Supplementary Information

Tirapazamine-Embedded Polyplatinum(IV) Complex: A Prodrug Combo for Hypoxia-Activated Synergistic Chemotherapy

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1. Experimental Section

1.1. Instrument and Characterizations. The platinum content in the polyplatinum(IV) complex-based nanogels and samples obtained outside of the dialysis bags in drug release experiments was measured on inductively coupled plasma mass spectrometer (ICP-MS, iCAP Qc-ICPMS, Thermoscientific, USA) and inductively coupled plasma atomic emission spectrometer (ICP-AES, iCAP 6300, Thermoscientific, USA). ^{1}H NMR spectra were measured by a Unity-300 MHz NMR spectrometer (Bruker) at room temperature. Ultravoilet-visible (UV-vis) absorption spectra were taken on a Shimadzu UV-vis spectrophotometer (UV-3600). Photoluminescence (PL) spectra were measured on a Perkin-Elmer LS-55 spectro-fluorometer. Mass spectroscopy (ESI-MS) measurements were performed on a Quattro Premier XE system (Waters) equipped with an electrospray interface (ESI). Size and size distribution of micelles were determined by dynamic light scattering (DLS, Zetasizer nano ZS, Malvern, UK). The morphology of the polyPPM nanoparticles was examined by transmission electronic microscopy (TEM, Tecnai G2spirit Biotwin) at an accelerating voltage of 120 kV.

1.2. Preparation of Pt(IV)-Based Prodrug Monomer (PPM). PPM was synthesized following a literature method.[1] Dry cis-[$PtCl_2(NH_3)_2$] (0.20 g, 6.6×10^4 mol) was suspended in water (5 mL) and a 10-fold excess of H_2O_2 (30% w/v, 7.0 mL, 6.0×10^3 mol) was added. The mixture was stirred for 1 h at 50 ^{o}C and a pale-yellow powder resulted. Recrystallization of cis, cis, trans-[$PtCl_2(OH)_2(NH_3)_2$] was performed *in situ*, collected and washed with cold water, ethanol and ether, and dried in a desiccator, yielding 0.109 g (3.27×10^4 mol, 49%). Then, the crosslinker Pt(IV) bis-urethan ethyl methacrylate was synthesized following a literature method with minor modification.[2] Cis, cis, trans-[$PtCl_2(OH)_2(NH_3)_2$] (200 mg, 0.60 mmol) was dissolved in anhydrous DMF (2 mL). After the addition of 122 mg 4-(dimethylamino)-pyridine (DMAP) as catalyst, 20 mg butylhydroxytoluene (BHT) as stabilizer and methacrylic acid-2-isocyanatoethylester (257 mg, 1.7 mmol), the mixture was further stirred for 24 h resulting in the formation of a homogeneous solution. The solution was filtered, and the desired product was precipitated by the addition of diethyl ether. The solid was collected by either filtration or centrifugation. To remove residual DMF, the solid was suspended in water for 30 min, isolated by centrifugation, resuspended in ethanol, isolated by centrifugation, resuspended in diethyl ether, isolated by centrifugation, and finally dried under vacuum. White solid. Yield: 0.153 g (48%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 6.90-6.77 (s, 2H), 6.77-6.30 (m, 6H), 6.14-5.98 (s, 2H), 5.82-5.62 (s, 2H), 4.16-3.85 (m, 4H), 3.24-

3.01 (m, 4H), 1.85 (dt, 6H). ESI-MS of PPM: $[M+H]^+$ calculated for $C_{14}H_{26}Cl_2N_4O_8Pt$, 644.08, found 644.0810.

1.3. The Synthesis of Polyplatinum(IV) Complex-based Polyprodrug Nanogels. The polyprodrug nanogels were prepared by reverse addition-fragmentation chain transfer polymerization (RAFT) using a 100 mL Schlenk flask as the reactor under nitrogen atmosphere. Briefly, 73.8 mg (0.25 mmol) of MPC, 537.6 mg (0.8 mmol) of PPM, 11.5 mg of DDMAT (3 mmol % to the monomers) and 1.7 mg of AIBN (1 mmol % to the monomers) and dissolved in 6 mL methanol and 2 mL DMSO was prepared in a 100 mL schlenk glass flask equipped with a reflux condenser, then the reactor contents were heated to 70 °C and kept under nitrogen atmosphere. The polymerization reaction was allowed to continue under nitrogen atmosphere with stirring for 6 h. The reaction mixture was subsequently cooled to 25 °C, while maintaining the stirring and nitrogen flow to prevent possible aggregation. Finally, the obtained nanogels were dialyzed for a week via a dialysis bag (molecular weight cut off 3500) to remove the unreacted reagents and impurities. ^{1}H NMR (400 MHz, D_2O) δ (ppm): 4.47-3.88 (-O-CH_2-), 3.81-3.51 (-N^+-CH_2-), 3.38-3.07 (-N-CH_2-), 2.74-2.51 (-N-CH_3), 1.40-1.04 (-C-CH_2-), 1.01-0.55 (-CH_3).

1.4. Drug Loading and *In Vitro* Release. The TPZ loaded in polyprodrug nanogels was determined by a Shimadzu UV-vis spectrophotometer (UV-3600). The absorbance at 500 nm was the characteristic absorption peak of TPZ. Then, the average particle diameter was determined by dynamic light scattering (DLS, Malvern Nano-ZS). Transmission electronic microscopy (TEM, Tecnai G2spirit Biotwin) was used to measure the morphology and size distribution of the polyprodrug@TPZ nanogels. Drug loading content (DLC) was determined by the following formula 1:

DLC (wt%) = [weight of loaded drug/weight of drug - incorporated polyprodrug] * 100% (1)

Pt species and TPZ release were measured by ICP-AES (for Pt) and UV absorbance (λ = 500 nm, TPZ). In detail, the polyprodrug@TPZ was dissolved in PBS (pH 7.4) or 10 mM GSH of PBS solution, which were kept in a shaking incubator at 37 oC. At timed intervals, 4 mL of external buffer was withdrawn from the reservoir and analyzed, and then 4 mL of the fresh PBS (pH 7.4) was added to keep the volume invariable. All measurements were performed in quadruplicate, and the releasing content was calculated by formula 2:

Releasing content (%) = (amount of drug in the releasing medium)/(amount of drug loaded into nanogels) * 100% (2)

1.5. Cell Lines and Cell Culture. The human lung carcinoma A549 cell lines were obtained from ATCC. Unless otherwise specified, cells were incubated at 37 °C in 5% CO_2 and grown in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were passed every 3 to 4 days and restarted from a frozen stock upon reaching passage number.

1.6. Transfection of NOX1 Small Interfering RNA Constructs into A549 Cells. Inhibition of NOX1 expression was assessed by reverse transcription (RT)-PCR analysis after transfection of A549 cells with NOX1 siRNA. The NOX1 siRNA construct is a pool of three sequences of siRNA as follows: duplex 1 sense strand, 5'-GCA ACC GUA CAC UGA GAA ATT-3'; duplex 2 sense strand, 5'-CCU AUC UCA UCC UGA GAA ATT-3'; and duplex 3 sense strand, 5'-GCU UCC AUC UUG AAA UCU ATT-3'. All siRNA constructs were purchased from Santa Cruz Biotechnology (USA). After grown in 60 mm dishes, A549 cells were transiently transfected with 100 nM NOX1 siRNA constructs by mixing with X-tremeGENE siRNA transfection reagent (Roche Applied Science). After incubation at 34 °C and 5% CO_2 for 36 h, A549 cells were further incubated with polyprodrug for different times. All samples were then prepared and analyzed for viability or western blot.

1.7. Immunocytochemistry. A549 cells were incubated with 20 μM Pt-equivalent polyprodrug for 12 h. Then, the culture medium were removed. A549 cells were rinsed, fixed with 4% paraformaldehyde in phosphate buffer saline solution for 20 min. After washed three times with PBS, Cells were pre-incubated 0.1% Triton X-100 of PBS buffer. A549 cells were then washed with PBS twice and blocked with PBS containing 1% BSA for 0.5 h, and followed by treating with primary antibodies in 1% BSA of PBS for 1.5 h. After rinses, A549 cells were treated with Alexa Fluor 568 (for anti-NOX1 anti-body) and Alexa Fluor 488 (for anti-NOXO1 antibody) secondary antibodies for 1.5 h. After rinsed with PBS for two times, A549 cells were further incubated with DAPI for nuclear image at room temperature for 5 min. Cells were observed under confocal microscopy using appropriate filters (Zeiss LSM-710 microscopy).

1.8. Western Blot Analysis. A549 cells and NOA1 siRNA transfected A549 cells (4×10^6 cells/dish) were plated in 60 mm culture dishes overnight, and followed by co-incubating with polyprodrug@TPZ (an equal Pt concentration of 10 μM) at 37 °C for 24 h. The untreated cells were served as controls. After washing with PBS three times, the Total Protein Extraction Kit (Bestbio Company, China) was used to extract the total proteins from A549 cells. Then, the protein samples (ca. 20-40 μg) were denatured in 5 × sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) sample buffers, and subjected to SDS-PAGE on 12% Tris-glycine gels. To verify the protein levels of the desired molecules, the blots were proved with the specific primary antibodies (dilution ratio: 1:500), followed by appropriate peroxidase-conjugated secondary antibodies (dilution ratio: 1:1500), and then visualized by using enhanced ECL western blotting detection reagents (Biosharp). The MultiGauge version 2.0 software was used to perform the densitometric analysis.

1.9. Animals and Tumor Model. The experimental protocols involving animals were approved by the Animal Ethics Committee of Shanghai Jiao Tong University and performed in compliance with the Guidelines for Care and Use of Laboratory Animals of Shanghai Jiao Tong University. Sprague-Dawley (SD) rats and BALB/c male nude mice were supplied by Chinese Academy of Sciences (Shanghai).

1.10. *In Vivo* Imaging. When the tumor volumes reached 100 mm^3, the BABL/c nude mice were divided into two groups randomly. Mice were injected with free Cy5.5, Cy5.5-loaded polyprodrug (200 mL, 200 mg/mL Cy5.5) via the tail vein, respectivly. The fluorescence signals of Cy5.5 were obtained by an ex/in vivo imaging system (CRI Maestro, USA) (ex: 690 nm; filter:735 nm).

1.11. Antitumor Study *In Vivo*. All nude mice (20-25 g, male, 4 weeks of age) were subcutaneously injected in the right backs with A549 cells suspension (200 μL, 2×10^6 cells) in RPMI medium, respectively. When the tumor volume reached approximately 50 mm^3, animals were randomly divided into the following 5 groups (n = 5 per group): Saline, TPZ, cisplatin+TPZ, polyprodrug and polyprodrug@TPZ groups. 200 μL saline containing with TPZ (2 mg/kg), cisplatin+TPZ (2 mg/kg for TPZ, 1.70 mg Pt/kg for cisplatin), polyprodrug (1.77 mg Pt/kg) and polyprodrug@TPZ (2 mg/kg for TPZ) were treated by the tail vein injection, respectively. Animals were injected at 5 different time points with an interval of 2 days (days 1, 3, 5, 7 and 9 after first treatment). After 21 days post first treatment, all A549 tumor bearing mice were sacrificed. Various tissues including tumor, heart, liver, lung, kidneys, and spleen were harvested, fixed, and assessed by terminal deoxy-nucleotidyl transferase-dUTP nick-end labeling (TUNEL) and histological examination. The volume of

tumor was calculated by formula 3: $V = ((\text{tumor width})^2 \times (\text{tumor length}))/2$ (3). Mortality, clinical status, body weight, and tumor growth curve of nude mice were carefully recorded.

1.12. Histological Examination. All the tissues (heart, kidneys, liver, lung, and spleen) were fixed in a formalin solution. In brief, the tissue samples were embedded in paraffin blocks, sectioned into 5 μm slices, and mounted onto the glass slides. After hematoxylin-eosin (H&E) staining, the sections were observed, and photos were taken using an optical microscopy. (Magnification: liver × 10; heart, spleen, lung, and kidney × 20) Statistical Analysis. Results are presented as mean or means ± standard deviation.

Supplementary Figures

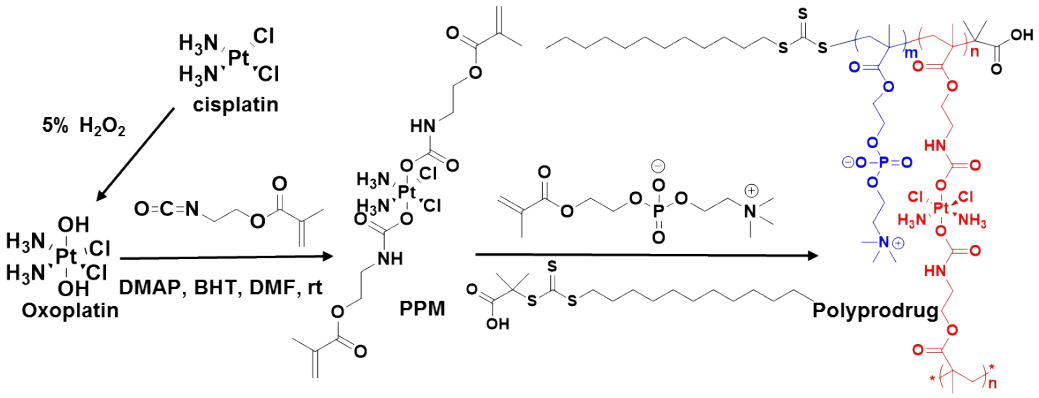

Fig. S1 Synthetic route of PPM and polyprodrug.

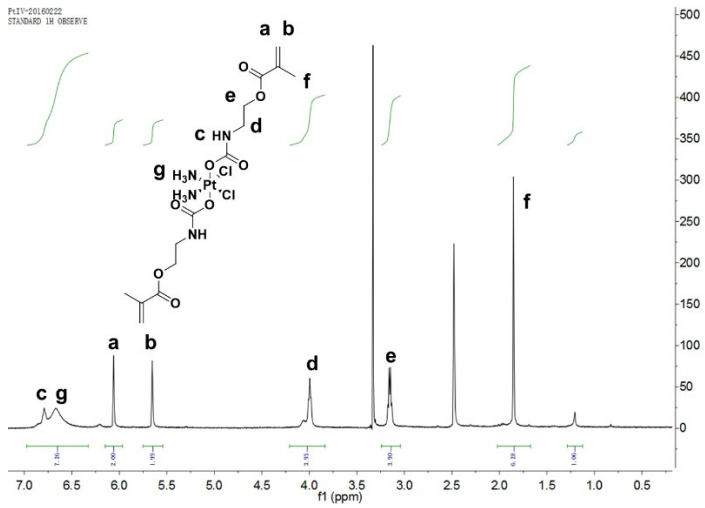

Fig. S2 ^{1}H NMR spectrum of Pt(IV) bis-urethan ethyl methacrylate in DMSO-d_6.

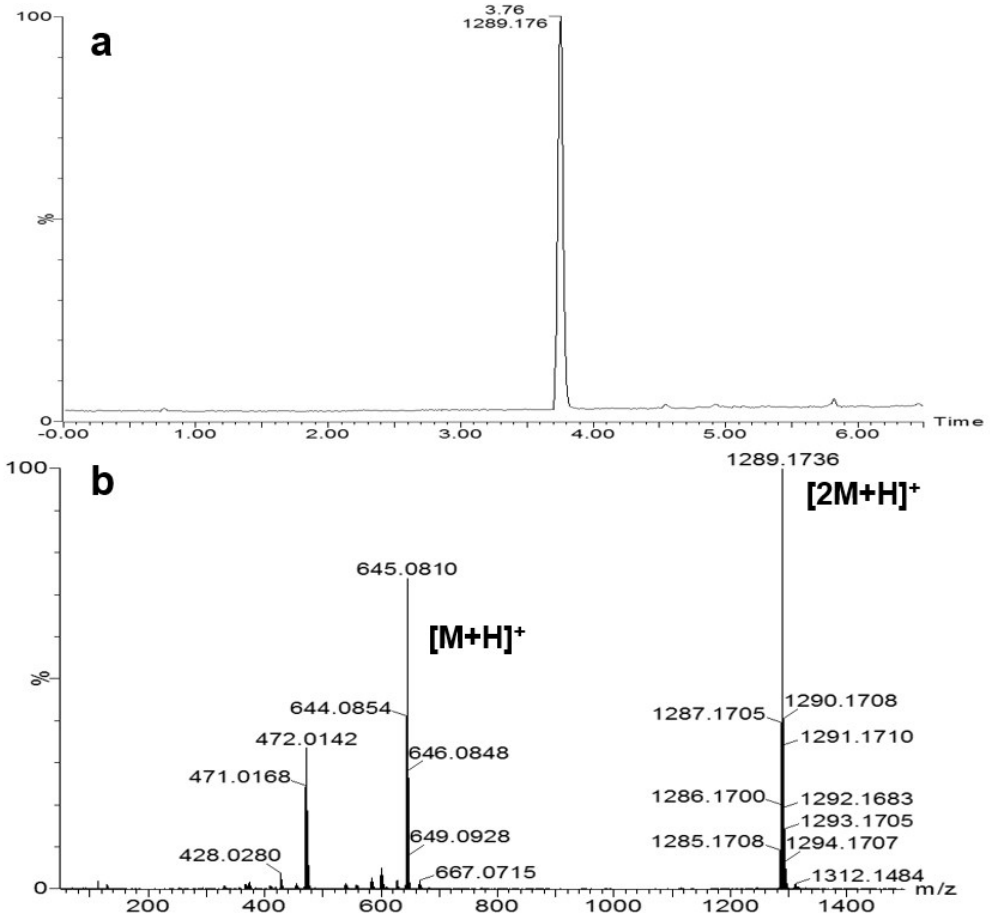

Fig. S3 HPLC-MS spectra of PPM.

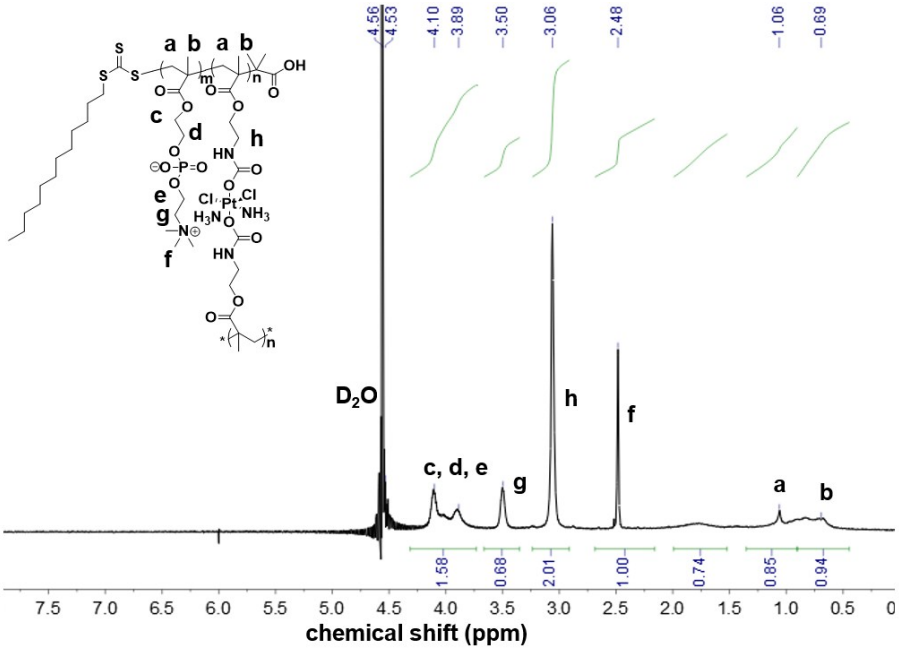

Fig. S4 ^{1}H NMR spectrum of polyprodrug in D_2O.

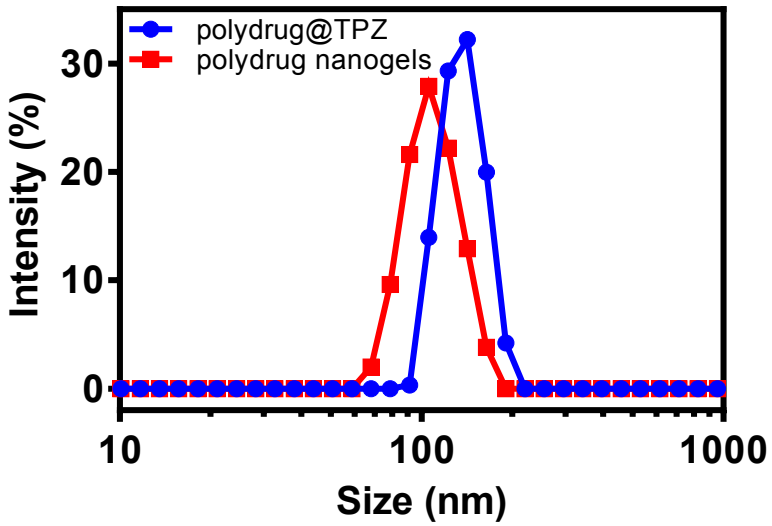

Fig. S5 DLS measurement of polyprodrug and polyprodrug@TPZ nanogels.

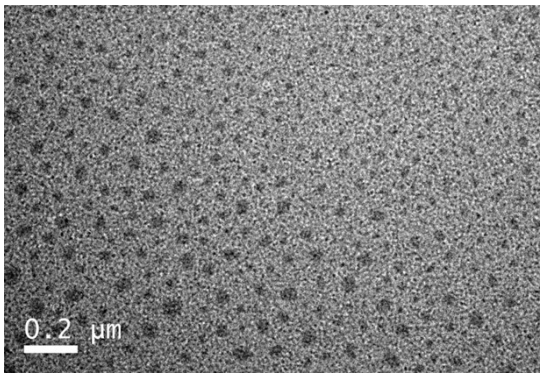

Fig. S6 TEM image of the polyprodrug@TPZ nanogels

Table S1. Pt content and size of polyprodrug and polyprodrug@TPZ nanogels

Sample	Pt(%)	Size (nm) DLS	Size (nm) TEM
polyprodrug	8.06	105.28 ± 20.8	98.1 ± 9.0
polyprodrug@TPZ	7.78	138.2 ± 14.6	123.1 ± 9.0

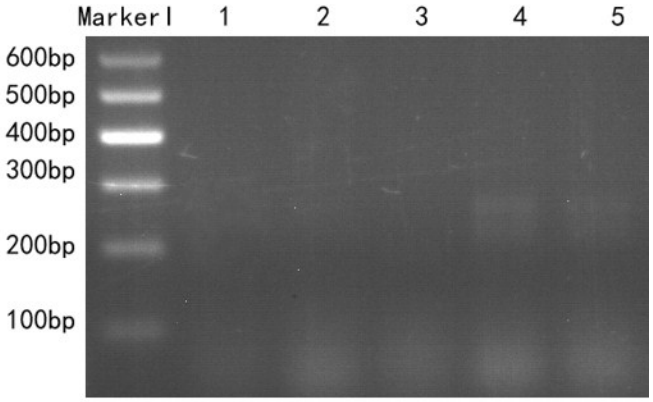

Fig. S7 The mRNA expression levels of NOX2 was examined by RT-PCR (1: 0 h, 2: 6 h, 3: 12 h, 4: 24 h, 5: 48 h). As shown in Fig. S7, NOX2 mRNA was not detected in A549 cell, indicating that polyprodrug cannot induce high expression of NOX2.

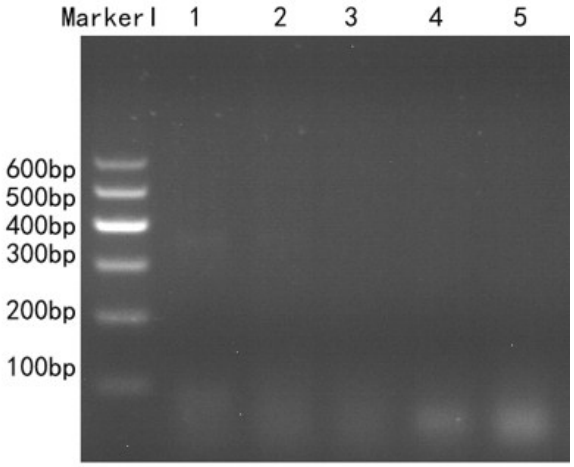

Fig. S8 The mRNA expression levels of NOX3 was examined by RT-PCR (1: 0 h, 2: 6 h, 3: 12 h, 4: 24 h, 5: 48 h). As shown in Fig. S8, NOX3 mRNA was not detected in A549 cell as well, indicating that polyprodrug cannot induce high expression of NOX3.

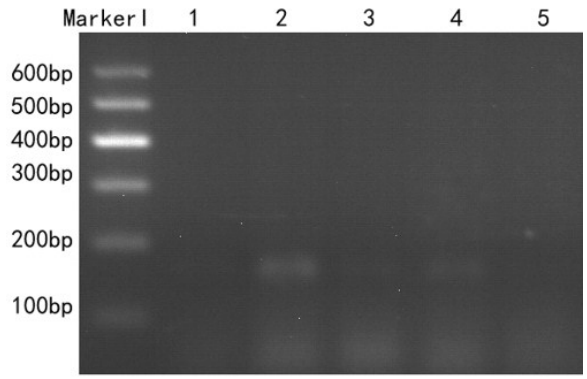

Fig. S9 The mRNA expression levels of NOX4 was examined by RT-PCR (1: 0 h, 2: 6 h, 3: 12 h, 4: 24 h, 5: 48 h). As shown in Fig. S9, the expression levels of NOX4 mRNA was not detectable.

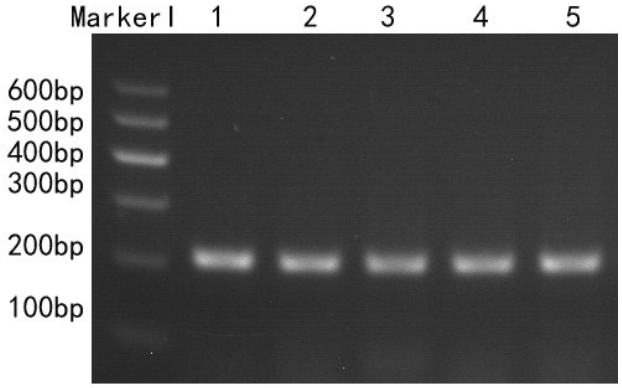

Fig. S10 The mRNA expression levels of $p47^{phox}$ was examined by RT-PCR (1: 0 h, 2: 6 h, 3: 12 h, 4: 24 h, 5: 48 h). As shown in Fig. S10, $p47^{phox}$ mRNA was detected in A549 cell. However, the level of $p47^{phox}$ expression was fixed as incubation times of polyprodrug increasing, indicating there was not relationship between polyprodrug and $p47^{phox}$.

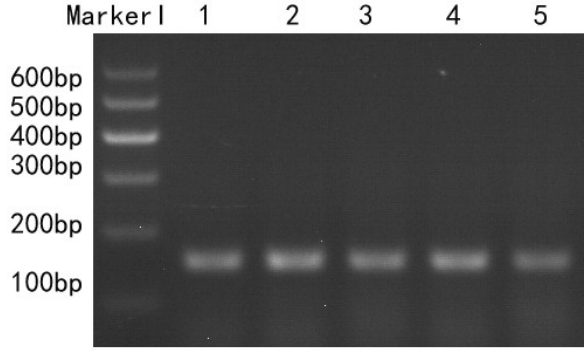

Fig. S11 The mRNA expression levels of $p67^{phox}$ was examined by RT-PCR (1: 0 h, 2: 6 h, 3: 12 h, 4: 24 h, 5: 48 h). As shown in Fig. S11, the mRNA expression levels of $p67^{phox}$ was not in a time-depentent manner, indicating that polyprodrug could not give rise to varition of $p67^{phox}$ mRNA levels.

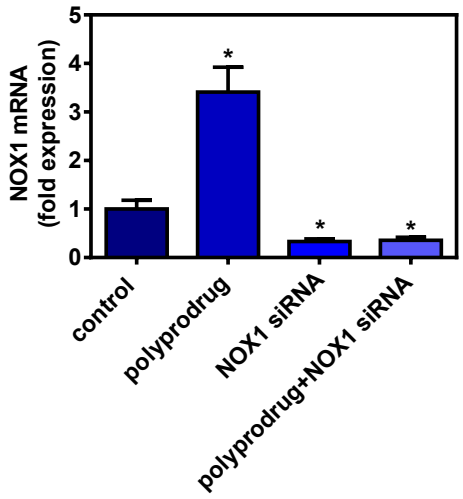

Fig. S12 A549 cells or NOX1 siRNA (5 nM, 36 h) transfected A549 cells were treated with polyprodrug for 12 h. NOX1 mRNA was measured by real-time PCR.

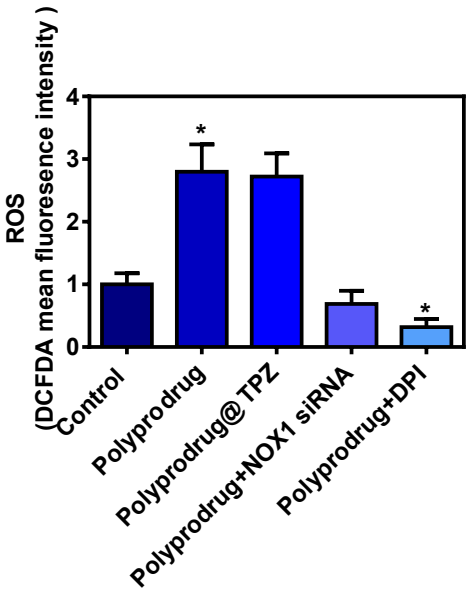

Fig. S13 The level of intracellular ROS was also monitored using a peroxide-sensitive fluorescent probe, DCFH-DA.

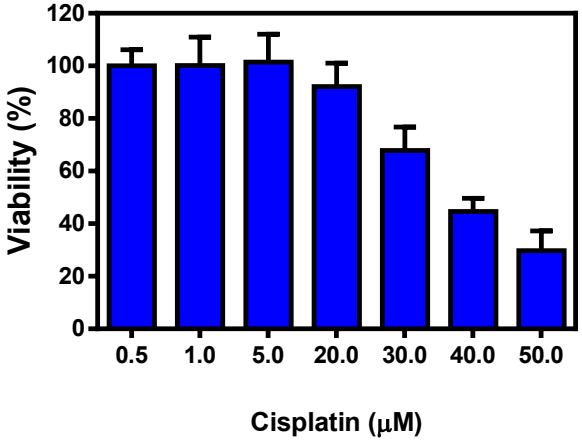

Fig. S14 The cytotoxicity in A549 cells treated with cisplatin.

Table S2. IC_{50} values for compounds tested on the A549 cell line after 24 h exposure

Sample	IC_{50} values (μg/mL of drugs)	IC_{50} values (μM)
TPZ	28.0	157
Pt	7.6	38.8
polyprodrug@TPZ	TPZ: 5.0	28
	Pt: 4.4	22.6

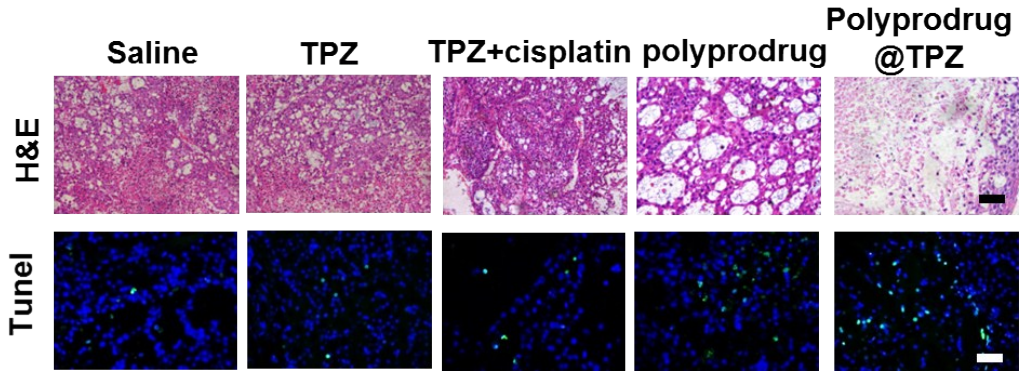

Fig. S15 Representative histological and TUNEL observations of A549 tumor sections (21 days after the first treatment) to assess the apoptosis of the tumor tissues after treatment. Scale bar = 100 μm.

References

[1] Barnes, K. R.; Kutikov, A.; Lippard, S. J. *Chem. Biol.* **2004**, 11, 557-564.

[2] Wilson, J. J.; Lippard, S. J. *Inorg. Chem.* **2011**, 50, 3103-3106.