Electronic Supplementary Material (ESI) for ChemComm.
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Supporting Information

Cross-linking of *S*-nitrosothiolated AIEgens inside cancer cells devoted to precisely monitor NO release and reverse chemo-resistance

Experimental section

Chemicals

Benzophenone was purchased from Tianjin Bodi Chemical Co., Ltd (Tianjin, China). 4-Aminobenzophenone was purchased from TCI (Shanghai, China). Zinc and mercury chloride were purchased from Chengdu Huaxia Chemical Reagent Co., Ltd. (Chengdu, China). Titanium tetrachloride, triphosgene, collidine, N,N-Diisopropylethylamine and trifluoroacetic acid were purchased from adams-beta (Shanghai, China). Rink amine resin (loading: 0.668 mmol/g), Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Cys(Trt)-OH, Fmoc-His(Trt)-OH, obenzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were obtained from GL Biochem. Ltd. (Shanghai, China) and used as received without any purification. 2-Mercaptoethanol, acetic acid, sulfanilamide and N-(1-naphtyl) ethylenediamine were obtained from Sigma–Aldrich (St.louis, MO, USA). Pyridine, piperidine, potassium carbonate and hydrogen peroxide were purchased from Chengdu Kelong Chemical Co., Ltd. (Chengdu, China). Thiazolyl blue and doxorubicin hydrochloride were purchased from Aladdin (Shanghai, China). Phosphotungstic acid 44-hydrate was purchased from Energy Chemical (Shanghai,China). DMSO was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Dulbecco's modified eagle medium (DMEM) was purchased from Gibco (Suzhou, China), fetal bovine serum (FBS) was purchased from Gibco (Auckland, New Zealand), pancreatin was purchased from Biosharp (Beijing,China), penicillin- streptomycin and phosphate buffer saline were purchased from Solarbio (Beijing, China). Anti-PGP antibody was purchased from Abcam (Cambridge, UK). ROS Assay Kit (DCFH-DA) and NO Assay Kit (DAF-FM DA) were purchased from Beyotime Biotechnology (Shanghai，China). The methanol and acetonitrile were of HPLC grade, whereas the other reagents were of analytical grade.

Instruments

Mass spectra were recorded on a Bruker 7.0T solariX Fourier Transform-ion Cyclotron Resonance-Mass spectrometry (FT-ICR-MS) for high-resolution mass spectra (HR-MS). Purification and analysis of peptides were conducted with SHIMADZU preparative reversed-phase high performance liquid chromatography and SHIMADZU high-performance liquid chromatography (Kyoto, Japan). The hydrodynamic diameter and size distribution of the micelles were measured by dynamic light scattering (DLS) (Omni multi-angle particle size and high sensitivity Zeta potential analyzer, Brookhaven, MS, USA). UV-Vis absorption spectra were recorded on Agilent Cary 60 spectrophotometer (Santa Clara, USA). Fluorescence spectra were measured on a Hitachi F-7000 fluorometer (Tokyo, Japan). The absorbance for MTT analysis was recorded on a microplate reader SpectraMax i3x (San Francisco, United States). Confocal laser scanning microscopy (CLSM) characterization was conducted with a confocal laser scanning biological microscope Leica TCS SP8 (Heidelberg, Germany). The detection of ROS was conducted with CytoFLEX Beckman Coulter Biotechnology Co., Ltd. (Suzhou, China). Transmission Electron Microscope (TEM) characterization was conducted with ThermoFisher Scientific Talos F200S (Waltham, USA). Slide samples were observed using upright microscope Leica DM6 (Heidelberg, Germany). Tumor tissues were cut with freezing microtome Leica CM1950 (Heidelberg, Germany).

Synthesis of TPE-NH_2

TPE-NH_2 was synthesized in reference to previous studies [1]. Zinc powder and anhydrous tetrahydrofuran (THF) were added to a three-neck flask under an Ar atmosphere. The suspension

was cooled to −10 °C in an ice-salt bath. $TiCl_4$ was slowly added using a syringe into the abovementioned suspension under stirring. The mixture that formed was reacted at r.t. for 1 h and refluxed for 3 h. Then, pyridine was injected at −10 °C. After stirring for 10 min, a solution of benzophenone and 4-aminobenzophenone in THF was added to the flask and the mixture continued to be refluxed for 3 h. Then, a 10% aqueous solution of K_2CO_3 was added to quench the reaction. The mixture was extracted with ethyl acetate. The combined organic layer was dried over Na_2SO_4. The solvent was removed under vacuum to give the crude product, which was further purified by silica chromatography using ethyl acetate/ petroleum ether as the eluent to give the desired product (yield: 10%).

Synthesis and Characterization of TPE-RSH

Peptide (GCGGCRK-NH_2) was synthesized by a standard solid-phase peptide synthesis (SPPS) method using rink amide resin (0.668 mmol/g). The amine acid couplings were conducted by adding protected amino acid, HBTU, HOBT, and DIPEA into resin and further reaction for 4h at room temperature. After Fmoc-group removal, the peptide then coupled to TPE-NH_2 using BTC and collidine in THF. TPE-RSH were cleaved from the resin using a TFA/TIS/DTT (95:2.5:2.5) cocktail, the mixture was concentrated and diethyl ether was added dropwise to give a white solid. TPESH is confirmed by LC-MS. Purification by preparative reversed-phase HPLC.

Synthesis of TPE-RSNO

TPE-RSH was dissolved in 0.5 M hydrochloric acid and then 2 equivalents of sodium nitrite were added, the reaction lasted for 1 hour at 4℃ in the dark. The accurate volume of NaOH 40%(W/V) was mixed with the solution to stop the reaction and obtain TPE-RSNO. The pH of the final solution is about 7.2 and the content of NO is detected by Griess-Saville methods (yield: 75%).

ROS-responsive fluorescence light up

100 μM TPE-RSNO in PBS (pH 7.4, 10 mM) was incubated with H_2O_2 (100 mM) at 37℃ at different time intervals to measure the change of fluorescence intensity. The PL spectra were collected from 380 to 620 nm under excitation at 320 nm.

In vitro release and fluorescence intensity of TPE-RSNO

The release profile of NO from TPE-RSNO was investigated in 10 mM phosphate buffer saline (PBS, pH 7.4) at 37 °C at time intervals (0.5, 1, 2, 4, 6, and 12 h) and detected using the Griess-Saville methods as previously described. Meanwhile, the fluorescence was also detected. The PL spectra were collected from 380 to 620 nm under excitation at 320 nm.

Size measurement using Dynamic Light Scattering (DLS).

The diameters of TPE-RSH and TPE-RSNO (100 μM) were measured by DLS. in the presence of H_2O_2 (100 mM), TPE-RSH and TPE-RSNO were separately incubated at 37°C, and their diameters were further measured.

Fluorescence microscope imaging

Samples that used in fluorescence microscope imaging were the same as those used in DLS measurements. After DLS measurements, 5 μL of solution was immediately added on slide, then the samples were covered with the coverslip and observed using fluorescence microscope.

TEM characterizations

Samples that used in TEM were same as those used in DLS measurements. After DLS measurements, 5μL of solution in each cuvette was immediately added on cupper grid. Solvent was evaporated. After dyeing and evaporating, the samples were observed using TEM.

Cell culture

Hepg2 and 293 cells were cultured in DMEM (basic) medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humid atmosphere

of 5% CO_2. Hepg2/ADR cells were cultured in DMEM (High Glucose) medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 μg/mL Doxorubicin hydrochloride at 37 °C in a humid atmosphere of 5% CO_2.

Measurements and calculation of ROS capacity.

Hepg2, Hepg2/ADR, and 293 cells were seeded in 12-well plates at a density of 40000 cells/well, and then washed 3 times with PBS until adherent. The cells were incubated with DCFH-DA (1:1000 diluted in RPMI-1640 medium without FBS) for 30 min. Then the cells were washed with PBS 3 times to remove excess dyes. Fluorescent intensity of DCFH was measured through CytoFLEX at 525 nm (excited at 480 nm).

Biocompatibility assay

The biocompatibility of TPE-RSH and TPE-RSNO was evaluated by MTT assay. 10000 293 cells were seeded overnight in the 96-well plate in DMEM medium. Various concentrations (5,10,20,50,100μM) of TPE-RSH or TPE-RSNO were added in each well for 24 hours. After incubation, MTT solution (0.5 mg/mL) was added into each well for 4 h at 37°C. Then the solution was removed and 100 μL dimethyl sulfoxide (DMSO) was added to each well. After 10 min of vibration mixing, the optical density (OD) at 490 nm was measured using a microplate reader. Each experiment was performed in triplicate.

half-maximum inhibition concentration (IC_{50}) of doxorubicin (dox) measurement

Hepg2/ADR cells were seeded overnight in 96-well plate (10000 cells/well) in DMEM medium. The medium in the cells was removed and washed with PBS, then TPE-RSNO (20 μM) was added in each well for 12 h, and dox was subsequently added for another 24 hours. Free dox treatment for 24 h without TPE-RSNO was the control group. After incubation, MTT solution (0.5 mg/mL) was added into each well for 4 h at 37°C. Then the solution was removed and 100 μL dimethyl sulfoxide (DMSO) was added to each well. After 10 min vibration mixing, the optical density (OD) at 490 nm was measured using a microplate reader. Statistical analysis of IC_{50} was carried out using Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA).

In vitro imaging and localization.

Approximately 30000 cells were seeded into 35 mm microscopy dishes and cultured overnight. The cells were incubated with different treatments. For the TPE-RSNO combine DOX group, TPE-RSNO was incubated for 4 h, then DOX was added for another 2 h. As for the TPE-RSH group, TPE-RSH was incubated for 6 hours. As for the DOX group, free dox was incubated for 2 h. PBS treatment was used as blank control. Next, the medium in the cells was removed, washed once with PBS for 10 min, and incubated with DAF-FM probe for 30 min, then washed three times with PBS, and the cells were fixed by 4% paraformaldehyde for 30 min. then washed three times with PBS for 10 min. The cells were further blocked with 5% BSA for 30 min at r.t., and incubated with P-glycoprotein antibody subsequently overnight in the dark box at 4℃, then washed three times with PBS for 10 min and incubated with Dy649 in the dark for 30 min, washed three times with PBS for 10 minutes, finally, added with the anti-quenching agent and observed under confocal laser scanning microscopy. For the signal of TPE, the excitation was 405 nm, and the emission filter was 466±10 nm. For the signal of DAF-FM, the excitation was 495 nm, and the emission filter was 515 nm. For the signal of dox, the excitation was 480 nm, and the emission filter was 550 nm. For the signal of P-glycoprotein secondary antibody, the excitation was 646 nm, and the emission filter was 674 nm.

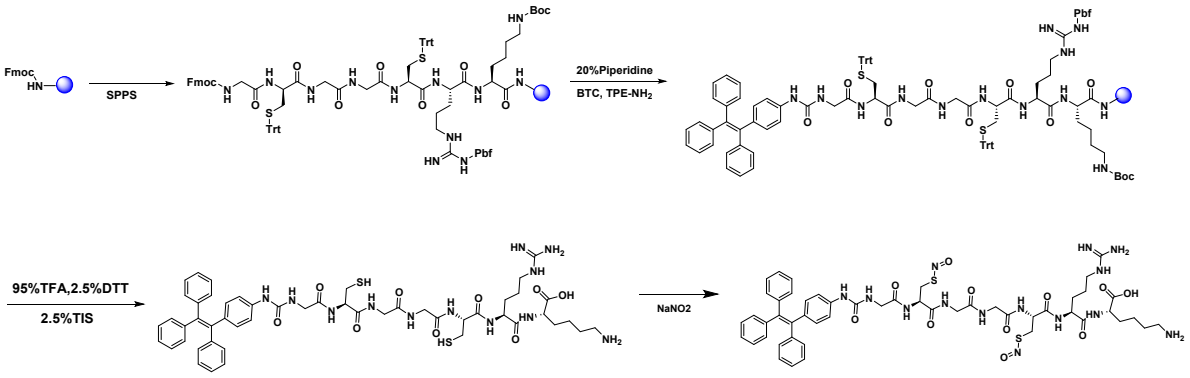
Therapeutic efficacy in Hepg2/ADR-tumor-bearing mice.

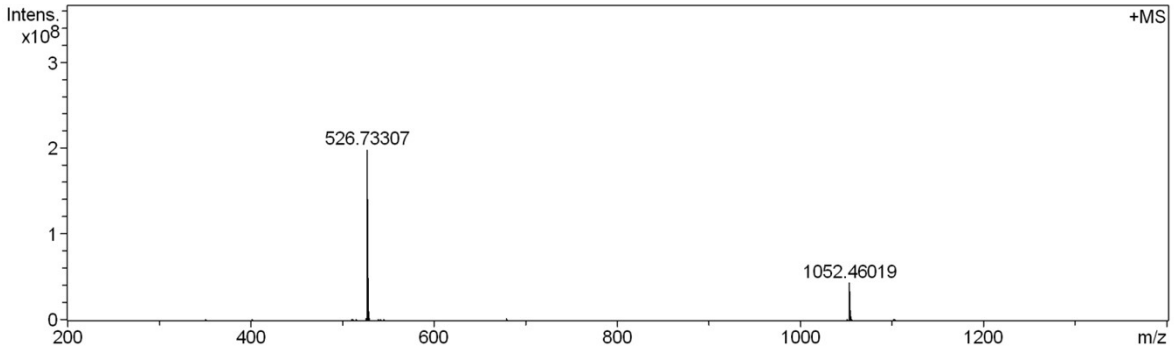
Male BALB/c nude mice, about 6−8 weeks old, were purchased from Beijing Huafukang Biotechnology Co., Ltd. (Beijing, China) and kept under specific pathogen-free conditions with free access to standard food and drinking water. All animal experiments were approved by the Third Military Medical Association for Animal Ethics. (Chongqing, China). Hepg2/ADR cells were injected into the right flank of mice. After the tumors had been allowed to develop to the adapted size, in vivo tumor suppression studies were carried out to examine the toxicity and tumor inhibition efficiency of TPE-RSNO, TPE-RSNO combined with DOX and free DOX. On the first day, mice were injected intratumorally with PBS (control group) and TPE-RSNO (400 μM, 50μL), and injected with PBS and free DOX (2 mg/kg) (n = 4) 12 h later. All the groups were treated only once.

The weights and tumor sizes were recorded daily at the same time. Tumor sizes were measured by a vernier caliper. Tumor volumes were calculated by the formula $(L \times W^2)/2$. L is the longest and W is the shortest tumor diameter (mm). On the 21st day, the mice were sacrificed, the tumor tissues were photographed and weighed.

Immunofluorescence of P-glycoprotein in tissues

To evaluate levels of P-glycoprotein in tumor cells, 6 μm thin sections of tumor tissues were cut in a cryostat and then incubated (12 h at 4°C) with the diluted anti-P-glycoprotein antibody. Subsequently, slices were washed with PBS and incubated for 1 h in secondary antibody, washed three times with PBS for 10 min, finally, added with the anti-quenching agent and observed under a microscope.





Meas. m/z	#	Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
526.73307	1	C 51 H 67 N 13 O 8 S 2	100.00	526.73330	0.43	0.21	79.0	25.0	even	ok
1052.46019	1	C 51 H 66 N 13 O 8 S 2	100.00	1052.45932	-0.83	-0.92	75.1	25.5	even	ok

Figure S1. Synthesis and characterization of TPE-RSNO. Mass spectrum. m/z 1052.46 corresponding to$[M+H^+]$ ion peak of TPESH. m/z 526.73 corresponding to$[M+2H^+]/2$ ion peak of TPE-RSH.

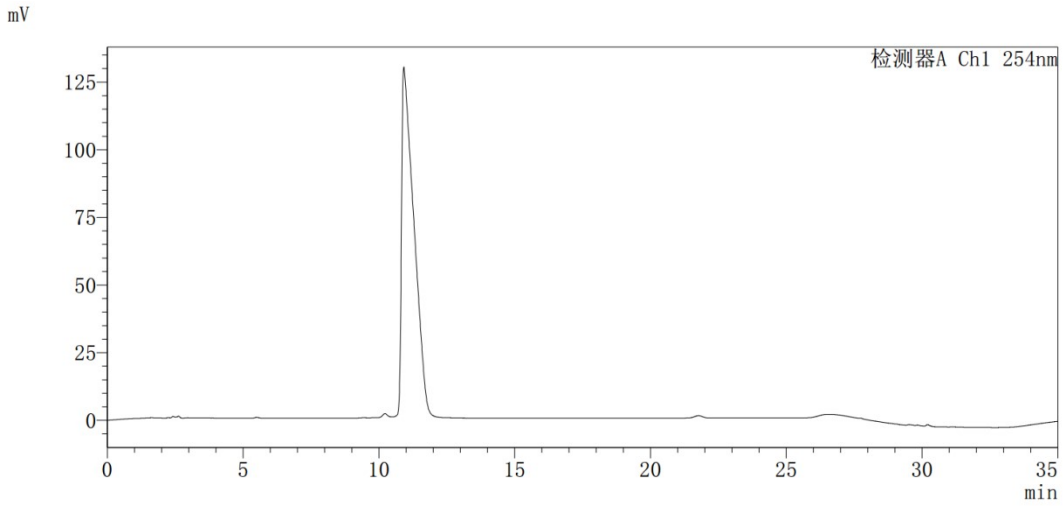


Figure S2. Characterizations of HPLC for TPE-RSH.

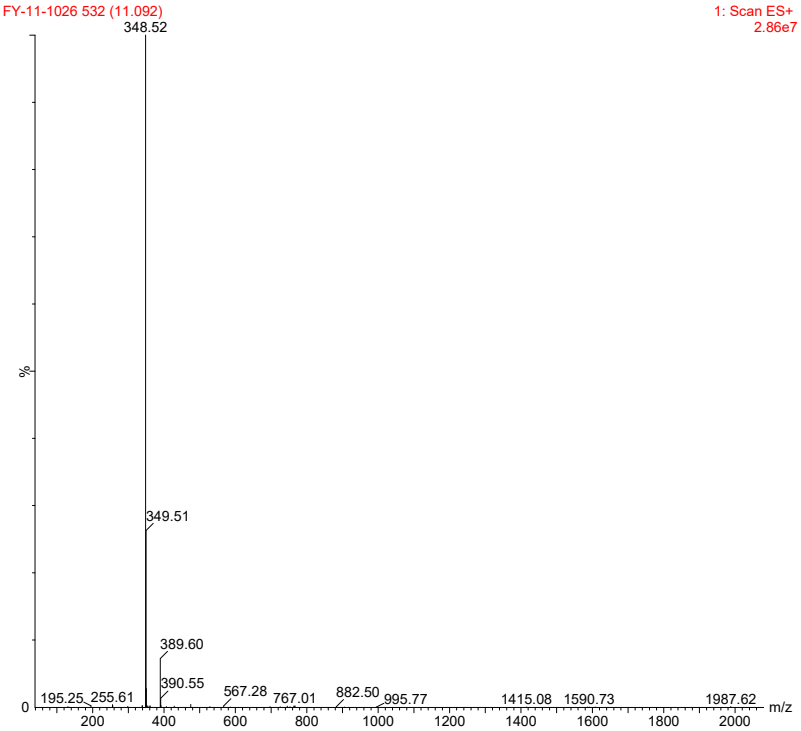


Figure S3. Mass spectrum. m/z 348.52 corresponding to[M+H+] ion peak of TPE-NH_2.

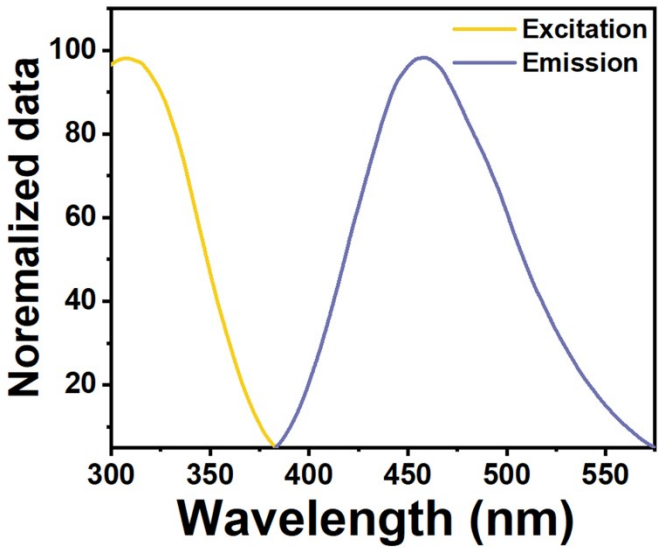


Figure S4. Emission and absorption spectra of TPE-RSNO.

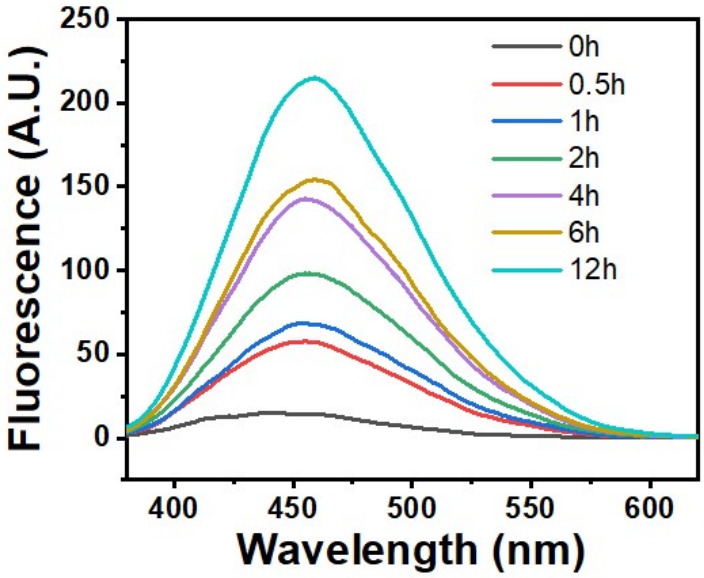


Figure S5. Time-dependent fluorescent emission spectra of TPE-RSNO in the presence of H_2O_2.

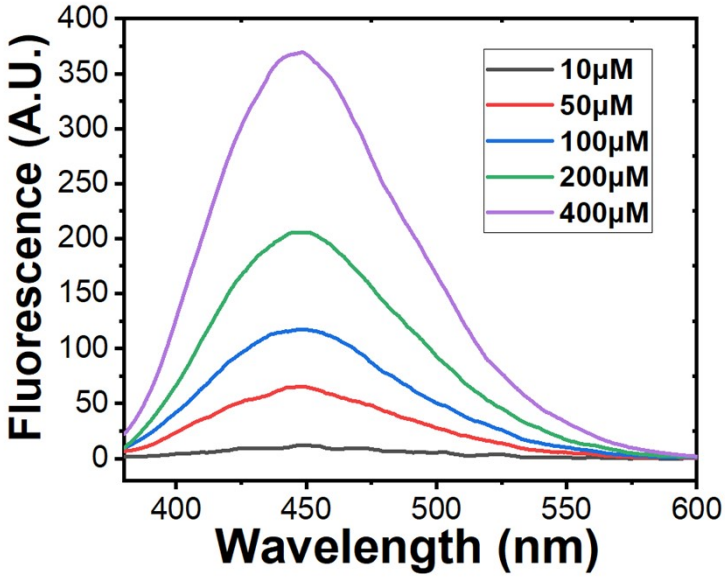


Figure S6. Concentration-dependent fluorescent emission spectra of TPE-RSNO in the presence of H_2O_2.

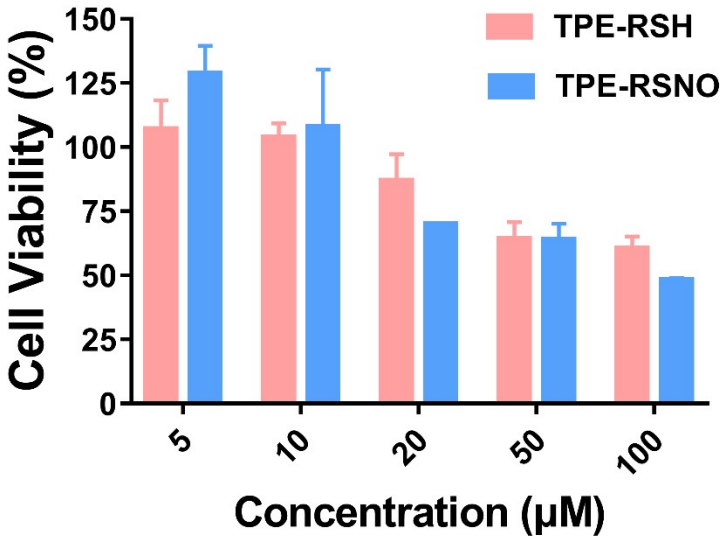


Figure S7. Biocompatibility of TPE-RSH and TPE-RSNO.

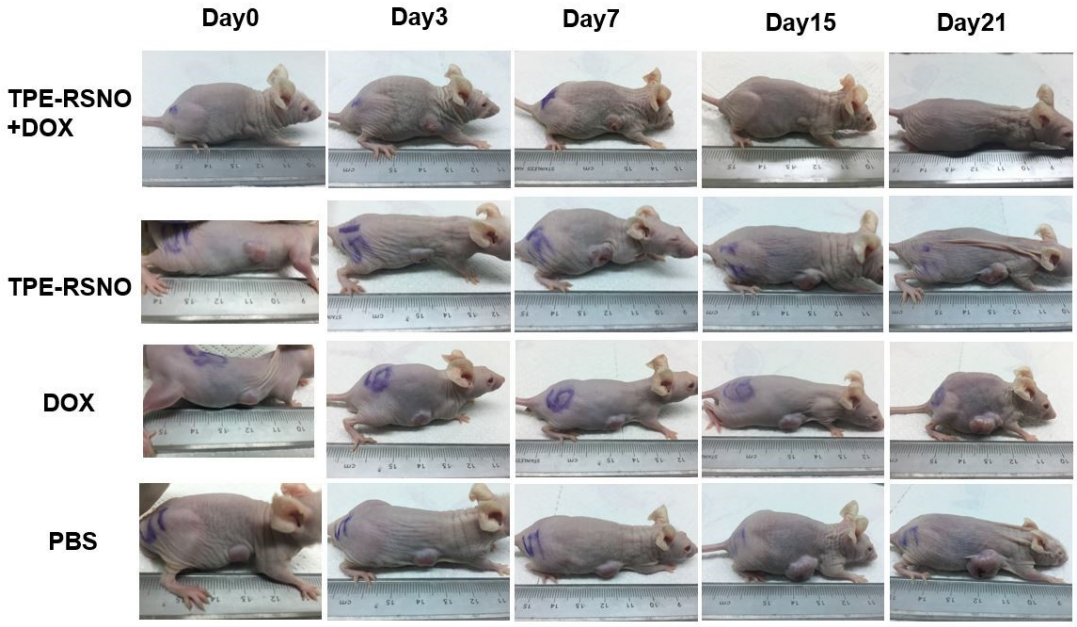


Figure S8. Photographs of Hepg2/ADR tumor-bearing nude mice and the tumors recorded during the antitumor studies (0, 3, 7, 15 and 21 days).

References

[1] Hu, Y.; Shi, L.; Su, Y.; Zhang, C.; Jin, X.; Zhu, X. A fluorescent light-up aggregation-induced emission probe for screening gefitinib-sensitive non-small cell lung carcinoma, *Biomater Sci* **2017**, *5*, 792-799.