Electronic Supplementary Material (ESI) for Dalton Transactions.
This journal is © The Royal Society of Chemistry 2020

Electronic Supplementary Information

DNA topoisomerases as additional targets for anticancer monofunctional platinum(II) complexes

Hongmei Zhang, Tao Yang, Yanqing Wang, Zenghui Wang, Zhenzhu Zhu, Zijian Guo and Xiaoyong Wang*

1. Supplementary figures and tables

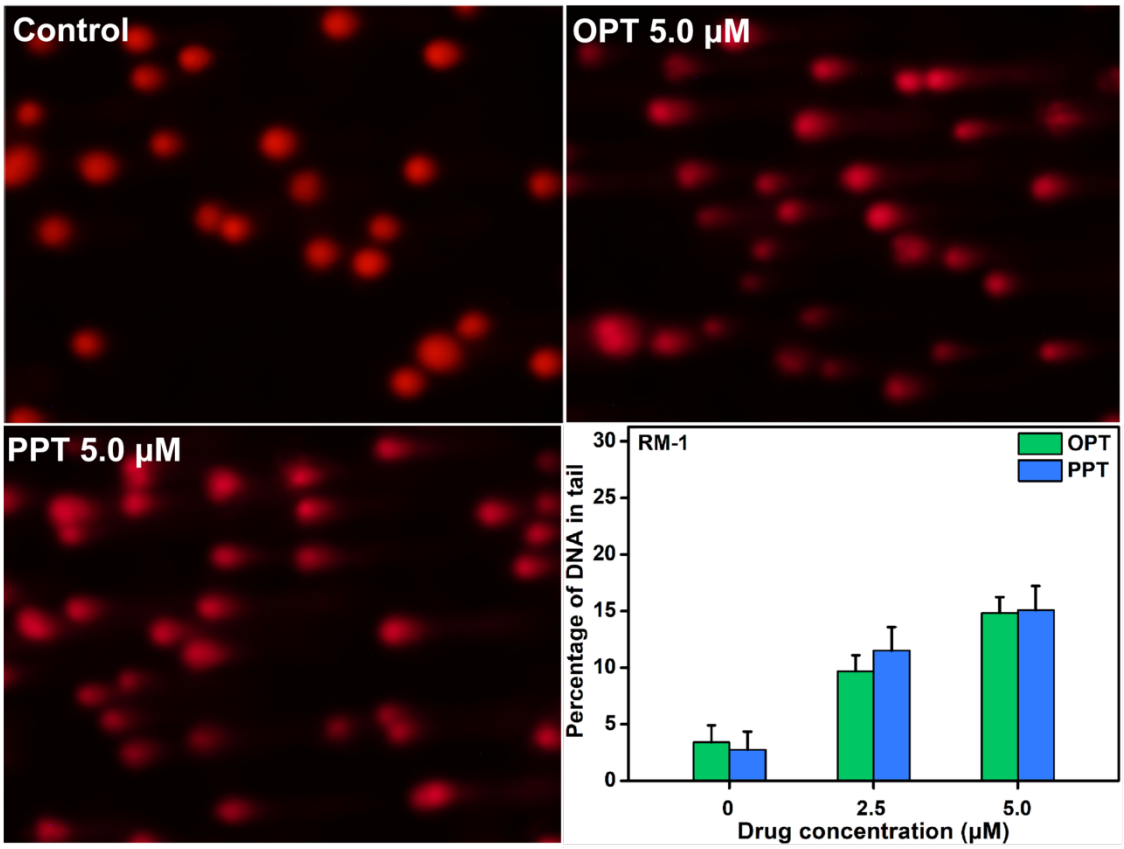

Fig. S1 Morphological appearance and quantification of DNA tails of RM1 cells after treatment with OPT and PPT, respectively, at different concentrations for 24 h. The data are presented as the means ± S.D. of three independent experiments.

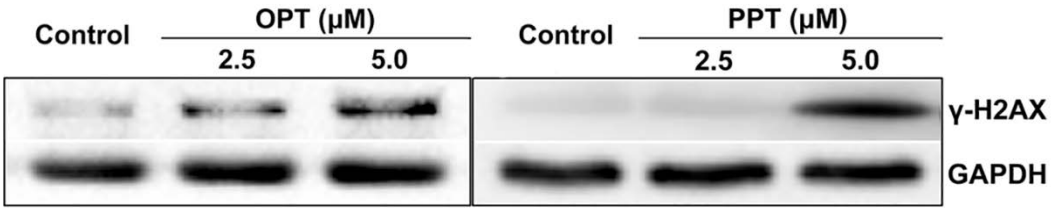

Fig. S2 Expression of γ-H2AX in RM-1 cells after exposure to OPT and PPT respectively for 24 h. GAPDH was used as a loading control.

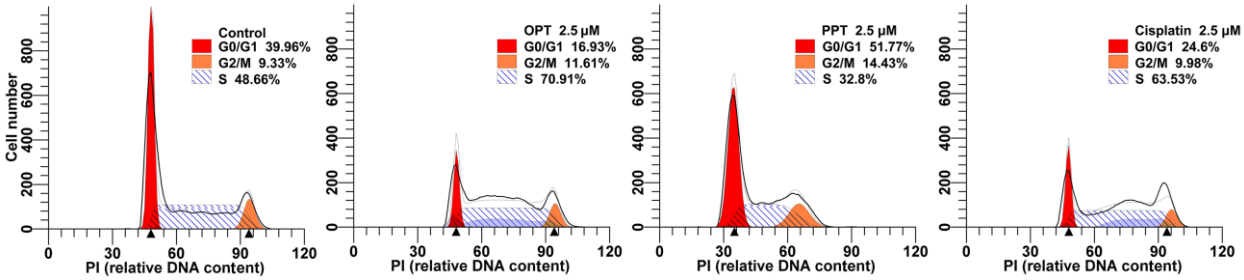

Fig. S3 Effect of OPT, PPT or cisplatin on the cell cycle of RM-1 cells after treatment for 24 h.

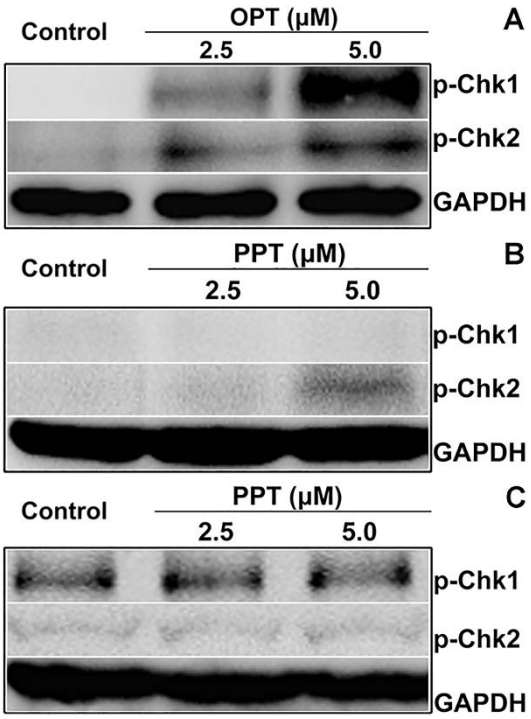

Fig. S4 Expressions of cell cycle checkpoint proteins p-Chk1 and p-Chk2 in RM1 cells after treatment with OPT (A) and PPT (B) respectively for 24 h, and in 786-O cells after treatment with PPT for 24 h (C).

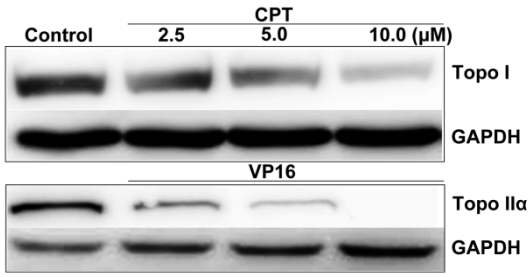

Fig. S5 Effect of CPT and VP16 on the expression of Topo I and Topo IIα in 786-O cells.

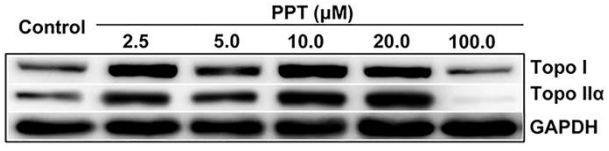

Fig. S6 Effect of PPT on the expression of Topo I and Topo II*α* in 786-O cells. GAPDH

was used as a loading control; data are presented as mean ± SD (n = 3).

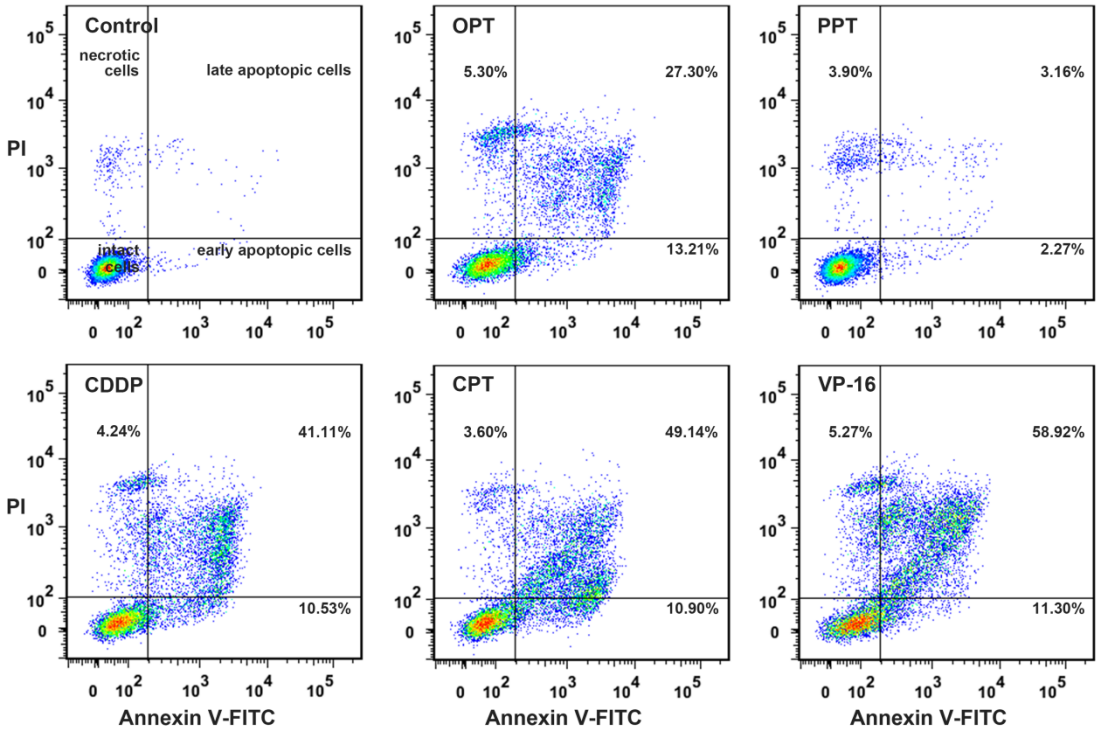

Fig. S7 Flow cytometric analysis of RM-1 cells after incubation with OPT, PPT, or CDDP (5 μM) for 48 h and subsequent staining with annexin V and PI. CPT and VP16 were used as positive controls for Topo I and Topo II*α* inhibitors, respectively.

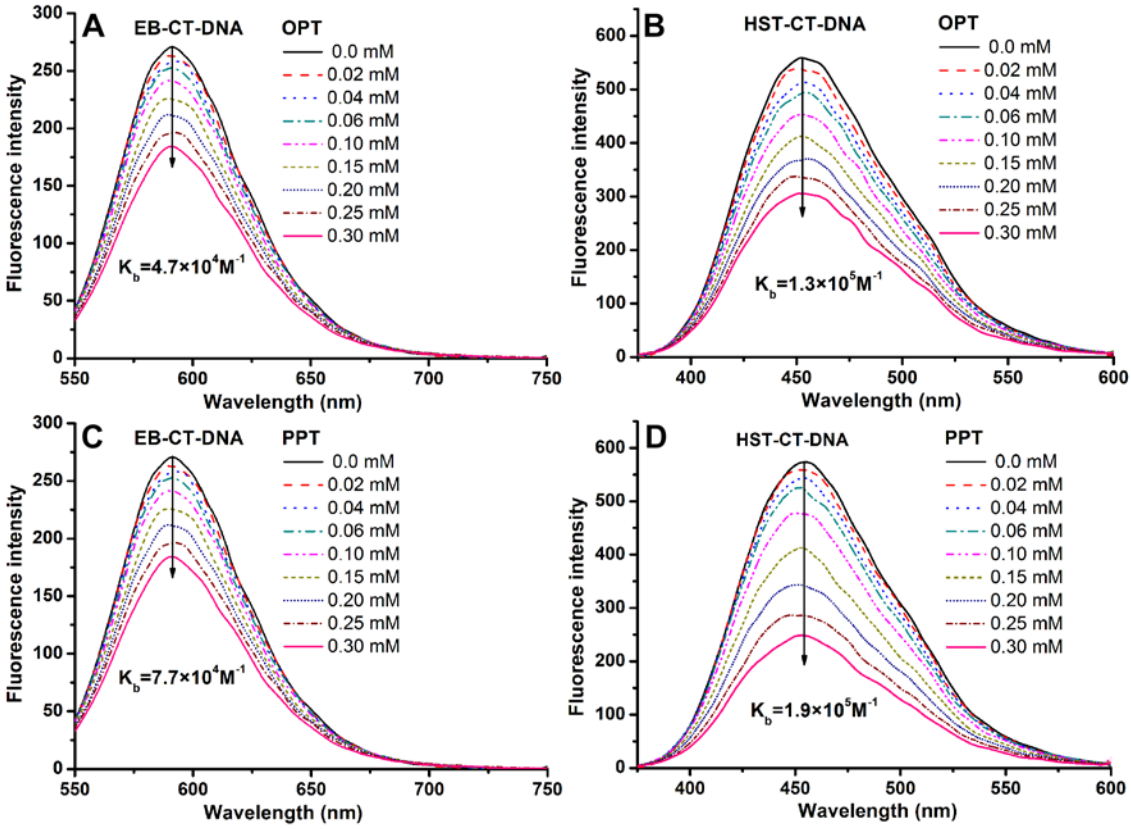

Fig. S8 Changes of fluorescence spectra upon titration of different concentrations of OPT or

PPT to the EB-CT-DNA (A, C) and Hoechst-CT-DNA (B, D) systems, which indicate the intercalative binding of OPT and PPT in the minor groove of calf thymus DNA.

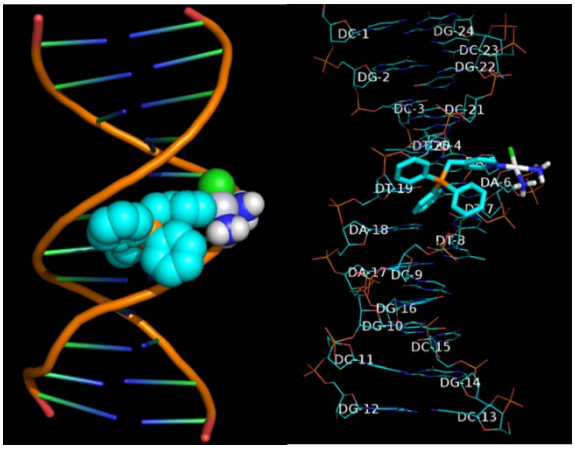

Fig. S9 Docking pose of PPT at the binding site of B-DNA.

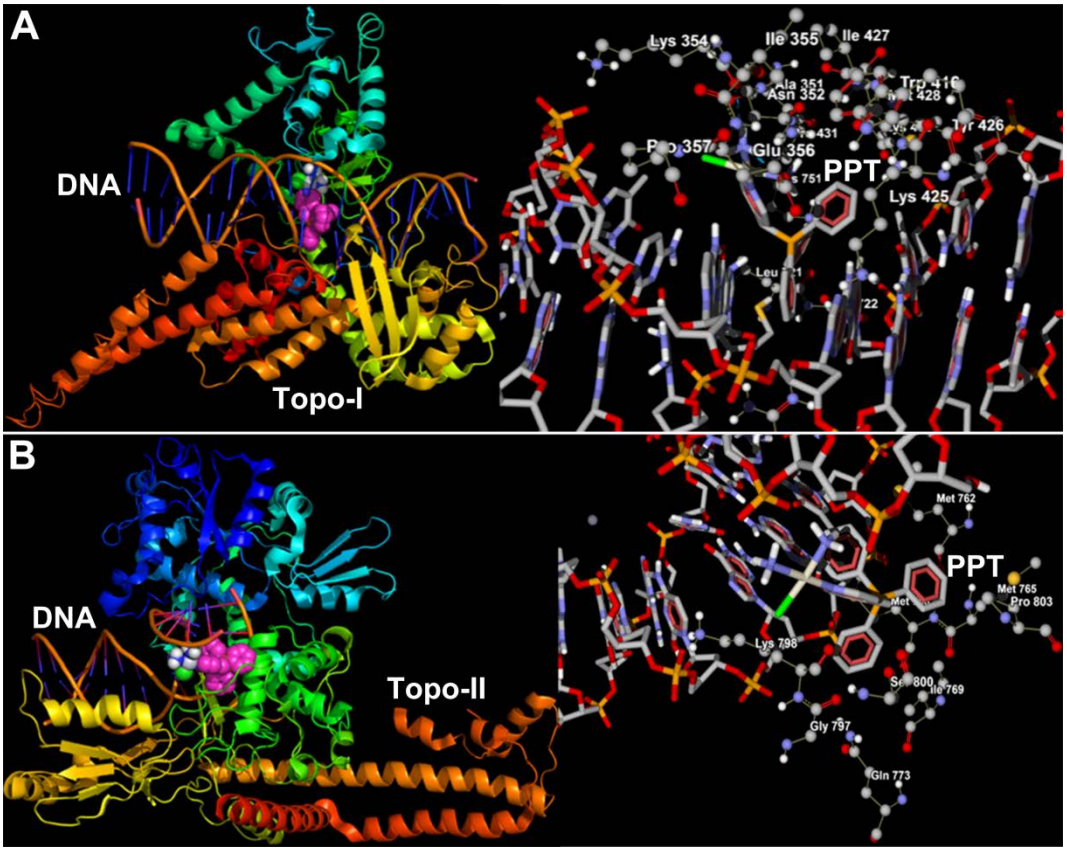

Fig. S10 The most possible binding sites of PPT with Topo I-DNA (A) and Topo II-DNA (B) complexes.

2. Experimental section

Chemicals and reagents

OPT and PPT were prepared as described previously.[1] Cisplatin was purchased from Shandong Boyuan Pharmaceutical Co., Ltd. China. Camptothecin (CPT), etoposide (VP-16), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetr-azoliumbromide (MTT),

ethidiumbromide (EB) and dimethysulfoxide (DMSO) were obtained from Sigma Aldrich. All reagents are of analytical grade and used without further purification unless specially noted. Purified human Topo IIα enzymes, Topo IIα assay kits, and unwinding kit were from TopoGen Inc. (Port Orange, FL). DNA Topoisomerase I and pBR322 DNA were purchased from Takara biotechnology (Dalian) Co., Ltd..

Cell culture

The human renal clear cell carcinoma cell line 786-O was purchased from KeyGen Biotech. Co. Ltd. (Nanjing, China). The murine prostate cancer cell line RM-1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics including 80 $U \cdot mL^{-1}$ penicillin and 80 $\mu g \cdot mL^{-1}$ streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. The cell suspensions were adjusted to a concentration of 1×10^6 viable cells/mL. Cells were cultured for about 24 h prior to treatment with the indicated concentrations of the complexes, and the vehicle control DMSO (final concentration was less than 0.1%, vol/vol).

Cytotoxicity

Cytotoxicity was measured using the MTT assay to evaluate the cytotoxicity of the complexes.[2] The cell suspensions were seeded in the 96-well plates, and after 24 h incubation, the cells were treated with various concentrations of OPT, PPT and cisplatin, respectively, for different time periods. After exposure to the drugs for a certain time, MTT (20 μL, 5 $mg \cdot mL^{-1}$ in PBS) solution was added to each well, and the plates were incubated at 37°C for another 4 h to allow MTT to metabolize into formazan crystals. The medium was removed and the MTT formazan was dissolved in DMSO (150 μL). The plates were shaken for 10 min and the optical density of each well was measured on a Varioskan Flash multimode reader (Tokyo, Japan) at 490 nm. Each test was performed in triplicate. Cell viability (%) and IC_{50} were calculated according to the ratio of $(OD_{test}–OD_{blank})/(OD_{contol}– OD_{blank}) \times 100\%$.

Cell cycle analysis

Cell cycle progression was analyzed by flow cytometry as previously described.[3] Briefly, cells were seeded in each well of a 6-well plate and incubated at 37°C in a 5% CO_2 humidified atmosphere overnight. Then the cells were incubated with OPT or PPT for 24 h. The attached cells were trypsinized and collected after mild centrifugation. The cells were washed with phosphate-buffered saline (PBS) and fixed in ice-cold 70% ethanol and stored at –20°C overnight. After washing twice with cold PBS, 100 μL of RNase A (KeyGenBiotech) was added and incubated at 37 °C for 30 min; 400 μL of propidium iodide (PI; KeyGenBiotech) was added to the cells and the cells were further incubated at 4°C for 30 min in the dark until measurements. The cell cycle analysis was performed on a BD LSRFortessa flow cytometry. The data were analyzed by the FlowJo 7.6.5 software (FlowJo LLC, Ashland, USA).

Topo I-mediated relaxation of supercoiled plasmid

DNA Topo I inhibitory activity was assessed by measuring the relaxation of negative supercoiled plasmid DNA pBR322. A mixture (20 μL) containing 250 ng of

supercoiled pBR322 plasmid DNA (Takara biotechnology, Dalian), 1 unit of recombinant human DNA Topo I (Takara biotechnology, Dalian), Tris-HCl (35 mM, pH 8.0), KCl (72 mM), $MgCl_2$ (5 mM), DTT (5 mM), spermidine (2 mM), and 0.01% bovine serum albumin (BSA) was incubated with or without OPT or PPT at 37°C for 30 min. Camptothecin (CPT) was used as a positive control. The reaction was terminated by 2 μL of 10% SDS and 4 μL of 6× loading buffer. The samples were loaded onto a 1% agarose gel and electrophoresed at 70 V for 3 h with TAE as the running buffer. The gels were stained with EB solution (0.5 $mg \cdot mL^{-1}$), destained with water, and photographed.

Topo II-mediated kinetoplast DNA (kDNA) decatenation

The effects of OPT and PPT on Topo II enzymatic activity were analyzed using a kinetoplast DNA (kDNA) assay according to the manufacturer's protocol. Briefly,120 ng of kDNA (TopoGen, USA) was incubated with 1U of Topo II (TopoGen, USA) in the presence or absence of OPT or PPT in a mixture (20 μL) containing Tris-HCl (250 mM, pH 8.0), NaCl (750 mM), $MgCl_2$ (50 mM), dithiothreitol (2.5 mM), and ATP (10 mM). Etoposide (VP-16) was used as a positive control. After 30 min at 37°C, 5× stop buffer was added and the samples were electrophoresed through a 1% agarose gel containing 1 × TAE buffer at 5 V/cm for 1 h. The gels were stained with EB, destained with water, and photographed by the Bio-Rad Gel-Doc XR imaging system.

Comet assay

Comet assay was performed using single-cell gel electrophoresis under alkaline conditions.[4] According to the manufacturer's protocol (KeyGenBiotech), 786-O or RM-1 cells were seeded in six-well plates and treated with different concentrations of OPT or PPT, and vehicle control (0.1% DMSO) for 24 h. The cells were washed twice with ice-cold PBS, harvested by trypsinization, and resuspended in PBS at a concentration of 1×10^6 cells/mL. The cell suspension (10 μL) was mixed with 0.7% LMA (75 μL, low melting point agarose in PBS, pH 7.4) and spread on microscopic slides precoated with 0.5% NMA (normal melting point agarose in PBS). A third layer of 0.7% LMA (75 μL) was applied over the layer of agarose with the cell suspension. After gel solidification, the slides were immersed in cold lysis buffer at 4 °C overnight, and then in the electrophoresis buffer (300 mM NaOH, 1mM Na_2EDTA) to unwind at room temperature for 20–60 min. Electrophoresis was performed in the same buffer at 25 V and 300 mA for 20–30 min. The slides were gently immersed in a neutralization buffer (0.4 mM Tris-HCl, pH 7.5) for three times, 10 min each time, and stained with 20 μL of PI. The slides were evaluated with a fluorescence microscope and images of 100 random selected cells were analyzed for each sample by the Comet Assay Software Project (CASP, http://casplab.com/). The percentage of DNA in tails was used to evaluate the DNA damage. Three independent experiments were performed.

RNA extraction and real-time PCR

The expression of DNA Topo I and Topo IIα mRNA in tumor cells was detected by quantitative real-time reverse transcription PCR as previously reported.[5] The total RNA was extracted from cells and further purified following the recommendations of the manufacturer (iScript^{TM}cDNA Synthesis Kit). Briefly, TRIzol Reagent (Invitrogen) was used to lyse the samples and harvest RNA from each cell line in triplicate, followed by

chloroform extraction and RNA precipitation with isopropyl alcohol. The isolated RNA was quantified using a Nano-Drop ND-1000 (Thermo Scientific, Waltham, USA). Complementary DNA (cDNA) was synthesized in a total volume of 20 μL, using 100 ng of RNA for each reaction. Real-time PCR was performed using gene expression assays (TaqMan; Applied Biosystems, Foster City, CA) and a Bio-Rad CFX96TM Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with the cDNA product and specific primers as previously described.[6,7] The primers used for Topo IIα were: forward 5'-GCGAGTGTGCTGGTCACTAA-3' and reverse 5'-ACAATTGGCCGCTAAACTTG-3'. The primers used for Topo I were: forward 5'-TGACAGCCCCGGATGAGA-3' and reverse 5'-TGCCAATCGAGCTGTTGCA-3'. GAPDH was used as the loading control (forward 5'-GAGTCCACTGGCGTCTTCAC-3' and reverse 5'-TTCACACCCAT GACGAACAT-3') to normalize the topoisomerase mRNA levels in the subsequent quantitative analysis. PCR was carried out in a final volume of 10 μL containing 1.0 μL topoisomerase forward and reverse each, 5.0 μL RT-PCR SuperMix, 1.0 μL cDNA, and 2.0 μL RNase-free water. The PCR reaction was pre-denaturing at 95 ℃ for 10 min, started with 30 s at 95°C, 30 s at 52°C and 30 s at 72°C for 35 cycles, with a final extension at 72 ℃ for 10 min. The reactions were performed in duplicate. The expression of specific mRNAs was analyzed by the $2^{-\Delta\Delta C_q}$ method. ΔC_q is the difference in C_q between the target gene and endogenous controls by subtracting the average C_q of controls. The fold-change for each sample relative to the control sample is $2^{-\Delta\Delta C_q}$.[8]

Western blot

Cells were treated with OPT or PPT for some time at 37°C and total protein was extracted by direct lysing cell pellets in lysis buffer as described previously.[9,10] For reversal of topoisomerase cleavable complexes, the drug-treated cells were incubated in fresh medium for another 30 min at 37°C prior to lysis. The cells were lysed with ice-cold modified lysis buffer (1% Nonidet P40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM of phenylmethanesulfonyl fluoride, dichlorodiphenyltrichloroethane, sodium pyrophosphate, β-glycerophosphate, sodium orthovanadate, sodium fluoride, EDTA, leupeptin, respectively) for 20 min on ice. To terminate the reactions, one fifth of 5×SDS-PAGE sample buffer was added to each sample. SDS-polyacrylamide gels (6–15%) were used for separating proteins. The proteins were immobilized onto PVDF membranes (Millipore, 0.22 μm) using a wet transfer blotter. After blotting, the membranes were blocked in 5% skim milk in PBST buffer (0.1% Tween-20 in PBS). Primary antibodies against γ-H2AX, p-ChK1 (Ser-345), p-ChK2 (Tr-68), Topo I and Topo IIα (Abcam, Cambridge, MA, USA) in appropriate dilutions were incubated with the membranes at 4°C overnight. GAPDH (Abcam, Cambridge, MA, USA) was used as an internal control. The blots were washed with PBST and incubated with secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. After washing with PBST, the blots were detected using immobilon western chemiluminescent HRP substrate (Millipore, Billerica, USA) and the membranes were scanned using a Chemiscope 3400 mini (Clinx science instrument co. Ltd).

Cell Apoptosis

Cells were incubated with different concentrations of the compounds and controls

for 48 h. Apoptosis was detected by flow cytometry using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, USA) according to the manufacturer's protocol. Briefly, cells were washed twice with cold PBS and resuspended in 100 μL of 1×binding buffer at a concentration of 1×10^5 cells/mL. Then the cells were stained with 5 μL of FITC Annexin V and 5 μL PI, gently mixed and incubated for 15 min at room temperature in the dark. Before testing, 400 μL of 1×binding buffer was added to each tube. Flow cytometry analyses were performed within 1 h. Data were analyzed with FlowJo 7.6.5 software. The experiments were repeated three times.

Molecular docking

Geometrical structures of OPT and PPT were optimized by using Gaussian 09 software with DFT B3LYP/LanL2DZ base.[11] The crystal structures of DNA (PDB, ID: 1BNA), Topo I/DNA (PDB, ID: 1SEU) and Topo II/DNA (PDB, ID: 4FM9) were obtained from the Protein Data Bank.[12,13,14] Autodock 4.2.3 and AutoDock tools 1.5.4 Programs were used to analyze the binding mode of DNA, Topo I/DNA and Topo II/DNA complexes with OPT or PPT.[15] In the calculation, both water molecules and the original compound that bound with DNA, Topo I/DNA or Topo II/DNA were removed from the crystal structures of DNA and Topo II. The calculated Kollman Charges and salvation parameters were then added. The grid volumes of DNA, Topo I/DNA and Topo II/DNA were set at 70 × 70 × 126, 126 × 126 × 126 and 126 × 126 × 126 grid points, respectively. The number of genetic algorithm (GA) runs were set at 100, and all other parameters were default settings. After the calculation, the Molegro Molecular Viewer software was used to analyze the docked results.[16]

References

1. Z. Z. Zhu, Z. H. Wang, C. L. Zhang, Y. J. Wang, H. M. Zhang, Z. J. Gan, Z. J. Guo and X. Y. Wang, *Chem. Sci.*, 2019, 10, 3089.
2. M. C. Alley, D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Czerwinski, D. L. Fine, B. J.Abbott, J. G. Mayo, R. H. Shoemaker and M. R. Boyd, *Cancer Res.*, 1988, 48, 589.
3. M. X. Lin, X. Y. Wang, J. H. Zhu, D. M. Fan, Y. M. Zhang, J. F. Zhang and Z. J. Guo, *Apoptosis*, 2011, 16, 288.
4. N. P. Singh, M. T. McCoy, R. R. Tice and E. L. Schneider, *Exp. Cell Res.*, 1988,175,184.
5. A. Romero, M. Martín, M. C. U. Cheang, J. A. L. García-Asenjo, B. Oliva, X. P. He, M. de la Hoya, J. Á. G. Sáenz, M. A. Fernández, E. D. Rubio, C. M. Perou and T. C. Llopis, *Am. J. Pathol.*, 2011, 178, 1453.
6. G. Cartei, A. Trestin, E. Colombrino, M. Nadai, S. N. Richter, L. Barzon,G. Palù and M. Palumbo, *Ann. Oncol.*, 2006, 17 (Sup. 5), v25.
7. N. Wang, M. F. Zhu, S. -W. Tsao, K. Man, Z. J. Zhang and Y. B. Feng, *Mol. Cancer*, 2013, 12, 119.
8. L. Guo, Q. Z. Li, Q. S. Xia, S. Dial, P. -C. Chan and P. Fu, *Food Chem. Toxicol.*, 2009, 47,433.
9. S. D. Desai, T. K. Li, A. Rodriguez-Bauman, E. H. Rubin and L. F. Liu, *Cancer Res.*, 2001, 61, 5926.
10. Y. F. He, Y. Ding, D. Wang, W. J. Zhang, W. Z. Chen, X. C. Liu, W. J. Qin, X. H. Qian, H. Chen and Z. J. Guo, *Chem. Sci.*, 2015, 6, 2074.
11. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, L.

R. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. G. Johnson, W. Chen, M. W. Wong, C. Gonzalez and J. A. Pople, *GAUSSIAN 09*, *Gaussian, Inc., Wallingford, CT*, 2013.
12. H. R. Drew, R. M. Wing, T. Takano, C. Broka, S. Tanaka, K. Itakura and R. E. Dickerson, *Proc. Natl. Acad. Sci. USA*, 1981, 78, 2179.
13. B. L. Staker, M. D. Feese, M. Cushman, Y. Pommier, D. Zembower, L. Stewart and A. B. Burgin, *J. Med. Chem.*, 2005, 48, 2336.
14. T. J. Wendorff, B. H. Schmidt, P. Heslop, C. A. Austin and J. M. Berger, *J. Mol. Biol.*, 2012, 424, 109.
15. G. M. Morris, R. Huey, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson, *J. Comput. Chem.*, 2009, 16, 2785.
16. R. Thomsen, M. H. Christensen, *J. Med. Chem.*, 2006, 49, 3315.