Electronic Supplementary Information (ESI)

A platinum(IV) prodrug aiming to defeat breast cancer through disrupting vasculature and inhibiting metastasis

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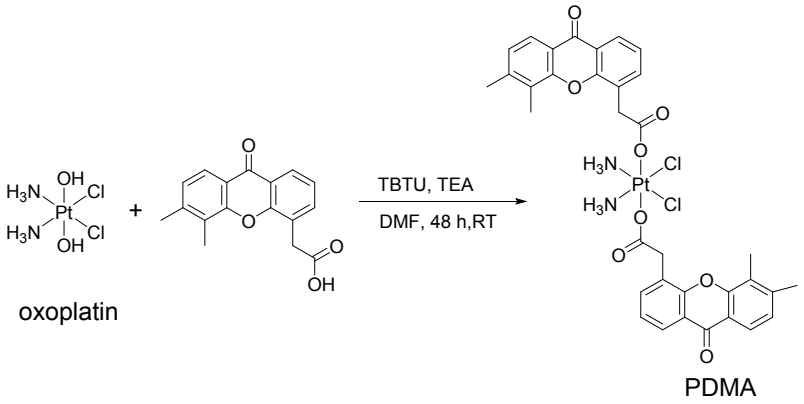
1. Supplementary Figures and Tables



Scheme. S1 Synthetic route to PDMA.

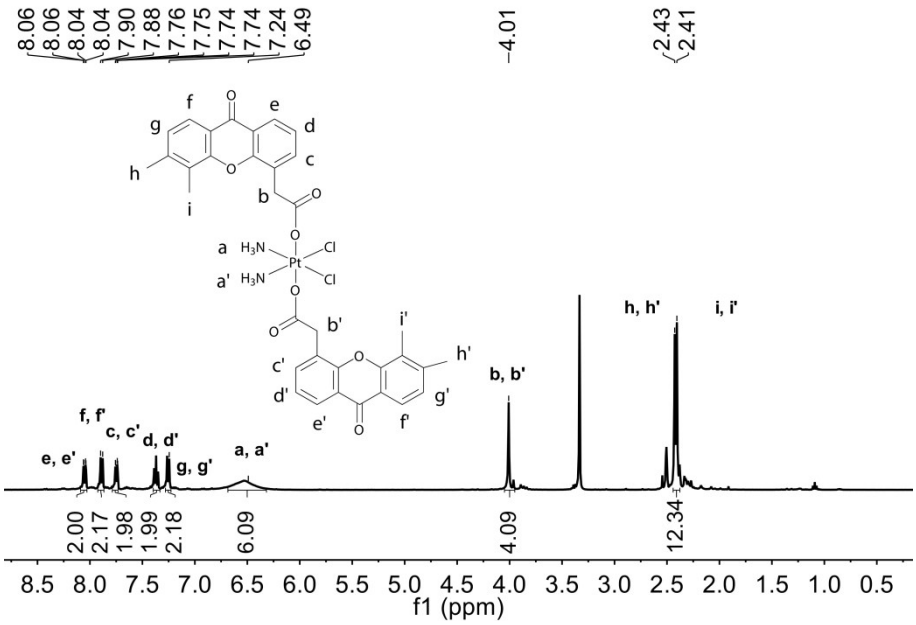


Fig. S1 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of PDMA in DMSO-d_6.

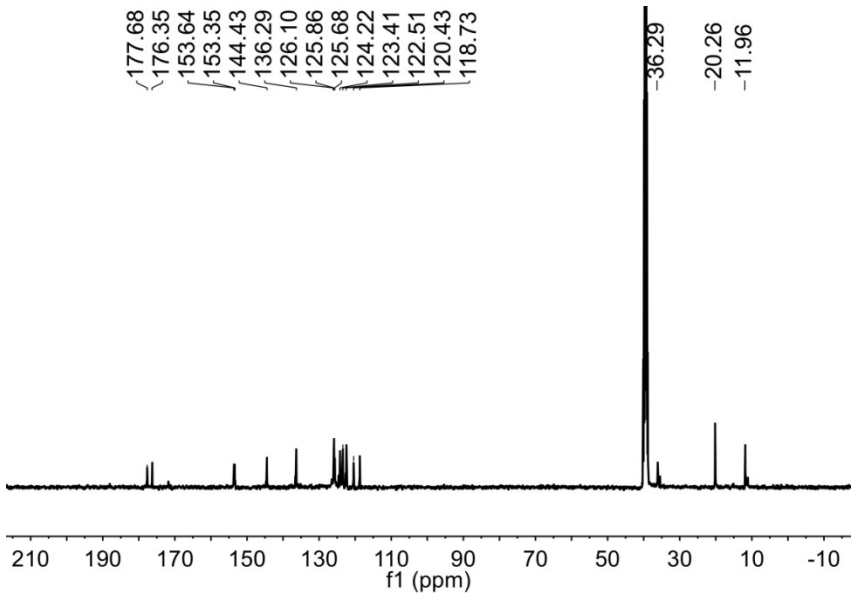


Fig. S2 ^{13}C NMR spectrum (101 MHz, DMSO-d_6) of PDMA in DMSO-d_6.

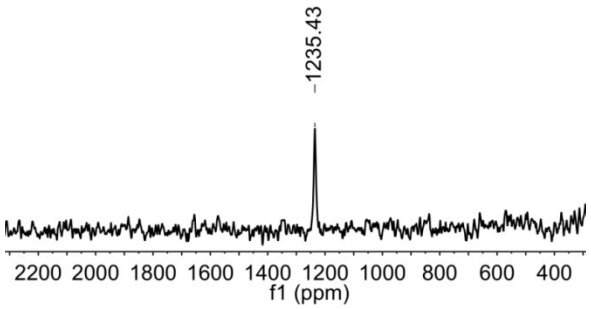


Fig. S3 ^{195}Pt NMR spectrum (86 MHz, DMSO-d_6) of PDMA in DMSO-d_6.

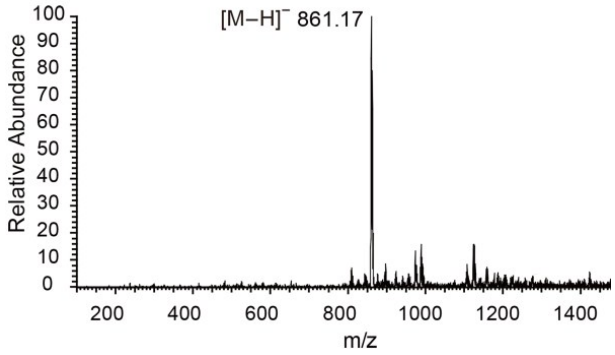


Fig. S4 ESI-MS spectrum of PDMA in methanol.

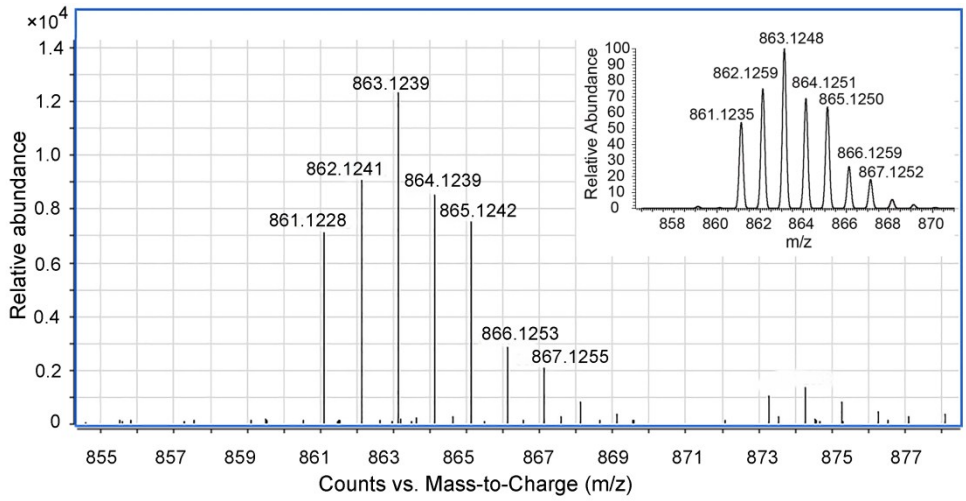


Fig. S5 HR-MS spectrum of PDMA **(**positive mode) in isotopic pattern. Inset is the simulated isotopic distribution pattern of the observed peak.

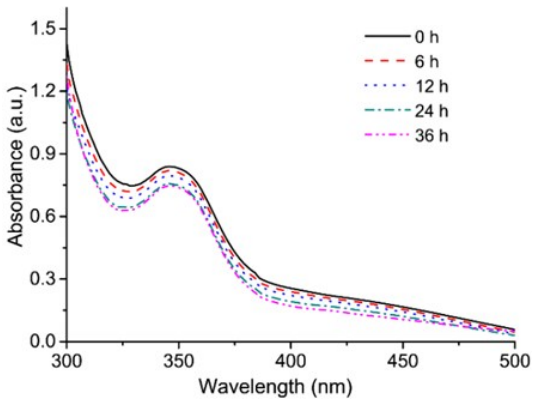


Fig. S6 Time-dependent UV-vis absorption spectra of PDMA in culture medium with 0.3% DMSO at 37 °C monitored at different time spans.

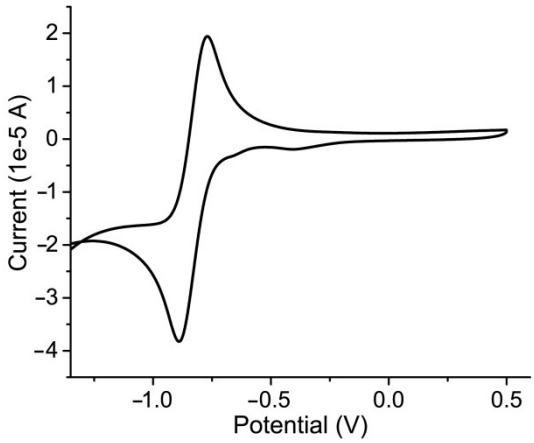


Fig. S7 Cyclic voltammogram of PDMA. Data was obtained using 0.1 M of (n-Bu_4N)PF_6 as the supporting electrolyte at room temperature.

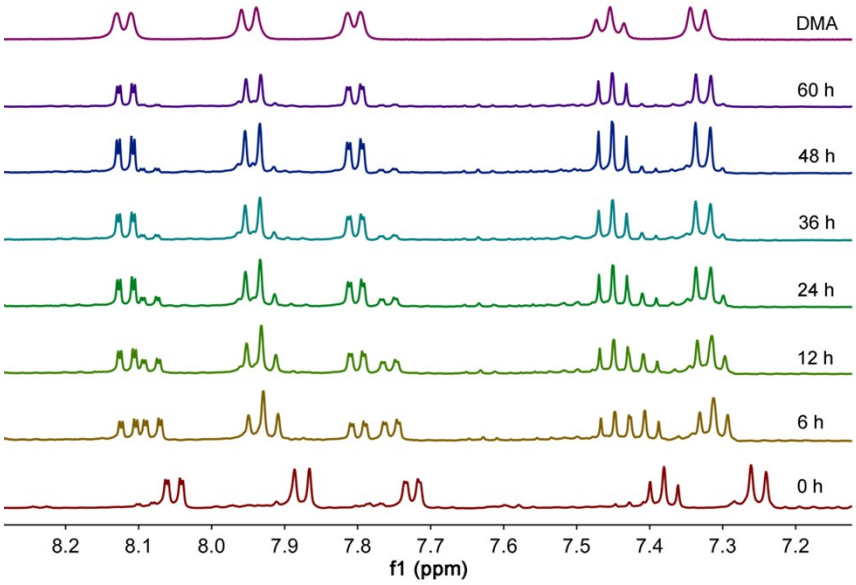


Fig. S8 Time-dependent ^{1}H NMR spectra for the reduction of PDMA (10 mM) by ascorbic acid (80 mM) after incubation at 37 °C.

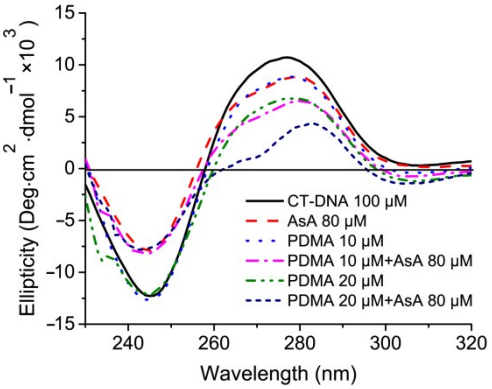


Fig. S9 CD spectra of CT-DNA (1.0×10^{-4} M) in the absence and presence of PDMA (10, 20 μM) with or without ascorbic acid (80 μM) after incubation at 37 °C for 24 h.

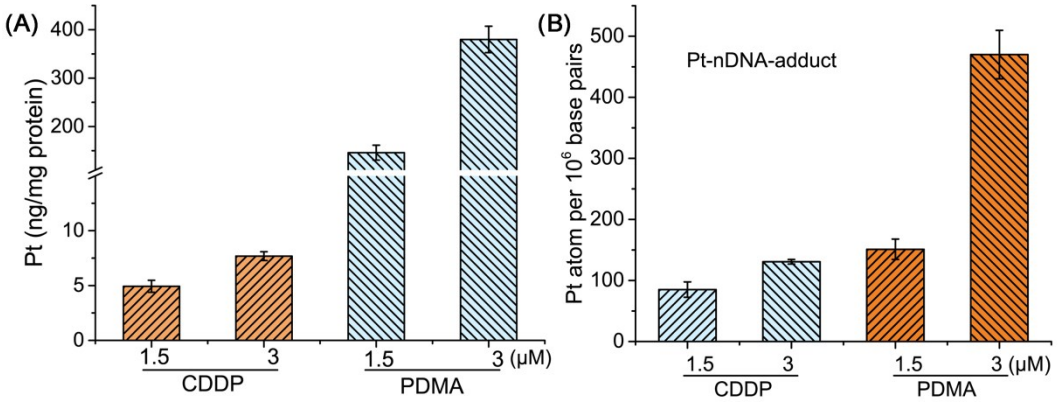


Fig. S10 Pt content (A) and Pt-nDNA adducts levels (B) in MDA-MB-231 cells after incubation with CDDP or PDMA (1.5, 3 μM) for 24 h.

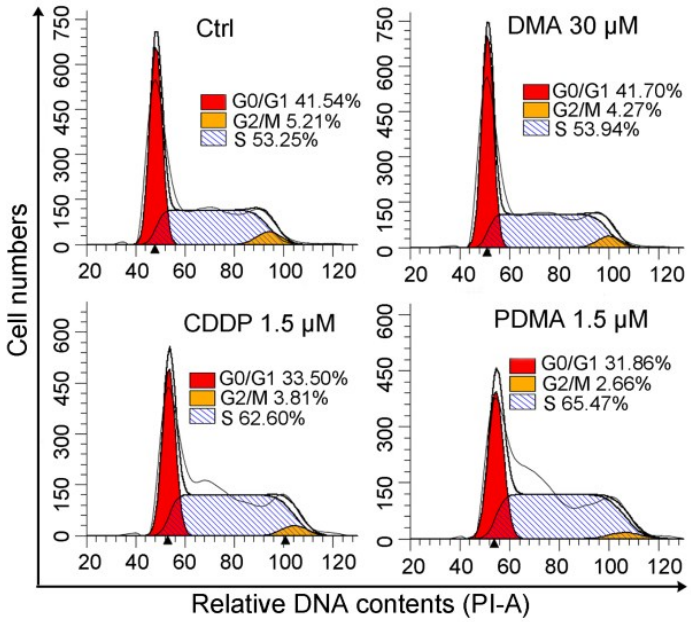


Fig. S11 Flow cytometry analysis of cell cycle distributions in MDA-MB-231 cells after treatment with PDMA, CDDP and ligand DMA for 24 h.

Table S1 IC_{50} values (μM) of PDMA and CDDP for HUV-EC cells at 24 and 72 h.

Compound	24 h	72 h
PDMA	32.2 ± 1.5	3.8 ± 0.4
CDDP+2DMA	30.5 ± 1.3	5.5 ± 0.7
CDDP	25.4 ± 1.4	2.2 ± 0.3
DMA	>100	>100

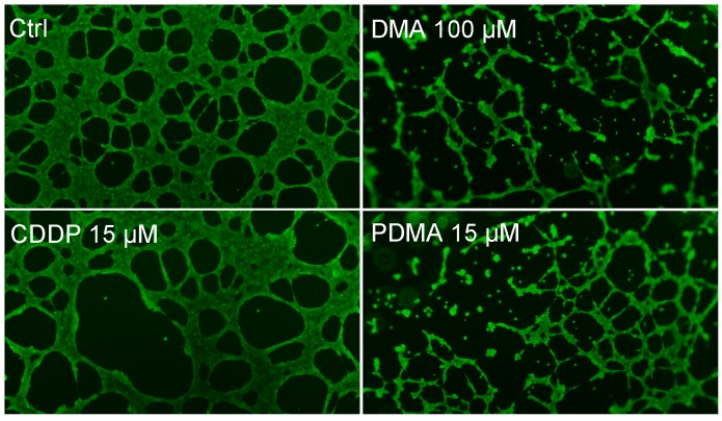


Fig. S12 Effect of different compounds on the tube formation of HUV-EC cells after incubation for 24 h.

Table S2 Proportion of embryo population with suppressed ISV by different compounds.

Compound	Suppressed ISV	Survival	Sample size
Control (0.25% DMSO)	0%	100%	10
CDDP (10 μM)	2%	100%	10
PDMA (10 μM)	44%	100%	10
DMA	55%	100%	10

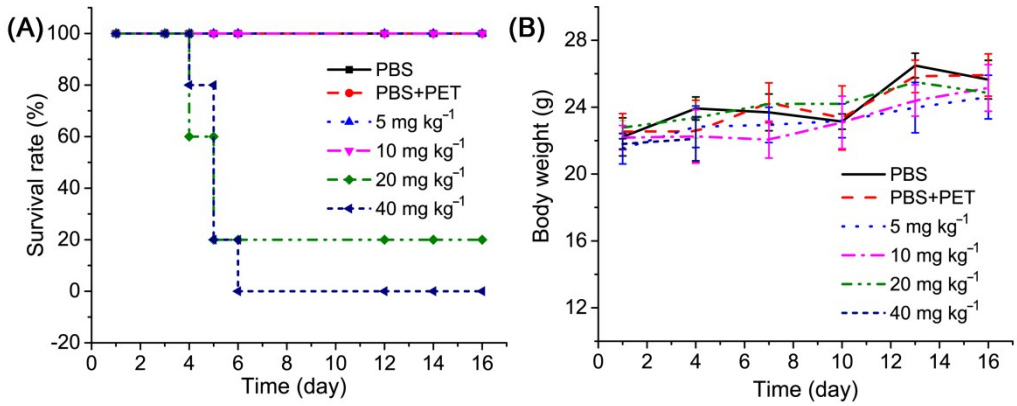


Fig. S13 Survival rate (A) and variations of the body weight (B) of the mice treated intravenously with different does of PDMA for 15 d.

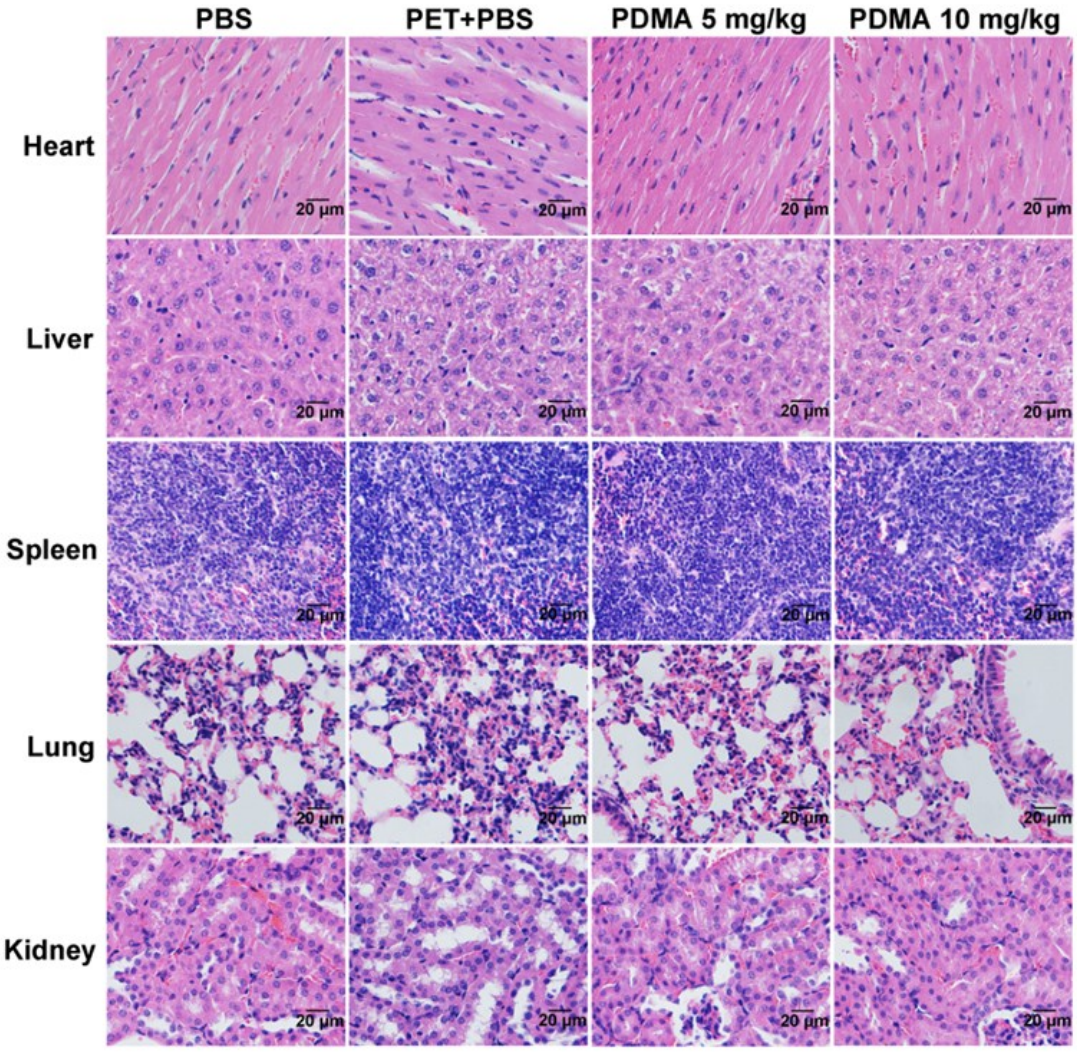


Fig. S14 H&E-stained images of heart, liver, spleen, lung and kidney sections collected from different mice after treatment with PBS, PET + PBS, and PDMA, respectively.

2. Experimental

2.1 Materials and methods

Cisplatin was obtained from Shandong Boyuan Pharmaceutical Co. Ltd. 5,6-Dimethylxanthenone-4-acetic acid (DMA) was purchased from Shanghai Shuya pharmaceutical technology Co., Ltd. Other chemicals and solvents were commercial reagent and used without further purification. O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU), triethylamine (TEA), and hydrogen peroxide (H_2O_2, 30%), N-dimethylformamide (DMF), dimethylsulfoxide (DMSO)

were purchased from J&K Scientific. The antibodies used for western blotting were purchased from Abcam. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) and calf thymus DNA (CT-DNA) were purchased from Sigma-Aldrich. Water was deionized and ultrafiltered by a Milli-Q apparatus (Millipore Corporation, China). *Cis*-diamminedichloro-*trans*-dihydroxyplatinum(IV) (oxoplatin) was prepared according to the literature procedure.[1] Genomic DNA mini preparation kit was purchased from Tiangen Biotech (Beijing) CO., Ltd. ^{1}H and ^{13}C NMR spectra were determined at 298 K on a BRUKER ADVANCE III HD 400 spectrometer using standard pulse sequences. Electrospray ionization mass spectra (ESI-MS) were obtained using an LCQ spectrometer (Finnigan). High resolution mass (HR-MS) spectrometric data were determined using an Agilent 6540Q-TOF HPLC-MS spectrometer. The isotopic distribution patterns of the observed species were simulated using the Isopro 3.0 program. Circular dichroism (CD) spectra were measured by a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Japan). The optical density (OD) of formazan was tested on Thermo Scientific Varioskan Flash. The inductively coupled plasma mass spectrometry (ICP-MS) data were obtained on ELAN 9000 ICP-MS (PerkinElmer Inc., U. S. A.). Elemental analysis (C, H and N) was performed using a CHN-O-Rapid elemental analyzer. The reduction potential was measured by cyclic voltammograms at room temperature on an electrochemical analyzer system (CHI 630D).

2.2 Cell lines

The human breast cancer MCF-7, MDA-MB-231, MDA-MB-435, and the human umbilical vein endothelial HUV-EC, as well as the human normal renal epithelial HK-2 cells lines were purchased from American Type Culture Collection (ATCC). MCF-7 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, (KeyGEN BioTECH) with 10% fetal bovine serum (FBS); MDA-MB-231 and MDA-MB-435 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with (KeyGEN BioTECH) 10% FBS; HK-2 cells were cultured in DMEM/F12 (KeyGEN BioTECH) with 12% FBS; HUV-EC cells were cultured in F-12K (Gibco) medium

supplemented with heparin (0.1 mg mL^{-1}), endothelial cell growth supplement 12 (0.03–0.05 mg mL^{-1}), 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). All cell lines were grown in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3 Synthesis of PDMA

c,c,t-$[Pt(NH_3)_2Cl_2(OH)_2]$ (100 mg, 0.30 mmol) were stirred in dry DMF (10 mL) with DMA (313.66 mg, 0.90 mmol, 3.0 equivalent), TEA (125 μL, 0.90 mmol), and TBTU (290 mg, 0.90 mmol) in the dark at room temperature for 48 h to give a light yellow solution. The obtained solution was concentrated by rotary evaporator to 5 mL. The solution was filtered and added dropwise to H_2O/ethanol (20 mL, 1:1) to get a light-yellow precipitate. The raw product was washed with methanol and ether in turn and then dried in vacuum. Yield: 145 mg, 35%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.05 (dd, J = 7.9, 1.4 Hz, 2H), 7.89 (d, J = 8.0 Hz, 2H), 7.79–7.73 (m, 2H), 7.37 (t, J = 7.6 Hz, 2H), 7.24 (s, 2H), 6.49 (s, 6H), 4.01 (s, 4H), 2.42 (d, J = 8.4 Hz, 12H) ppm. ^{13}C NMR (101 MHz, DMSO-d_6) δ 177.68, 176.35, 153.64, 153.35, 144.43, 136.29, 126.10, 125.86, 125.68, 124.22, 123.41, 122.51, 120.43, 118.73, 36.29, 20.26, 11.96 ppm. ^{195}Pt NMR (86 MHz, DMSO-d_6) δ –1235.43 ppm. MS (ESI^-) m/z: found (calcd): 861.1667 (861.1617) for $[M–H]^-$. HRMS m/z: found (calcd): 863.1239 (863.1248) for $[M+H]^+$. Elemental analysis: found (calcd, %) for $C_{34}H_{32}Cl_2N_2O_8Pt$: C, 47.85 (47.34); H, 4.01 (3.74); N, 3.24 (3.25).

2.4 Stability

PDMA was dissolved in culture medium at 5×10^{-5} M with 10% FBS containing 0.3% DMSO. UV-vis absorption spectrum of the complex was determined on a Specord 200 UV-visible spectrophotometer under physiological conditions.

2.5 NMR titrations of PDMA with ascorbic acid

^{1}H NMR titrations were carried out in the presence of ascorbic acid (Ac). PDMA (10 mg in 400 μL of DMSO-d_6) was mixed with ~200 μL of Ac (8 equivalents) and

spectra were recorded at 0, 6, 12, 24, 36, 48, 60 h.

2.6 Cyclic voltammetry measurement

Cyclic voltammogram of PDMA was measured in a three-electrode system including glassy carbon as the working electrode, a Pt wire as the auxiliary electrode, and saturated calomel electrode as the reference electrode. Deaeration of PDMA solution (0.1 mM in DMF) was accomplished by passing a stream of nitrogen through the solution for 10 min. [n-Bu_4N][PF_6] (0.1 M) was used as the supporting electrolyte. Trace amounts of ferrocene were added as an internal reference. The scan rate was set to 100 mV s^{-1}. The reduction potential was calculated according to the cyclic voltammogram.

2.7 Octanol/water partition coefficient (Log $P_{o/w}$)

The lipophilicity of PDMA was measured in an octanol/buffer system using the shake-flask method and UV spectroscopy. Solutions of PDMA (50, 100, 150, 200 μM) containing DMSO (1%) were prepared in the PBS (pH 7.4) presaturated with octanol. Equal volumes (2.0 mL) of the solution and octanol presaturated with PBS were mixed and shaken at room temperature for 2 h and then separated into two phases by centrifugation. The concentration of the solute in the aqueous phase was determined by spectrophotometry (λ_{max} = 347 nm). According to the law of mass conservation, the drug concentration of the corresponding octanol phase and the lipo−hydro partition coefficient $P_{o/w}$ ($P_{o/w} = C_o/C_w = A_o/A_w$, where A stands for absorbance) were calculated. The log *P* values were calculated from an average of three independent measurements.

2.8 DNA binding

CT-DNA stock solution was prepared by dissolving DNA in Tris-HCl buffer solution (5 mM Tris-HCl, 50 mM NaCl, pH 7.4), which was stored at 4 °C overnight and used within one week. The concentration of CT-DNA was determined by nanodrop spectrophotometer at 260 nm, taking 6600 M^{-1} cm^{-1} as the molar absorption coefficient. The ratio of the UV absorbance at 260 and 280 nm (A260/A280) was

calculated as 1.89, indicating that the DNA solution is sufficiently free of protein. Samples were prepared by incubating CT-DNA (100 μM) with different concentrations of PDMA in the absence or presence of ascorbic acid at 37 °C for 24 h in the dark. CD spectra were recorded in the range of 220–320 nm using a scan speed of 10 nm min^{-1}.

2.9 Cytotoxicity assay

MCF-7, MDA-MB-231, MDA-MB-435 and HK-2 cells (3000–5000 cells/well) were seeded on a 96-well plate (Corning) in 100 μL of desired medium and incubated at 37 °C in humidified incubator with 5% CO_2 overnight. Stock solution of cisplatin was prepared in PBS while PDMA and DMA stock solutions were prepared in DMSO. The stock solutions were diluted in complete medium (DMSO concentration < 0.5%). The cells were treated with the complexes respectively at different concentrations (0.25–64 μM). After 72 h, MTT (20 μL, 5 mg mL^{-1} in PBS) was added into each well and incubated at 37 °C for 4 h. The medium in each well was replaced with DMSO (150 μL) to solubilize formazan, and the plate was shaken gently to homogenize the mixture of the solution. The absorbance of the solution in each well was measured at 570 nm by microplate reader (Thermo Scientific Varioskan Flash). Cytotoxicity data was fitted to a sigmoidal curve. IC_{50} values were interpolated from the dose-dependent curves. The reported IC_{50} values are the average of three independent experiments.

2.10 Cellular Pt uptake and DNA platination

MDA-MB-231 cells were seeded in 6-well plate (Corning) at a density of 10^5 cells/mL and incubated overnight under standard growth condition. The culture media for the cells were replaced with fresh growth media. The cells were then treated with fresh growth media containing CDDP or PDMA (1.5, 3 μM) for 24 h. The cells were harvested with trypsin and washed with PBS three times, and then were digested by concentrated nitric acid (100 μL) at 95 °C for 2 h, hydrogen peroxide (30%, 50 μL) at 95 °C for 1.5 h, concentrated hydrochloric acid (50 μL) at 95 °C until total volume

was less than 50 μL. The solution was diluted to 1 mL with water, and the content of Pt was measured by ICP-MS. The average of three parallel experimental data was reported as the final result.

2.11 Cell cycle analysis

Propidium iodide (PI) was used for staining analysis. MDA-MB-231 cells (2×10^5 cells) were seeded in a 6-well plate and cultured overnight. CDDP and PDMA were added to the culture medium respectively to reach the specific concentrations (1.5, 3 μM). After incubation for 24 h, the cells were collected, centrifuged at 1000 r min^{-1} for 5 min, and washed with cold PBS three times. The cells were fixed in ethanol (70%) at 4 °C overnight, and then were centrifuged to get rid of ethanol and further washed twice with PBS, incubated with RNase A solution (100 μL) at 37 °C for 30 min. After incubation, PI (400 μL) (KeyGEN BioTECH) was added and incubated at 4 °C in the dark for 30 min. Finally, samples were washed with PBS and resuspended in PBS (500 μL). Flow cytometry (BD LARFortessaTM, USA) was used to detect cells in different phases of the cell cycle. Cell cycle profiles were modeled using Modfit LT software (Verity Software House, Topsham, ME, USA).

2.12 Apoptotic assay

MCF-7 cells (2×10^5 cells/well) were plated in 6-well plates and cultured overnight at 37 °C. After incubation with various concentrations of PDMA (1.5, 3 μM), CDDP (1.5, 3 μM) and the mixture of CDDP and ligand (3 μM) for 48 h, the cells were transferred to 1.5 mL centrifuge tubes. According to the instruction of the FITC Annexin V Apoptosis Detection Kit, the cells were washed twice with cold PBS and resuspended in binding buffer (100 μL) at a concentration of 1×10^5 cells. The cells were incubated at room temperature in the presence of Annexin V-FITC (5 μL) for 30 min in the dark, and then were stirred gently and incubated with PI (5 μL) in the dark for 10 min. After the addition of binding buffer (400 μL), the cells were immediately analyzed on a flow cytometer.

2.13 DNA damage and expression of apoptosis-related proteins

MDA-MB-231 cells were seeded in 10 cm plates and treated with PDMA and CDDP (1.5, 3 μM) respectively for 48 h. The suspended and attached cells were collected and lysed by ice-cold cell lysis buffer with protease and phosphatase inhibitor. The cell lysates were incubated on ice for 30 min and centrifuged at 12,000 g for 15 min. Protein concentrations were then measured using protein assay reagents, and equal amounts of protein per lane was applied in SDS-PAGE and electrophoretically transferred onto a PVDF membrane. The membrane was blocked with 5% bovine serum albumin (BSA) and incubated overnight at 4 °C with specific primary antibodies, including anti-human γH_2AX, caspase-3, caspase-9 and PARP antibodies. The membrane was washed with TBST buffer three times, incubated with appropriate secondary antibody for 1 h at room temperature. The immunoblots were visualized by enhanced chemiluminescence kits (Millipore Corporation, USA). Protein levels were determined by ChemiScope series (Clinx Science Instruments Co, Ltd).

2.14 Tube formation assay and anti-vascular activity

Tube formation assay was performed with μ-Slide Angiogenesis (ibidi) according to the manufacturer's instruction. Corning matrigel (Basement Membrane Mat, 354234) was thawed on ice. μ-Slide and pipet tips were precooled in the refrigerator. After addition of matrigel (10 μL) to each inner well, the μ-Slide was placed in a humid petri dish prepared with wet tissues and put into the incubator for polymerization (30 min). Meantime, HUV-EC cell suspension (6×10^5 cells/mL) was prepared and added to each well (50 μL). After 30 min, the medium in the well was replaced with medium containing DMA (100 μM), CDDP, and PDMA (15 μM), respectively. After incubation for 24 h, the supernatant was discarded, and calcein AM (KeyGEN BioTECH) (20 μL, 10 μM) diluted with PBS was added into each well. The μ-Slide was incubated in the dark for 30 min at room temperature, and washed with PBS for three times. The pictures were taken by OLYMPUS IX71 inverted microscope (λ_{ex} = 485 nm, λ_{em} = 529 nm).

The determination of anti-vascular activity was similar to the procedure described

above except that after the cell suspension (50 μL) was added to each well, the incubation was lasted for 4 h at 37 °C, when the endothelial cells (HUV-EC cells) had reorganized to form tubelike structures.

2.15 Angiogenesis assay on zebrafish

Transgenic zebrafish of the strain *Tg(fli1a:EGFP)* with a casper background was raised under standard conditions at 27–28 °C. After manual dechorionation at 24 h post fertilization (hpf) the embryos were transferred into 6 cm plates at 8 embryos/well in 10 mL of E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 0.01% methylene blue, pH 7.2) and treated with DMA (50 μM), PDMA (10 μM), and CDDP (10 μM), respectively, for 24 h. The development of SIV (subintestinal veins) was documented by confocal imaging at 48 hpf after being tranquilized by Tricaine (0.042 mg mL^{-1}, Sigma aldrich). Fishes were captured by a Carl Zeiss LSM710 inverted confocal microscope (λ_{ex} = 488 nm, λ_{em} = 509 nm) with a Fluar 2.5 x/0.12NA objective to monitor the formation of vessel.

2.16 Wound-healing assay

Culture-inserts (ibidi) were placed in a 24-well plate (Corning). Suspension of MDA-MB-231 cells (3×10^5 cells/mL) was prepared by DMEM containing 12% FBS and seeded in 24-well plates. Cell suspension (70 μL) was seeded into each well of the culture insert and incubated at 37 °C for 24 h. The culture-inserts were then removed by sterile tweezers gently, creating a cell-free gap of 500 μm. Cells were incubated in DMEM with 1% FBS in the absence or presence of CDDP or PDMA at 37 °C under 5% CO_2 with different times. Images of the wound were photographed at 0 and 48 h.

2.17 Acute toxicity

Female mice (20 ± 2 g) were purchased and housed in cages at 22 ± 2 °C temperature and a 12 h light-darkness cycle with standard food and water available ad libitum. The cages were cleaned at regular interval. Different concentrations of PDMA (5, 10, 20, 40 mg kg^{-1}) were dissolved in PET (polyethylene glycol

400/ethanol/Tween-80, 6:3:1, v/v/v) with 2% DMSO and suspended in PBS. The mice were injected with identical PBS (200 μL) or with solvent control (PET : PBS = 2 : 8, 200 μL) every three days. The experiment groups were injected intravenously with PDMA solutions in PBS (200 μL). The changes in body weight were recorded and the survival rate of the mice was calculated over a period of 15 days. Finally, the LD_{50} values of PDMA were calculated. All the experimental procedures on mice were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nanjing University and experiments were approved by the Institutional Animal Care and Use Committee of Nanjing University.

2.18. H&E staining

Histopathological examination was performed using hematoxylin and eosin (H&E) staining. Mouse organs (heart, liver, spleen, lung and kidney) were collected in 4% paraformaldehyde for proper fixation and then embedded in paraffin using tissue embedding machine. Sections were prepared orderly by dewaxing, dehydration and H&E staining. The tissue morphology was then observed under fluorescence microscopy (Olympus BX41, Japan).

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[1] R. K. Pathak and S. Dhar, *Chem. Eur. J.*, 2016, **22**, 3029–3036.