Supplementary Information (SI)

Triphenylamine Flanked Furan-diketopyrrolopyrrole for Multi-imaging Guided Photothermal/Photodynamic Cancer Therapy

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Experimental Section

Materials

All commercial chemicals and materials were purchased from Sigma-Aldrich and used without further purification unless especial explanations. 2',7'-dichloro fluorescein diacetate (DCFH-DA), 4',6-diamidino-2-phenylindole (DAPI), N-acetyl-L-cysteine (NAC) and cells were purchased from Institute of Biochemistry and Cell Biology, SIBS, CAS (China). Fetal bovine serum and 3-4, 5-dimethyl-2-thiazoly l-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from GIBCO.

Characterization

Intermediate products and FDPP-TPA were characterized via $^1$H NMR, $^{13}$C NMR spectra (Bruker DRX NMR spectrometer), fluorescence spectra (HITACHI, Japan), UV-vis-NIR spectrophotometer (Shimadzu, Japan), scanning electron microscope (SEM), transmission electron microscopy (TEM, JEM-2010FEF) and dynamic light scattering (DLS, Malvern Zeta Sizer, Nano-ZS, UK). Other apparatus were optical fiber coupled 660 nm diode-laser, confocal fluorescence microscope (Olympus IX 70), flow cytometry BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA), multispectral photoacoustic tomography (MSOT) system and IR thermal camera (FLIR Systems, Inc., Wilsonville, OR, USA). Animal procedures were performed in a strict accordance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication no. 85-23 Rev. 1985). Besides, all experiments were approved by the research center for laboratory animals of Yangzhou university of traditional Chinese medicine (Yangzhou, China). For the protection of human subjects, the investigators adhered to the policies of applicable law.
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Synthesis of FDPP-1

Tert-amyl alcohol (50 mL) and potassium tert-butoxide (8.00 g, 71.3 mmol) were added into a 100 ml two neck flask under N\textsubscript{2} atmosphere. The mixture was heated to 110 °C. Then, dimethyl succinate (2.93 g, 19.8 mmol) and 4-cyanofuran (5.00 g, 53.7 mmol) in tert-amyl alcohol (10 mL) were injected. After one hour, methanol (3 mL) was extracted from the mixture and the reaction system was cooled to 65 °C. After two hours, methanol (80 mL) was added to terminate the reaction. Ten minutes later, acetic acid (5 ml) was injected to reaction system and kept overnight. The crude product was washed with warm water and methyl alcohol to acquire FDPP-1 (3.52 g, yield: 27%). \textsuperscript{1}H NMR (500 MHz, DMSO) \(\delta\) 8.62 (d, \(J = 4.3\) Hz, 2H), 8.58 (d, \(J = 4.2\) Hz, 2H), 8.0 (t, \(J = 7.6\) Hz, 2H), 7.24 (t, \(J = 7.2\) Hz, 2H). \textsuperscript{13}C NMR (126 MHz, DMSO) \(\delta\) 161.12, 146.76, 143.74, 131.24, 116.68, 113.56, 112.83, 107.57, 41.03, 37.82.

Synthesis of FDPP-2

FDPP-1 (2.68 g, 10 mmol), potassium carbonate (4.15 g, 30 mmol) and 1-Bromo-iso-octane (5.80 g, 30 mmol) were dissolved in N, N-dimethyl formamide (200 mL) and magnetically stirred at 120 °C for one day. Then washed the crude product with saturated salt water. At last, the residue was purified by a silica column (DCM / PE, V/V=6:1) to obtain FDPP-2 (2.55 g, yield: 50%). \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\) 8.33 (d, \(J = 3.6\) Hz, 2H), 7.61 (s, 2H), 6.85 (m, 2H), 4.04 (d, \(J = 7.3\) Hz, 4H), 1.76 (s, 2H), 1.54 (s, 4H), 1.40 – 1.18 (m, 16H), 1.02 – 0.81 (m, 10H). \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\) 145.91, 120.87, 114.97, 48.02, 40.99, 30.60, 28.71, 23.78, 22.99, 15.65, 10.56.
Synthesis of FDPP-TPA

FDPP-2 (1.01g, 2mmol), 4-Bromotriphenylamine (3.24g, 10mmol), potassium carbonate (0.69g, 5mmol), pivalic acid (0.15g, 1.5mmol) and palladium acetate (0.09g, 0.4mmol) were dissolved in dimethylacetamide (5 mL). The mixture was reacted under the nitrogen condition for 10 hours. Then washed the crude product with saturated salt water. At last, the residue was purified by a silica column (DCM/PE, V/V=6:1) to offer FDPP-TPA (1.58g, yield: 80%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 8.47 (s, 4H), 7.59 (d, J= 8.5 Hz, 2H), 7.34 (d, J= 2.8 Hz, 2H), 7.24 (d, J= 13.4, 5.7 Hz, 8H) 6.81-6.69 (m, 16H), 2.79 (d, J = 11.6 Hz, 4H), 2.1-0.9 (m, 30H). \(^1\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 166.22, 159.60, 149.12, 147.57, 145.27, 138.89, 129.13, 127.92, 124.92, 124.37, 123.81, 123.51, 122.55, 108.43, 103.36, 48.58, 33.27, 31.26, 29.91, 23.36, 22.93, 13.78, 10.36.

Preparation of FDPP-TPA NPs

FDPP-TPA NPs were prepared by re-precipitation. 200 μL (2 mg/mL) FDPP-TPA solution (dissolved in THF) were dropwise added into deionized water (5 mL) at room temperature by magnetic stirring. During this process, the FDPP-TPA molecules self-assembled into FDPP-TPA NPs through hydrophobic interactions and π-π stacking. After stirring for five minutes, the THF was removed by bubbling nitrogen.

Morphology Analysis and Characterization

The microstructure and size of FDPP-TPA NPs were characterized by SEM and TEM. SEM samples were prepared by depositing FDPP-TPA NPs on silicon substrate and operated by FEI quanta 200F microscope. TEM samples were prepared by drop-casting FDPP-TPA NPs on copper grids and operated by JEOL JEM-1011 microscope. The sizes of the FDPP-TPA
NPs were recorded by dynamic light scattering (DLS) measurement on instrument of Malvern Zetasizer. Ultraviolet-visible (UV-vis) and fluorescence spectrum were operated by quartz cuvettes with optical path-length of 1 cm (wavelength range: 300-800 nm).

**Photothermal Performance of FDPP-TPA NPs**

Photothermal performance of FDPP-TPA NPs was recorded as previously work.\(^1\)\(^-\)\(^2\) Aqueous dispersion of FDPP-TPA NPs (2 mL) were irradiated with 660 nm laser at different concentrations (20, 40, 60 and 80 μg/mL) and different power densities (0.25, 0.5, 0.75 and 1.0 W/cm\(^2\)) for 10 min using IR thermal camera and BMIR software to monitor temperature changes and thermal images.

**Photothermal Conversion (PTC) Efficiency**

To measure the PTC efficiency of FDPP-TPA NPs, aqueous dispersion of FDPP-TPA NPs (2 mL) was irradiated with 660 nm laser (1.0 W/cm\(^2\)) for 10 min. The laser was shut off immediately when the temperature reached to a plateau. The temperature was recorded by IR thermal camera every 20 s. The same execution of deionized water was acted as a contrast test.

PTC efficiency was calculated by the following equation:

\[
\eta = \frac{hS (T_{\text{max}} - T_{\text{surr}}) - Q_0}{I (1 - 10^{-A730})}
\]

1) h represents heat transfer coefficient; S represents sample container surface area; \(T_{\text{max}}\) represents steady-state maximum temperature; \(T_{\text{surr}}\) represents ambient room temperature; \(Q_0\) represents energy input by the same solvent without NPs after the same laser irradiation. To calculate the \(hS\), \(\theta\) was defined:
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\[ \theta = \frac{T - T_{sur}}{T_{max} - T_{sur}} \quad 2) \]

Therefore the hS was calculated by the following equation:

\[ \tau_s = \frac{C_d m_d}{hS} \quad 3) \]

\( \tau_s \) represents characteristic thermal time constant; the heat capacity \( c_d \) of water was 4.2 J g\(^{-1}\) k\(^{-1}\); \( m_d \) represents the mass of the solution (g). \( Q_0 \) was calculated using the following equation:

\[ Q_0 = hS \left( T_{max} - T_{sur} \right) \quad 4) \]

Calculation of Singlet Oxygen Quantum Yield

Singlet oxygen quantum yield was calculated by \( \Phi_{\Delta(X)} = \Phi_{\Delta(MB)} \times (S_X/S_{MB}) \times (F_{MB}/F_X) \), in which the subscripts MB and X represent the methylene blue and FDPP-TPA, respectively. \( F \) represents absorption correction factor calculated by \( F = 1 - 10^{-OD} \) (OD represents absorbance of the MB and FDPP-TPA at 653 nm). \( S \) represents slope of absorbance plot of DPBF (416 nm) vs irradiation time.

Cell Culture and Incubation Conditions

HeLa cancer cells were obtained from the Institute of Biochemistry and Cell Biology, SIBS, CAS (China) and cultured in fresh Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% inactivated fetal bovine serum (FBS) and 1% (penicillin and streptomycin) under a humidified atmosphere with 5% CO\(_2\) and 95% air at 37 °C.

In vitro Photo-induced Cancer Cell Killing

For assessment photo-induced cancer cell killing in vitro, HeLa cells were seeded into two 96-well plates in 200 μL medium at a density of about \( 1 \times 10^5 \) cells per well (the replication
well number is five). The fresh complement medium with concentrations of FDPP-TPA NPs (0, 4, 8, 12, 16, 20, 24 and 28 μg/mL) was added into this two plates after 24 hours. One of the two plates was irradiated with a 660 nm laser (1 W/cm², 5 minutes) and the other was still kept in dark after 12 hours. The cells were continually incubated for 12 hours. Then the MTT solution (5 mg/mL, 20 µL) was added to each well and incubated for another 4 hours. After this DMSO (150 µL) was added to dissolve the purple precipitate. Absorbance intensity was measured at the optical densities (O.D) of 492 nm with a microplate reader. The mean cell viability and standard deviation for the parallel five wells for each concentration were calculated. Cell viability values were calculated by Cell viability (%) = absorbance of experimental group / the absorbance of control group ×100%.

**In vitro Cellular Uptake of FDPP-TPA NPs**

For the fluorescent cell imaging, HeLa cells were seeded into a confocal culture plate. After 24 hours, the fresh complement medium of 13 μg/mL FDPP-TPA NPs aqueous solution was added. The cells were stained with DAPI for 4 min at room temperature after 12 hours. The images were monitored by a laser scanning up-conversion luminescence microscope equipped.

**Intracellular ROS Assay**

HeLa cells were also seeded into confocal culture plates and adhered for 24 hours. Then cells were incubated in culture medium containing 13 μg/mL of FDPP-TPA NPs (2 mL) for another 24 hours in dark condition. After that, the cells were incubated in the DCFH-DA in dark condition for 20 min. At last, DAPI was used to stain. After the laser irradiation, cell imaging was performed with a laser scanning up-conversion luminescence microscope.
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equipped (excited wavelength: 405 nm, collection from 420 to 520 nm).

**Apoptosis Study by Flow Cytometry**

HeLa cells were seeded into 12-well plates and divided into 4 groups. Control group (without FDPP-TPA NPs) and three treatment groups (8, 12 and 16 μg/mL) were all irradiated with 660 nm laser (1 W/cm²). After incubation for 12 hours, the cells were stained with propidium iodide and annexin V FITC. The samples were measured by flow cytometer BD LSRFortessa.

**The Scratch-wound Healing Assay**

HeLa Cells were seeded into a 6-well plate and adhered for 80% confluence. A denuded diameter area was created by a yellow tip. Then the cells were washed with PBS (three times) and further incubated with different concentrations of FDPP-TPA NPs for 100% confluence (except the denuded area). After this, pictures were taken as initial contrast (0 h). Next, the cells were irradiated by 660 nm laser (1 W/cm²) light activation. At last, pictures were taken by microscope at different time after irradiation (12 h, 24 h and 48 h).

**Animals and Tumor Model**

18 nude mice (18-20 g weight, five weeks aged) were obtained from Yangzhou University (Comparative Medicine Centre). The HeLa tumors were inoculated by injection with 200 μL of PBS containing 4×10⁶ HeLa cells at left front leg subcutaneous. All the mice were carefully placed in the cages in ventilated animal rooms with free access to water and commercial laboratory complete food. *In vivo* experiments were carried out when the tumor volumes approached 150-200 mm³.

**Photoacoustic Imaging**
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For photoacoustic (PA) imaging in vitro, FDPP-TPA NPs in deionized water with various concentrations (0, 10, 20, 40 and 80 μg/mL) were measured with multispectral photoacoustic tomography system. For PA imaging in vivo, the mice must be anaesthetized by chloral hydrate solution. After the intravenous injection of FDPP-TPA NPs, the contrast data at 0, 3, 6, 9 and 12 h were also recorded using the MSOT system with excitation wavelength of 660 nm.

**In vivo Phototherapy**

The HeLa tumors bearing mice were randomly divided into three groups (saline only, NPs only and NPs with laser, n=6 / group). When the tumor volumes were about 150-200 mm$^3$, mice in the saline group were injected with 100 μL normal saline. Mice in NPs only and NPs with laser groups were injected with 100 μL DPPCN-Fc NPs. After 6 hours of the injection, the solid tumors of the NPs with laser group were irradiated with an optical fiber coupled 660 nm diode-laser for 8 min (laser beam diameter: 5 mm, power density:1 W/cm$^2$). The infrared thermal imaging camera was used to take thermal images. Tumor volumes and body weight of each group were recorded by caliper and digital scale for 18 days (one time every two days). Tumor size was calculated by formula of length × width × width / 2.

**Histology Sample Preparation**

After 18 days treatment, all mice were killed to harvest the tumors and major organs (liver, heart, lung, spleen, and kidney) for haematoxylin and eosin (H&E) staining. Finally, the slices were observed under optical microscopy.

**References:**

**Figure S1.** (a) UV-vis-NIR absorbance spectra of FDPP-TPA NPs before/after laser illumination (PBS, 660 nm, 1.0 W/cm², 10 min). (b) SEM image of FDPP-TPA NPs. Inset: TEM image of FDPP-TPA NPs after illumination in PBS. (c, d) DLS size distribution of FDPP-TPA NPs before and after illumination (PBS, 660 nm, 1.0 W/cm², 10 min).
Figure S2. Cell viability of HeLa cells in different conditions (660 nm laser, 1.0 W/cm², 5 min).