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Delivering a Photosensitive Transplatin Prodrug to Overcome Cisplatin Drug Resistance

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Materials and Methods

Materials

$MPEG_{114}$-b-PCL_{20}-b-PLL_{10} was synthesized as previously described in our lab [1]. TP1 was synthesized as previously described [2,3]. Rhodamine B (RhB), 4',6-diamidino-2-phenylindole (DAPI), N hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), 2′-deoxyguanosine 5′-monophosphate sodium salt hydrate (5'-dGMP) (D9500-100MG) and sodium azide were purchased from Sigma-Aldrich. Cisplatin (purity 99%) was bought from Shandong Boyuan Pharmaceutical Co. Ltd., China. All other chemicals and solvent were used as received.

General Measurements

^{1}H NMR spectra were measured by a Unity-300MHz NMR spectrometer (Bruker) at room temperature. Matrix-assisted laser-desorption ionization and time-of-flight mass spectroscopy (MALDI-TOF-MS, Waters, USA) was used to study the chelation of transplatin and TP2 in the presence of 5'-dGMP upon UVA irradiation. Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 6300, Thermoscientific, USA) was used to determine the total platinum contents in M(Pt) and samples obtained outside of the dialysis bags in drug release experiments. Size and size distribution of micelles were

determined by DLS with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology, USA). The morphology of M(Pt) was measured by TEM performed on a JEOL JEM-1011 electron microscope. Particle size and zeta potential measurements were conducted on a Malvern Zetasizer Nano ZS.

UVA light source and irradiation conditions

A bank of six UVA light tubes bought from Huaqiang Eletronics (Nanjing, China) with 18 W light sources (1.8 mW/cm^2, λ_{max} = 365 nm) were used for all *in vitro* irradiation including the drug releases, irradiated sample preparation for MALDI-TOF-MS and *in vitro* MTT assays.

Synthesis of TP1

TP1 was synthesized as previously described[2,3].

Synthesis of TP2

TP1 (0.334 g, 1 mmol) was dissolved in 5 ml DMSO in a flask protected from light, to which 0.1 g succinic anhydride were added. The reaction mixture was kept stirred overnight and then DMSO was removed by lyophilization to get the crude product TP2. TP2 was then dissolved in 5 ml methanol and precipitated by excess of ether, washed 3 times with ether (3× 50 mL), and re-crystallized in acetone.

Conjugation of TP2 onto biodegradable polymers

TP2 (0.447 mg, 1 mmol) was dissolved in the 10 ml pure water in a flask, to which 2 mmol EDC and 2 mmol NHS were added and the reaction mixture were vigorously stirred for 10 min at room temperature. $MPEG_{114}$-b-PCL_{20}-b-PLL_{10} (500 mg) in 1 mL DMF was added to the flask. The reaction mixture was stirred at room temperature for another 4 h and then subjected to filtration and the filtrate was dialyzed against water to remove unreacted platinum drugs within a dialysis bag(molecular weight cutoff: 3500 Da) and byproducts of coupling agents. The aqueous solution of the polymer-drug conjugate was then lyophilized for use.

Preparation of M(Pt)

The micellar nanoparticles of M(Pt) were prepared by a nano-precipitation method . Briefly, polymer-transplatin(IV) conjugate (2 mg) was dissolved in DMF (1 ml) and then de-ionized water (5 ml) was added in drop-wise. The mixture was put in dialysis bag (molecular weight cut off: 3500 g/mol) and dialyzed against de-ionized water for 1 day and lyophilized to powder.

Drug release from M(Pt)

M(Pt) (5 mg) was dissolved in PBS (10 mL, 0.1 M, pH 7.4), and then put into a pre-swelled dialysis bag (3,500 MWCO), that was then immersed into PBS (100 mL) at 37 °C. Samples were kept in the dark or under UVA lamps. At desirable time intervals, 1.5 mL was withdrawn from the dialysate and measured for Pt using ICP-OES. After sampling, fresh PBS (1.5 mL) was added to the dialysate.

Study of the chelation of transplatin and TP2 with 5'-dGMP Pupon UVA irradiation

a) For transplatin: transplatin (5 mg) was dissolved in an aqueous solution of 5'-dGMP (1 mM, 10 ml);

b) For TP2, TP2 (5 mg) was dissolved in an aqueous solution of 5'-dGMP (1 mM, 10 ml) under UVA irradiation for 1 h;

Aliquots of the reaction mixture in both a) and b) were collected for MALDI-TOF-MS study in positive mode.

Cell source and growth conditions

A2780 (cisplatin sensitive human ovarian cancer cells) and A2780DDP (cisplatin acquired resistant human ovarian cancer cells), SKOV-3 (cisplatin intrinsic resistant ovarian cancer) were obtained from Huazhong University of Science and Technology (Hubei, China) and grown as previously described.

Photo-toxicity of light source

The light source and intensity was described in previous section. A2780 and A2780DDP cells were seeded in 96-well plate at a density of 5000 cells/well overnight. UVA light was used for irradiation of the 96-well plates for 1 h with one plate in the dark as a control. The cells were then left growing for another 48 h. After that, 20 μL MTT (5 mg/ml) were added to each well and the cells were further incubated for another 4 h. Then 100 μL acidified SDS solution was added to each well overnight. Absorbance at 570 nm was read.

Cell viability studies

A2780, A2780DDP, SKOV-3 cells were seeded in 96-well plate at a density of 5000 cells/well overnight. All the drugs including cisplatin, transplatin, TP1, TP2 and M(Pt) were freshly prepared in RPMI-1640 culture media and filtered by 0.2 μm filter. Then the drug concentrations were measured by ICP-OES. The cells were treated with cisplatin and M(Pt) at a concentration range from 0.1 to 400 μM in triplicates. For transplatin, TP1 and TP2, the range was set from 0.1 to 800 μM. All the drug concentrations above were based on platinum. For the UVA irradiation, cells plates were incubated for 4 h in the dark, UVA irradiated

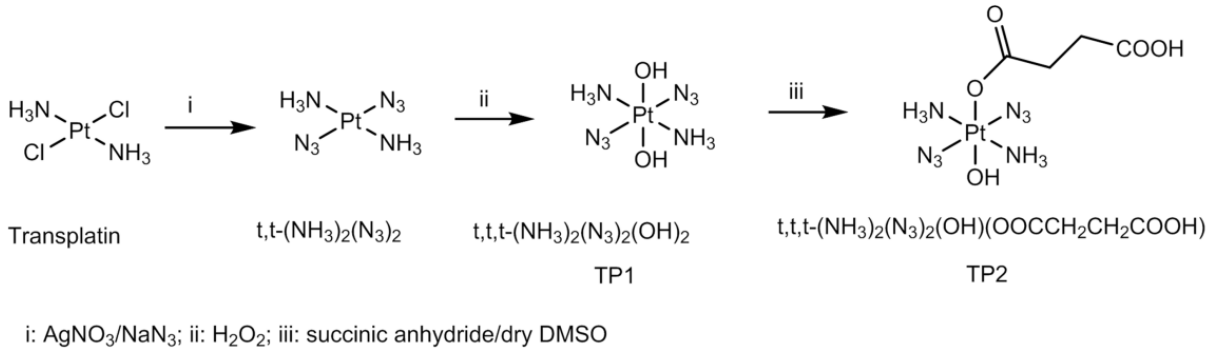
for 1 h, then incubated in the dark for an additional 43 h. For control samples, incubation were performed in the dark for 48 h. Cytotoxicity was then assessed using an MTT assay as described previously [4]. The above MTT assay was repeated for 3 times. Then the IC_{50} values were derived from the dose-response curve of each drug. IC_{50} values were shown as mean value ± standard deviation.

Intracellular uptake of drugs

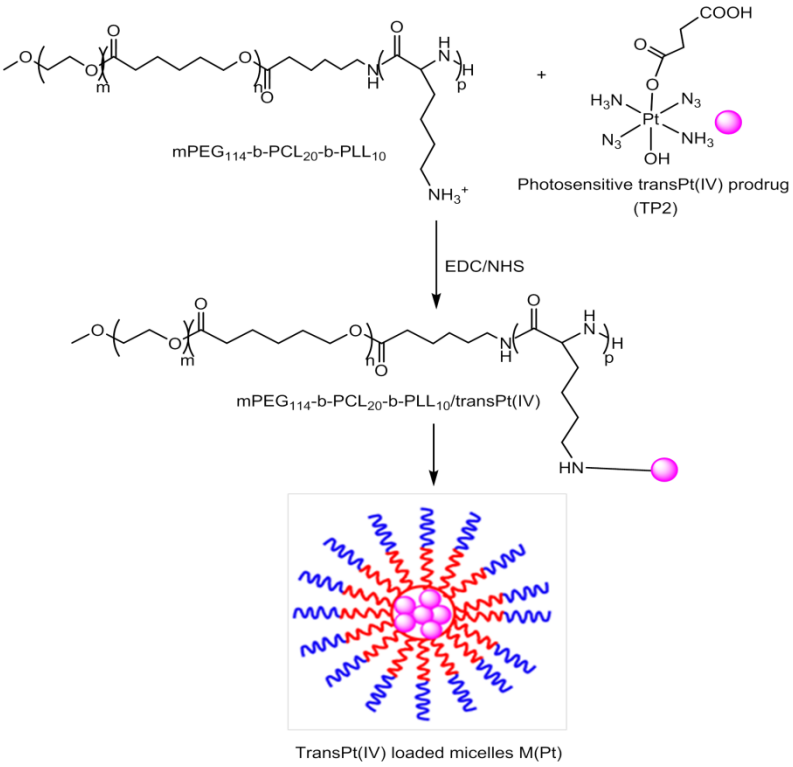
A2780 (cisplatin sensitive human ovarian cancer cells) and A2780DDP (cisplatin acquired resistant human ovarian cancer cells) were obtained from Huazhong University of Science and Technology (Hubei, China) and grown as previously described, seeded in 6-well plates at a density of 1×10^6 cells/well overnight, and then treated with Pt drugs which were pre-dissolved in RPMI-1640 culture media (cisplatin, transplatin, TP1, TP2 and M(Pt) in triplicates at an equal concentration of 40 μM based on platinum at 37 °C for 4 h or 8 h. The cells were then washed with PBS for 5 times, counted and lysed. Thereafter, the Pt content in the cancer cells was measured via ICP-MS and the uptake of Pt was expressed as ng Pt/million cells. The above drug uptake experiments were repeated for 3 times. Pt uptake values were shown as mean value ± standard deviation.

Confocal Laser Scanning Microscopy

A2780 and A2780DDP cells were seeded in 6 well plates at a density of 1×10^5 cells/well and then the cells were separately incubated with RhB labeled M(Pt) at 15 μg/ml (based on RhB) for 1 h at 37 °C. After that, cells were thoroughly washed with PBS, fixed, stained by DAPI, sealed with glycerin and observed by CLSM (Olympus, FV-1000).



Scheme S1. Synthesis of photosensitive transPt(IV) prodrugs t,t,t-$(NH_3)_2(N_3)_2(OH)_2$ (TP1) and t,t,t-$(NH_3)_2(N_3)_2(OH)(OOCCH_2CH_2COOH)$ (TP2).



Scheme S2. Conjugation of TP2 to biodegradable polymer with pendant amino groups to prepare polymer-transPt(IV) conjugate and preparation of its micelles M(Pt).

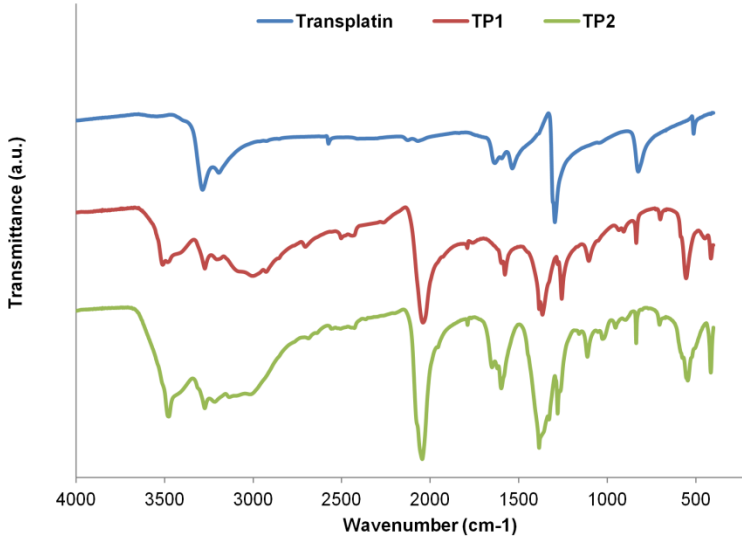


Figure S1. Characterization of transplatin, TP1 and TP2 by IR.

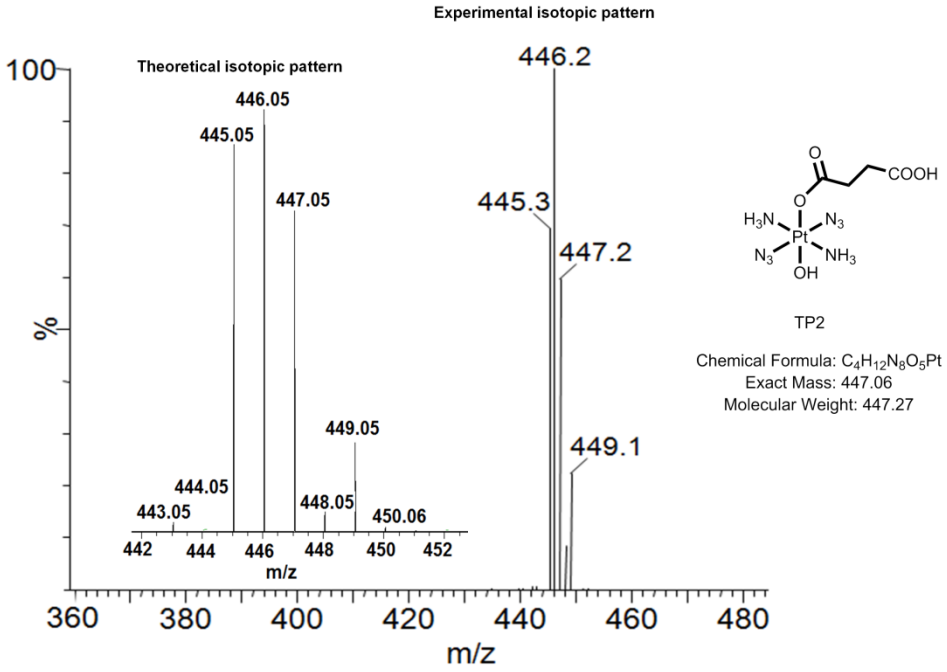


Figure S2. Characterization of TP2 by ESI-MS (negative mode). The peak at m/z=446.2 can be attributed to the molecular ion of TP2. Moreover, the theoretical isotopic pattern (insets) coordinates to the experimental isotopic pattern.

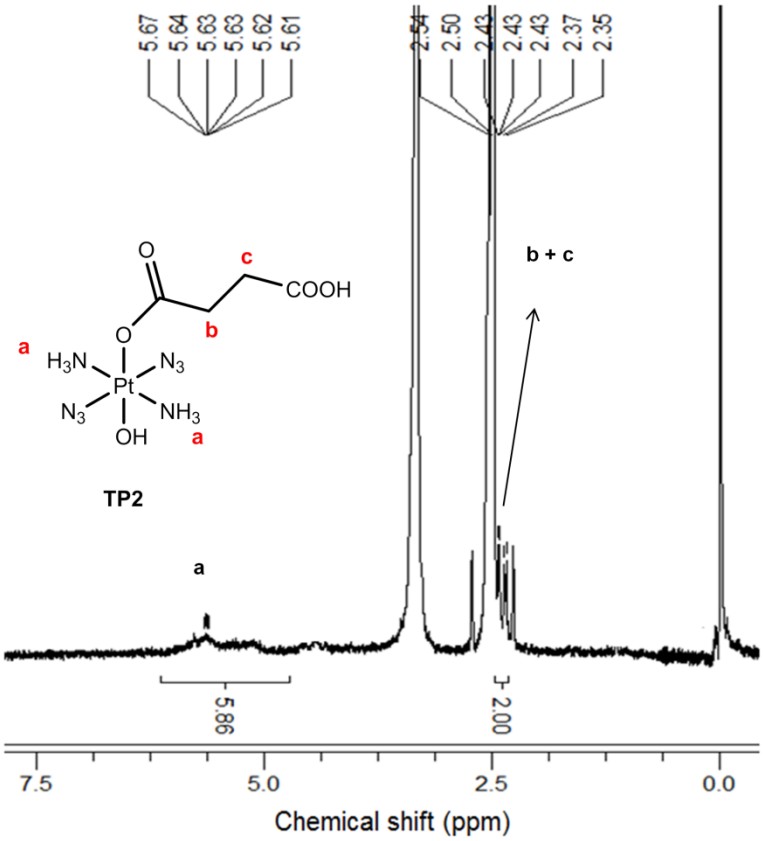


Figure S3. ^{1}H NMR of TP2 in d_6-DMSO.

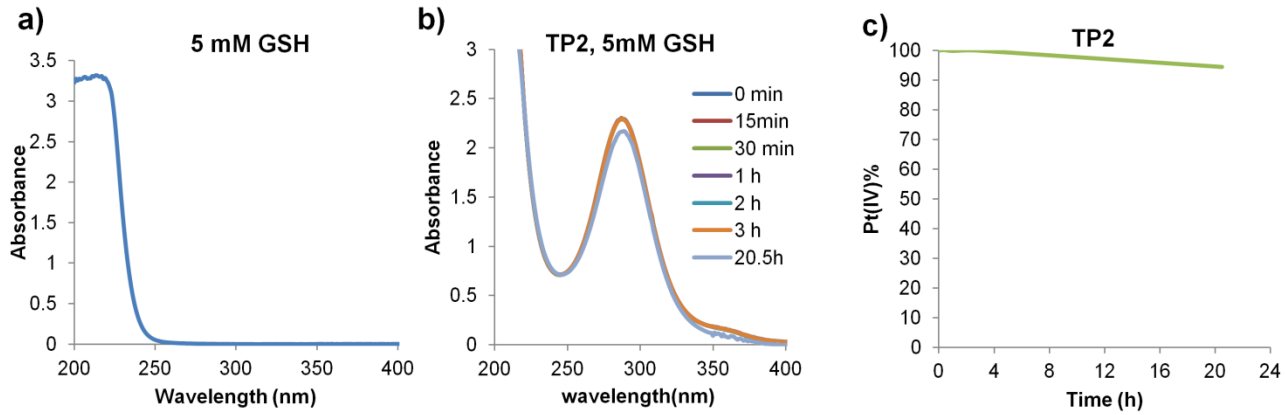


Figure S4. The stability of TP2 in 5 mM GSH. UV-vis curve of 5 mM GSH (a) and TP2 in the presence of 5 mM GSH. The reduction kinetics of TP2 was shown in (c). The reduction percentage of TP2 was shown as the decrease in the peak value at 286 nm

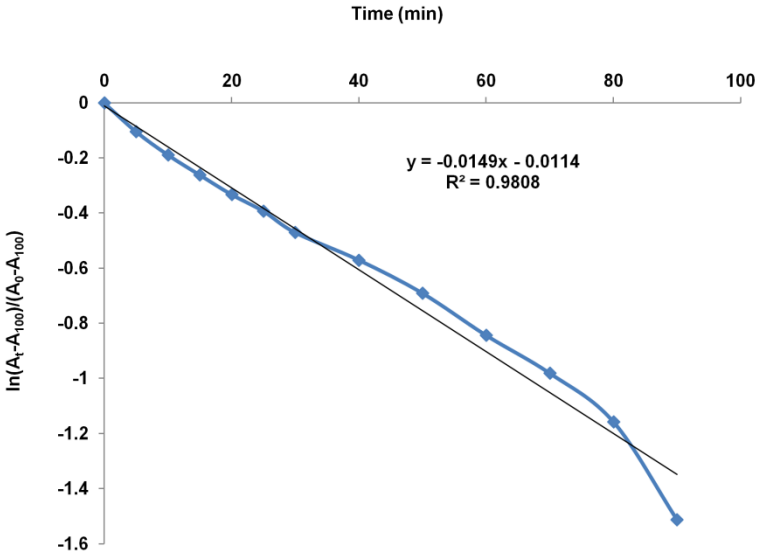
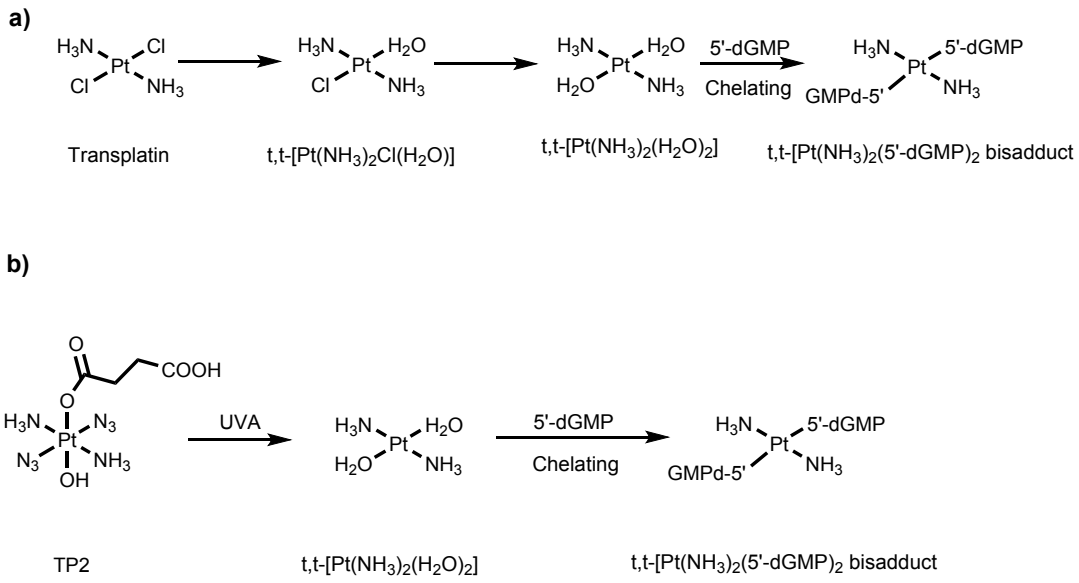
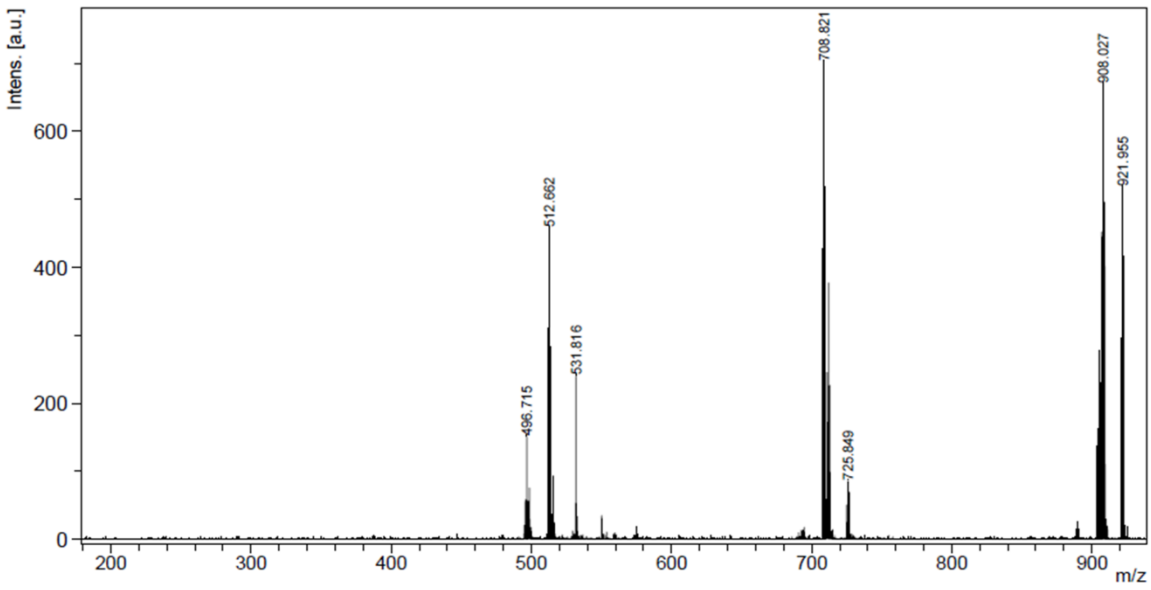


Figure S5. The photo-reduction kinetics of M(Pt). A_t, A_0 and A_{100} were the peak absorbance at 286 nm at time t min, 0 min and 100 min respectively. The reduction of M(Pt) follows a first order kinetics in the first 80 min.



Scheme S3. Formation of 5'-dGMP adducts with transplatin and TP2. (a) transplatin undergoes a two step hydrolysis and then chelates with 5'-dGMP to form bisadduct.(b) TP2 can be reduced to hydrolyzed cisplatin upon UVA irradiation. The hydrolyzed cisplatin then chelates with 5'-dGMP to form bisadduct.

a)



b)

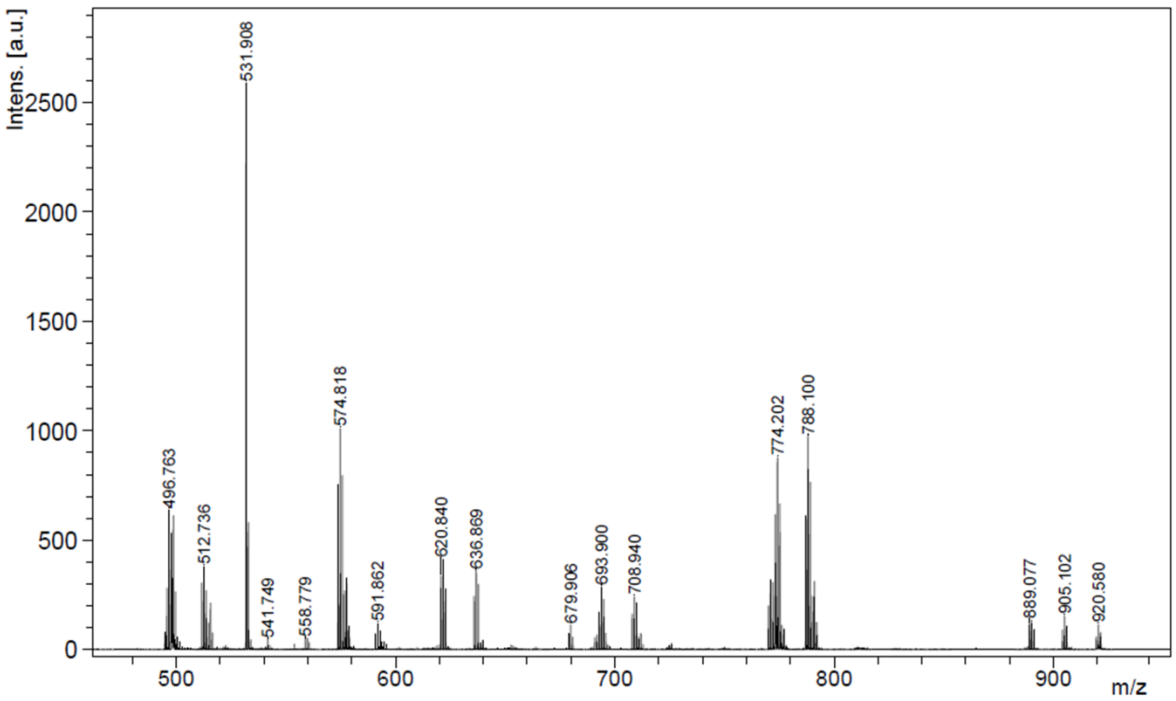


Figure S6. Chelation of 5'-dGMP with transplatin in the dark (a) and TP2 upon UVA irradiation (b) by MALDI-TOF-MS in positive mode. The peaks at m/z=920.58, 905.10, 889.07 can be attributed to bisadduct ($(NH_3)_2Pt(5'\text{-}dGMP)_2$, m/z=920.58) and fragments formed from it by losing of one or two $–NH_3$ (i.e., $(NH_3)Pt(5'\text{-}dGMP)_2$, m/z=905.10; $Pt(5'\text{-}dGMP)_2$, m/z=889.07).

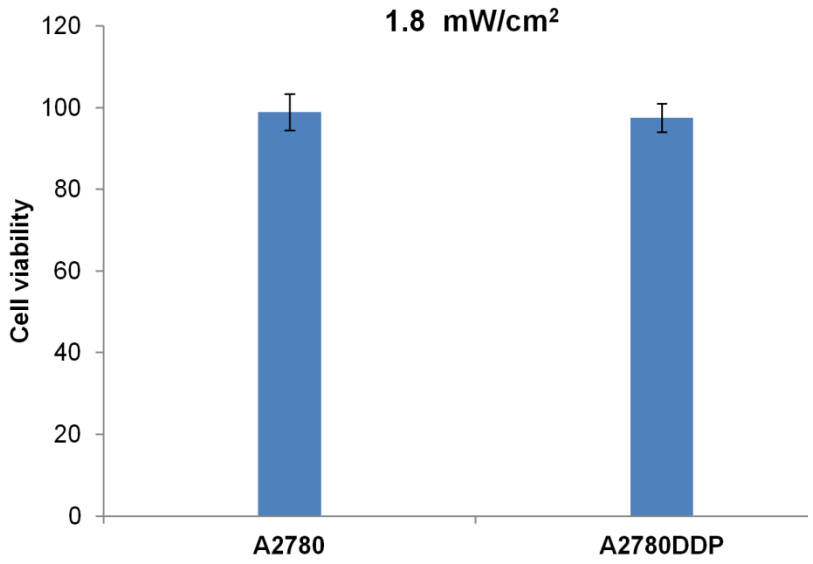


Figure S7 Photo toxicity of the UVA irradiation (1.8 mW/cm^2) for 1 h on A2780 and A2780DDPcells.

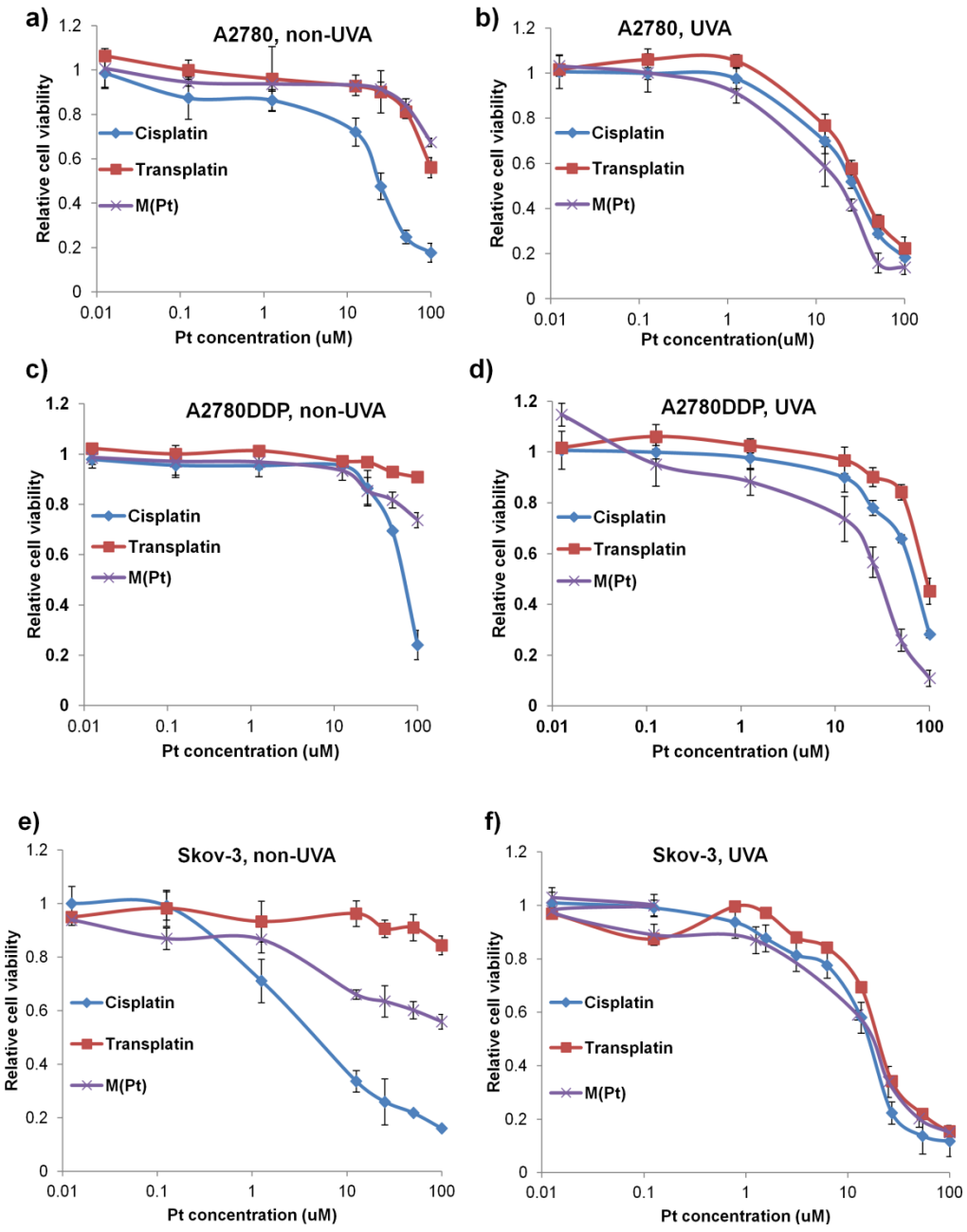


Figure S8. Representative cell viability curves of cisplatin, transplatin and M(Pt) on A2780 a,b), A2780DDP (c,d) and SKOV-3 (e, f) cancer cells in the dark (UVA-off) and upon UVA irradiation (UVA).

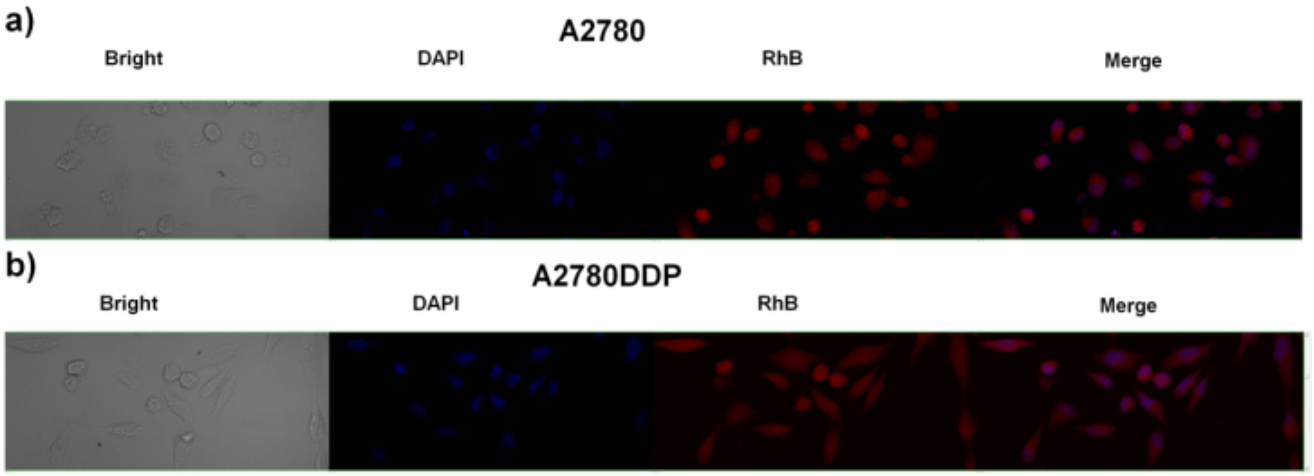


Figure S9.Uptake of M(Pt) micelles by confcocal laser scanning. M(Pt) micelles were labelled with Rhodamine B. Cell nucleus were stained by DAPI. After 1 h treatment by M(Pt/RhB), A2780 and A2780DDP cells were imaged by CLSM (Olympus, FV-1000).

Table S1. IC_{50} values of cisplatin, transplatin, TP1, TP2 and M(Pt) in the dark and upon UVA irradiation. All the experiments were repeated with 3 times with 4 replicates in each concentration. Values are shown as mean± standard deviation.

Drug	A2780(μM)		A2780DDP(μM)	
	Non-UVA	UVA	Non-UVA	UVA
Cisplatin	23.6±3.5	26.7±2.7	72.4±5.8	70.1±6.3
Transplatin	121±8.4	32.2±4.1	430±9.4	94.2±7.6
TP1	354±27.1	276±14.5	550±35	426±21
TP2	298±18.4	214±8.9	412±19.8	334±23
M(Pt)	157±7.9	46.5±5.4	216±11	30.4±4.5

Table S2. Intracellular uptake of cisplatin, transplatin, TP2 and M(Pt).

Drug	A2780(ng Pt/million cells)		A2780DDP(ng Pt/million cells)		Uptake fold	
	4 h	8 h	4 h	8 h	4 h	8 h
Cisplatin	69.8±5.1	99.4±8.2	6.4±0.9	30.9±4.3	10.9	3.2
Transplatin	10.4±3.4	39.5±4.2	5.9±1.6	28.9±5.1	1.7	1.4
TP2	35.1±3.2	76.0±5.4	28.0±3.1	65.0±11.7	1.3	1.2
M(Pt)	510.2±23.5	960±57.3	410±22.0	728±38.1	1.2	1.3

Reference:

[1] R. G. Qi, S. Liu, J. Chen, H. H. Xiao, L. S. Yan, Y. B. Huang and X. B. Jing, *J. Control. Release*, 2012, **159**,251-260.

[2] N. J. Farrer, J. A. Woods, V. P. Munk, F. S. Mackay and P. J. Sadler, *Chem. Res. Toxicol*., 2010, **23**, 413-421.

[3] F. S. Mackay, J. A. Woods, H. Moseley, J. Ferguson, A. Dawson, S. Parsons and P. J. Sadler. *Chem. Eur. J.,* 2006, **12**, 3155-3161.

[4] H. Xiao, G.T. Noble, J.F. Stefanick, R. Qi, T. Kiziltepe, X. Jing and B. Bilgicer, J. Control. Release, 2014, **173**, 11-7