Supporting Information

A Facile PEG/Thiol-Functionalized Nanographene Oxide Carrier with Appropriate Glutathione-Responsive Switch

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Experimental Section

Materials

Graphite powder (Aldrich, 99.99+%), potassium permanganate ($KMnO_4$, Aldrich, 99%), sulfuric acid (H_2SO_4, Aldrich, 95~98%), sodium nitrate ($NaNO_3$, Aldrich, 99%), paclitaxel (PTX, Aldrich, 99%), 7-ethyl-10-hydroxycamptothecin (SN38, Aldrich, 99%), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl, Aldrich, 99%), fluorescein isothiocyanate (FITC, TCI, 97%), glutathione (GSH, Aladdin, 98%), 2-aminoethanethio (Aladdin, 98%) and MTT assay (Dojindo, Japan) were used as received. Six-armed PEG with six amino end groups (6-armed PEG-NH_2, M_n = 10,000 g/mol) was purchased from Ponsure Biotech. Inc. (Shanghai, China). RPMI 1640 and DMEM medium were purchased from GIBCO/Invitrogen, USA, and supplemented with 10% fetal bovine serum (FBS, BI Biological Industries Ltd., Israel) and 1% penicillin-streptomycin (10,000 U/mL penicillin and 10 mg/mL streptomycin, Solarbio Life Science, China).

Measurements

All ^1H and ^{13}C NMR analyses were performed on a JEOL resonance ECZ 400S spectrometer (400 MHz) in $CDCl_3$ and DMSO-d_6, tetramethylsilane (^1H NMR) was used as internal standard. FT-IR spectra are recorded on a Nicolet AVATAR-360 FT-IR spectrophotometer with a 4 cm^{-1} resolution. UV/vis absorption spectra were acquired by a Hitachi U-2910 spectrophotometer. X-ray photoelectron spectroscopy (XPS) was recorded on an EscaLab 250Xi photoelectron spectrometer.

Thermogravimetric analysis (TGA) was conducted on a TA Discovery TGA 55 thermal analysis system in N_2 with a heating rate of 10°C/min. Elemental analysis was carried out on a Vario EL III system. Electrospray ionization mass spectrometry (ESI-MS) and high-resolution mass spectrometry (HR-MS) were measured by an Agilent LC/MSD SL system and a Thermo Fisher Scientific LTQ FT Ultra system, respectively. Hydrodynamic diameter (D_h) was measured by dynamic light scattering (DLS) with a Malvern Nano-ZS90 Zetasizer at room temperature. Atomic force microscope (AFM) images were taken by a JPK Nano Wizard Sense system in the AC mode of dropping the sample solution onto the freshly exfoliated mica substrate. High-performance liquid chromatography (HPLC) analysis was performed on an Agilent 1260 Infinity II system. Relative cell viability was recorded at the absorbance of 450 nm by using a Tecan GENios Pro microplate reader. Cellular uptake images were taken by an Olympus BX51 fluorescence microscope.

Synthesis of 2-(Pyridin-2-yldisulfanyl) Ethanol

A CH_2Cl_2 (10 mL) solution of 2-mercaptoethanol (0.87 g, 11.1 mmol) was added dropwise at room temperature to a CH_2Cl_2 (20 mL) solution of 2,2-dithiopyridine (4.89 g, 22.2 mmol) followed by stirring at room temperature for 4 h so that the initial colorless solution turned yellow. After rotary evaporation, 2-(pyridin-2-yldisulfanyl) ethanol **1** (0.9 g, 40% yield) was obtained as a yellow oil via gel chromatography (silica). ^{1}H NMR ($CDCl_3$): δ (ppm): 2.92 (t, 2H), 3.77 (t, 2H), 7.14 (m, 1H), 7.41 (m, 1H), 7.57 (m, 1H), 8.52 (dd, 1H). All data are consistent with reported values.[1]

Synthesis of 4-Nitrophenyl 2-(Pyridin-2-yldisulfaneyl)ethyl Carbonate

2-(Pyridin-2-yldisulfanyl) ethanol **1** (187 mg, 1.0 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL) followed by adding anhydrous pyridine (158 mg, 2 mmol), 4-dimethylaminopyridine (DMAP, 5 mg, 0.04 mmol) and 4-nitrophenyl chloroformate (403 mg, 2.0 mmol). The mixture was stirred at room temperature overnight, then washed with 2 M HCl and brine followed by drying over Na_2SO_4. After rotary evaporation, 4-nitrophenyl 2-(pyridin-2-yldisulfaneyl)ethyl carbonate **2** (326.2 mg, 93% yield) was obtained as a pale-yellow oil via silica gel chromatography. ^{1}H NMR ($CDCl_3$): δ (ppm): 3.14 (t, 2H), 4.55 (t, 2H), 6.90 (m, 1H), 7.36 (d, 2H), 7.65 (t, 1H), 8.11 (d, 1H), 8.28 (d, 2H), 8.49 (d, 1H). All data are consistent with reported values.[2]

Synthesis of Paclitaxel-2-(Pyridin-2-yldisulfaneyl)ethyl Carbonate

4-Nitrophenyl 2-(pyridin-2-yldisulfaneyl)ethyl carbonate **2** (131.6 mg, 0.374 mmol), PTX (943 mg,0.374 mmol), DMAP (2.3 mg, 0.019 mmol) and dry CH_2Cl_2 (25 mL) were stirred at room temperature overnight so that the initial colorless solution turned yellow. After rotary evaporation, paclitaxel-2-(pyridin-2-yldisulfaneyl)ethyl carbonate **3** (0.9 g, 40% yield) was obtained as a slightly yellow solid by silica gel chromatography.[3] ESI-MS $[M+H]^+$: 1067.3.

Synthesis of Benzothiophen-2-one

Hydrogen peroxide (H_2O_2, 30%, 11.2 mL) was added dropwise to an ethanol (60

mL) solution of thianaphthene-2-boronic acid (6.18 g, 17 mmol) under stirring and the mixture turned red. After stirring at room temperature for 8 h, the mixture was concentrated under reduced pressure, diluted with water and extracted with CH_2Cl_2. The combined organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Benzothiophen-2-one **4** (5.0053 g, 98% yield) was obtained a pink solid by flash silica gel chromatography. ^{1}H NMR ($CDCl_3$): δ (ppm): 4.05 (s, 2H), 7.18-7.45 (m, 4H). All data are consistent with reported values.[4]

Synthesis of 2-(2-Mercaptophenyl) Acetic Acid

Benzothiophen-2-one **4** (480 mg, 3.04 mmol) was added into a mixture of 1 M KOH (15 mL) and THF (5 mL). The mixture was stirred at 60°C for 14 h. After cooled to room temperature, pH of the solution was adjusted to 2.0 by adding 5 M HCl. The mixture was then extracted with CH_2Cl_2 three times, and the organic portion was dried over Na_2SO_4 followed by filtration and concentration *in vacuo*, affording 2-(2-mercaptophenyl) acetic acid **5** (512 mg, 65% yield) as a pale yellow waxy solid. ^{1}H NMR ($CDCl_3$): δ (ppm): 3.72 (s, 2 H), 7.07-7.43 (m, 3H), 7.55 (m, 1H). All data are consistent with reported values.[5]

Synthesis of 2-(2-(Pyridin-2-yldisulfanyl)phenyl) Acetic Acid

2-(2-Mercaptophenyl) acetic acid **5** (100 mg, 0.594 mmol) was dissolved in methanol (0.5 mL) followed by adding 1,2-di(pyridin-2-yl) disulfane (131 mg, 0.594 mmol). The mixture was stirred at room temperature overnight followed by

concentration *in vacuo*. The crude product was purified by silica gel chromatography, yielding 2-(2-(pyridin-2-yldisulfanyl)phenyl) acetic acid **6** (135 mg, 82% yield) as a waxy yellow solid. ^{1}H NMR ($CDCl_3$): δ (ppm): 3.98 (s, 2H), 7.18-7.33 (m, 4H), 7.67-7.76 (m, 3H), 8.43 (m, 1H). All data are consistent with reported values.[6]

Synthesis of Paclitaxel-2-(2-(pyridin-2-yldisulfanyl)phenyl) Acetate

Disulfide linker of 2-(2-(pyridin-2-yldisulfanyl)phenyl) acetic acid **6** (85 mg, 0.373 mmol) was dissolved in CH_2Cl_2 (20 mL) containing PTX (319 mg, 0.374 mmol) and DMAP (2.3 mg, 0.019 mmol). The solution was cooled to 0°C followed by adding dicyclohexylcarbodiimide (DCC, 77.2 mg, 0.374 mmol) in CH_2Cl_2 (4 mL) dropwise. The mixture was then warmed to room temperature and stirred overnight. After concentration *in vacuo*, the crude product was purified by silica gel chromatography, yielding paclitaxel-2-(2-(pyridin-2-yldisulfanyl)phenyl) acetate **7** (290 mg, 80% yield) as a white crystalline powder.[5] ESI-MS $[M+H]^+$: calcd. 1114.3, found. 1114.6.

Synthesis of SN38-2-(2-(pyridin-2-yldisulfanyl)phenyl) Acetate

Disulfide linker of 2-(2-(pyridin-2-yldisulfanyl)phenyl) acetic acid **6** (85 mg, 0.373 mmol) was dissolved in CH_2Cl_2 (20 mL) containing SN38 (146.7 mg, 0.374 mmol) and DMAP (6.0 mg, 0.05 mmol). The solution was cooled to 0°C followed by adding dicyclohexylcarbodiimide (DCC, 77.2 mg, 0.374 mmol) in CH_2Cl_2 (4 mL) dropwise. The mixture was then warmed to room temperature and stirred overnight. After concentration *in vacuo*, the crude product was purified by silica gel chromatography,

yielding SN38-2-(2-(pyridin-2-yldisulfanyl)phenyl) acetate **8** as a fait yellow crystalline powder. ^{1}H NMR ($CDCl_3$): δ (ppm): 1.01 (t, 3H), 1.39 (t, 3H), 1.87 (q, 2H), 3.19 (q, 2H), 4.07 (m, 2H), 5.28-5.70 (m, 4H), 7.25-7.40 (m, 5H), 7.66 (m, 1H), 7.70 (m, 2H), 7.93 (m, 2H), 8.45 (m, 1H), 8.56 (m, 1H). HR-ESI-MS $[M+H]^{1+}$: calcd. 652.17, found 652.1582. All data are consistent with reported values.[5]

Preparation of Nanographene Oxide

Graphene oxide (GO) was prepared from graphite powder through the modified Hummer's method.[7,8] Graphite powder was oxidized by concentrated H_2SO_4, $NaNO_3$ and $KMnO_4$ at 0°C and 35°C each for 3 h followed by adding H_2O_2 (30%) slowly while the black solution turned yellow. The resulting suspension was then washed with distilled water by filtration and finally subjected to dialysis for removing residual salts and acids. Nanographene oxide (NGO) was prepared via the breakage of GO by an ultra-sonication process. The as-prepared GO suspension was subjected to ultra-sonication by a SONICS VCX750 instrument operated at 20 KHz for 1 h in an ice bath,[9] affording NGO.

Preparation of PEG-NGO

GO aqueous dispersion was pre-treated with NaOH followed by adding 6-armed PEG-NH_2 and EDC·HCl for sonication at room temperature for 1 h, ultimately the mixture was kept stirring vigorously at room temperature for 1 day. PEG-NGO was finally obtained by purifying the crude product by dialysis (MW_{cutoff} = 14 kDa)

against double-distilled water for 7 days to remove unbound 6-armed PEG-NH_2.[10]

Preparation of PEG-NGO-SH

PEG-NGO aqueous dispersion (20 mL, 0.05 wt%) was mixed with 10 mg of cysteamine and 5 mg of KOH and the homogeneous dispersion was vigorously stirred at 80°C for 1 day. Finally, the resulting product was dialyzed (MW_{cutoff} = 3.5 kDa) against double-distilled water for several days to remove residual salts and bases.[11]

Preparation of PEG-NGO-S-S-PTX

PEG-NGO-SH aqueous dispersion (0.05 wt%, 20 mL) was mixed with 10.0 mg of paclitaxel-2-(2-(pyridin-2-yldisulfanyl)phenyl) acetate **7** in ethanol (20 mL), and the mixture was vigorously stirred at room temperature for 1 day. Finally, the residual product salts were removed via ultra-filtration (MW_{cutoff} = 10 kDa), and the mixture was re-dissolved in water by ultra-sonication with a concentration of ~0.2 mg/mL.

Preparation of PEG-NGO-S-S-SN38

PEG-NGO-SH aqueous dispersion (0.05 wt%, 20 mL) was mixed with 12.5 mg of SN38-2-(2-(pyridin-2-yldisulfanyl)phenyl) acetate **8** in ethanol (20 mL), and the mixture was vigorously stirred at room temperature for 1 day. Finally, the residual product salts were removed via ultra-filtration (MW_{cutoff} = 10kDa), and the mixture was re-dissolved in water by ultra-sonication with a concentration of ~0.2 mg/mL.

Preparation of PEG-NGO-S-S-PTX/SN38

PEG-NGO-SH aqueous dispersion (0.05 wt%, 20 mL) was mixed with 10.5 mg of paclitaxel-2-(2-(pyridin-2-yldisulfanyl)phenyl) acetate **7** and 10.5 mg of SN38-2-(2-(pyridin-2-yldisulfanyl)phenyl) acetate **8** in ethanol (20 mL), and the mixture was vigorously stirred at room temperature for 1 day. Finally, the residual product salts were removed via ultra-filtration (MW_{cutoff} = 10kDa), and the mixture was re-dissolved in water by ultra-sonication with a concentration of ~0.2 mg/mL.

Stability of Drug Delivery System

The stability of drug delivery system (DDS) dispersion was investigated by measuring the mean diameter of DDS NPs in dispersed medium. Briefly, DDS (0.25 mg/mL) was incubated in PBS solution (pH = 7.4) and aqueous media at 4°C, respectively, and the particle size was measured after 30 days to examine the long-term stability.[12] The long-term stability was also studied in FBS and DMEM.

***in vitro* Drug Release**

DDS (20 mL, 250 μg/mL) was dialyzed against PBS (80 mL, pH = 7.4 or 5.0, 10.0 mM) containing either 10 mM or 0 mM of GSH using a dialysis membrane (MW_{cutoff} = 7 kDa). At each interval, 200 μL of solution outside the dialysis membrane was sampled. The concentration of PTX in the solution was determined by HPLC.

Another way for drug release behavior *in vitro* was as follows: DDS (20 mL, 250 μg/mL) was dialyzed against PBS (80 mL, pH = 5.0, 10 mM) containing GSH (10 mM) using a dialysis membrane (MW_{cutoff} = 7 kDa). At each interval, the total

solution (80 mL) outside the dialysis membrane was withdrawn and replaced with the same volume of fresh PBS (80 mL, pH = 7.4, 10 mM) containing GSH (10 mM). The concentration of PTX in the solution was determined by HPLC.[7]

Cell Culture

A549 human lung cancer cells and 293T human renal epithelial cells were supplied by Shanghai Institute of Cell Biology, Chinese Academy of Sciences. They were cultured at 37°C under a humid 5% CO_2 atmosphere in RPMI 1640 and DMEM medium, respectively, supplemented with 10% FBS and 1% penicillin-streptomycin.

Cellular Uptake

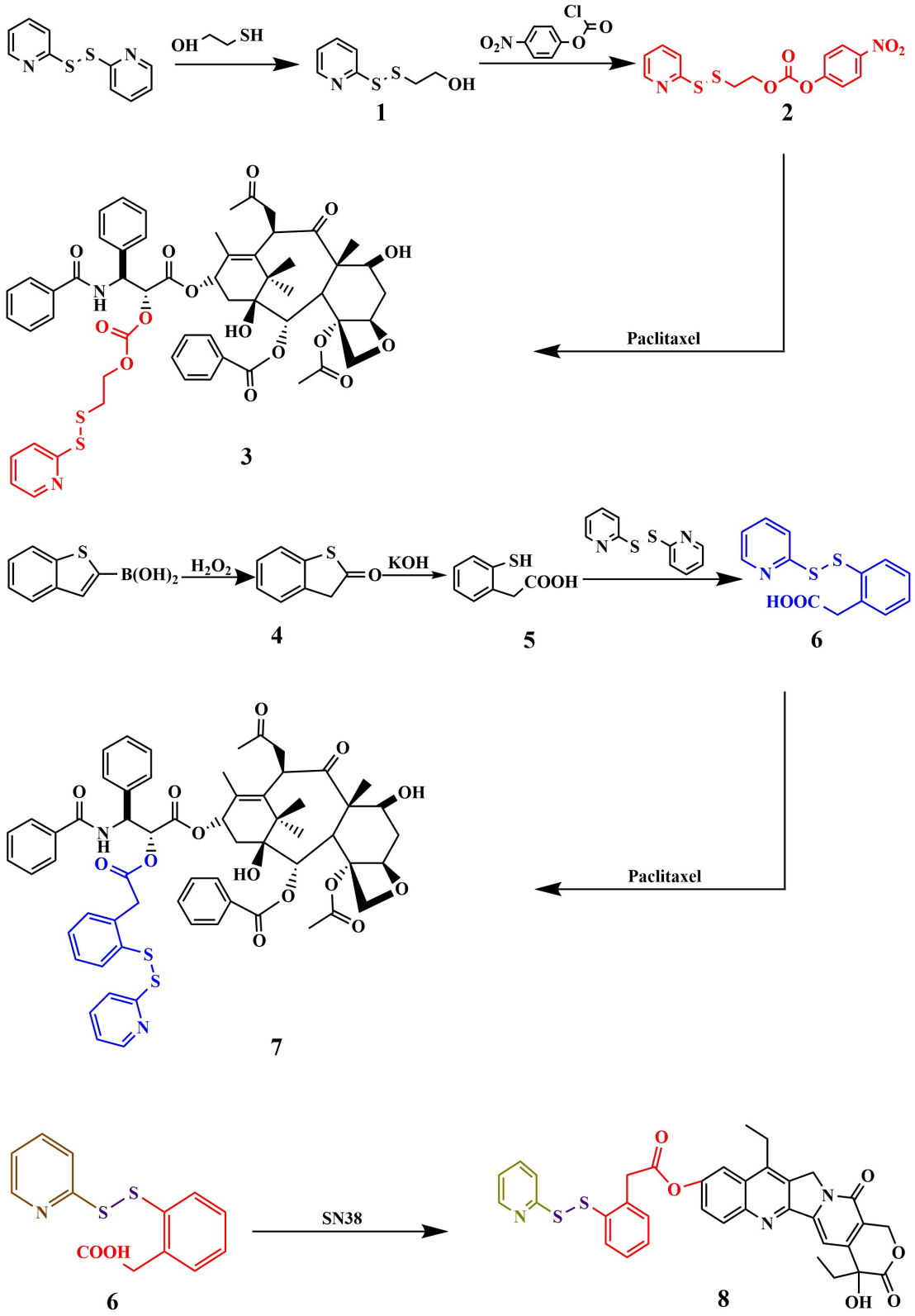
PEG-NGO-S-S-PTX was labeled by FITC by mixing 1 mL of FITC aqueous solution (0.5 mg/mL) with 8 mL of PEG-NGO-S-S-PTX aqueous suspension (3.5 mg/mL). Free FITC was removed by dialysis against double-distilled water. The resulting fluorescein-labeled PEG-NGO-S-S-PTX nanocomplex, FITC-PEG-NGO- S-S-PTX, was stored at 4°C. A549 and 293T cells were plated on a 20 mm glass round coverslip in 6-well plates and allowed to adhere overnight. A549 and 293T cells were incubated with FITC-PEG-NGO-S-S-PTX for 1, 3 and 6 h and washed with PBS three times. The cells were then imaged under an inverted Olympus BX51 fluorescence microscope. For the cellular uptake of PEG-NGO-S-S- PTX/SN38, it was similar with the preceding part of PEG-NGO-S-S-PTX but without the loading of FITC.

Cell Viability Assay

A549 and 293T cells were plated in 96 well plates at a density of 5×10^3 cells per well in 100 μL of culture medium (RPMI 1640 for A549) and added with desired amounts of free PTX and SN38 (dissolved in DMSO and diluted in PBS), PEG-NGO, PEG-NGO-S-S-PTX, PEG-NGO-S-S-SN38 and PEG-NGO-S-S-PTX/SN38. The relative cell viability was measured by WST assay using MTT. After continuous incubation for 12, 24, 36, 48, 60 and 72 h, absorbance was measured at 450 nm using a Tecan GENios Pro microplate reader.

Statistical Analysis

All the *in vitro* experiments were performed at least three times with three replicates each time (N = 3 and n = 3). The results are expressed as the mean±standard deviation (SD). Variables were compared by independent samples student's *t* test. The significance was set to probabilities of *$p<0.05$, **$p<0.01$ and ***$p<0.001$ when compared to controls.



Scheme S1. Synthesis of disulfide-modified PTX derivatives and disulfide-modified SN38 derivative.

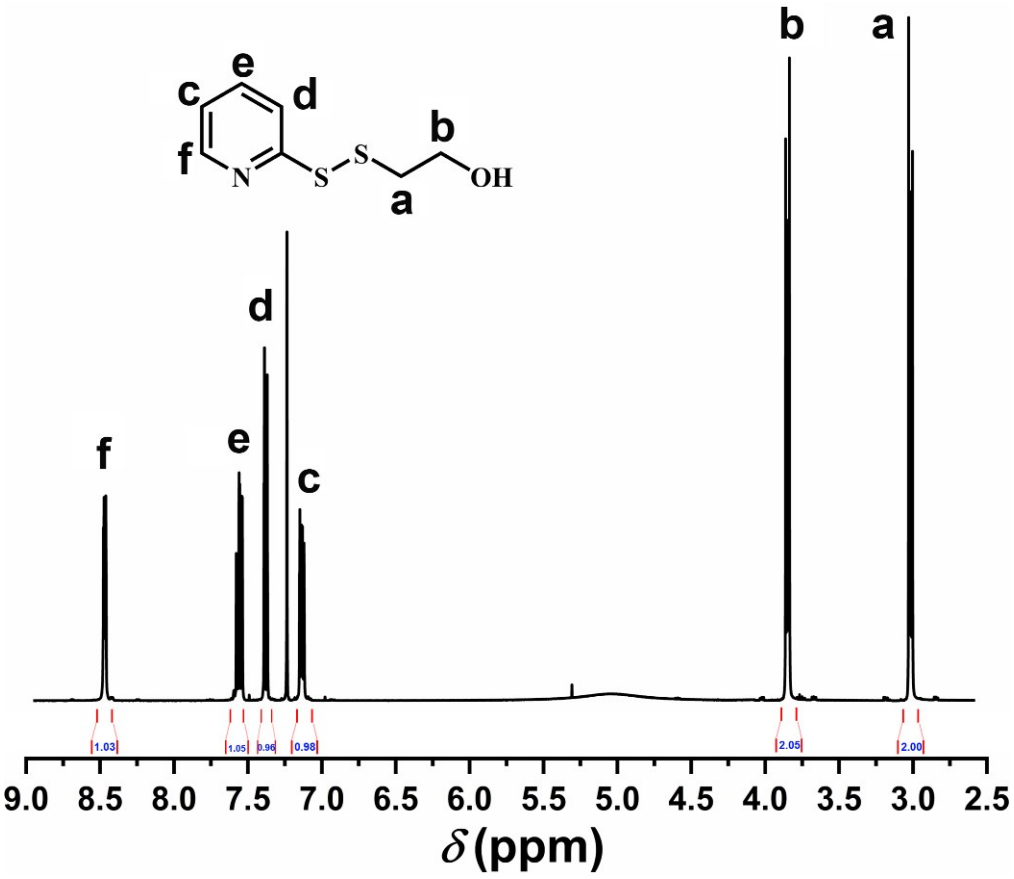


Figure S1. ^{1}H NMR spectrum of 2-(pyridin-2-yldisulfanyl) ethanol **1** in $CDCl_3$.

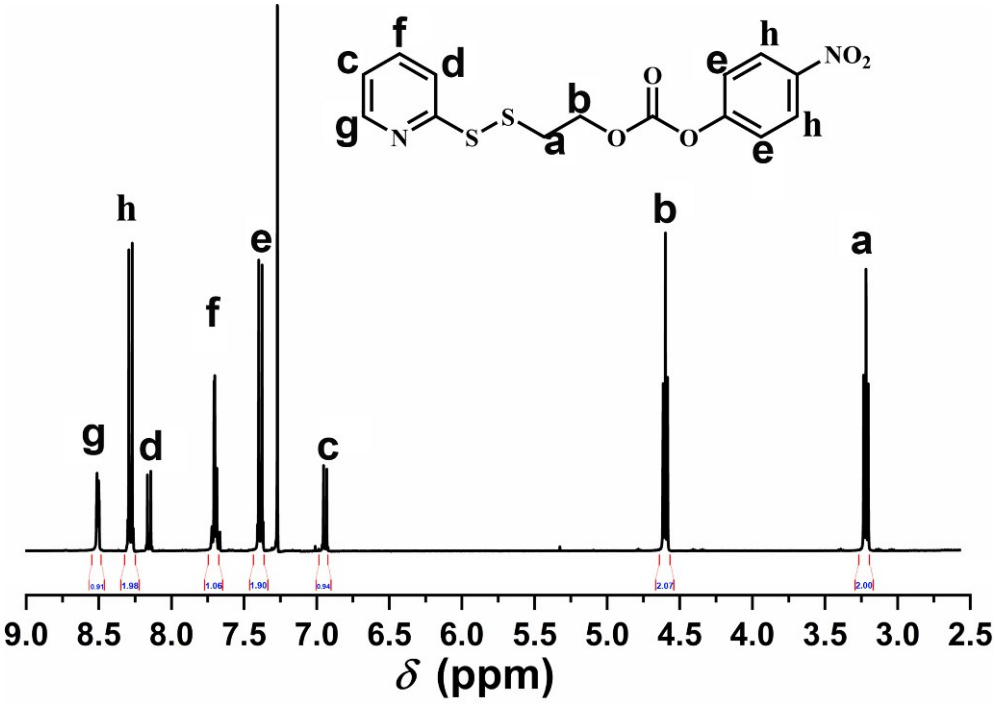


Figure S2. ^{1}H NMR spectrum of 4-nitrophenyl 2-(pyridin-2-yldisulfaneyl)ethyl carbonate **2** in $CDCl_3$.

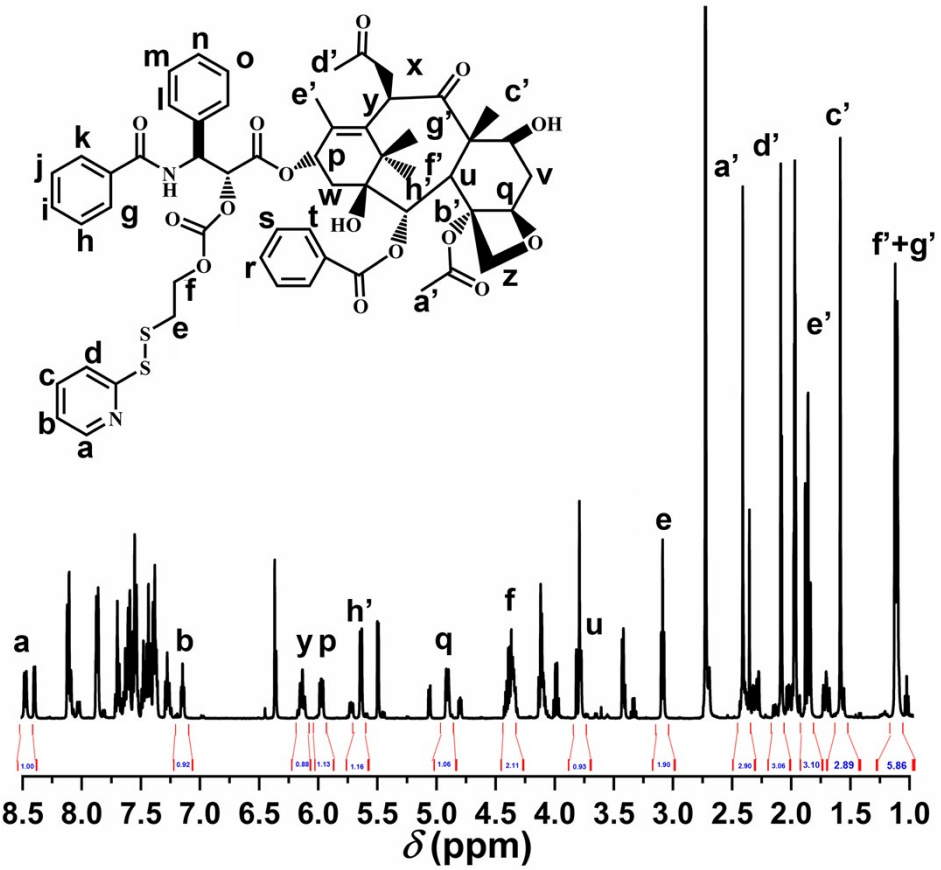


Figure S3. ^{1}H NMR spectrum of paclitaxel-2-(pyridin-2-yldisulfaneyl)ethyl carbonate **3** in DMSO-d_6.

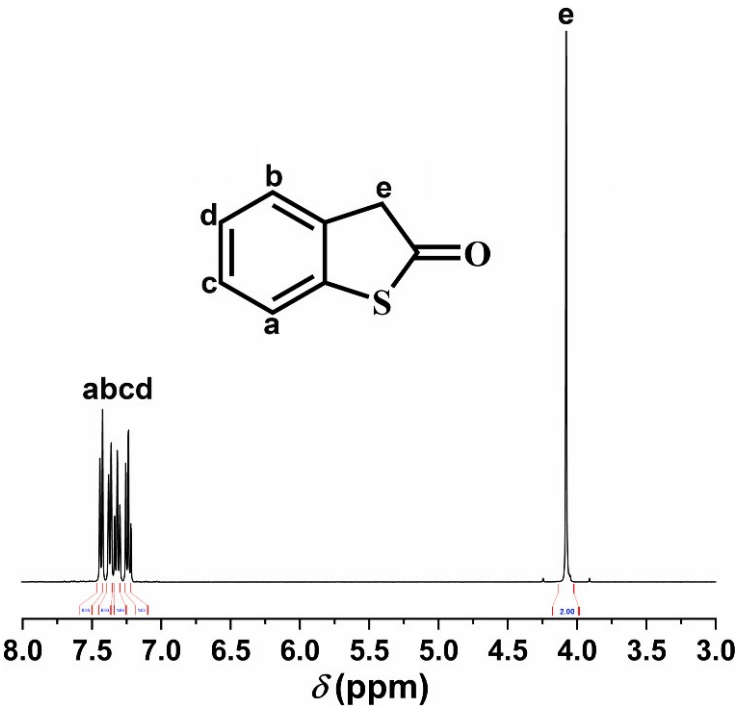


Figure S4. ^{1}H NMR spectrum of benzothiophen-2-one **4** in $CDCl_3$.

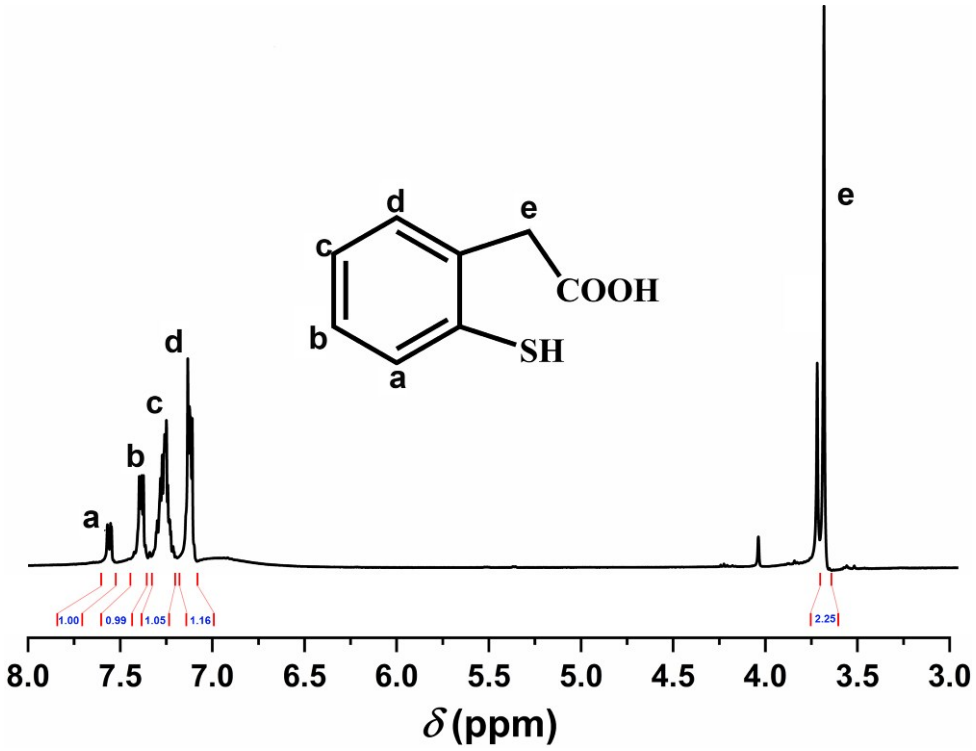


Figure S5. ^{1}H NMR spectrum of 2-(2-mercaptophenyl) acetic acid **5** in $CDCl_3$.

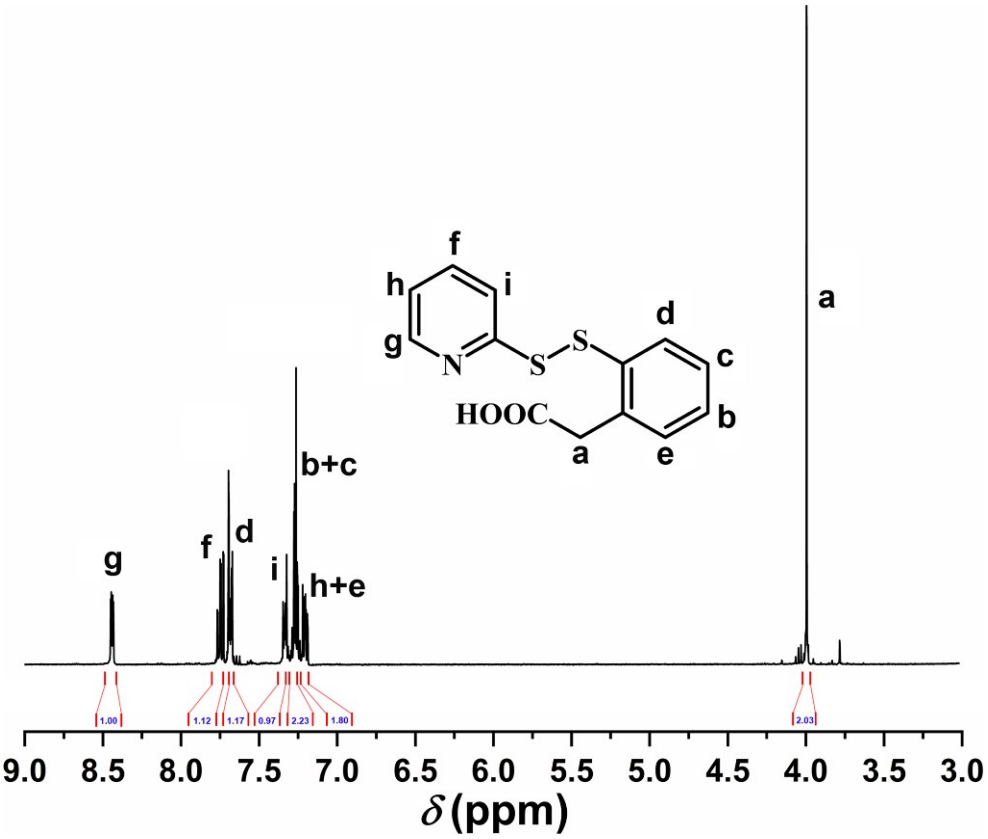


Figure S6. ^{1}H NMR spectrum of 2-(2-(pyridin-2-yldisulfanyl)phenyl) acetic acid **6** in $CDCl_3$.

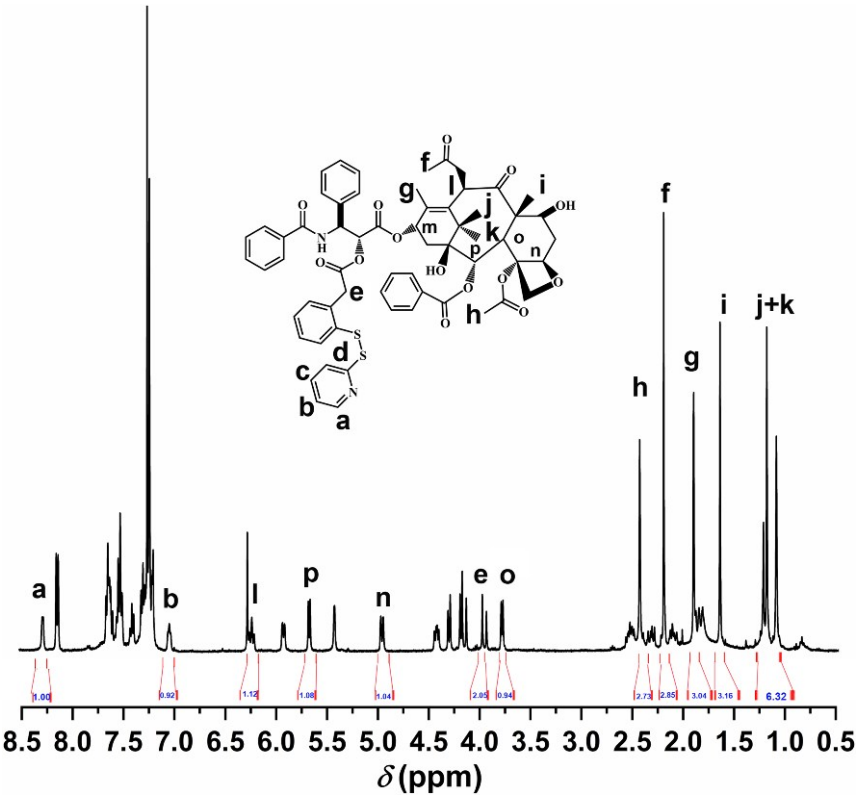


Figure S7. ^{1}H NMR spectrum of paclitaxel-2-(2-(pyridin-2-yldisulfanyl)phenyl) acetate **7** in $CDCl_3$.

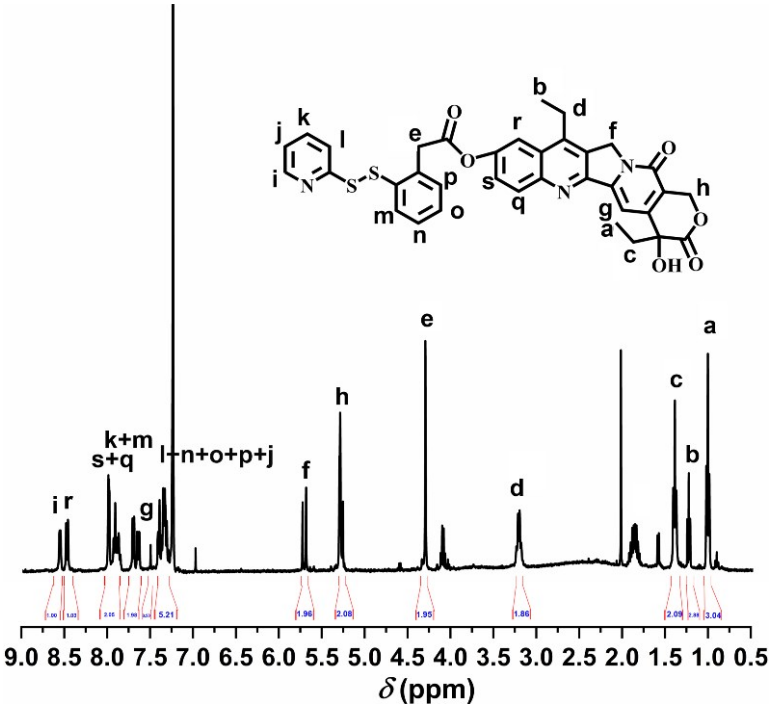


Figure S8. ^{1}H NMR spectrum of SN38-2-(2-(pyridin-2-yldisulfanyl)phenyl) acetate **8** in $CDCl_3$.

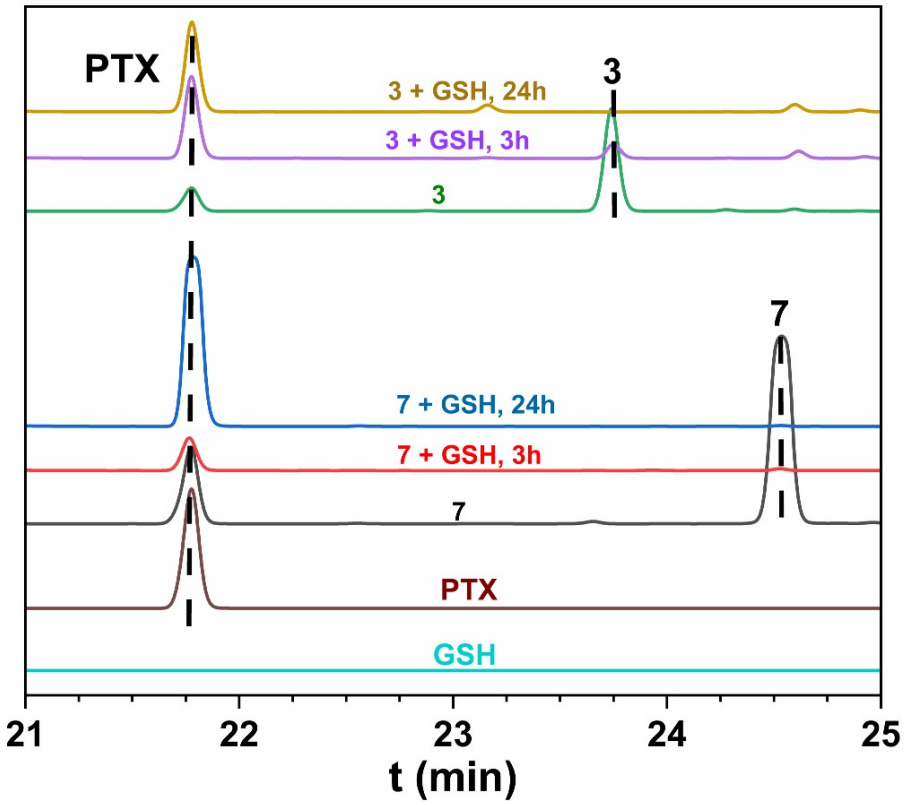
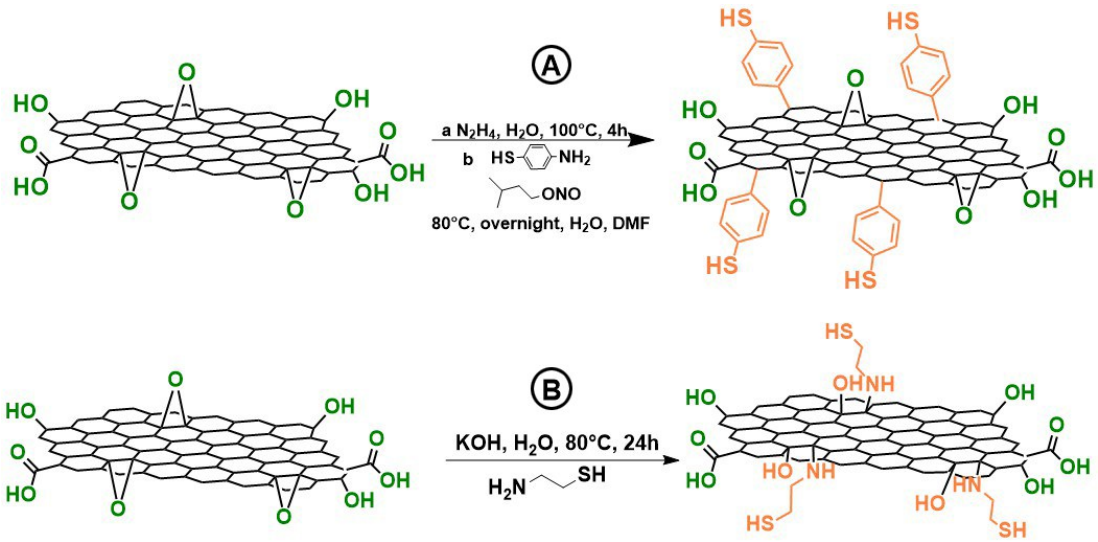


Figure S9. HPLC curves of release of pristine PTX from disulfide-modified PTX derivatives **3** and **7** in GSH solution (2 mg/mL, pH = 7.4).



Scheme S2. Synthesis of NGO-SH via diazonium (A) or epoxy ring opening (B) reaction.

Table S1. Sulfur content of NGO-SH[a]

entry	S%
diazonium addition	10.90%
ring-opening reaction	7.89%

[a] Obtained from elemental analysis.

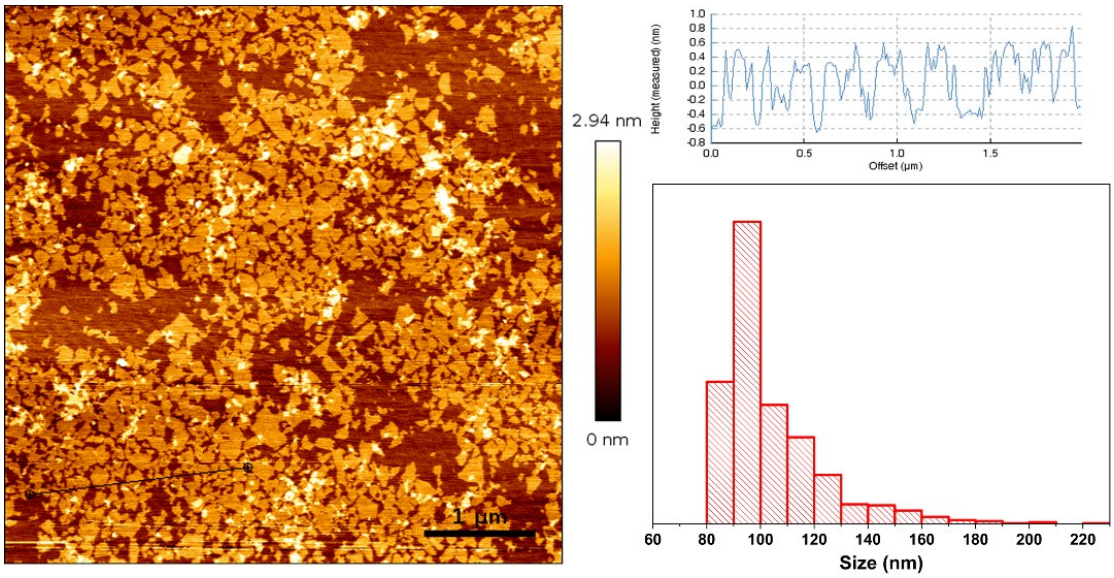


Figure S10. AFM image of NGO.

Figure S11. Aqueous dispersion of PEGylated NGO-SH.

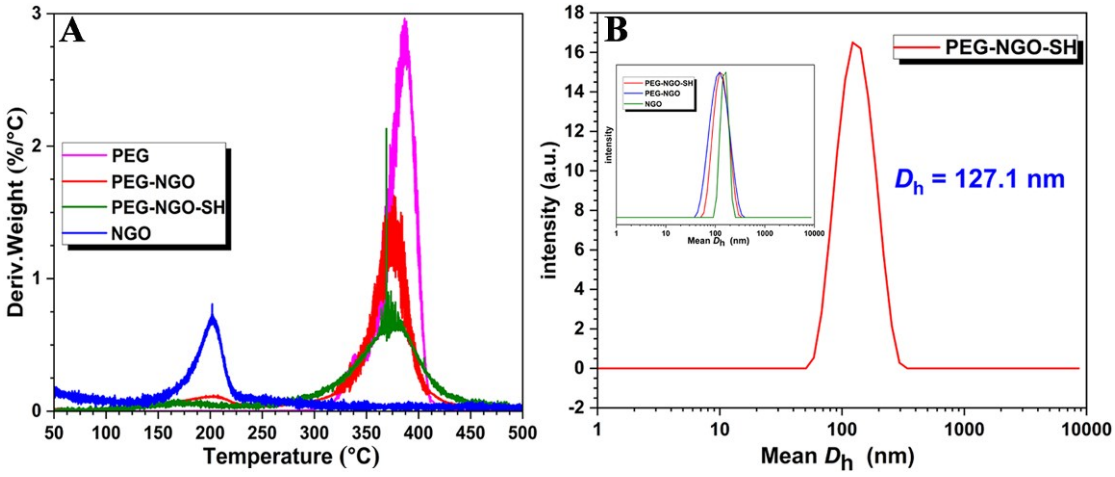


Figure S12. (A) DTG (in N_2) curves of NGO, PEG, PEG-NGO and PEG-NGO-SH with a heating rate of 10°C/min; (B) Hydrodynamic diameter distributions of NGO, PEG-NGO and PEG-NGO-SH.

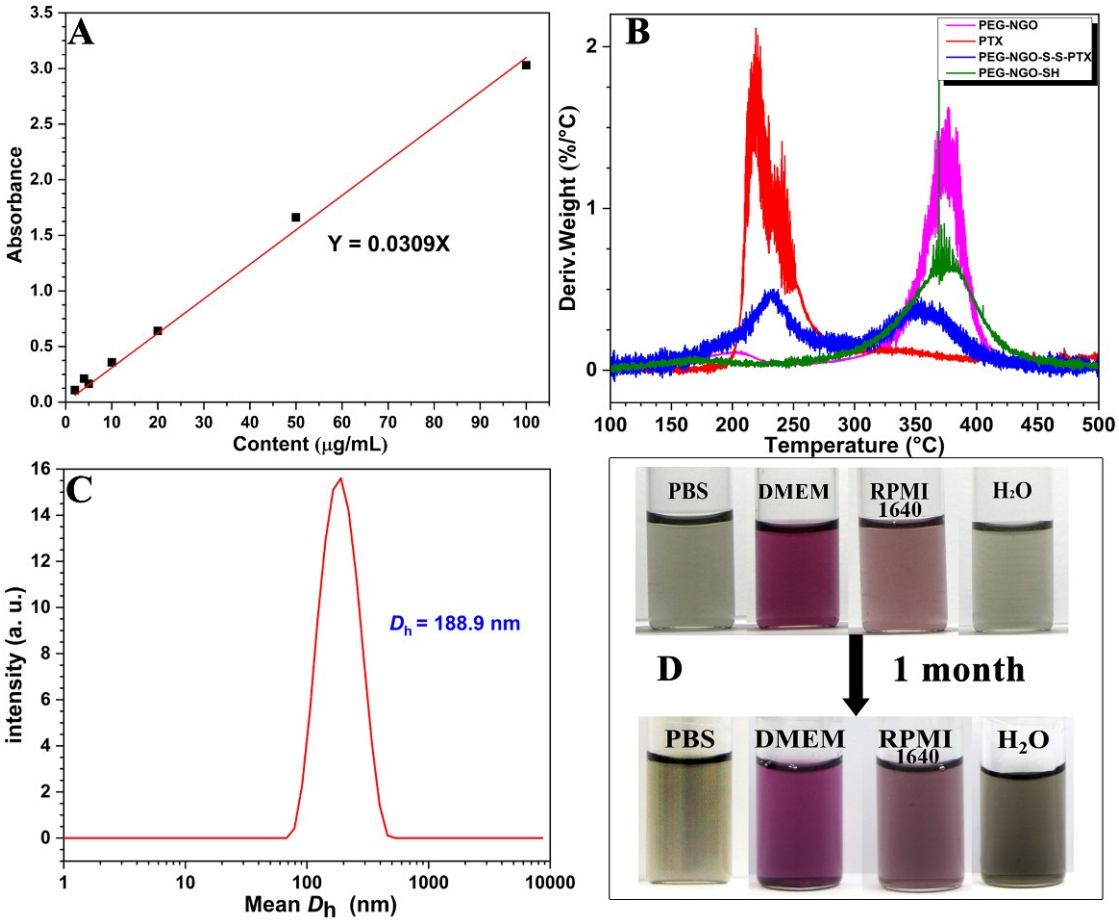


Figure S13. (A) UV/vis standard absorbance curve of PTX at 230 nm; (B) DTG (in N_2) curves of PTX, PEG-NGO, PEG-NGO-SH and PEG-NGO-S-S-PTX with a heating rate of 10°C/min; (C) Hydrodynamic diameter distribution of PEG-NGO-S-S-PTX and PEG-NGO-SH; (D) Colloidal stability of PEG-NGO-S-S-PTX in water, DMEM, RPMI 1640 and PBS solutions before and after placing for 1 month.

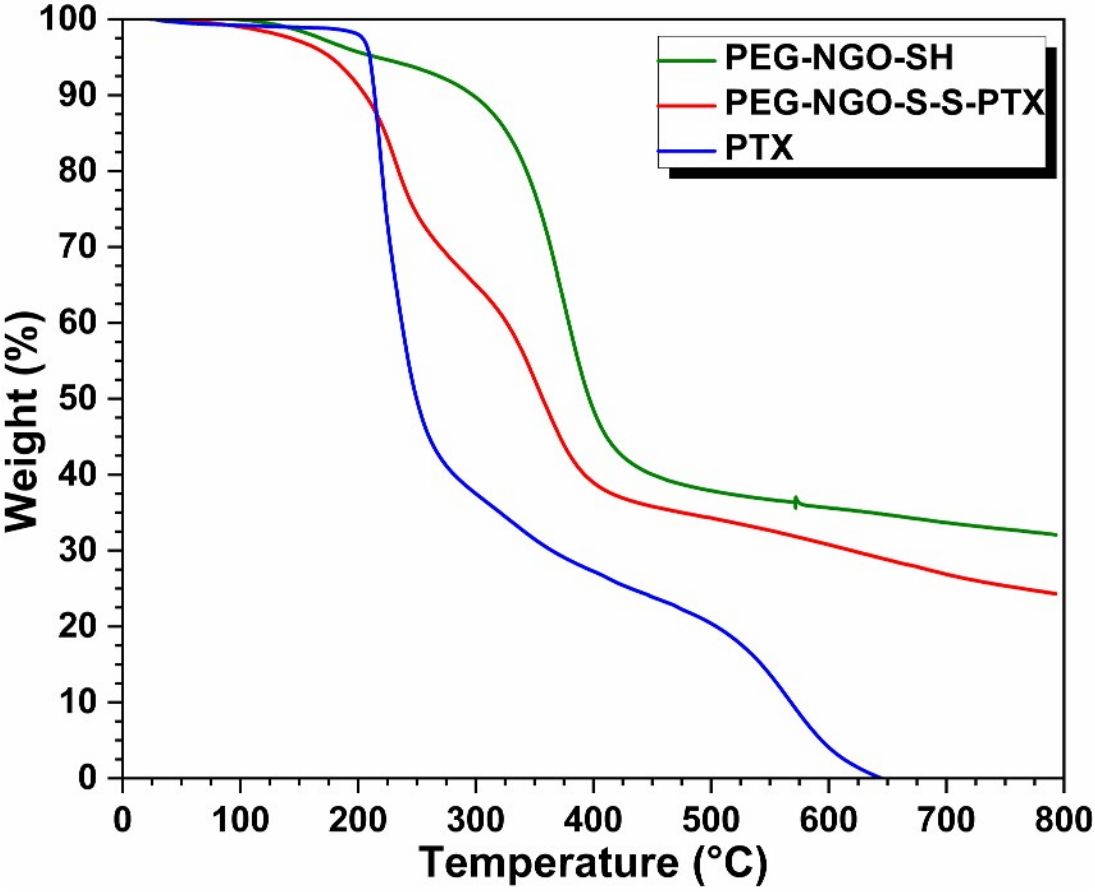


Figure S14. TGA curves (in N_2) of PTX, PEG-NGO-SH and PEG-NGO-S-S-PTX with a heating rate of 10°C/min.

The loading ratio of PTX in PEG-NGO-S-S-PTX was also determined by TGA. It can be seen from Figure S12 that the mass loss of PEG-NGO-SH was obviously accelerated in the range between 200°C and 400°C (olive line) while the mass loss of

PTX was phased over two sections starting at 200°C and 500°C (blue line), respectively. For the curve of PEG-NGO-S-S-PTX (red line), the mass loss of PEG-NGO-S-S-PTX was phased over three sections originating from PEG-NGO-SH and PTX. PEG-NGO-S-S-PTX was found to have a 63.7% weight loss at 450°C in N_2 (red line), whereas the corresponding weight loss for PTX (blue line) and PEG-NGO-SH (olive line) were 76.2% and 59.8%, respectively. Thus, we can estimate the weight percentage of PTX in PEG-NGO-S-S-PTX according to the following equation set (x and y are weight percentage of PTX and PEG-NGO-SH, respectively) and the result is that PEG-NGO-S-S-PTX contains 23.8 wt% of PTX and 76.2 wt% of PEG-NGO-SH.

$$\begin{cases} 0.762x + 0.598y = 0.637 & (1) \\ x + y = 1 & (2) \end{cases}$$

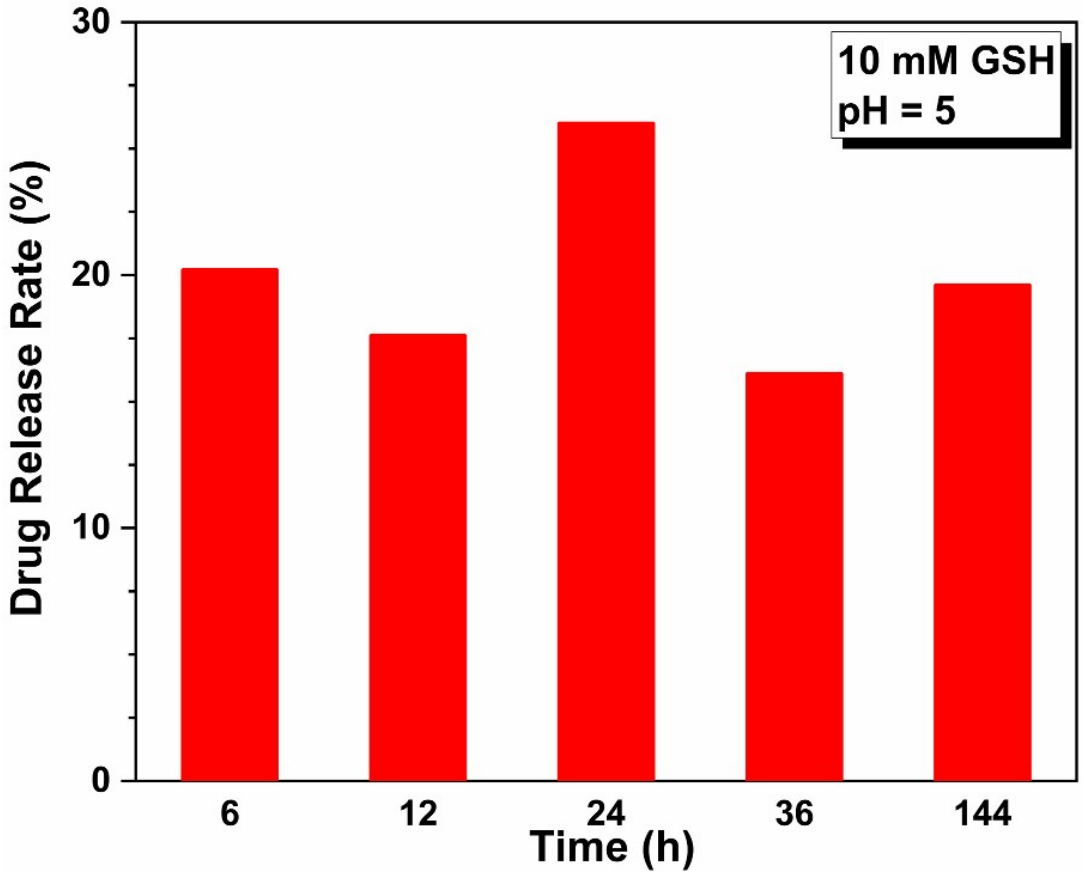


Figure S15. PTX release profile from PEG-NGO-S-S-PTX in fresh PBS buffer (pH = 5.0) containing GSH (10 mM), PBS was updated at certain intervals.

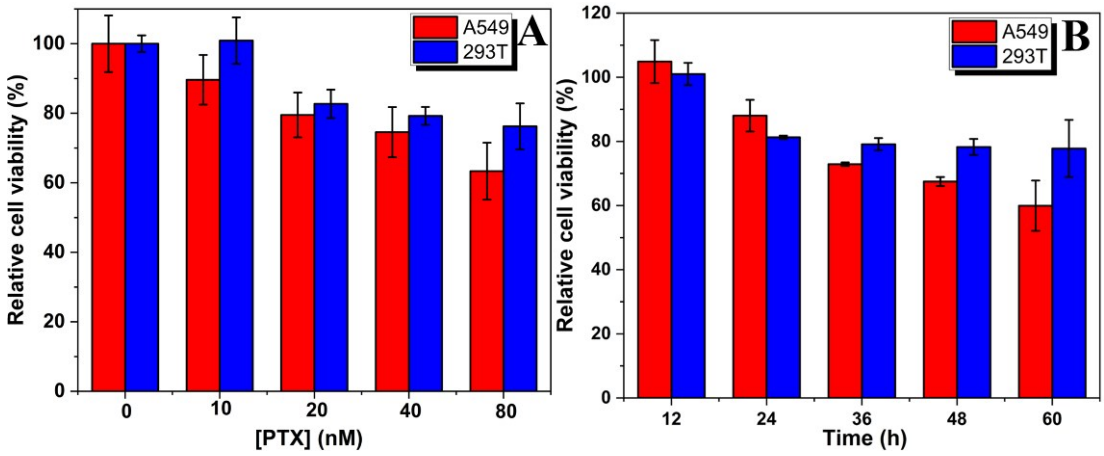


Figure S16. Relative cell viability of A549 and 293T cells after treatment with PTX in different (A) concentrations of PTX for 72 hours and (B) time at a constant PTX concentration of 80 nM. The experiments were repeated three times, all with similar results. Data are presented as the mean ± SD (n = 3).

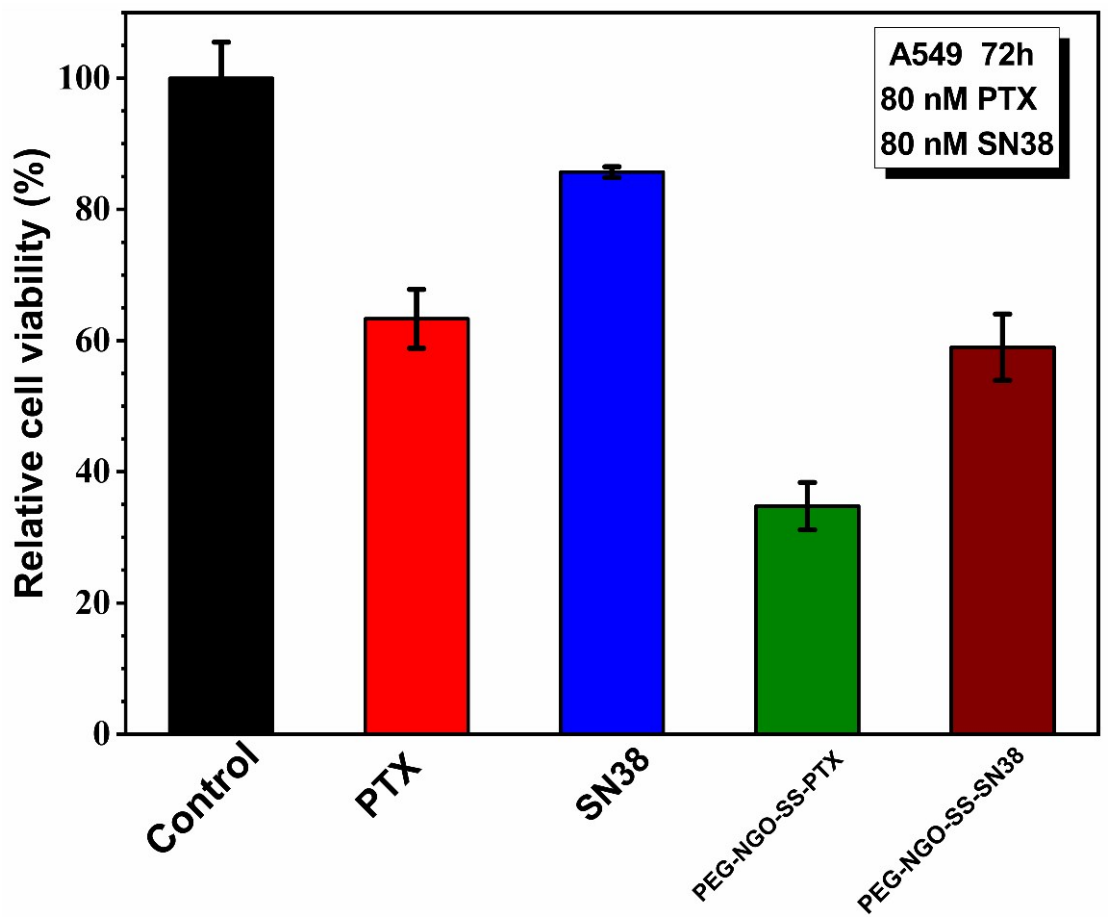


Figure S17. Relative cell viability of A549 cells after treatment with PTX, SN38, PEG-NGO-S-S-PTX and PEG-NGO-S-S-SN38 at a constant PTX/SN38 concentration of 80 nM for 72 hours. The experiments were repeated three times, all with similar results. Data are means as ± SD (n = 3).

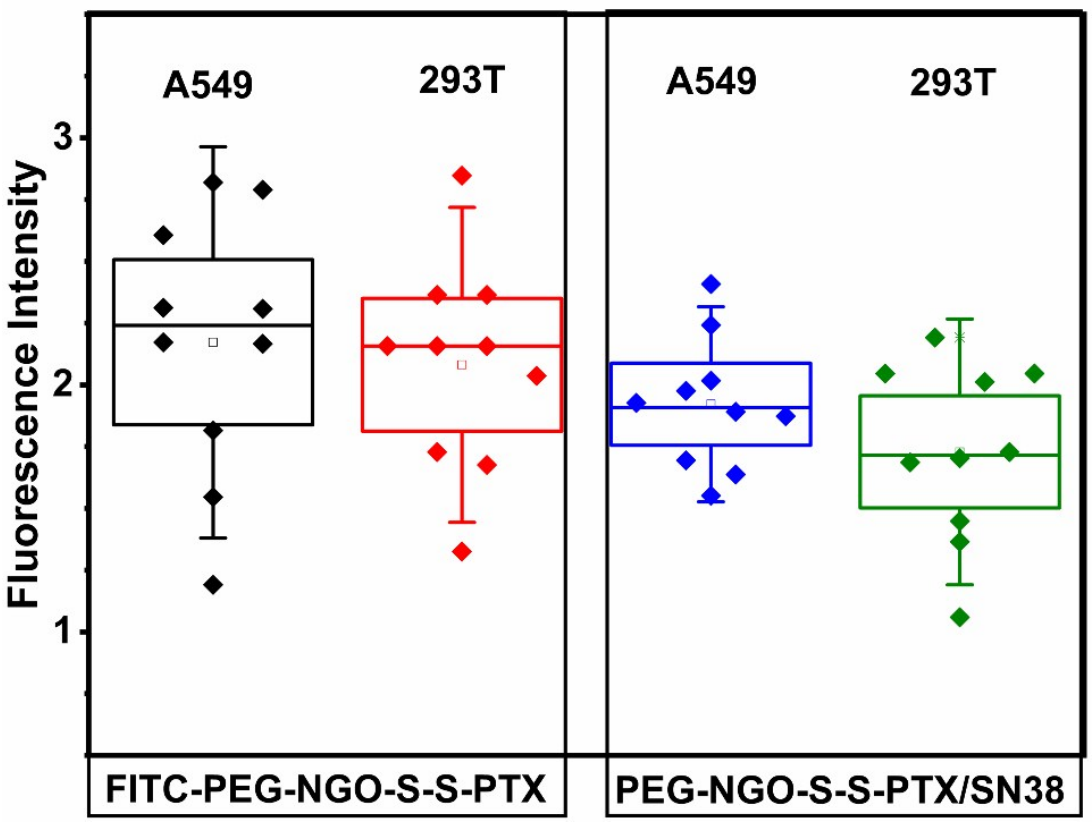


Figure S18. Quantitative flow cytometry of A549 and 293T cells incubated with FITC-PEG-NGO-S-S-PTX and PEG-NGO-S-S-PTX/SN38.

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