Supplementary Information

Supramolecular Palladium Complexes Based on Guanidinium Pillar[5]arene for Cancer Therapy

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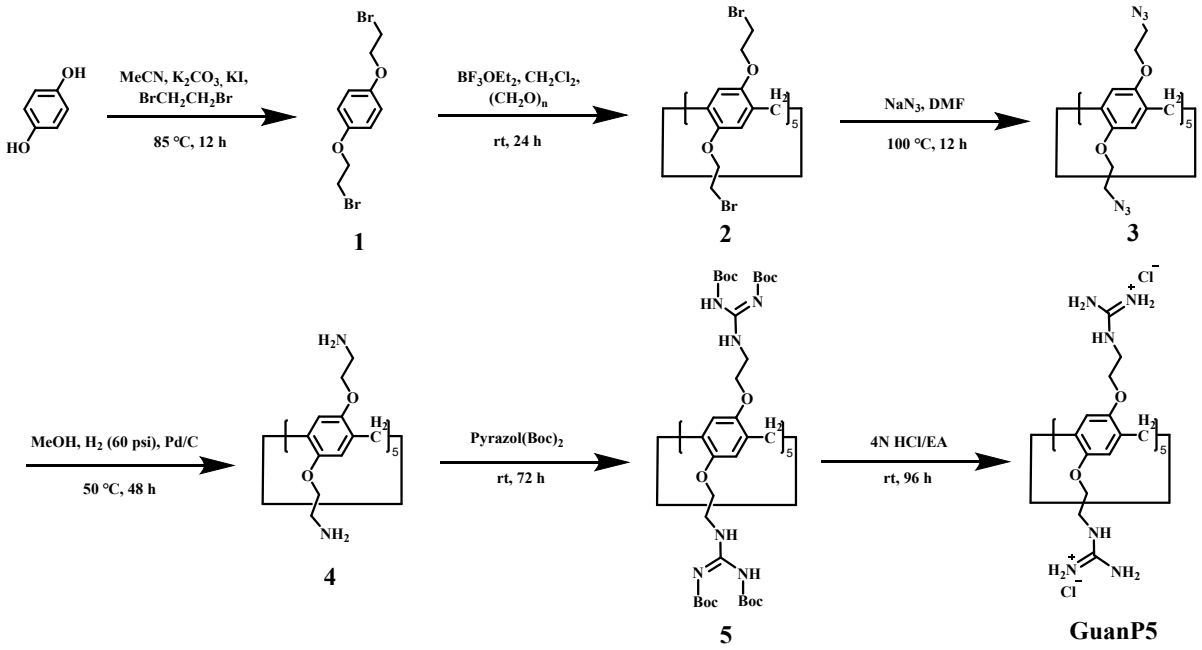
Experimental section

Reagents and Materials. Unless otherwise stated, all reagents used were of analytical grade and required no further purification. Water used in this work was ultrapure water. 1, 2-dibromoethane (98%), potassium iodide (99%), polyformaldehyde ((99%), hydroquinone (99%), boron trifluoride diethyl etherate (98%), Potassium tetrachloropalladate (K_2PdCl_4, 99%) and N, N-di-BOC-1H-1-guanidine pyrazole (99%) were purchased from Aladdin Chemical Reagent Co., Ltd. Adriamycin (DOX, 98%) was purchased from Tianjin Heowns Biochemical Technology Co., Ltd. Glutathione (GSH) was purchased from Sigma-Aldrich Co., Ltd. Hydrochloric acid, dichloromethane, methanol, ethyl acetate, and petroleum ether were all purchased from Chengdu Kelong Chemical Reagent Factory. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Aladdin Reagent Co., Ltd. (China). Dimethyl sulfoxide (DMSO) was from Chengdu Kelun Chemical Reagent Co., Ltd. Glutathione (GSH, 99%) and potassium bromide (KBr) were both acquired from Adamas Chemical Reagent Co., Ltd. Fetal bovine serum (FBS) was from Solarbio Science & Technology Co., Ltd. Trypsin was purchased from Ligen Biotech Co., Ltd. Penicillin-streptomycin solution was obtained from Beyotime Biotechnology Co., Ltd. Media (RPMI 1640, DMEM, Gibco) were sourced from Thermo Fisher Scientific Inc. The apoptosis detection kit and JC-10 dye were both acquired from Lanji Technology Co., Ltd.

Instruments. The ^1H NMR spectra were obtained using an Avance Neo 400 MHz spectrometer. UV–Vis spectra were acquired on a Shimadzu 1750 UV–visible spectrophotometer. TEM and SEM images were captured on a TECNAI G2 SPIRITBIO and a Nano SEM-450 instrument, respectively. DLS measurements were conducted using a Malvern ZEN3600 apparatus. FTIR spectra were recorded using a Vertex70 spectrometer. The confocal laser scanning microscopy (CLSM) images were captured utilizing a Revolution WD spinning disk confocal microscope. The flow cytometry data were obtained using a BD FACSAriaTM III flow cytometer. Inductively

coupled plasma optical emission spectroscopy (ICP-OES) and X-ray photoelectron spectroscopy (XPS) data were tested with Agilent 5110 (OES) and Thermo Scientific K-Alpha, respectively.

Synthesis and characterization of GuanP5



Scheme S1 Synthetic route of GuanP5.

GuanP5 was synthesized according to the reported method[1].

Synthesis of Compound **1**: Hydroquinone (0.50 g, 4.5 mmol), potassium iodide (2.51 g, 18.1 mmol), 1, 2-dibromoethanes (4.36 g, 27.3 mmol) and 25 mL acetonitrile were added to an oven-dried round-bottled flask, mixed and stirred under nitrogen protection for 12 h by reflux. After the reaction, acetonitrile in the reaction mixture was removed by a rotavapor. The residue was dissolved in methylene chloride and washed with deionized water three times, the organic phase was collected and dried with anhydrous Na_2SO_4. Then the solvent was dried via a rotavapor and the crude was purified by silica gel column chromatography (PE: DCM = 5:1) to give Compound **1** as a white solid (150 mg, 29%).^{1}H NMR (400 MHz, $CDCl_3$) δ 6.86 (s, 4H), 4.24 (t, J = 6.3 Hz, 4H), 3.61 (t, J = 6.3 Hz, 4H) ppm.

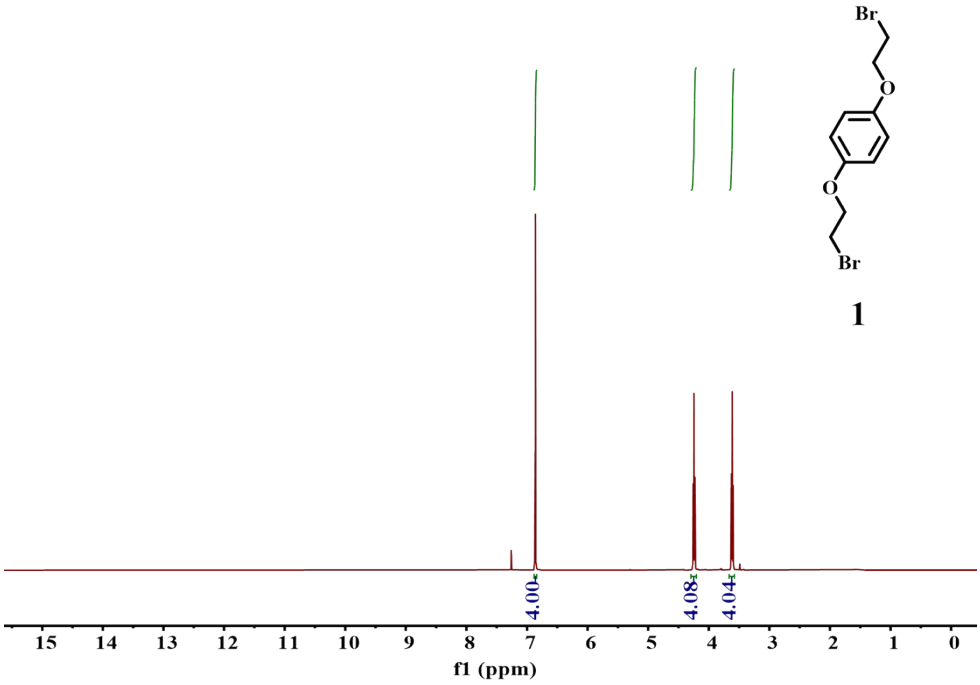


Fig. S1 ^{1}H NMR spectrum of Compound **1** (400 MHz, $CDCl_3$).

Synthesis of Compound **2**: Compound **1** (1.00 g, 3 mmol) and polyformaldehyde (0.3 g, 10 mmol) were dissolved in 1, 2-dichloroethane (100 mL) solution, and boron trifluoride diethyl etherate (500 μL, 4 mmol) was added under the protection of nitrogen, and the reaction was stirred at room temperature for 24 h. After the reaction, a saturated sodium carbonate solution (100 mL) was added to quench the reaction. The organic phase was extracted with dichloromethane and washed with deionized water three times. The organic phase was dried with anhydrous Na_2SO_4 and then the solvent was removed by rotation, and the crude was purified by silica gel column chromatography (PE: DCM = 1:1) to give Compound **2** as a white solid (600 mg, 58%).^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.91 (s, 10H), 4.23 (t, J = 5.7 Hz, 20H), 3.84 (s, 10H), 3.63 (t, J = 5.7 Hz, 20H) ppm.

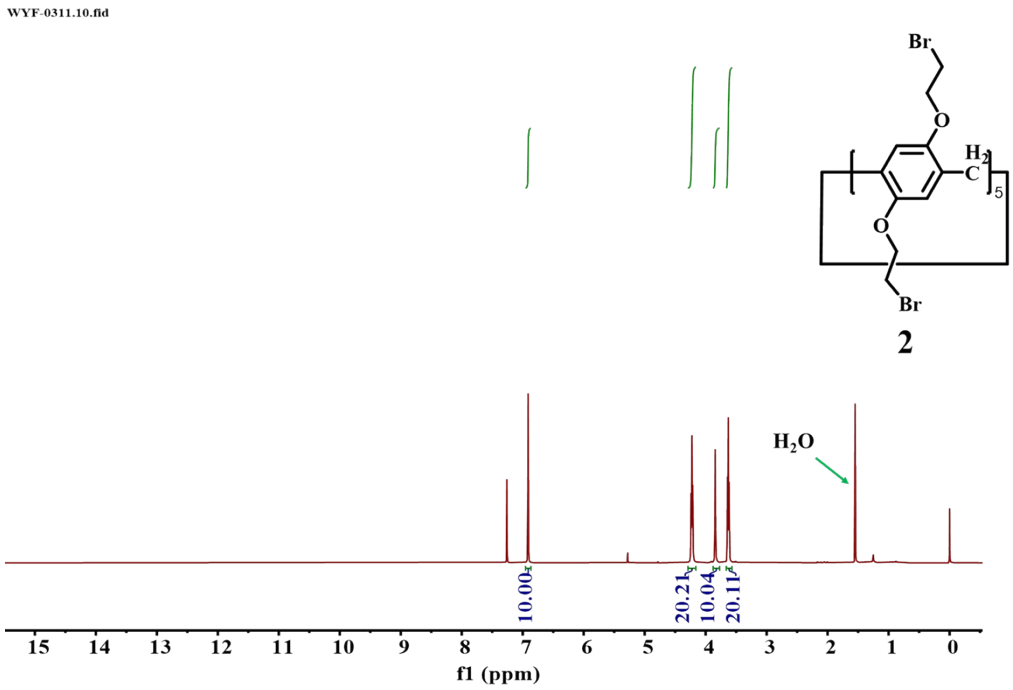


Fig. S2 ^{1}H NMR spectrum of Compound **2** (400 MHz, $CDCl_3$).

Synthesis of Compound **3**: An anhydrous DMF (5 mL) was added to a round-bottled flask containing NaN_3 (193 mg, 3 mol) and Compound **2** (100 mg, 0.06 mmol) under a nitrogen atmosphere. The reaction mixture was stirred at 100 ℃ for 12 h, then cooled to room temperature and quenched with 80 mL water. The precipitate was filtered and washed with water 3 times, and then purified by silica gel chromatography (PE: DCM = 4:3) to give Compound **3** as a white solid (74 mg, 95%).^{1}H NMR (400 MHz, $CDCl_3$) δ 6.91 (s, 10H), 4.23 (t, J = 4.9 Hz, 20H), 3.84 (s, 10H), 3.63 (t, J = 4.8 Hz, 20H) ppm.

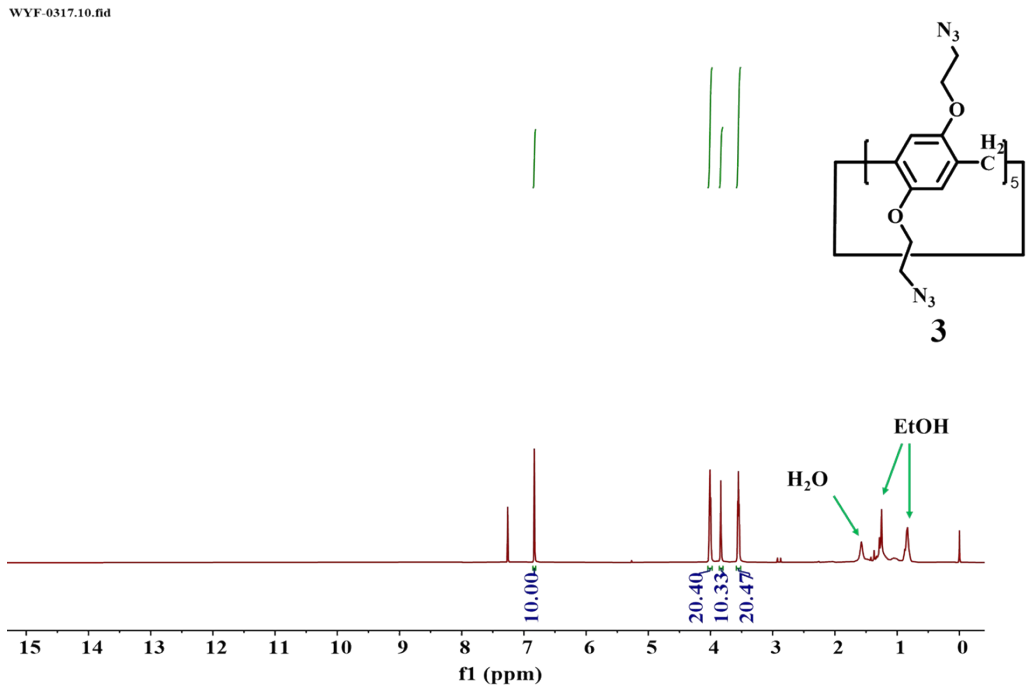


Fig. S3 ^{1}H NMR spectrum of Compound **3** (400 MHz, $CDCl_3$).

Synthesis of Compound **4**: Compound **3** (300 mg, 0.23 mmol) and Pd/C (10%, 50 mg) in 10 mL methanol were placed in a high-pressure reactor and stirred at 50℃ in a hydrogen atmosphere (60 psi) for 48 h. After the reaction, the reaction mixture was cooled to room temperature, filtered, collected and concentrated to obtain Compound **4** as a white solid (219 mg, 92%).^{1}H NMR (400 MHz, CD_3OD) δ 6.77 (s, 10H), 3.89 (m, 30H), 2.98 (s, 20H) ppm.

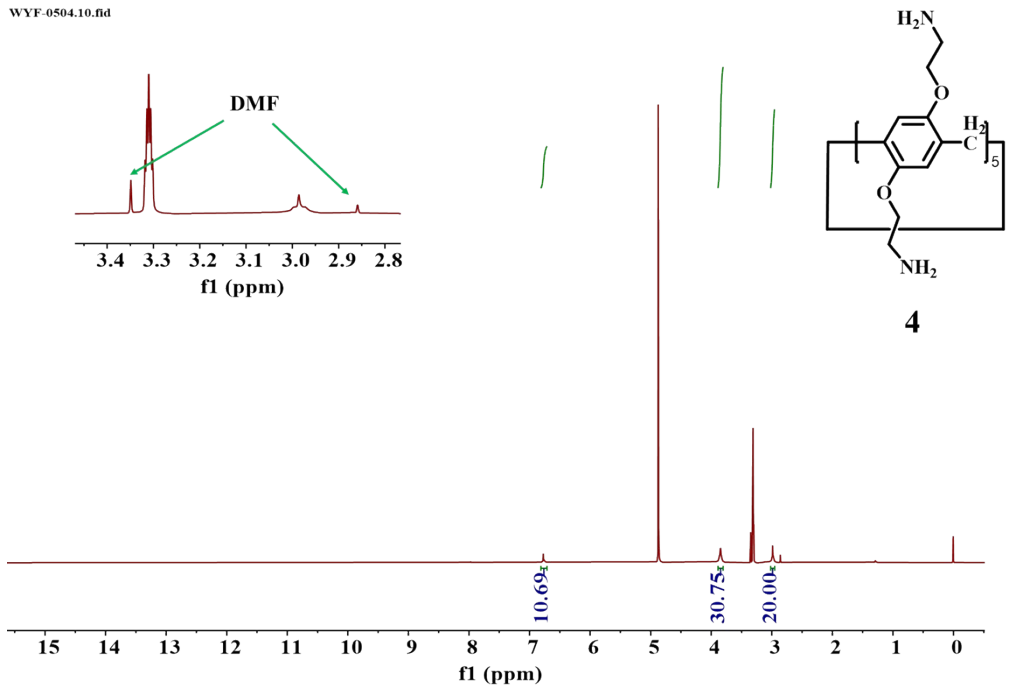


Fig. S4 ^{1}H NMR spectrum of compound **4** (400 MHz, CD_3OD).

Synthesis of Compound **5**: N, N-di-BOC-1H-1-guanidine pyrazole (1.71 g, 5.5 mmol) was added to DMF (20 mL) solution of Compound **4** (0.52 g, 0.5 mmol) under N_2 protection. The reaction mixture was stirred at room temperature for 3 days, then concentrated under reduced pressure. The resulting residue was dissolved in 30 mL CH_2Cl_2 and washed with water three times. The organic phase is collected and concentrated under reduced pressure. The crude was then purified by silica gel chromatography (DCM: MeOH = 100:1) to give Compound **5** as a white solid (0.73 g, 42%).^{1}H NMR (400 MHz, $CDCl_3$) δ 11.53 (s, 10H), 8.89 (t, J = 5.1 Hz, 10H), 6.80 (s, 10H), 4.06 (m, 40H), 3.64 (m, 10H), 1.47 (m, 180H) ppm.

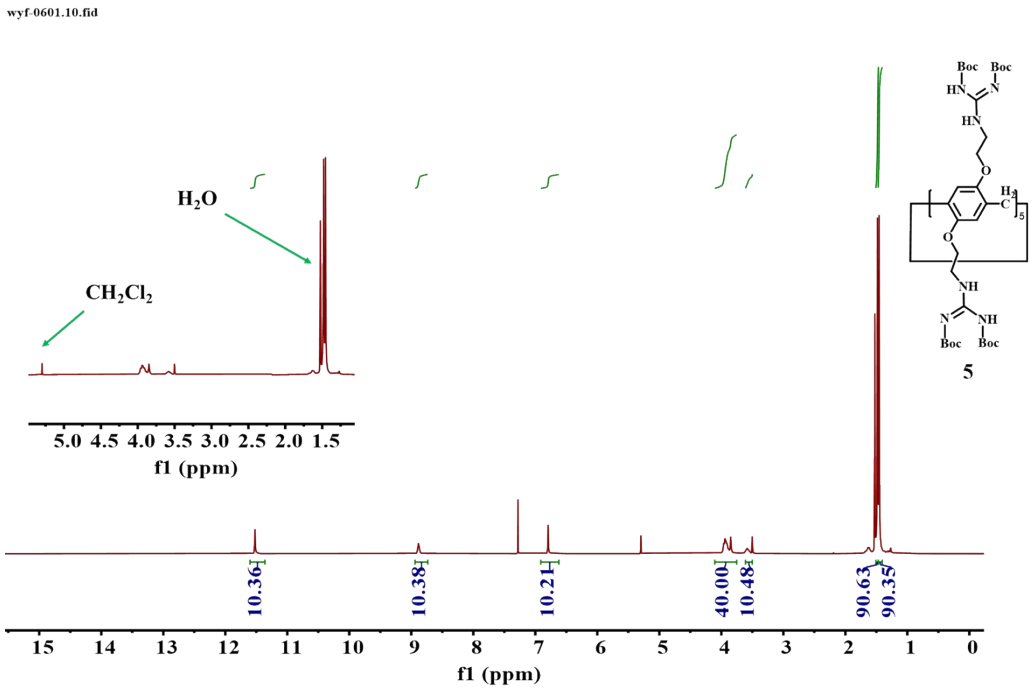


Fig. S5 ^{1}H NMR spectrum of Compound **5** (400 MHz, $CDCl_3$).

Synthesis of GuanP5: Compound **5** (0.34 g, 0.1 mmol) was dissolved in a mixture of 20 mL MeOH and 10 mL $CHCl_3$ under N_2 protection, and 0.3 mL 4N HCl/EA was added to the solution. The resulting reaction mixture was stirred at room temperature for 4 days. The residue obtained under reduced pressure was dissolved in 0.5 mL methanol, followed by an addition of 100 mL ethyl ether. The precipitate was filtered and collected to give GuanP5 as a white solid (173 mg, 95%).^{1}H NMR (400 MHz, CD_3OD) δ 6.83 (s, 10H), 4.08 (m, 20H), 3.87 (m, 30H) ppm.

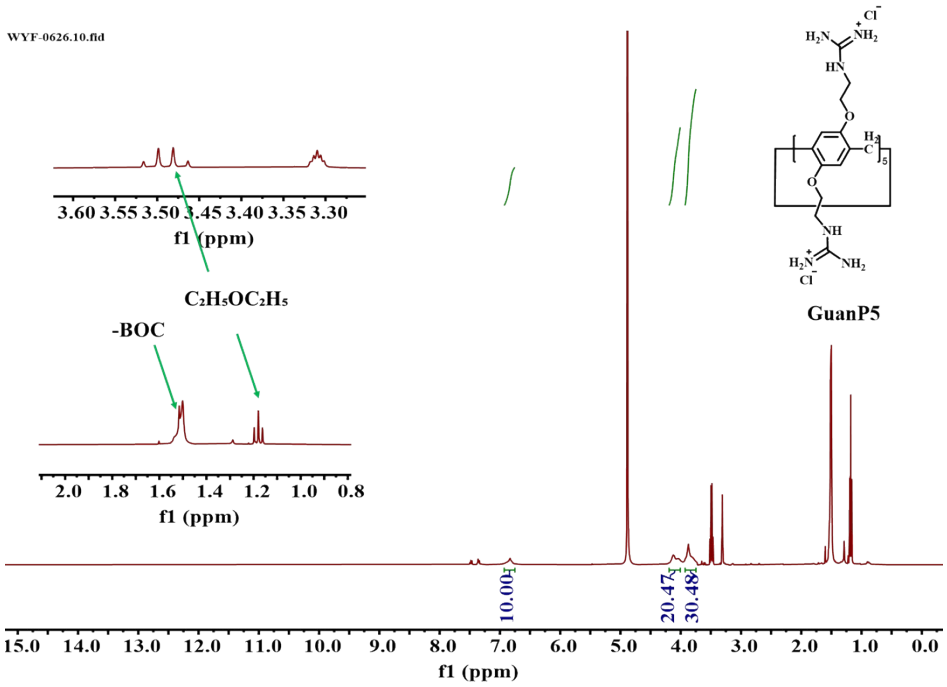


Fig. S6 ^{1}H NMR spectrum of GuanP5 (400 MHz, CD_3OD).

Preparation of G-Pd

GuanP5 and K_2PdCl_4 were mixed in aqueous solution according to the molar ratios of 1:1, 1:2, 1:5, and 1:10 (total 1 mL). After ultrasonic mixing, the particle size distribution was determined using a ZEN3600 nanometer laser particle size analyzer through dynamic light scattering. GuanP5 alone can self-assemble into 500 nm nanoparticles in water with good dispersion (PDI = 0.193). When GuanP5 was mixed with K_2PdCl_4 at a 1:1 molar ratio, the particle size of the nanoparticles was reduced to 400 nm, and the dispersion was slightly decreased (PDI = 0.201). When the molar ratio is 1:5, the particle size is further reduced to 210 nm and the dispersion is improved (PDI = 0.137). However, when the molar ratio reached 1:10, a precipitate was formed. These results reveal the effect of the GuanP5 to K_2PdCl_4 ratio on nanoparticle formation and stability. After optimizing the experimental conditions, we found that palladium-complexed nanoparticles (G-Pd) could be prepared successfully when the molar ratio is GuanP5:K_2PdCl_4 = 1:5.

The Critical Aggregation Concentration (CAC) was determined by the surface tension method. The surface tensions of G-Pd in different concentrations (0, 2, 4, 6, 8,

10, 12, 14, 16, 18, 20 μg/mL) were measured by a surface tensiometer. The CAC of G-Pd nanoparticle in aqueous solution was calculated according to the obtained surface tension fitting.

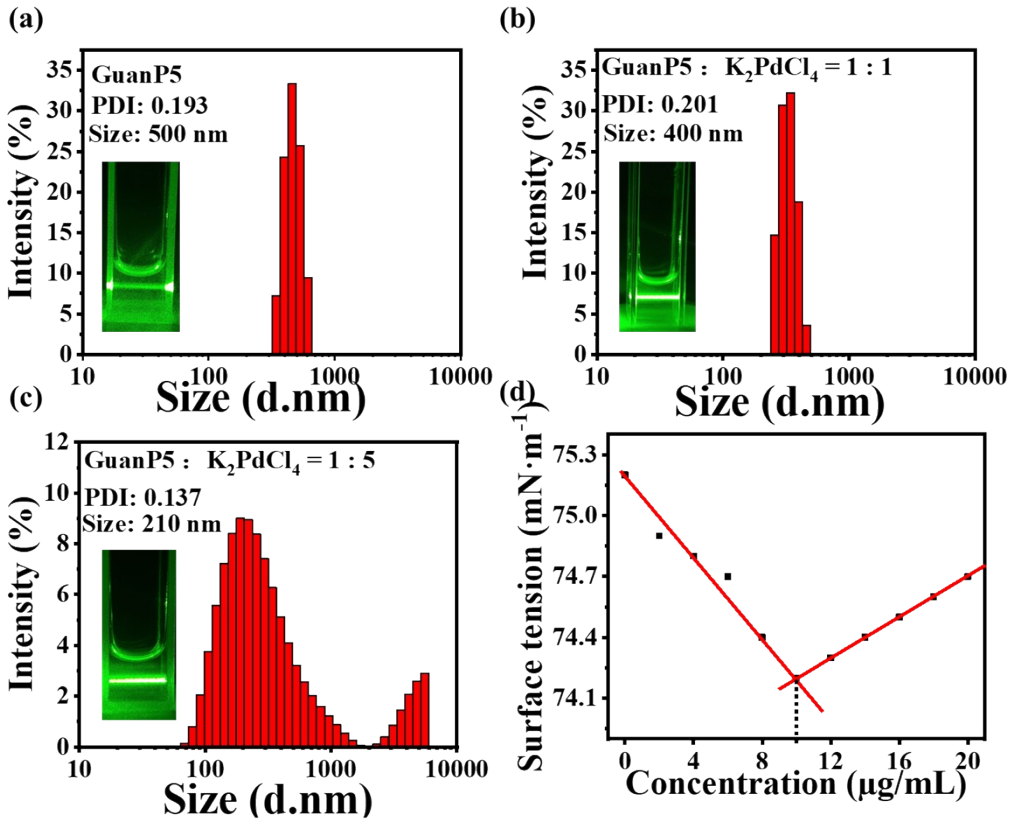


Fig. S7 (a-c) Hydrodynamic diameter distribution and PDI of nanoparticles formed by GuanP5 and K_2PdCl_4 in water at different molar ratios (GuanP5: K_2PdCl_4 = 1 : 0, 1: 1, 1: 5). (d) Critical aggregation concentration of G-Pd.

Preparation of G-Pd@DOX

G-Pd and DOX were dissolved in pure water with a mass ratio of 1:5 to prepare DOX-loaded nanoparticles (G-Pd@DOX). 2 mg of G-Pd and 10 mg of DOX were dissolved in 2 mL of water, respectively under ultrasonication. The DOX solution was then added to the G-Pd aqueous solution, and the mixture was ultrasonicated for 30 min and left to stand overnight. The color of the solution changed from yellow to pink. After the reaction, the mixture was centrifuged and washed with pure water three times and then dialyzed with a dialysis bag (molecular weight cut-off point (MWCO) = 1000 Da) to remove excess DOX until DOX could not be detected in the dialysate. Finally, the

G-Pd@DOX nanoparticles were centrifuged and dispersed in 1 mL of ethanol and stored for future use.

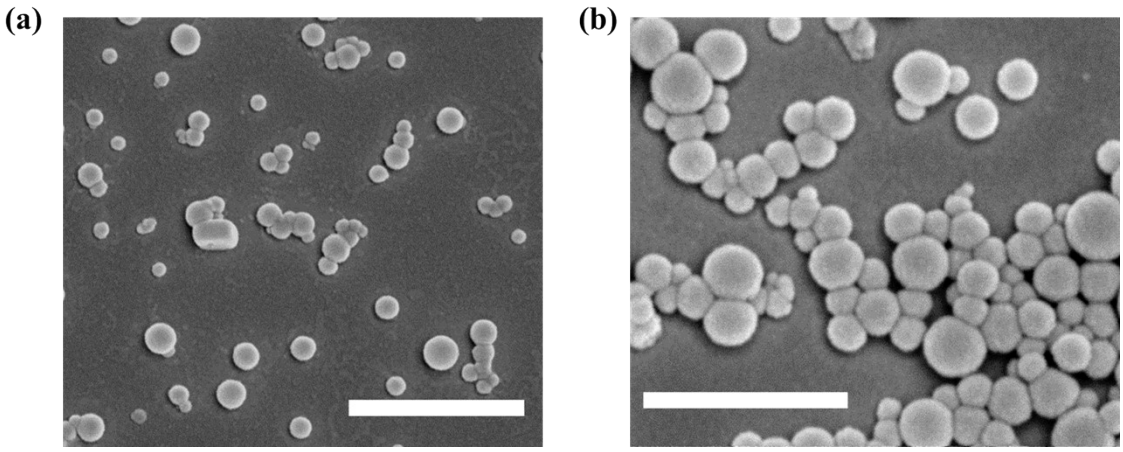


Fig. S8 Scanning electron microscope image of (a) G-Pd (Scale bar: 1 μm) and (b) G-Pd@DOX (Scale bar: 500 nm).

Subsequently, the concentration-absorbance curve for DOX based on the maximum absorption at 488 nm was obtained to facilitate the calculation of loading capacity and loading efficiency of DOX into G-Pd. The loading capacity and efficiency were calculated via the following formula:

$$Loading\ Efficiency = \frac{m_0 - m_s}{m_0} \times 100\% \quad (2\text{-}1)$$

$$Loading\ Capacity = \frac{m_0 - m_s}{m_t} \times 100\% \quad (2\text{-}2)$$

Here, m_0, m_s, and m_t stand for the weight of free DOX feeding amount, DOX in the supernatant, and DOX-loaded G-Pd, respectively.

Drug release: Place G-Pd@DOX in a dialysis bag and immerse it in 15 mL of 10 mM GSH solution. Stir the mixture at room temperature. At specified intervals (1, 2, 4, 6, 8, 10, 12, and 24 h), measure the distinctive peak of the solution outside the dialysis bag at a wavelength of 488 nm. Lastly, transfer the solution back into its initial container.

The stability of G-Pd and G-Pd@DOX

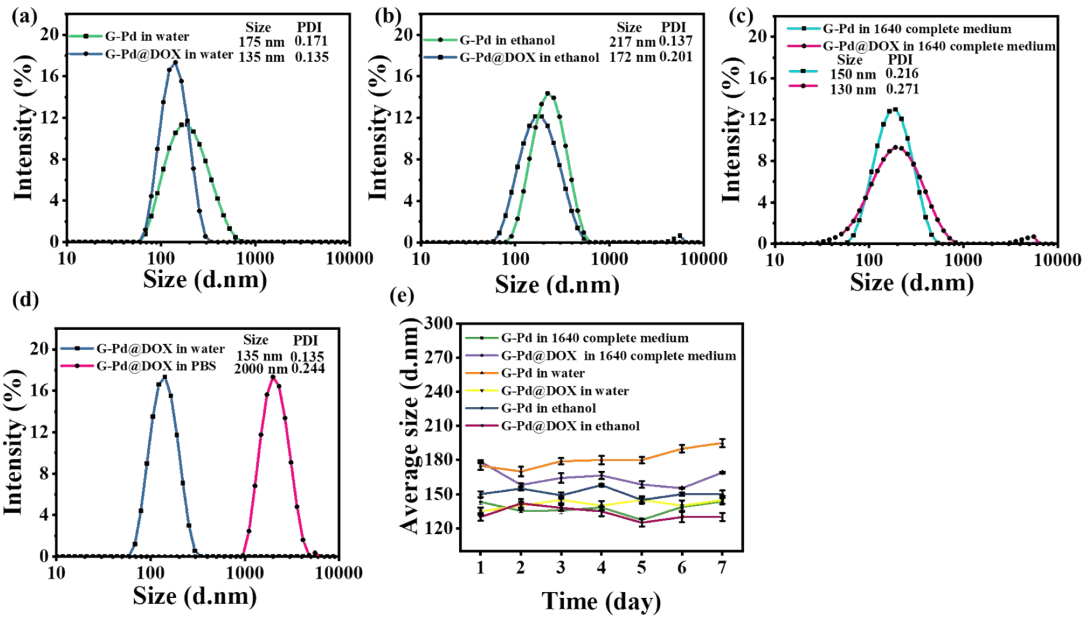


Fig. S9 Hydrodynamic diameter distribution and PDI of G-Pd (40 μg/mL) and G-Pd@DOX (50 μg/mL) in (a) water, (b) ethanol, (c) complete 1640 medium, and (d) PBS. (e) The average hydrodynamic diameter of G-Pd and G-Pd@DOX in complete 1640 medium, water and ethanol. Data are presented as the mean ± SD (n = 3).

The UV-vis absorption spectra of G-Pd and G-Pd@DOX

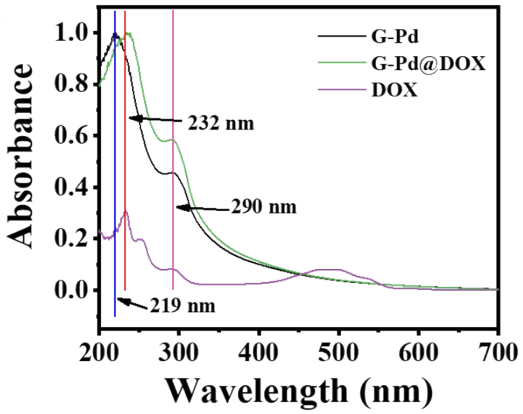


Fig. S10 UV-vis absorption spectra of G-Pd (40 μg/mL), G-Pd@DOX (50 μg/mL) and DOX in water.

Cell culture

Human hepatoma carcinoma (HepG2) cell line, human normal liver (HL7702) cell line, and human breast cancer (MCF-7 and MDA-MB-468) cell lines used in this work were all sourced from Jiangsu KeyGEN Biotech Co., Ltd. The cells were cultured in 1% (v/v) antibiotics and 10% (v/v) FBS supplemented medium under 5% CO_2 at 37 °C. The culture medium was replaced daily, and sub-culturing of the cells was performed when they reached 90% confluence.

Cellular uptake

To validate cellular uptake, HepG2 cells (2×10^5) were seeded in a 6-well plate and cultured for 24 h, followed by incubation with 50 μg/mL G-Pd@DOX for various durations (0, 1, 2, 4, 6, and 12 h). Fluorescence intensity at 488 nm was then measured using flow cytometry, providing quantitative data on cellular internalization. Additionally, HepG2 cells were seeded on glass bottom dishes, cultured for 24 h, and incubated with G-Pd@DOX for 2, 4, and 12 h, respectively. After incubation, cells were washed with PBS to eliminate non-internalized nanoparticles, fixed with formaldehyde, and stained with DAPI. Confocal laser scanning microscopy (CLSM) was employed to visualize the intergration of G-Pd@DOX into HepG2 cells, while Image-Pro Plus software was used to analyze intracellular DOX fluorescence (DOX: λ_{ex} = 488 nm, λ_{em} = 590 nm) intensity.

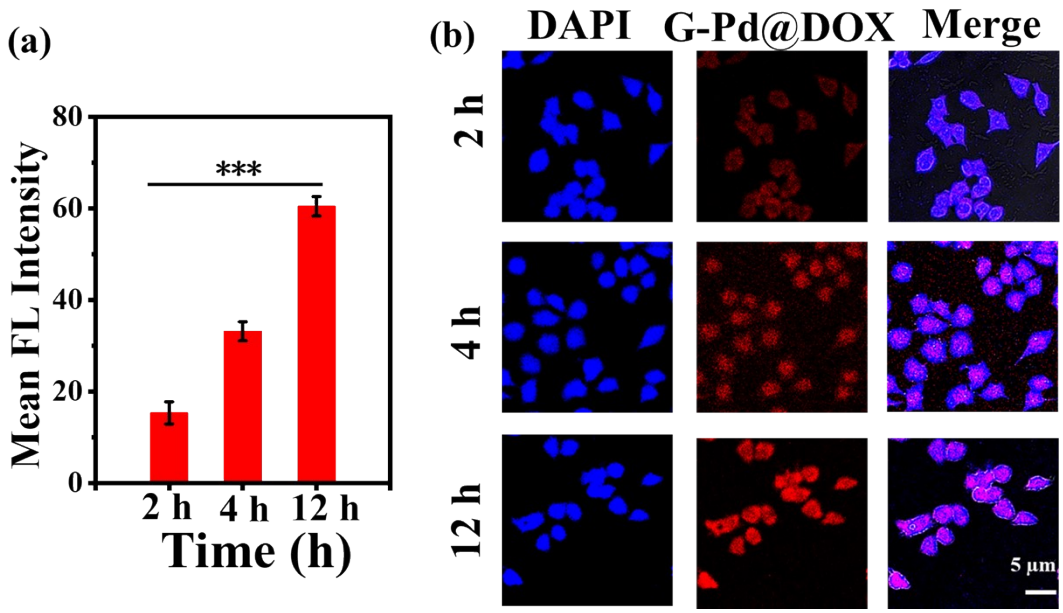


Fig. S11 (a) Quantitative analysis of the mean fluorescence intensity at each time point of (b). (b) CLSM images of HepG2 cells after co-incubation with G-Pd@DOX for 2, 4, and 12 h (scale bar: 5 μm). Data are presented as the mean ± SD (n = 3, ***P < 0.001).

Study on mechanism of Cellular uptake

The following conditions were used to investigate the endocytosis mechanism of nanoparticles: Chlorpromazine (Chl) as a clathrin-mediated endocytosis inhibitor, Genistein (Gen) as a caveolae-mediated endocytosis inhibitor, NaN_3 as an ATP inhibitor, and incubation at 4 ℃ as a condition to inhibit cell membrane fluidity. First, HepG2 cells were inoculated in a confocal dish at a density of 2×10^5 cells per well and cultured overnight. Subsequently, the cells were divided into groups: PBS, Chl, Gen, NaN_3, and 4 ℃. 50 μg/mL Chl, 200 μg/mL Gen, and 10 mM NaN_3 were added to the respective groups and incubated for 1 h. The last group was incubated at 4 ℃ for 1 h. Then, 50 μg/mL G-Pd@DOX nanoparticles were added to each group and cultured in a 37 ℃ incubator or a 4 ℃ refrigerator for 4 h. After incubation, the cells were washed three times with PBS to remove unbound nanoparticles. Finally, confocal laser scanning microscope (CLSM) was used to detect the intracellular fluorescence intensity

of DOX (DOX: λ_{ex} = 488 nm, λ_{em} = 590 nm).

Cytotoxicity assay

Cytotoxicity against HepG2, MCF-7, HL7702, and MDA-MB-468 cells was evaluated using MTT assays. Cells were seeded into 96-well plates with complete 1640 or DMEM medium (5 × 10^3 cells/well, 100 μL per well). After 24 h, the medium was replaced with fresh medium containing various concentrations of DOX (HepG2, MCF-7, and HL7702: 0, 0.5, 1.25, 2.5, 5, 10 μg/mL; MDA-MB-468: 0, 0.125, 0.25, 0.5, 1.25, 2.5 μg/mL), G-Pd (HepG2, MCF-7 and HL7702: 0, 2, 5, 10, 20, 40 μg/mL; MDA-MB-468: 0, 0.5, 1, 2, 5, 10 μg/mL) and G-Pd@DOX (HepG2, MCF-7 and HL7702: 0, 2.5, 6.25, 12.5, 25, 50 μg/mL; MDA-MB-468: 0, 0.625, 1.25, 2.5, 6.25, 12.5 μg/mL) for an additional 24 h or 48 h of incubation. The control group was cultured in a medium without any sample. Subsequently, the medium was replaced with MTT-containing medium (0.5 mg/mL). After 4 h, the MTT-containing medium was removed, and 100 μL of DMSO was added. The absorbance at 490 nm was then measured using a microplate reader. Five replicate wells were set up for each experimental group.

The cytotoxicity of K_2PdCl_4 towards HepG2, MCF-7, HL7702, and MDA-MB-468 cells was evaluated using the MTT assay. The cells were seeded into 96-well plates containing complete 1640 or DMEM medium (5 × 10^3 cells per well, 100 μL per well). After 24 h, the medium was replaced with fresh medium containing varying concentrations of K_2PdCl_4 (0, 0.277, 0.692, 1.385, 2.77, 5,54, 11.08, 16.62 μg/mL), followed by an incubation period of either 24 h or 48 h. The control group was maintained in a medium without any sample. Subsequently, the medium was exchanged with MTT-containing medium (0.5 mg/mL). After 4 h, the MTT-containing medium was removed, and 100 μL of DMSO was added. The absorbance was then measured at 490 nm using a microplate reader. Each experimental group consisted of five replicate wells to ensure data reliability.

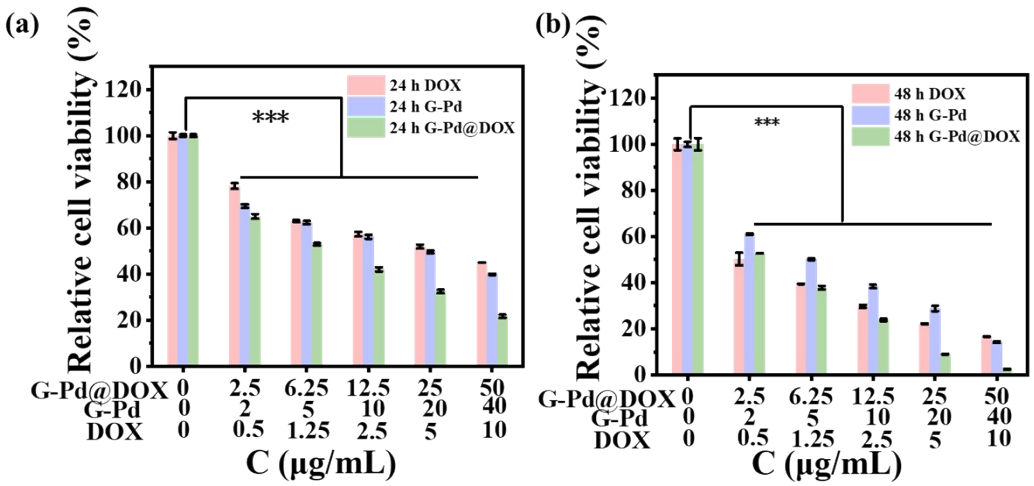


Fig. S12 Relative cell viability of MCF-7 cells after co-culture with different concentrations of DOX, G-Pd, and G-Pd@DOX for (a) 24 h and (b) 48 h. Data are presented as the mean ± SD (n = 5, ***P < 0.001).

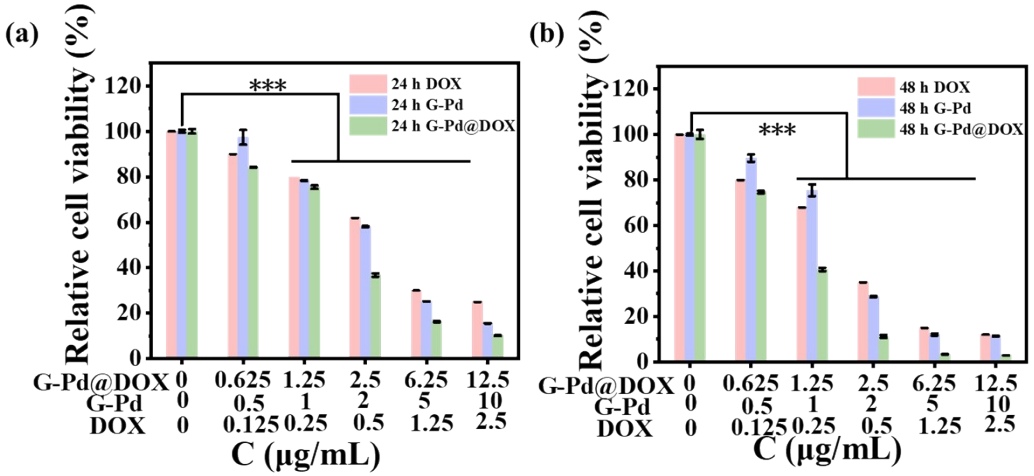


Fig. S13 Relative cell viability of MDA-MB-468 cells after co-culture with different concentrations of DOX, G-Pd, and G-Pd@DOX for (a) 24 h and (b) 48 h. Data are presented as the mean ± SD (n = 5, ***P < 0.001).

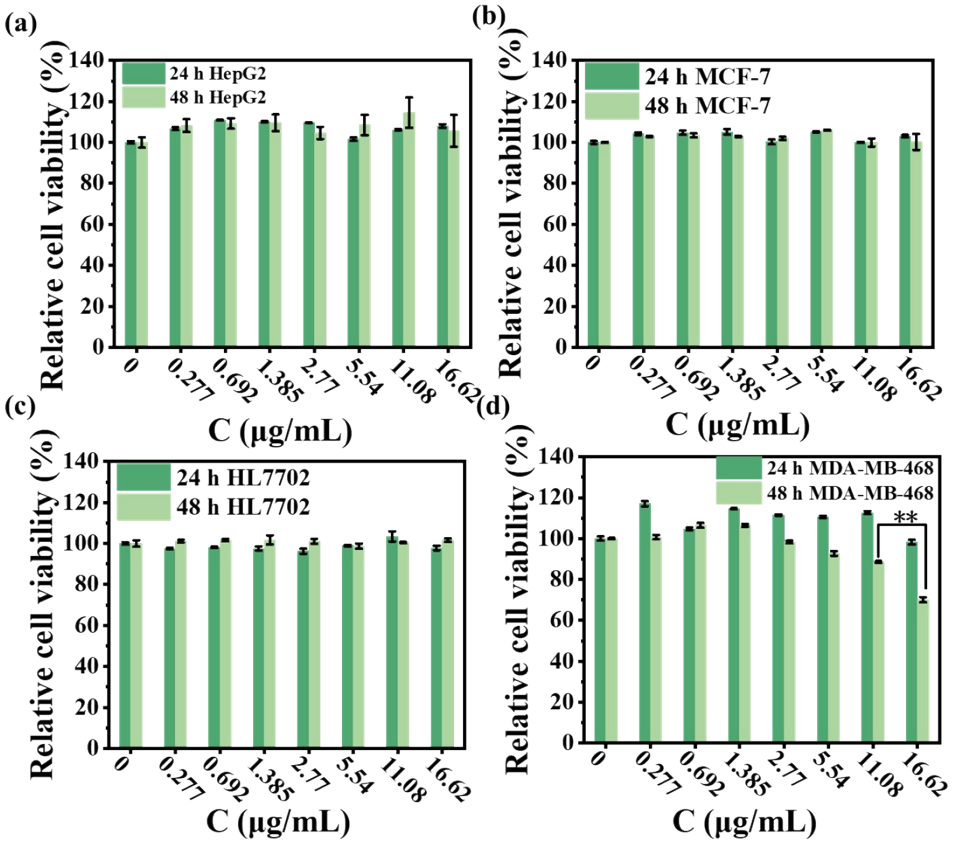


Fig. S14 Relative cell viability of (a) HepG2, (b) MCF-7, (c) HL7702, (d) MDA-MB-468 cells after co-culture with different concentrations of K_2PdCl_4 for 24, 48 h. Data are presented as the mean ± SD (n = 5, $^{**}P < 0.01$).

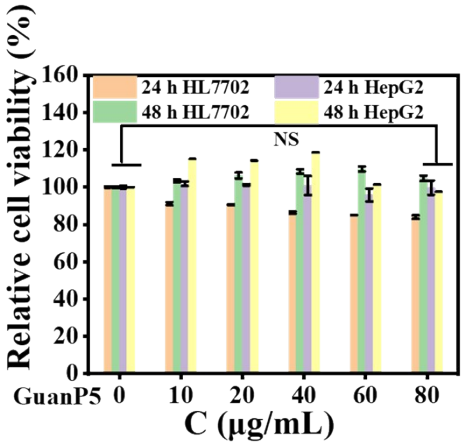


Fig. S15 Relative cell viability of HepG2, HL7702 cells after co-culture with different concentrations of GuanP5 for 24, 48 h. Data are presented as the mean ± SD (n = 5, NS means no significance).

Mitochondria damage

To evaluate mitochondrial membrane potential, JC-10 was used as a detection probe. HepG2 cells were seeded into 35 mm dishes with glass coverslips containing 1.5 mL of complete 1640 medium at a density of 2×10^5 cells/dish and cultured for 24 h. The culture medium was then replaced, and the experimental groups were incubated with 40 μg/mL G-Pd and 50 μg/mL G-Pd@DOX for 24 h. After incubation, the culture medium was carefully removed, and the procedure outlined in the manufacturer's instructions for JC-10 dye was followed. Subsequently, images were acquired using a confocal laser scanning microscope.

Live-dead cell staining

HepG2 cells were cultured in a 35 mm glass-bottom confocal dish at a density of 1.0×10^5 cells per dish for 24 h. Once the cells adhered to the dish, the original medium was replaced and the cells were divided into four groups: PBS, DOX, G-Pd, and G-Pd@DOX. The PBS group was cultured with a complete 1640 medium, while the other groups were treated with drugs (DOX: 10 μg/mL, G-Pd: 40 μg/mL, G-Pd@DOX: 50 μg/mL) in a volume of 1.5 mL medium for incubation over a period of 24 h. After incubation, the medium was removed and replaced with a staining solution composed of Calcein-AM (2 μM) and PI (4 μM) to stain the cells. Following staining at room temperature for 30 minutes, excess staining solution was discarded, and the cells were washed three times with PBS. Finally, confocal microscopy analysis was performed on these stained cells.

Apoptosis assays

HepG2 cells were seeded and cultured in six-well plates for 24 h. After this initial culturing period, the original culture medium was removed and replaced with a fresh medium containing either PBS, DOX, G-Pd, or G-Pd@DOX. Subsequently, the HepG2 cells were incubated for a further 24 h. Following incubation, the cells were washed three times with PBS (pH 7.4, 2 mL). The cells were then harvested, washed again three

times with PBS (pH 7.4, 2 mL), and resuspended in 500 μL of PBS (pH 7.4). Annexin V-FITC (5 μL) and PI (10 μL) were added to the cell suspension. The cells were incubated in the dark for 15 min at room temperature and analyzed by flow cytometry.

References

1. S. Guo, Q. Huang, Y. Chen, J. Wei, J. Zheng, L. Wang, Y. Wang and R. Wang, *Angew. Chem. Int. Ed*., 2021, **60**, 618-623.