-cooling processes.

Figure S7. (a) Cellular uptake of BDP-CR and BDP in 4T1 cells. (b) Intracellular average fluorescence intensity at different time points after incubation with BDP or BDP-CR. Scale bars: 30 μm
Figure S8. Confocal microscopic images of BDP-CR co-localized with various commercially available organelle trackers in 4T1 cells. 4T1 cells were incubated with BDP-CR (2.0 μM) for 4 h and then with (b) LysoTracker Green (LTG), (f) MitoTracker Green (MTG) and (j) Hoechst 33342 (Hoechst) for 30 min. (c) Merged image of (a) and (b). (g) Merged image of (e) and (f). (k) Merged image of (i) and (j). (d) Co-localization scatterplots of (c). PC: 0.86. (h) Co-localization scatterplots of (g). PC: 0.27. (l) Co-localization scatterplots of (k). PC: 0.02. Scale bars: 30 μm.
Figure S9. Dark toxicity and phototoxicity effects for MCF-7 (a and b), Hela (c and d) and B16 (e and f) cells treated with CR, BDP or BDP-CR at different concentrations following 671 nm light (100 mW/cm²) irradiation for 5 min.

Figure S10. Calcein AM and propidium iodide co-staining fluorescence imaging in MCF-7, Hela and B16 cells. Scale bars: 400 μm.
**Figure S11.** Cell viability of 4T1 cells treated with BDP-CR at different concentrations under 671/808 nm light (100 mW/cm²) irradiation for 5 min. Statistical significance: **P < 0.01** and ***P < 0.001.

**Figure S12.** Confocal fluorescence images of Annexin VFITC/PI-stained 4T1 cells with different treatments. λ<sub>ex</sub>: 488 nm, λ<sub>em</sub>: 505-545 nm (FITC); 620-700 nm (PI). Scale bars: 30 μm.
Figure S13. Flow cytometry analysis by Annexin V-FITC and PI staining of 4T1 cells following different treatments. Cells were incubated under different conditions and divided into six groups, including (1) control; (2) 671 nm light; (3) CR; (4) BDP-CR; (5) BDP plus 671 nm light irradiation and (6) BDP-CR plus 671 nm light irradiation.

Figure S14. *In vivo* fluorescence imaging of 4T1 tumor-bearing BALB/c mice after injection of BDP and BDP-CR at 0.5, 1, 2, 4 and 6 h.
Figure S15. (a) Mouse and tumor imaging of group “BDP+671 nm light” and “BDP-CR+671 nm light” at the 14th day, respectively. (b) Tumor weight from different groups of tumor-bearing mice. ***P < 0.001, ****P < 0.0001 determined by Student’s t test.

Figure S16. Digital photos of tumors harvested from each group on day 21 post treatments.
Figure S17. In vivo biosafety assay. H&E staining of heart, liver, spleen, lung, and kidney of mice from different treatment groups after 21 days of treatment. Scale bar = 100 μm.
Figure S18. The hemolysis rate of CR, BDP and BDP-CR at different concentrations.
Each point represents the mean±S.D. (n=3).

Figure S19. ESI-HRMS spectrum of compound 1.
Figure S20. ESI-HRMS spectrum of compound 2.

Figure S21. ESI-HRMS spectrum of compound CR.
Figure S22. $^1$H NMR spectrum of CR.

Figure S23. $^{13}$C NMR spectrum of CR.
Figure S24. ESI-MS spectrum of compound 3.

Figure S25. ESI-MS spectrum of compound 4.
Figure S26. ESI-MS spectrum of compound 5.

Figure S27. MALDI-HRMS spectrum of compound BDP.
Figure S28. $^1$H NMR spectrum of BDP.

Figure S29. $^{13}$C NMR spectrum of BDP.
Figure S30. MALDI-HRMS spectrum of compound BDP-CR.

Figure S31. $^1$H NMR spectrum of BDP-CR.
Figure S32. $^{13}$C NMR spectrum of BDP-CR.

Supporting Reference

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