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Supporting Information

Design, synthesis and evaluation of novel π-π stacking nano-intercalator as anti-tumor agent

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Experimental section

1. General

Sprague Dawley rats and ICR mice were purchased from the Animal Center of Peking University. Work performed was based on a protocol reviewed and approved by the ethics committee of Capital Medical University. The committee assures that the welfare of the animals was maintained in accordance to the requirements of the Animal Welfare Act and in accordance to the NIH Guide for Care and Use of Laboratory Animals. Statistical analyses of all the biological data were carried out by use of ANOVA. P-values<0.05 were considered statistically significant.

The protected L-amino acid and reagents were available commercially (Sigma-Aldrich Corp, St Louis MO, USA). Anhydrous solvents were dried and purified by standard methods prior to use. Column chromatography was performed on silica gel of 200-300 mesh. The purity of the intermediates and the products was measured with thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC, C18 column 4.6×150 mm; Waters Corporation, Milford, MA, USA), and was greater than 97%. Reactions were monitored by TLC on glass plates coated with silica gel with a fluorescent indicator. Melting points were determined on a XT5 hot stage apparatus (Beijing Keyi Electro-Optic Factory, Beijing, PR China) and uncorrected.

Proton nuclear magnetic resonance (^{1}HNMR) spectra were recorded on a Bruker 500 MHz spectrometer, with ~5 mg of THPDTPI in 0.5 mL of deuteron dimethyl sulfoxide (DMSO-d6), and tetramethylsilane (TMS) was used as internal reference. The probe temperature was regulated to 298 K, and the spectra were recorded by using a simple pulse-acquire sequence zg30. Typical acquisition parameters consisted of 64 K points covering a sweep width of 16447 Hz, a pulse width (pw90) of 8.63 μs, and a total repetition time of 24 s to ensure full relaxation of the ^{1}H resonances. Digital zero filling to 64 K and a 0.3 Hz exponential function were applied to the FID before Fourier transformation.

Electrospray ionization (ESI) mass spectrometry (MS), ESI-MS, was measured on a ZQ 2000 (Waters Corp) and a 9.4 T solariX Fourier transform (FT) ion cyclotron

resonance (ICR), FT-ICR, mass spectrometer (Bruker Corp, Billerica, MA, USA), with an ESI/matrix-assisted laser desorption/ionization (MALDI) dual ion source.

2. ^{1}HNMR spectrum of THPDTPI

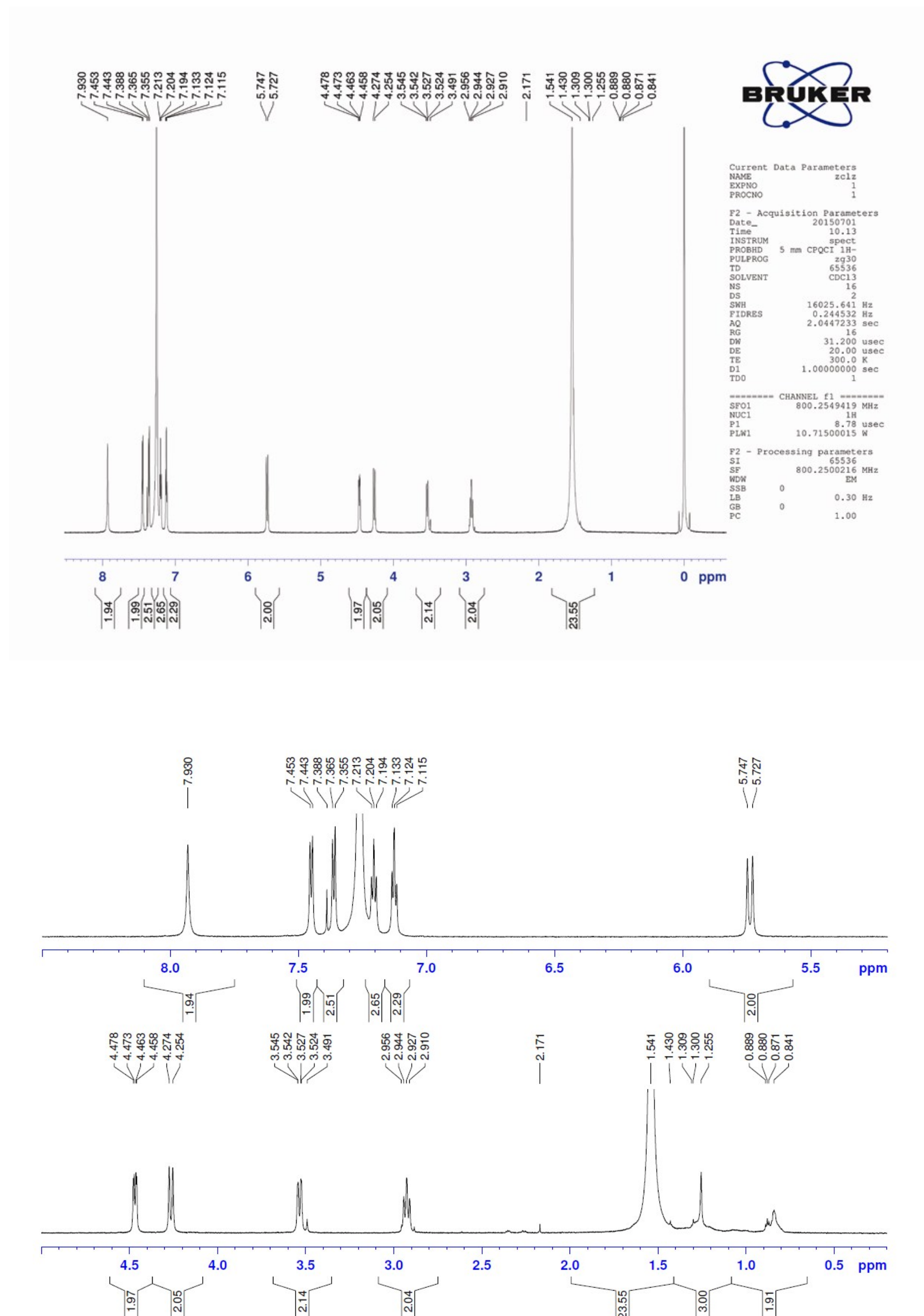

Fig. S1 ^{1}H NMR (800 MHz, $CDCl_3$): δ= 7.930 (s, 2H), 7.448 (d, *J* = 8.0 Hz, 2H), 7.360 (d, *J* = 8.0 Hz, 2H), 7.204 (t, *J* = 8.0 Hz, 2H), 7.124 (t, *J* = 8.0 Hz, 2H), 5.737 (d, *J* = 16.0 Hz, 2H), 4.468 (dd,

J = 12.0 Hz, *J* = 4.0 Hz, 2H), 4.264 (d, *J* = 16.0 Hz, 2H), 3.535 (dd, *J* = 14.4 Hz, *J* =2.4 Hz, 2H), 2.927 (t, *J* = 13.6 Hz, 2H).

3. ^{13}CNMR spectrum of THPDTPI

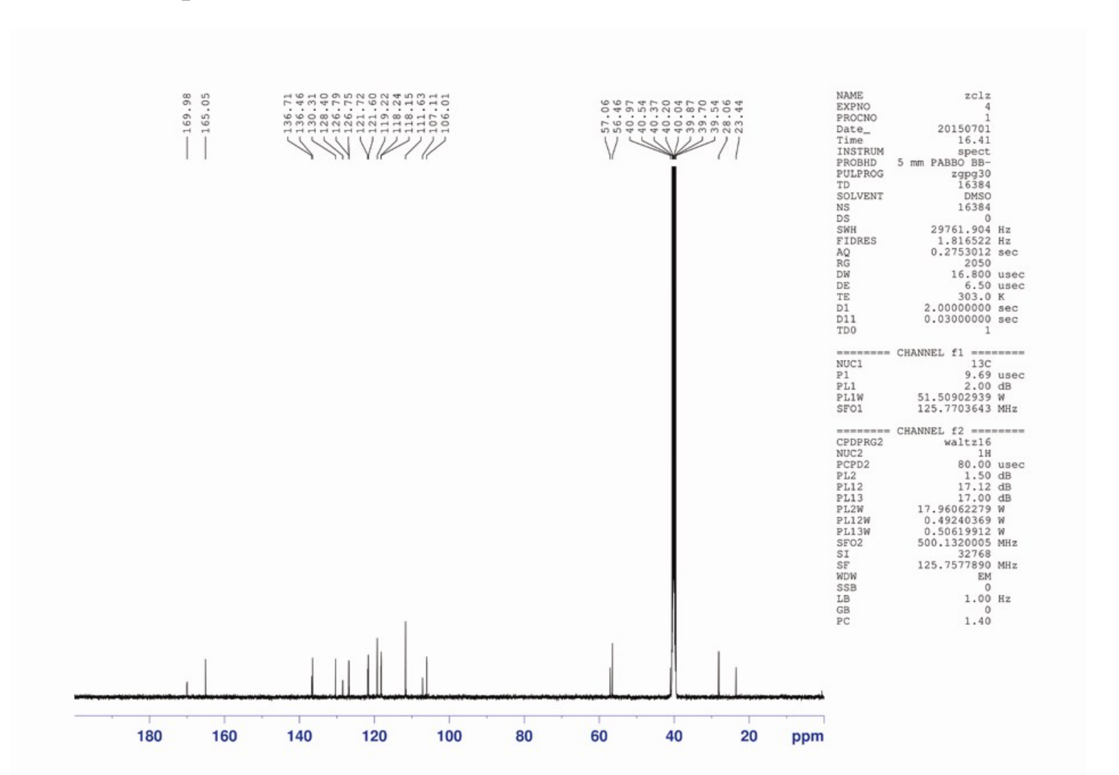

Fig. S2 ^{13}C NMR (125 MHz, DMSO-d6): δ= 169.98, 165.05, 136.71, 136.46, 130.31, 128.40, 126.75, 121.72, 121.60, 119.22, 118.24, 118.15, 111.63, 107.11, 106.01, 57.06, 56.46, 40.97, 28.06, 23.44.

4. NOESY 2D ^{1}HNMR of THPDTPI

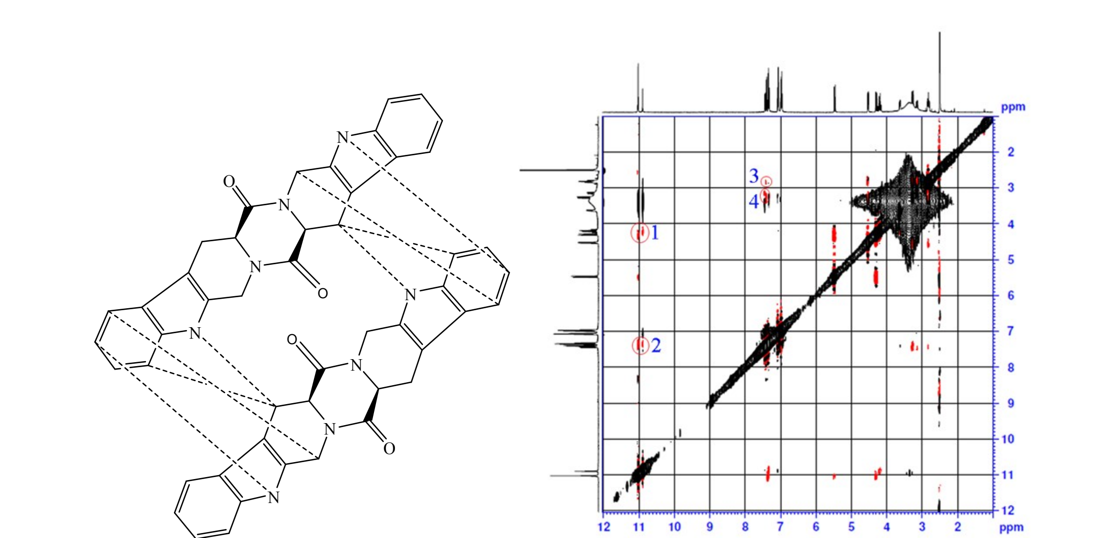

Fig. S3 NOESY 2D ^{1}HNMR spectrum: the cross-peaks labeled with red circles define intermolecular interaction of THPDTPI.

5. ESI(-)/FT-MS spectrum of THPDTPI

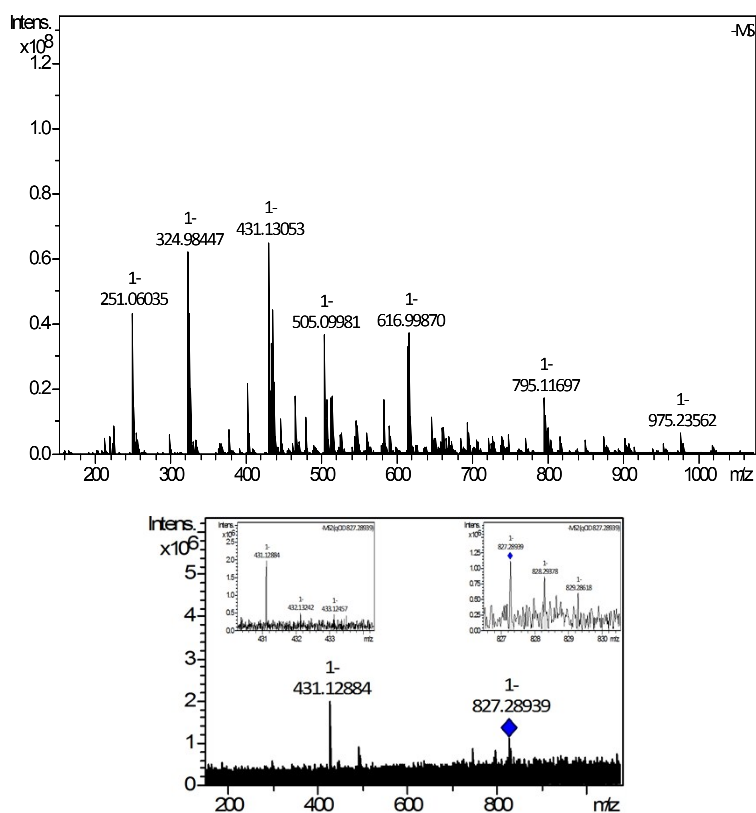

Fig. S4 ESI(-)/FT-MS full scan spectrum (up) and qCID ion scan spectrum (down).

6. HPLC purity of THPDTPI

An Agilent Technologies 1200 Series HLPC (high-performance liquid chromatogra phy) system (Agilent Technologies, Santa Clara, CA, USA) was used to measure the HPLC purity of THPDTPI. The sample was separated on a Waters XTerra C18 reversed-phase column (2.1×150 mm, 5 μm; Waters Limited, Hertfordshire, UK) protected by a guard column of the same material (5×10 mm, 5 μm). The column thermostat was maintained at 40 °C. To the column, 5 μL of a solution of THPDTPI in ultrapure water was injected for analysis. The mobile phase consisted of water and acetonitrile (5:95). The flow rate was 0.2 mL/minute. The column was washed with water and methanol (35:65), and equilibrated to initial conditions for 15 minutes. Ultraviolet (UV) absorption spectra were recorded online. The UV detector was set to a scanning range of 200-400 nm, and a wavelength of 254 nm was used to monitor THPDTPI. The chromatogram was recorded and gave THPDTPI a retention time of 5.704 minutes and 98.9% purity.

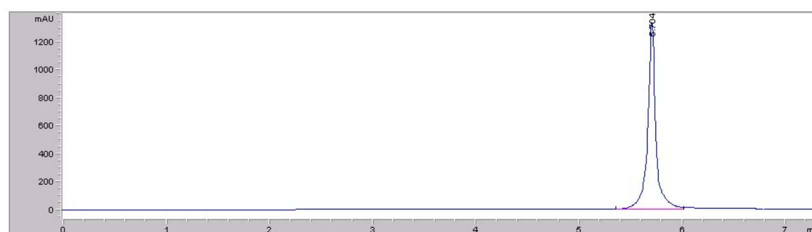

Fig. S5 HPLC program of THPDTPI.

7. Faraday-Tyndall effect of THPDTPI

To explore the nano-property of the aqueous solution of THPDTPI Faraday-Tyndall effect was tested, 1 nM solution of THPDTPI in pH2.0 and pH7.0 ultrapure water were irradiated with laser beam of 650 nm. In addition, the zeta potential and size thereof were determined on a Malvern's Zetasizer (Nano-ZS90; Malvern Instruments) with the DTS (Nano) Program.

8. Theoretically predicted nanoparticle size of THPDTPI

To theoretically predict the formation and the size of the nanoparticles the mesoscale simulation software was used to perform the calculation. The molecule of THPDTPI was built and optimized simply in the Visualizer window. "Beads" were constructed from atomistic simulations and placed at the center-of-mass of groups of the atoms corresponding to particular parts of a molecule.

9. TEM image of THPDTPI

The shape and size of THPDTPI were imaged with transmission electron microscopy (TEM, JSM-6360 LV, JEOL, Tokyo, Japan). An aqueous solution of THPDTPI (pH 7.0) was dripped onto a formvar-coated copper grid. After drying in air the grid was heated at 35 °C for 24 h. The grids were viewed under TEM to image the shape and size of the nanospecies by counting >100 species in the regions selected randomly, and all grids were viewed in triplicate. The TEM was operated at 80 kV (the electron beam accelerating voltage). Images were recorded on imaging plate (Gatan Bioscan Camera Model 1792; Gatan, Inc., Pleasanton, CA, USA) with 20eV energy windows at 6,000- 400,000× and were digitally enlarged.

10. SEM image of THPDTPI

The shape and size of the precipitates of THPDTPI were imaged with scanning electron microscopy (SEM, JEM-1230; JEOL) at 50 kV. With double-sided tape

(Euromedex, Souffelweyersheim, France) the precipitates were attached to a copper plate, which were coated with 20 nm gold-palladium using a JEOL JFC-1600 Auto Fine Coater. The coater was operated at 15 kV, 30 mA, and 200 mTorr (argon) for 60 s. The copper plates were viewed under SEM to image the shape and size of the precipi- tates by counting >100 species in the regions selected randomly, and all plates were viewed in triplicate. Images were recorded on an imaging plate (Gatan Bioscan Camera Model 1792; Gatan, Inc.) with 20 eV energy windows at 100-10,000× and were digitally enlarged.

11. AFM image of nanoparticles of THPDTPI

Atomic force microscopy (AFM) images were obtained using the contact mode on Nanoscope 3D AFM (Veeco Instruments, Inc., Plainview, NY, USA) under ambient conditions. Samples of rat plasma alone and rat plasma plus THPDTPI were used for recording the images, and all samples were imaged in triplicate.

12. Generation of 3D structure for docking

The 2D structure of THPDTPI was sketched in ChemDraw Ultra 10.0, converted to 3D conformation in Chem3D 10.0, and then energy minimized in Discovery Studio 3.5 with a Merck molecular force field (Merck & Co.) until the minimum RMS reached 0.001 in Chem3D Ultra 10.0. The energy optimized conformations in the whole conformational space of THPDTPI were sampled with systematic search and BEST method of Discovery Studio 3.5, which were practiced with a SMART minimizer using CHARMM force field. The energy threshold was set to 20 kcal/mol at 300 K. The maximum minimization steps were 200 and the minimization root mean squared (RMS) gradient was 0.1 Å. The maximum generated conformations were 255 with a RMS deviation (RMSD) cutoff of 0.2 Å. Top 10 energy optimized conformations of THPDTPI were used for the docking to DNA.

13. Docking toward active site of DNA

Software AutoDock 4 was used to perform the molecular docking of 10 energy optimized conformations of THPDTPI toward DNA. The DNA was treated as rigid and prepared by AutoDockTools 1.5: merging nonpolar hydrogens and assigning gasteiger charges and autodock elements. The 10 energy optimized conformations of

THPDTPI were treated as rigid ligands and prepared by AutoDockTools 1.5: merging nonpolar hydrogens, assigning gasteiger charges, finding root and aromatic carbons, detecting rotatable bonds, and setting torsions. The grid box dimensions were set to 50 Å × 50 Å × 50 Å with a grid spacing of 0.375 Å. The Lamarckian genetic algorithm (LGA) was used to find the appropriate binding positions, orientations, and the conformations of THPDTPI in the active site pocket of P-selectin. The global optimization was started with parameters of a population of 300 randomly positioned individuals. The maximum number of energy evaluations was increased to 2.5×10^7, and the maximum number of generations in the LGA algorithm was increased to 2.7×10^5. The Solis and Wets local search was performed with a maximum number of 3000. During the molecular docking experiments, 200 runs were carried out for each ligand. The resulted 200 conformations of each ligand were scored by the lowest binding energy and clustered with an rms tolerance of 2.0 Å.

14. *In vivo* anti-tumor assay

Male ICR mice (purchased from Capital Medical University) were maintained at 21°C with a natural day/night cycle in a conventional animal colony. Mice were 10 weeks old at the beginning of the experiment. S180 ascites tumor cells were subcutaneously injected to form solid tumors. To initiate subcutaneous tumors, cells obtained in ascitic form from tumor-bearing mice were serially transplanted once per week. Subcutaneous tumors were implanted under the skin at the right armpit by injecting 0.2 mL of normal saline (NS) containing 2×10^6 viable tumor cells. Twenty-four hours after implantation, mice were randomly divided into treatment groups (12 per group). Treatments were: THPDTPI (0.01, 0.1 and 1.0 μmol/kg/day), doxorubicin (Dox, positive control, 2μmol/kg/day), 0.5% carmellose sodium (CMCNa, vehicle, negative control, 0.2 mL/mouse/day). THPDTPI and CMCNa were orally given every day for 7 days. Dox was intraperitoneally injected every day for 7 days. Mice were weighed daily. Twenty-four hours after the last administration, mice were weighed, sacrificed by ether anesthesia, and dissected to immediately obtain and weigh the tumor.

15. Acute toxicity assay

Male ICR mice were purchased from Laboratory Animal Center of Capital Medical University for determining acute toxicity. The mice were maintained at 21 °C with a natural day/night cycle in a conventional animal colony and were 10 weeks old at the beginning of the experiment. Twelve hours after fasting, mice were randomly divided into eight groups (6 per group). The mice were orally administered with the suspension of THPDTPI in CMCNa (dose: 100 μmol/kg, 10000 times of the minimal effective dose) or CMCNa (0.5%, 0.2 mL/mouse). Four hours or 7 days after the administration the mice received ether anaesthesia and were sacrificed to sample the blood and organs for toxic analysis. Besides, THPDTPI and CMCNa treated the S180 mice have similar organ weights that are shown in Figure S6 of the ESI.†. This means that the administration THPDTPI induces no organ toxicity, and consequently induces no Dox-like heart toxicity in particular.

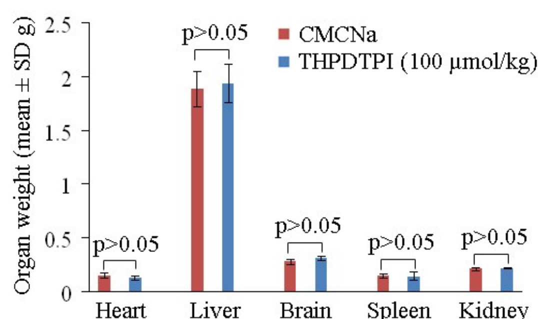

Fig. S6 Organ weight of THPDTPI and CMCNa treated S180 mice.

16. Determination of serum ALT, AST and Cr

To estimate the effect of THPDTPI on liver and kidney function the blood of ICR mice receiving acute toxicity assay was centrifuged to obtain serum for measuring alanine transaminase (ALT), aspartate transaminase (AST) and creatinine (Cr) by using alanine aminotransferase assay kit, aspartate aminotransferase assay kit and creatinine assay kit, respectively, on a microtiter plate reader within 15 min and recording O.D. value. All kits were purchased from Nanjing Jiancheng Bioengineering Institute. According to the standard curve the serum concentrations of ALT, AST and Cr were calculated (n=12). Data were statistically analyzed by t-test. P-values < 0.05 were considered statistically significant.

17. UV spectrum assay reflecting the interaction of THPDTPI and CT DNA

UV spectrum assay can visualize the intercalation of THPDTPI towards CT DNA, and this assay was performed. In brief, after recording the UV spectra (Shimadzu 2550 spectrophotometer, 260-400 nm wavelength) of THPDTPI (3 mL, 0.9 μM, in pH 7.4 PBS) in the presence or absence of CT DNA (in pH 7.4 PBS, final concentration: 0, 0.8, 1.6, 2.4, 3.2, 4.0 μM) or the UV spectra of CT DNA (3 mL, 200 μM, in pH 7.4 PBS) in the presence or absence of THPDTPI (in pH 7.4 PBS, final concentration: 0, 1.6, 3.0, 4.1, 5.1, 6.0, 6.7, 7.4 μM) were recorded to monitor the intercalation of THPDTPI towards CT DNA and to identify the intercalation model.

18. Fluorescent spectrum assay for THPDTPI intercalating towards CT DNA

Fluorescent spectrum assay can visualize the intercalation of THPDTPI towards CT DNA, and this assay was performed. In brief, after recording the fluorescent spectra (Shimadzu RF-5310PC spectrofluorometer, 286 nm of fluorescence excitation wavelength) of THPDTPI (2 mL, 0.18 μM, in pH 7.4 PBS) in the presence or absence of CT DNA (in pH 7.4 PBS, final concentration: 0, 4.5, 13.7, 18.5, 23.7, 28.1μM) or the fluorescent spectra of solutions of CT DNA (20 μL, 80 μM, in pH7.4 PBS) in the presence or absence of THPDTPI (in pH 7.4 PBS, final concentration: 0, 0.2, 0.4, 0.5, 0.7, 0.9 μM)were recorded to monitor the intercalation of THPDTPI towards CT DNA and to identify the intercalation model.

19. Relative viscosity assay for THPDTPI intercalating towards CT DNA

The intercalation of THPDTPI towards DNA was mirrored with the relative viscosity of DNA, and the data were recorded on Ubbeholde viscometer immersed in a thermo-stated water bath maintained at 25 °C. In the assay to a solution of CT DNA in PBS (13 mL, 80 μM) the solutions of THPDTPI in PBSwere added for keeping the ratio of [THPDTPI]:[CT DNA] in the range of 0-0.7 to form the samples, and the flow time of the samples were measured after a thermal equilibrium time of 5 min. The flow time of each sample was calculated. The relative viscosity of CT DNA in the presence and absence of THPDTPI were calculated from the equation, $\eta = (t - t_0)/t_0$, wherein t_0 and t were the observed flow time in the absence and presence of THPDTPI. Data are presented as $(\eta/\eta_0)^{1/3}$ versus binding ratio, wherein η is the viscosity of CT DNA in

the presence of THPDTPI and η_0 is the viscosity of CT DNA alone.

21. CD spectrum assay for THPDTPI intercalating towards CT DNA

In CD experiments a solution system (pH 7.4) consisting of a solution of CT DNA in PBS buffer (final concentration, 14 μM) and a solution of THPDTPI in PBS buffer (final concentration, 0, 36, 72, 108 μM) were incubated at 37 ºC for 3 h, and the spectra were tested according to a standard procedure.

22. In vitro anti-proliferation assay

In vitro cell viability assays were carried out using 96-well microtiter culture plates and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] staining, according to the standard procedures. S180 and HeLa cells (5×10^4 cells/mL) were grown in DMEM medium [containing 10% (v/v) fetal calf serum, 60 μg mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin]. Stock solutions of THPDTPI were prepared in DMSO and diluted with culture medium to desired concentrations. Cultures were propagated at 37 °C in a humidified atmosphere (with 5% CO_2) for 24 hours, and then THPDTPI was added (0.01, 0.1, 1, 5, 10 and 50 μM). After 48 hours of treatment, MTT solution was added (5 μg/mL; 25 μL per well), and cells were incubated for an additional 4 hours. The optical density was measured at 570 nm by a micro-plate reader, after adding 100 μL of DMSO to dissolve the MTT-formazan product (n = 3).