Supporting Information

Hydrogen sulfide triggered theranostic agent for imaging and cancer therapy

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Materials and equipment

All chemicals were available commercially and used without further purification. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400. ESI-MS was determined on an LCQ electrospray mass spectrometer. UV-visible absorption spectra were recorded on a PerkinElmer Lambda 365 UV-vis spectrophotometer. Fluorescence measurements were performed on Hitachi Fluorescence Spectrophotometer F-4600. The transmission electron microscopy (TEM) images were obtained using a JEOL JEM-1011 transmission electron microscope. Fluorescence confocal imaging was carried out on a confocal microscope (A1, Nikon, Japan). Flow cytometry analysis was performed with a BD FACSverse Cell Analyzer. Western blotting experiments were conducted on the Mini-Protean Tetra System (BIORAD, Power PacTM HC, Singapore). The western blotting signal was enhanced by Tanon High-sig ECL Western Blotting Substrate and visualized by Tanon 5200 Multi. The type of Fourier transform infrared spectrometer was Bruker Tensor 27.

Sodium hydrosulfide (NaHS) was firstly prepared as a stock solution (20 mM) for further testing, other analytes including cysteine, homocysteine, glutathione, methionine, lysine, proline, valine, ethanoylaminoethanoic acid, NaClO, H_2O_2, TBHP, $NaNO_2$, $Na_2S_2O_3$, Na_2SO_3, NaCl, KCl, $FeCl_2$, $CuCl_2$, $ZnSO_4$, $MgCl_2$, $CaCl_2$, Na_2CO_3, $NaHCO_3$, Na_2SO_4, $NaHSO_4$, NaH_2PO_4, Na_2HPO_4, NaOAc were dissolved by dilution. PNF was dissolved in DMSO to get a 5 mM stock solution. The final concentration of PNF was settled at 10 μM with 5% DMSO in PBS buffer (10 mM, 1 mM CTAB, pH 7.4). After incubation with the various analytes for 1 h at 37 °C in PBS buffer, the emission spectrum was measured and scanned from 435 nm to 800 nm at 1200 nm/min, and both excitation and emission slit widths were 10 nm, λ_{ex} =415 nm.

Synthesis and characterization

3-amino-1,8-naphthalic anhydride (1). A solution of tin(II) chloride (4.783 g, 21.2 mmol) in concentrated HCl (32%, 3.5 mL) was added dropwise to a stirring suspension of 3-nitro-1,8-naphthalic anhydride (1.009 g, 4.1 mmol) in ethanol (2 mL), and the resulting suspension was stirred and refluxed for 2 h, before being cooled to room temperature. The precipitated product was collected by filtration, washed sequentially with water, ethanol, and ether. The resulting red-brown solid was dried in a vacuum oven to a constant weight (0.801g, yield, 90.0%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.17 – 8.08 (m, 2H), 7.98 (d, J = 2.3 Hz, 1H), 7.67 (dd, J = 8.3, 7.3 Hz, 1H), 7.40 (d, J = 2.3 Hz, 1H).

(5-amino-2-[2-(dimethylamino)ethyl]-1H-benz[de]isoquinoline-1,3(2H)-dione), Amonafide (ANF). A suspension of 300 mg of 3-amino-1,8-naphthalic anhydride (300 mg, 1.4 mmol) and 1,1-dimethylethylenediamine (130 mg, 1.47 mmol) was dissolved in 6.5 mL ethanol and then was heated to reflux for about 2.5 h. The solution was slowly cooled at room temperature. The precipitate was observed and then the solids were isolated by vacuum filtration and washed initially with 1 mL of ethanol

and then with 1 mL of n-hexane in two portions. The resulting yellow solid was dried in a vacuum oven to a constant weight (280 mg, yield, 71.0%).^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10 – 8.01 (m, 2H), 7.97 (d, J = 2.3 Hz, 1H), 7.61 (dd, J = 8.2, 7.3 Hz, 1H), 7.29 (d, J = 2.2 Hz, 1H), 6.01 (s, 2H), 4.16 (t, J = 6.8 Hz, 2H), 2.60 (t, J = 6.6 Hz, 2H), 2.28 (s, 6H).

N-(2-(dimethylamino)ethyl)-3-azido-1,8-naphthalimide (PNF). ANF (100 mg, 0.35 mmol) was dissolved in acetonitrile (2 mL), water (2 mL), concentrated H_2SO_4 (2 mL) and cooled to 0 °C in an ice bath. $NaNO_2$ (48.3 mg, 0.7 mmol) in H_2O (0.5 mL) was then added dropwise. The solution was stirred for 30 min followed by the addition of NaN_3 (45.5 mg, 0.7 mmol) in H_2O (1 mL) and stir overnight at room temperature. The solution was extracted with ethyl acetate, washed with Na_2CO_3, $Na_2S_2O_3$, brine, dried over $MgSO_4$, and concentrated. The resulting pale yellow solid was dried in a vacuum oven to a constant weight (67 mg, yield, 61.0%). ^{1}H NMR (400 MHz, MeOD) δ 8.55 (dd, *J* = 7.3, 1.0 Hz, 1H), 8.36 (dd, *J* = 8.3, 0.7 Hz, 1H), 8.24 (d, *J* = 2.3 Hz, 1H), 8.11 (d, *J* = 2.3 Hz, 1H), 7.87 (dd, *J* = 8.2, 7.4 Hz, 1H), 4.63 – 4.50 (m, 2H), 3.67 – 3.53 (m, 2H), 3.07 (s, 6H). 13C NMR (101 MHz, MeOD) δ 164.34, 163.92, 139.69, 133.53, 132.92, 130.25, 127.86, 125.46, 123.87, 122.96, 122.09, 121.87, 56.10, 42.85, 35.29. ESI-MS: $C_{16}H_{16}N_5O_2$ [M + H^+], Calculated: 310.13, Found: 310.17.

Determination of the fluorescence quantum yield

The fluorescence quantum yield Φ_u was estimated through the participation ratio method, where using the ethanol solution of rhodamine B (10 μM, Φ = 0.69, λ_{ex} = 365 nm) for the sample and reference. Through testing the absorption and fluorescence spectra of PNF (10 μM) and ANF (10 μM), the fluorescence quantum yield was calculated using the equation as follows:

$$\Phi_u = [(A_s F_u n^2)/ (A_u F_s n_0^2)]\Phi_s.$$

Φ_s is the quantum yield of the reference substance, A_s and A_u represents the absorbance of the reference and testing sample at the excitation wavelength, F_s and F_u refer to the integrated emission band areas under the same conditions, n and n_0 are the solvent refractive indexes of determined and reference, respectively. In the process of detection should control the absorbance to be lower than 0.05.

PNF: Φ = 0.0025; ANF: Φ = 0.14

The detection limit of probe

The emission spectrum of 10 μM PNF in PBS (10 mM, 5% DMSO, 1mM CTAB, pH 7.4) was collected for 20 times to determine the background noise σ. The linear regression curve was then fitted according to the data in the range of NaHS from 0 to 10 equiv. and obtained the slope of the curve, then the detection limit was calculated

using the following equation:

$$\text{The limit of detection (LOD)} = 3\sigma/k$$

Where σ is the standard deviation of eleven blank measurements, and k is the slope of the linear equation. The detection limit (3σ/k) was then determined to be 0.33 μM.

UFLC analysis

UFLC analysis of PNF after incubation without or with NaHS at 310 K for different time was performed on a Prominence UFLC system (SHIMADZU Co. Ltd., JPN). Running conditions were as follows: mobile phase composition was MeOH/H_2O: 90/10; temperature of 303 K; ACE Excel 5 C18 column of 4.6*250 mm; and flow rate of 1 mL/min.

The measurement of lipophilicity

Lipophilicity was presented as log $P_{o/w}$ values, which were determined by the flask-shaking method. An aliquot of a stock solution of the sample in aqueous NaCl (0.9% w/v and saturated with octanol) was added to an equal volume of octanol (saturated with 0.9% NaCl, w/v), and the mixture was shaken overnight at 60 rpm to allow partitioning at 298 K. After the sample was centrifuged at 3000 rpm for 10 min, the probe content of the organic and aqueous phases was determined by UV absorbance. Log *P* was defined as the logarithmic ratio of probe concentrations in the organic and aqueous phases.

Cell viability assay

The cytotoxicity was evaluated using the MTT colorimetric cytotoxicity assay (for anchorage-dependent cell) or cell counting kit-8 assay (for floating cell). Cells were placed in 96-well plates (5000 cells/well). After 18 h, cells were treated with different concentrations of compounds The IC_{50} value was defined as the concentration of compound required to inhibit cell viability by 50%, compared to cells treated with the maximum amount of DMSO (1%) and considered to be 100% viable.

Cell cycle analysis

A549R cells were seeded in 6-well cell plates and PNF with desired concentrations was added after incubation at 37 °C for 18 h, in an atmosphere of 5% CO_2 and 95% air. After treatment with PNF for 24 h at 37 °C, cells were harvested, washed twice with cold PBS, and fixed with 70% ethanol overnight at 4 °C. The fixed mixture was washed

twice with PBS, incubated with propidium iodide (PI, 50 μg/mL) for 30 min after pre-treatment with RNase A (100 μg/mL) for 10 min, washed with PBS twice, and subjected to flow cytometric analysis to evaluate the impact of A549R on the cell cycle arrest.

Transmission electron microscopy

The morphology of A549R was investigated using TEM. A549R cells were plated in 10 cm dishes and cultured for at 37 °C for 18 h. The cells were incubated with PNF (5 μM) for 24 h and harvested. The obtained pellets were fixed with 2.5% glutaraldehyde at 4 °C overnight, washed several times with PBS, and further fixed with 1% OsO4. The samples were dehydrated sequentially using solutions of acetone (50%, 75%, 90% and 100%) prior to impregnation in increasing concentrations (25%, 50%, 75% and 100%) of resin in acetone over a period of 24 h. Then samples were cut into small segments with ultramicrotome, which were mounted onto copper grids, followed by staining with 2% aqueous uranyl acetate and lead citrate. The sections were washed with distilled water and observed under transmission electron microscopy (JEOL JEM-1011) after drying.

Western blot analysis

In the Western blot study, A549R cells were treated with the desired concentration of PNF, collected and washed twice with ice-cold PBS. The pellets were lysed in 50-100 μL RIPA lysis buffer [100 μL, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitor cocktail] containing 1mM PMSF on ice for several min. Cell debris was removed by centrifugation at 13000 rpm for 20 min at 4 °C. The liquid supernatant containing the proteins was collected, and the total protein content of each sample was quantified by using a BCA Protein Assay Kit (Beyotime). About 30-40 micrograms of proteins from each sample was reconstituted in loading buffer [100 mM DTT, 1×protein loading dye] and heated at 95 °C for 5 min. Electrophoretic analysis of supernatants was carried out by 12% SDS-PAGE and transferred to PVDF (Millipore, 0.22 μm) membranes in Towbin buffer containing 0.033% SDS. PVDF membranes were blocked at ambient temperature in the buffer (5% skim milk/0.1% Tween-20/PBS) for 1 h. The primary antibodies of interest were used to immerse the membranes at 4 °C overnight. The membranes were washed with PBST (0.1% Tween-20/PBS) three times and incubated with peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG as secondary antibody in washing buffer (PBST) at room temperature for 1 h. Equal loading of protein was confirmed by comparison with Tubulin and GAPDH expression. Western blotting signal was enhanced by Tanon High-sig ECL Western Blotting Substrate and visualized by Tanon 5200 Multi.

3D tumor spheroids viability assays

The spheroids were prepared with A549R cells by seeding 1,500 cells/well in an Ultra-Low Attachment 96-well plate (Corning). The 5-day spheroids were incubated in a normal medium with PNF and cisplatin at the desired concentration. The spheroid growth was monitored using a live-cell phase-contrast microscope (Axio Observer, Zeiss).

3D tumor spheroids imaging

The spheroids were prepared with A549R cells by seeding 1,500 cells/well in an Ultra-Low Attachment 96-well plate (Corning). For imaging, the 5-day spheroids were incubated in a normal medium with PNF at the desired concentration for 48 h. Then, spheroids were washed twice with PBS, stained with AO/EB following the manufacturer's instructions, and fixed in 4% paraformaldehyde. Spheroids were placed in a glass-bottom dish and imaged at different depths (z-stacking) with a confocal scanning microscopy system (AO: λ_{ex} = 488 nm and λ_{em} range 500-550 nm, EB: λ_{ex} = 561 nm and λ_{em} range 570-620 nm).

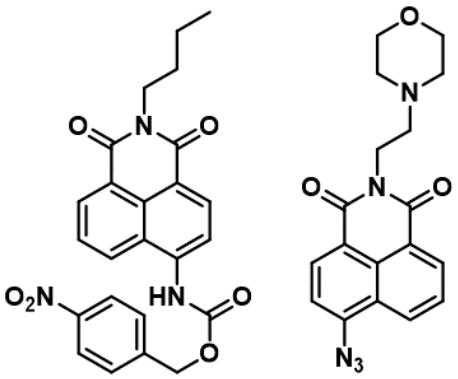
Supplementary tables and figures

Table S1 IC_{50} values of ANF and PNF against different cell lines with cisplatin as the control for 48 h.

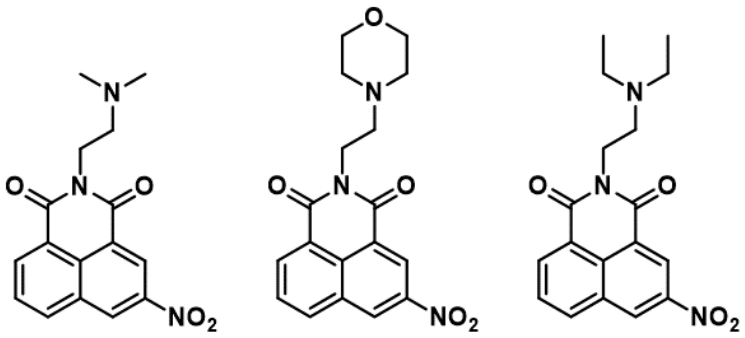
Cell lines	IC_{50} (μM)		
	ANF	PNF	Cisplatin
A549	3.5 ± 0.2	1.7 ± 0.1	10.2 ± 0.4
A549R	16.6 ± 0.6	2.8 ± 0.1	29.8 ± 1.7
A2780	13.4 ± 0.7	4.7 ± 0.1	3.2 ± 0.1
A2780R	27.6 ± 1.1	8.4 ± 0.3	28.6 ± 0.5
HCT116	12.7 ± 0.1	2.3 ± 0.2	15.3 ± 0.6
A261	4.8 ± 0.2	1.3 ± 0.1	6.0 ± 0.4
U87MG	19.1 ± 0.1	6.5 ± 0.1	11.9 ± 0.5
MCF-7	12.8 ± 0.1	5.2 ± 0.1	11.9 ± 0.2
K562	7.8 ± 0.5	2.0 ± 0.1	5.2 ± 0.1
NB-4	6.2 ± 0.1	1.7 ± 0.1	3.5 ± 0.1
HeLa	11.2 ± 0.4	5.4 ± 0.1	8.6 ± 0.4

Table S2 IC_{50} values of PNF and ANF towards A549R cells, with cisplatin as the control for 12 h, 24 h, and 48 h.

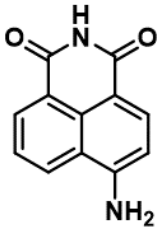
Time	IC_{50} (μM)		
	ANF	PNF	Cisplatin
12 h	78.6 ± 2.7	63.3 ± 1.9	100.2 ± 3.3
24 h	37.6 ± 0.4	7.45 ± 0.3	36.9 ± 0.7
48 h	16.6 ± 0.6	2.8 ± 0.1	29.8 ± 1.7



H_2S probes
Chem. Soc. Rev., 2015, **44**, 4596-4618

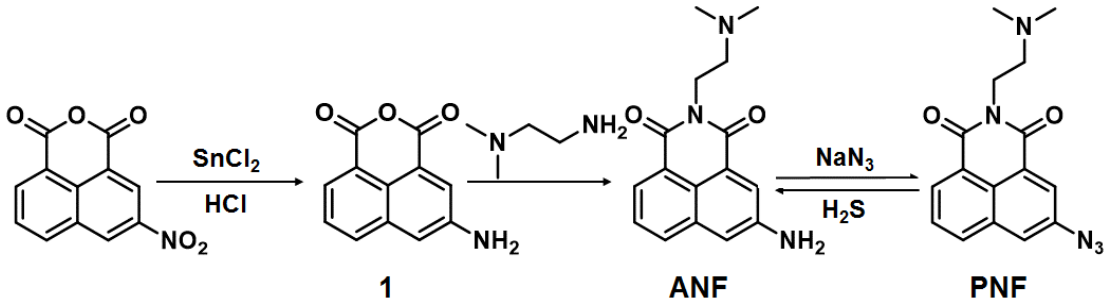


anticancer agents
J. Med. Chem., 1984, **27**, 1677–1682

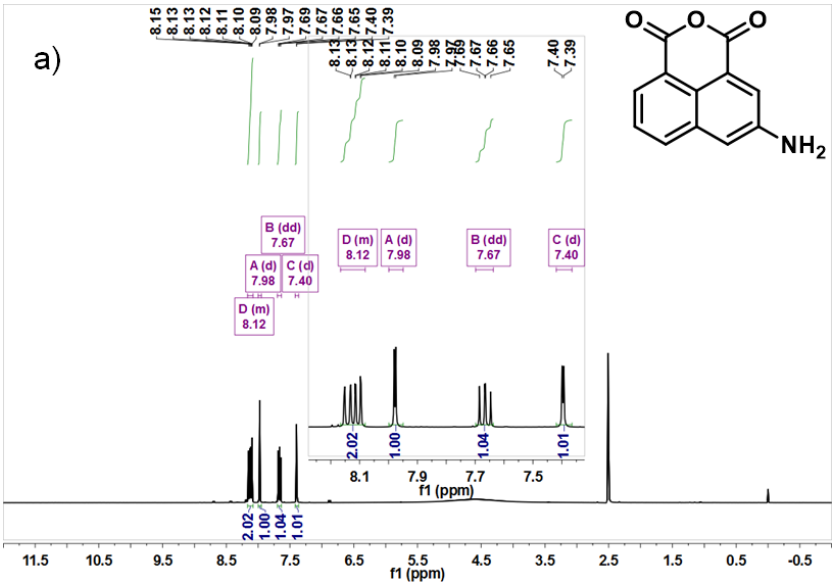


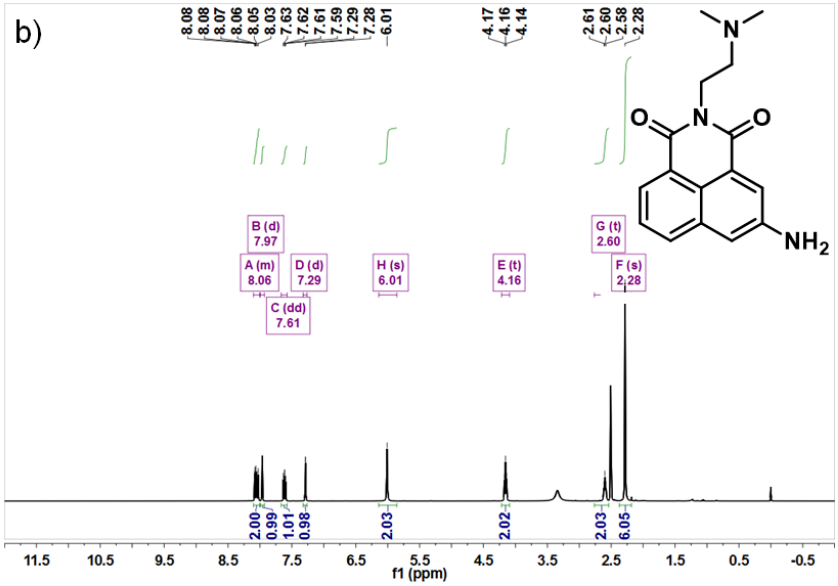
neuroprotective agent
Free Radical Research, 2009, **43**, 400-408

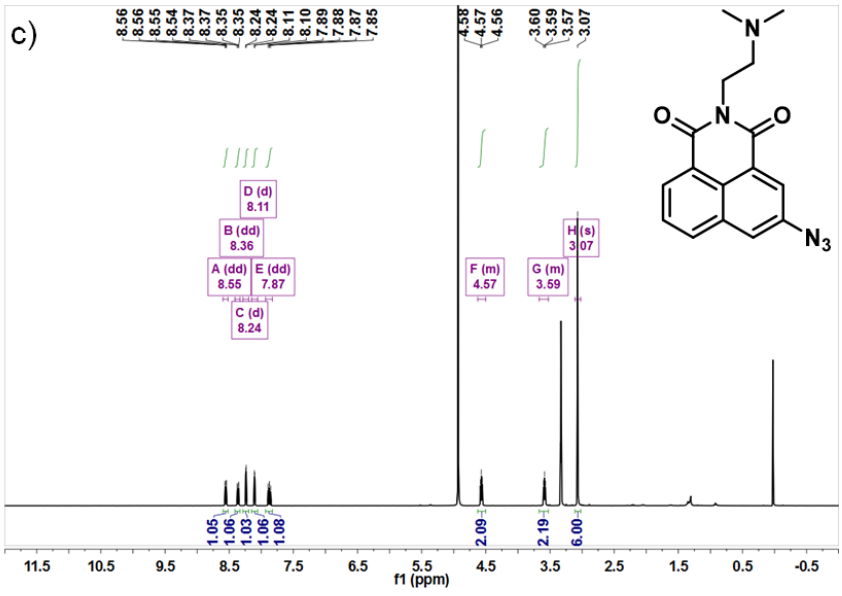
Scheme S1 Previous reported 1,8-Naphthalimide-derivatives can be used as probes, anticancer and neuroprotective agents.



Scheme S2 Chemical synthetic of PNF.


a)
B (dd) 7.67
A (d) 7.98
C (d) 7.40
D (m) 8.12
D (m) 8.12
A (d) 7.98
B (dd) 7.67
C (d) 7.40
2.02
1.00
1.04
1.01
8.1
7.9
7.7
7.5
f1 (ppm)
2.02
1.00
1.04
1.01
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
-0.5
f1 (ppm)
O
O
O
NH2


b)
B (d) 7.97
A (m) 8.06
D (d) 7.29
C (dd) 7.61
H (s) 6.01
E (t) 4.16
G (t) 2.60
F (s) 2,28
2.00
0.99
1.01
0.98
2.03
2.02
2.03
6.05
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
-0.5
f1 (ppm)
N
O
N
O
NH2


c)
D (d) 8.11
B (dd) 8.36
A (dd) 8.55
E (dd) 7.87
C (d) 8.24
F (m) 4.57
G (m) 3.59
H (s) 3.07
1.05
1.06
1.03
1.06
1.08
2.09
2.19
6.00
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
-0.5
f1 (ppm)
N
O
N
O
N3

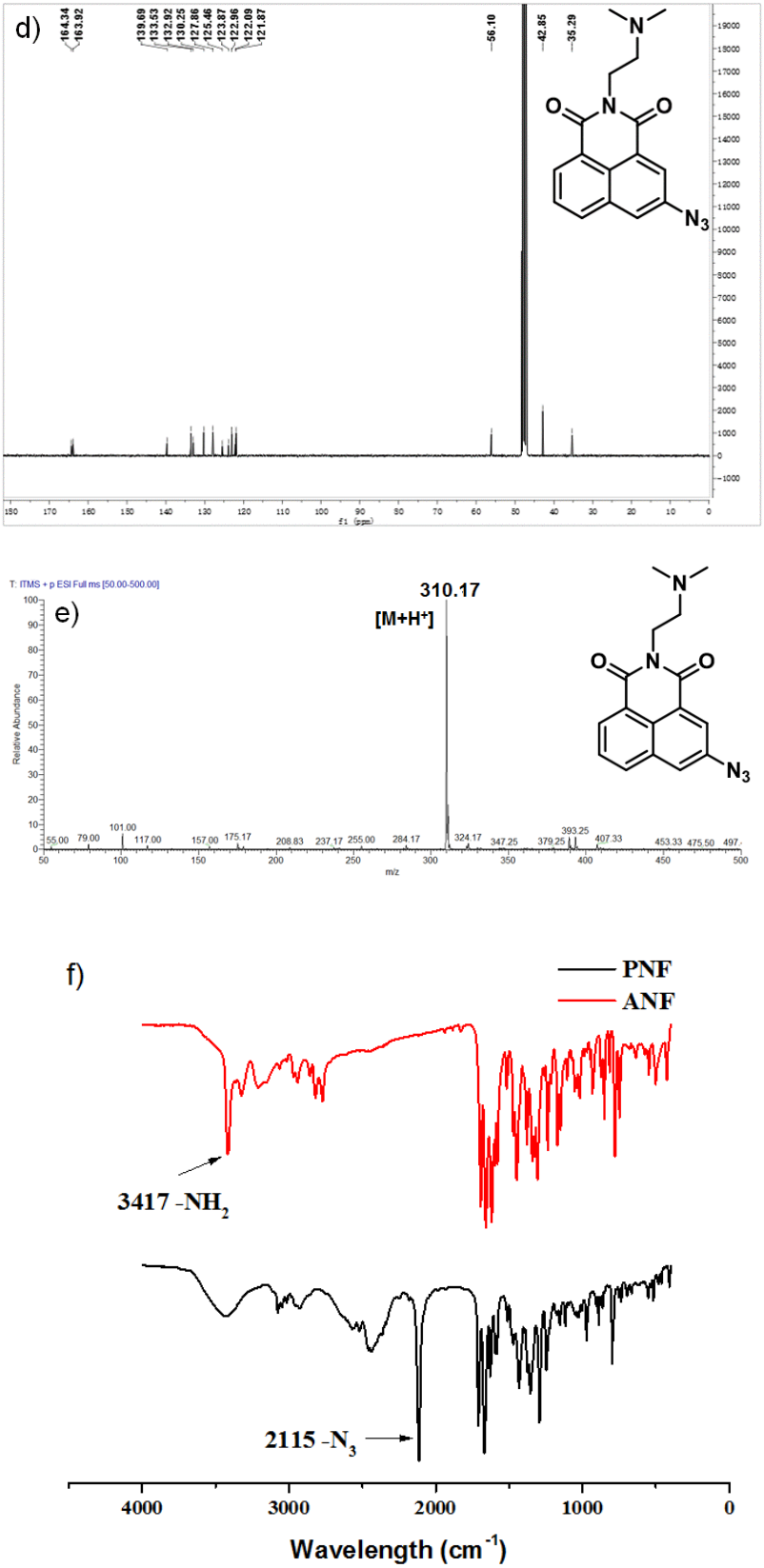


Figure S1 (a) ^{1}H NMR of compound **1**. (b) ^{1}H NMR of compound ANF. (c) ^{1}H NMR of compound PNF. (d) ^{13}C NMR of compound PNF. (e) ESI-MS of compound PNF. (f) IR spectra of compound ANF and PNF.

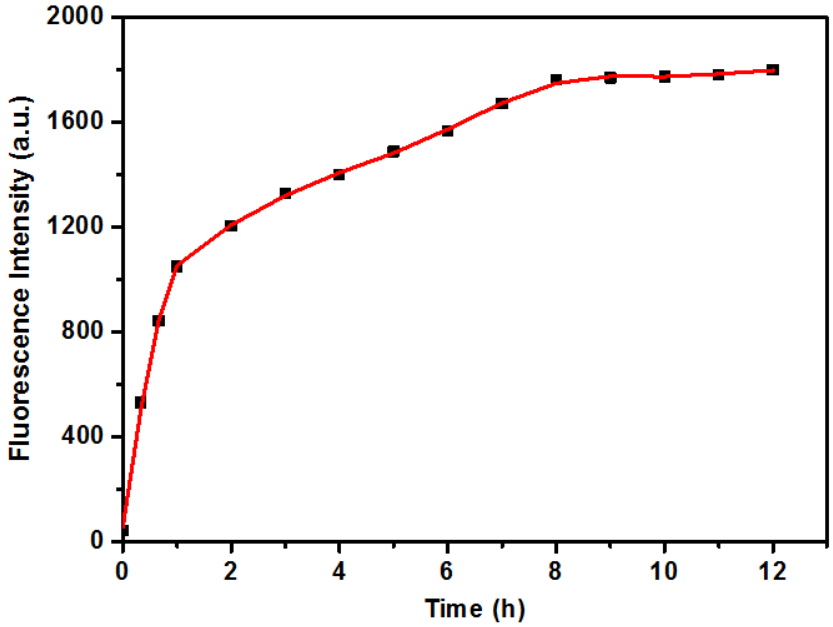


Figure S2 Time-dependent fluorescence intensity of PNF (10 μM) at 598 nm incubated with NaHS (100 μM) in PBS buffer (10 mM, 5% DMSO, 1 mM CTAB, pH 7.4) at 37 ℃. Excitation wavelength: 415 nm, emission wavelength: 598 nm, excitation and emission slit widths = 10 nm.

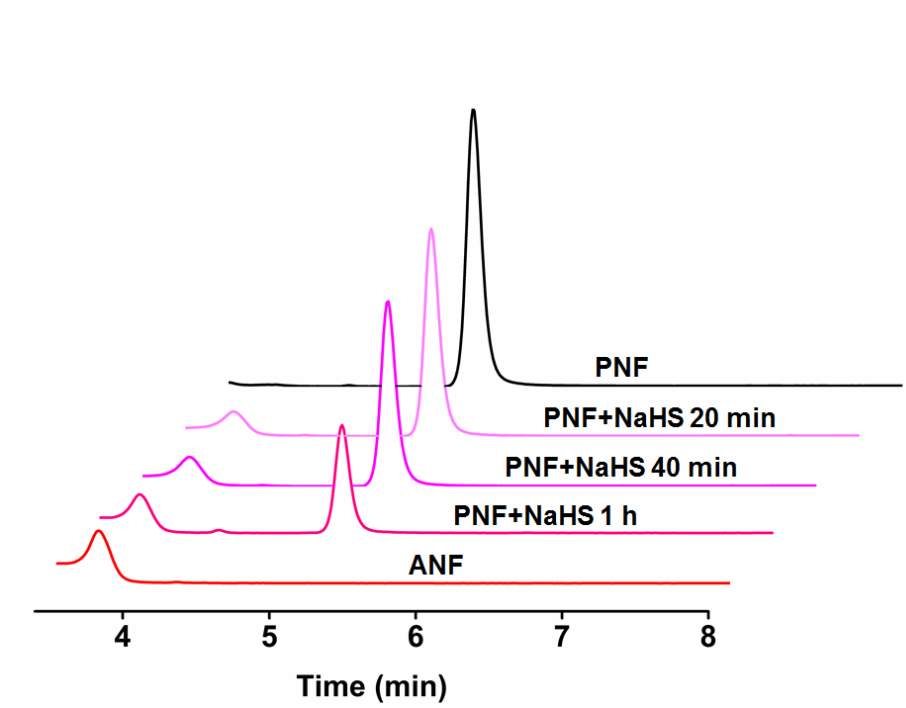


Figure S3 UFLC analysis of the reaction solution from the incubated mixture of PNF (10 μM) with NaHS (100 μM) for different time in PBS buffer (10 mM, 5% DMSO, 1mM CTAB, pH 7.4) at 37 ℃.

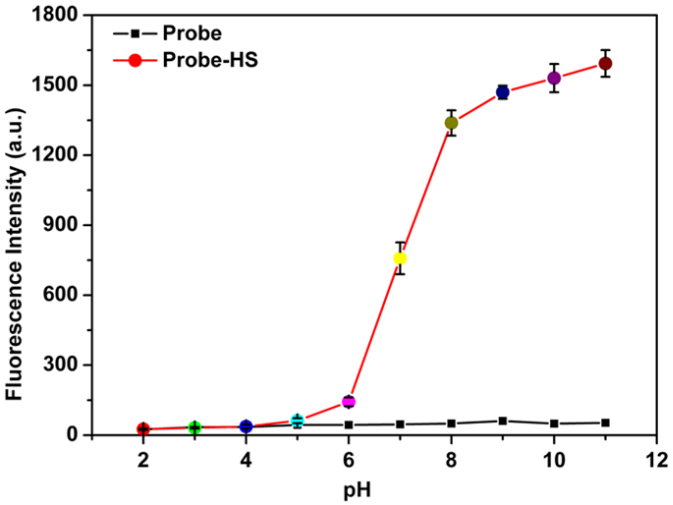


Figure S4 Fluorescence intensity changes of PNF (10 μM) at 598 nm in the absence or presence of NaHS (100 μM) in PBS buffer (10 mM, 5% DMSO, 1mM CTAB, pH 7.4) with various pH conditions at 37 ℃ for 1 h. All the data represent the average of three independent experiments.

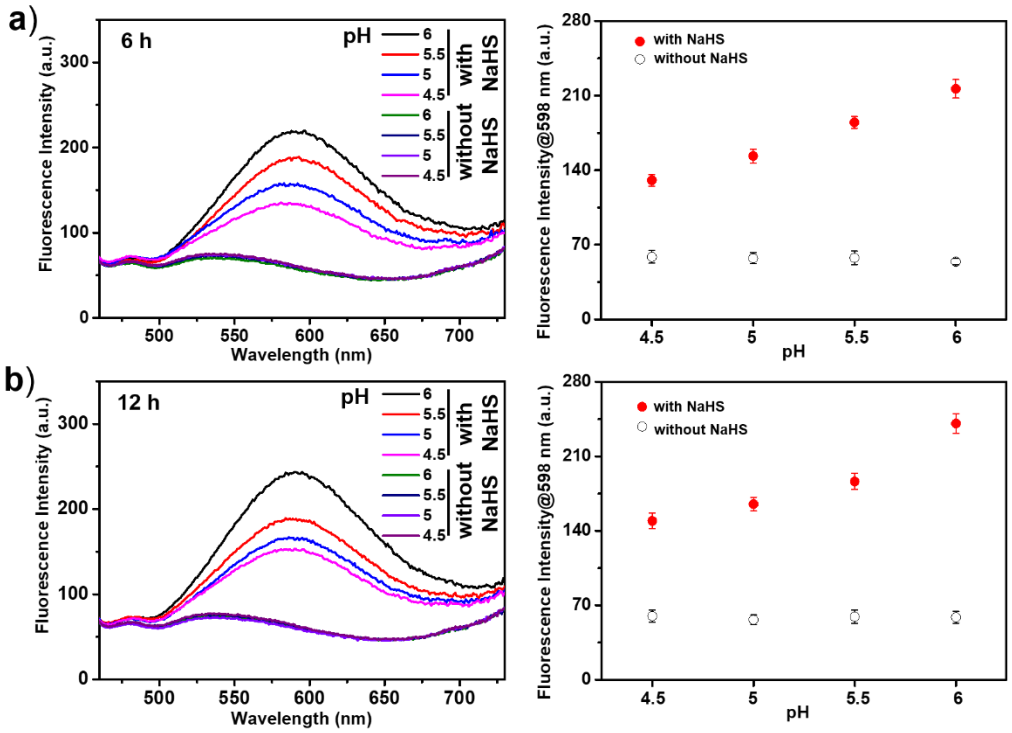


Figure S5. In the absence or presence of NaHS (100 μM), PNF (10 μM) was incubated in PBS buffer (10 mM, 5% DMSO, 1 mM CTAB) at different pHs (pH 4.5, 5, 5.5, 6) for 6 h (a) or 12 h (b) at 37 °C, respectively, and the changes of fluorescence intensity at 598 nm were observed. The error bars were ± SD (n = 3). Excitation: 415 nm, slit/slit: 10 nm/10 nm.

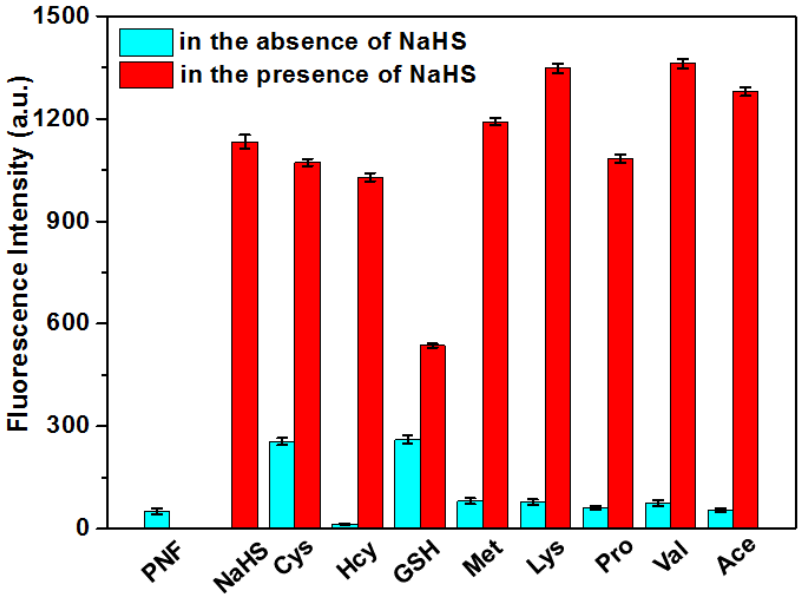


Figure S6 Fluorescence intensity of PNF (10 μM) at 598 nm in the absence/presence of NaHS (100 μM) under the condition of various amino acids in PBS buffer (10 mM, pH 7.4, 5% DMSO) at 37 °C for 1 h. In the absence of NaHS: Cys (200 μM); Hcy (200 μM); GSH (5 mM); Met (200 μM); Lys(200 μM); Pro (200 μM); Val (200 μM); Ace (200 μM); In the present of NaHS: Cys (1 mM); Hcy (1 mM); GSH (5 mM); Met (1 mM); Lys(1 mM); Pro (1 mM); Val (1 mM); Ace (1 mM). All the data represent the average of three independent experiments.

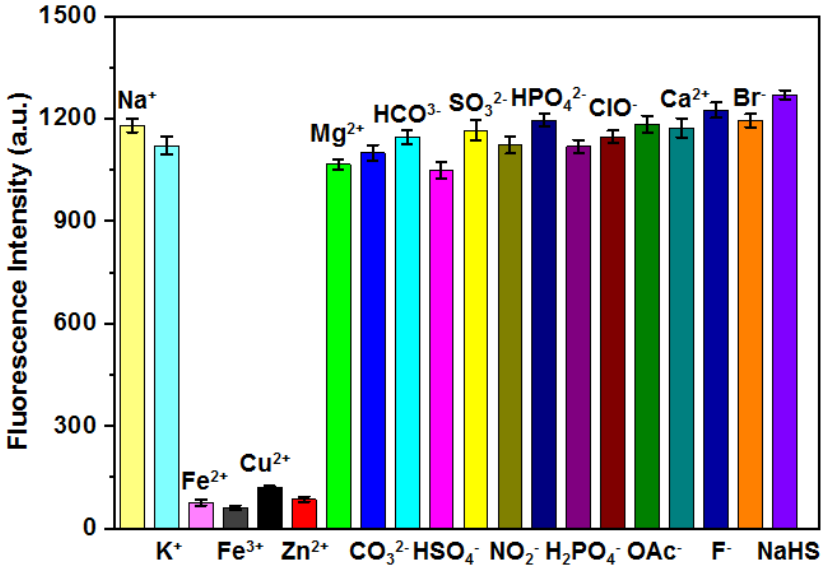


Figure S7 Fluorescence intensity of PNF (10 μM) at 598 nm towards NaHS (100 μM) in the presence of reactive oxygen species (200 μM ClO^-), reactive nitrogen species (NO_2^-, 200 μM), sulphur-containing inorganic ions (200 μM SO_3^{2-}) and inorganic salts salts (1 mM NaCl, 1 mM KCl, 100μM $FeCl_2$, 100μM $FeCl_3$, 100μM $CuCl_2$, 100μM $ZnSO_4$, 1 mM $MgCl_2$, 200 μM Na_2CO_3, 200 μM $NaHCO_3$, 200 μM $NaHSO_4$, 200 μM Na_2SO_3, 200 μM Na_2HPO_4, 200 μM NaH_2PO_4, 200 μM NaOAc, 1 mM $CaCl_2$, 200 μM KF, 200 μM KBr). All the data represent the average of three independent experiments.

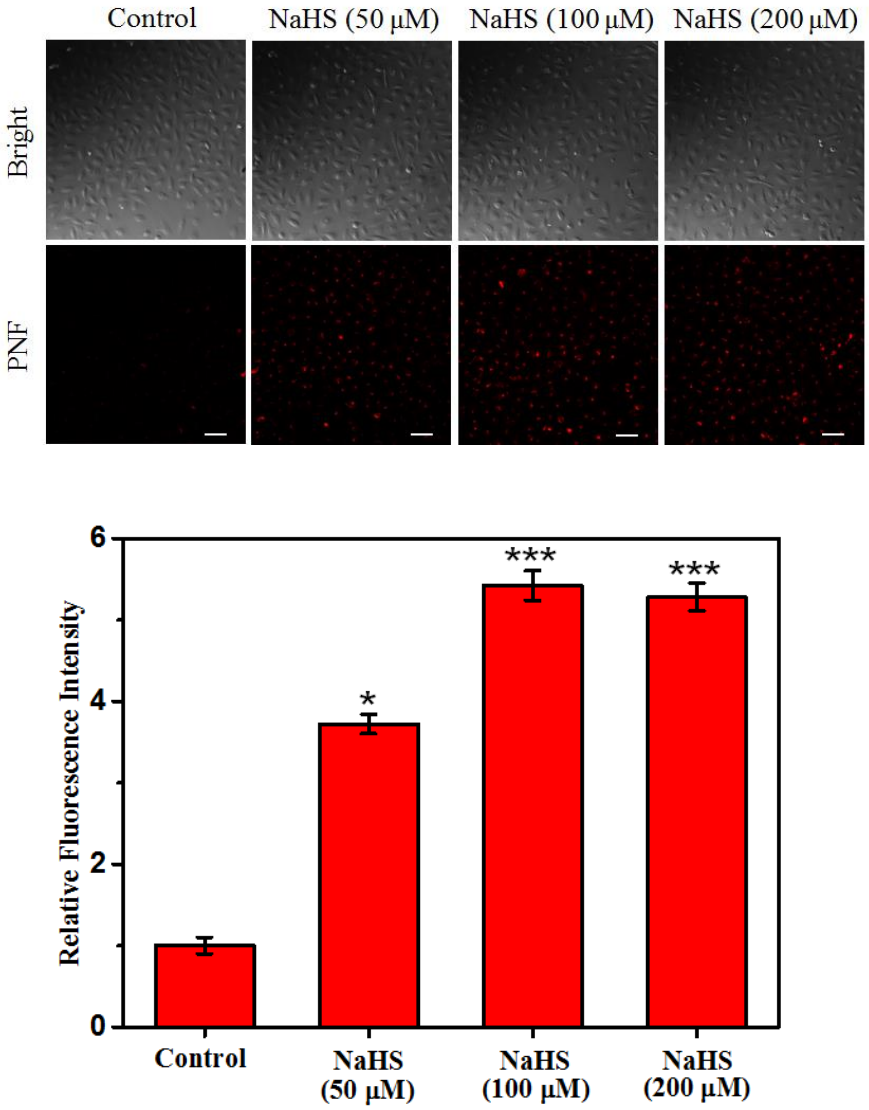


Figure S8 (Up) Confocal fluorescence images for monitoring exogenous H_2S. A549R cells were incubated first with PNF (10 μM), then treated with different concentrations of NaHS (50 μM, 100 μM, 200 μM) for 1 h. λ_{ex} = 405 nm, collected at 570–620 nm. Scale bar: 100 μm. (Down) Relative fluorescence intensity (*R*) output of groups according to the corresponding images of cells. All the data represent the average of three independent experiments, the error bars were ± standard deviation (SD). Statistical analyses performed with a two-tailed Student's *t*-test with unequal variance, **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001

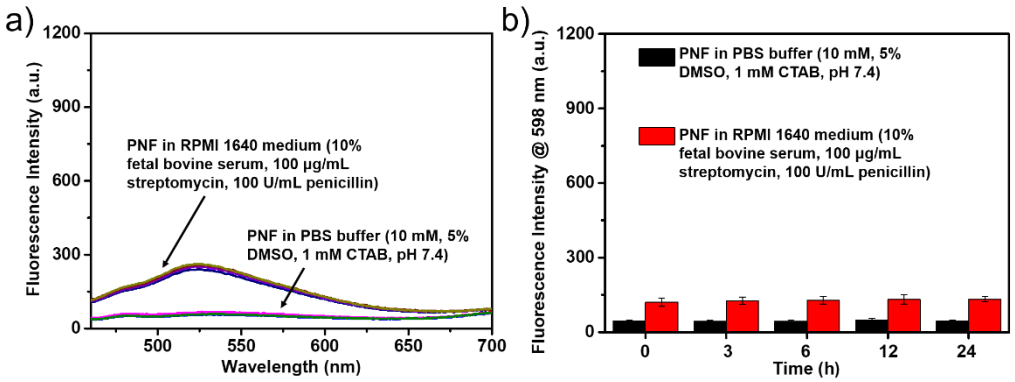


Figure S9 Stability studies of PNF in different experimental environments. (a) Fluorescence spectra of prodrug PNF (10 μM) in PBS buffer (10 mM, 5% DMSO, 1 mM CTAB) or RPMI 1640 medium after incubation for 0-24 h, respectively. (b) Changes of fluorescent intensity at 598 nm after incubation for 0-24 h. The error bars were ± SD (n = 3). Excitation: 415 nm, slit/slit: 10 nm/10 nm.

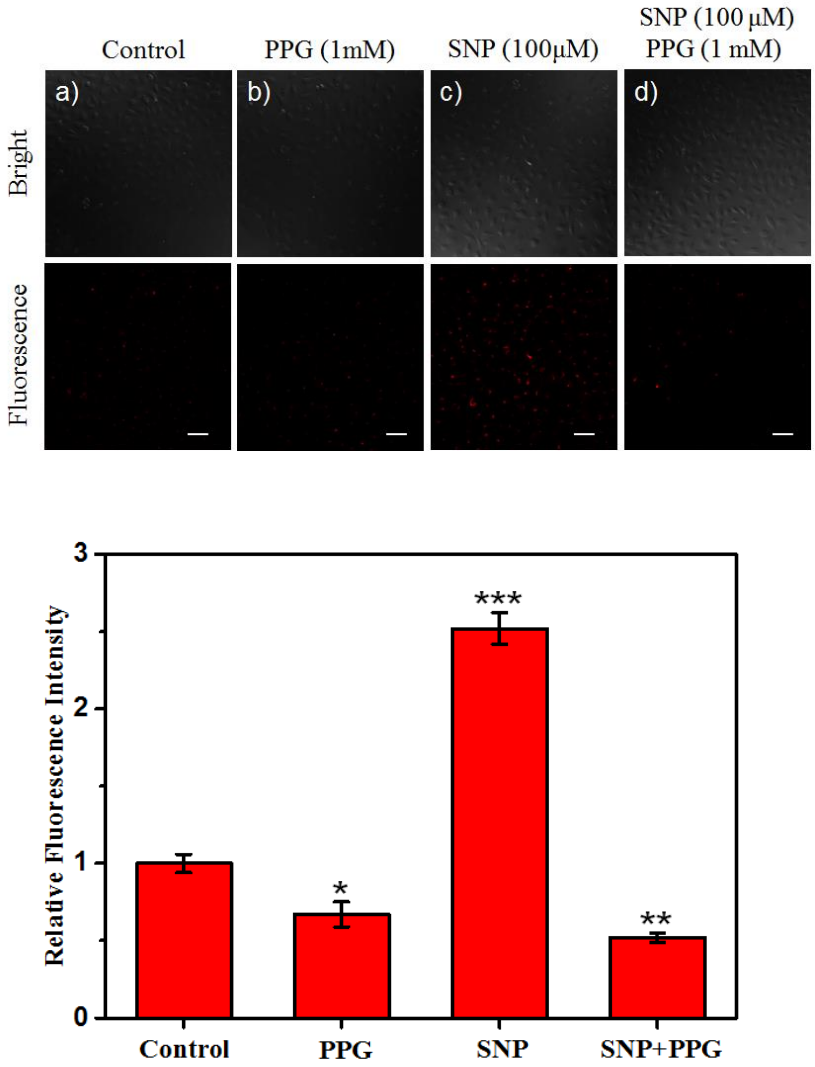


Figure S10 (Up) Confocal fluorescence images of endogenous H_2S in living A549R cells with PNF (10 μM) upon excitation at 405 nm. (a) Untreated cells. (b) Cells were pretreated with 1 mM PPG for 30 min, and then incubated with PNF (10 μM) for 1 h. (c) Cells were pre-stimulated with SNP (100 μM) for 30 min, then incubated with PNF (10 μM) for 1 h. (d) Cells were co-treated with SNP (100 μM) and PPG (1 mM) for 30 min, and then cells were incubated PNF (10 μM) for another 1 h before imaging. Scale bar: 100 μm. (Down) Relative fluorescence intensity (*R*) output of groups according to the corresponding images of cells. All the data represent the average of three independent experiments, the error bars were ± standard deviation (SD). Statistical analyses performed with a two-tailed Student's *t*-test with unequal variance, **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001.

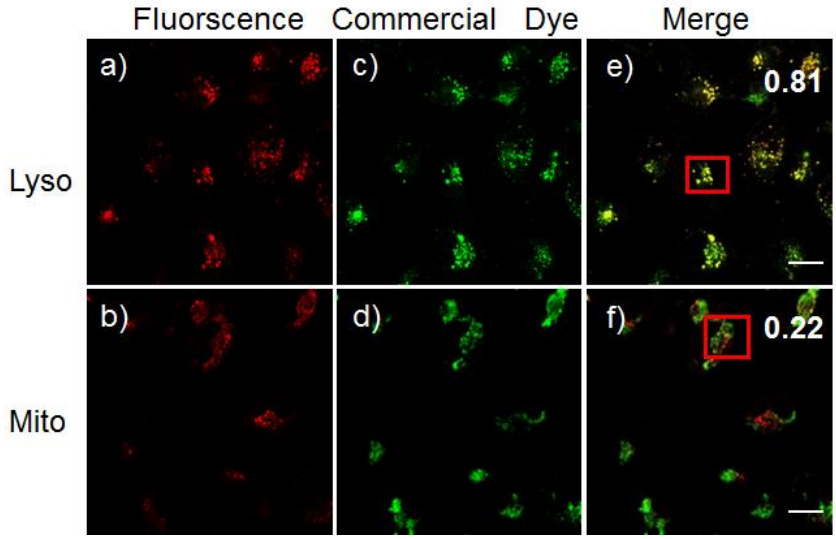


Figure S11 Intracellular localization in living A549R cells incubation with PNF for 12 h. (a, b) PNF (10 μM, λ_{ex} = 405 nm and λ_{em} range 570−620 nm); (c) LysoTracker Green (2 μM, λ_{ex} = 488 nm and λ_{em} range 500−550 nm); (d) MitoTracker Green (0.2 μM, λ_{ex} = 488 nm and λ_{em} range 500−550 nm); (e, f) Merged images. Scale bar = 20 μm.

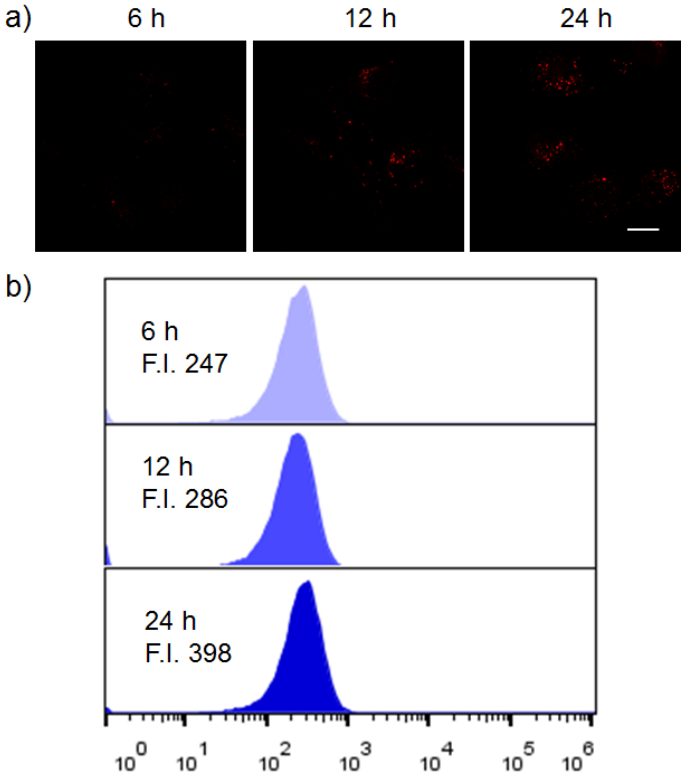


Figure S12 (a) A549R cells were treated with PNF (5 μM) for 6 h, 12 h, 24 h before imaging. Red channel: collected at 570–620 nm, λ_{ex} = 405 nm, scale bar = 20 μm. (b) Flow cytometry analytic results of the relative ratio of fluorescence intensity.

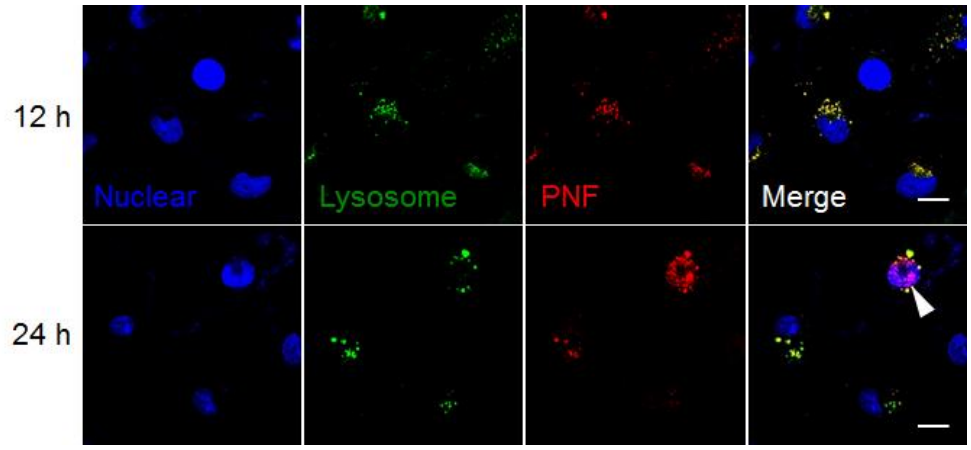


Figure S13 Fluorescence images of PNF-treated A549R Cells. Cells were treated with PNF (5 μM, λ_{ex} = 405 nm and λ_{em} range 570−620 nm) for 12 h or 24 h. All the tested cells were stained with LysoTracker Green (2 μM, λ_{ex} = 488 nm and λ_{em} range 500−550 nm) and Hoechst33324 (1X, λ_{ex} = 405 nm and λ_{em} range 425−475 nm) White arrow: the transportation of activated PNF from the lysosomes to the nucleus.

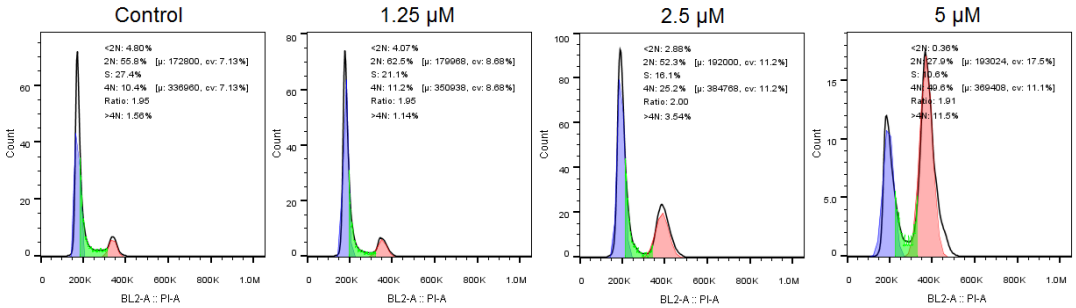


Figure S14 Flow cytometry data for cell cycle distribution of PNF-treated A549R cells for 24 h.

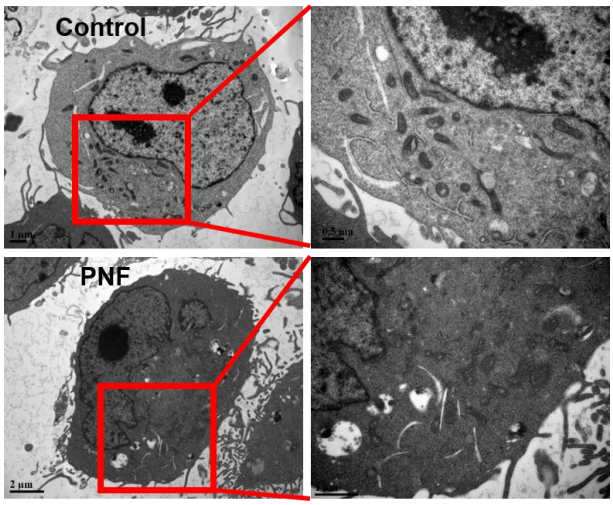


Figure S15 Representative TEM images of A549R cells treated with or without PNF (5 μM) for 24 h.

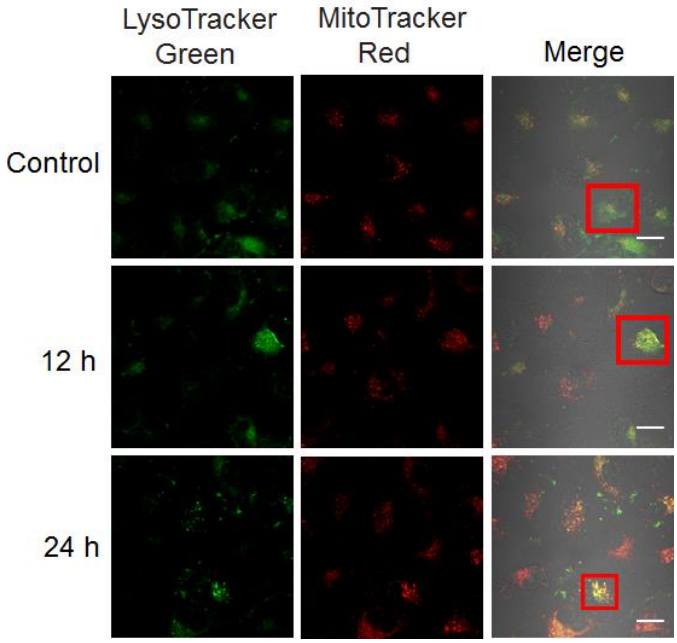


Figure S16 Confocal microscopy images of A549R cells treated with PNF (5 μM) for 12 h and 24 h, respectively. MitoTracker Red (0.3 μM, λ_{ex} = 561 nm, λ_{em} range 570−620 nm); LysoTracker Green (2 μM, λ_{ex} = 488 nm, λ_{em} range 500−550 nm). Scar bar: 20 μm.

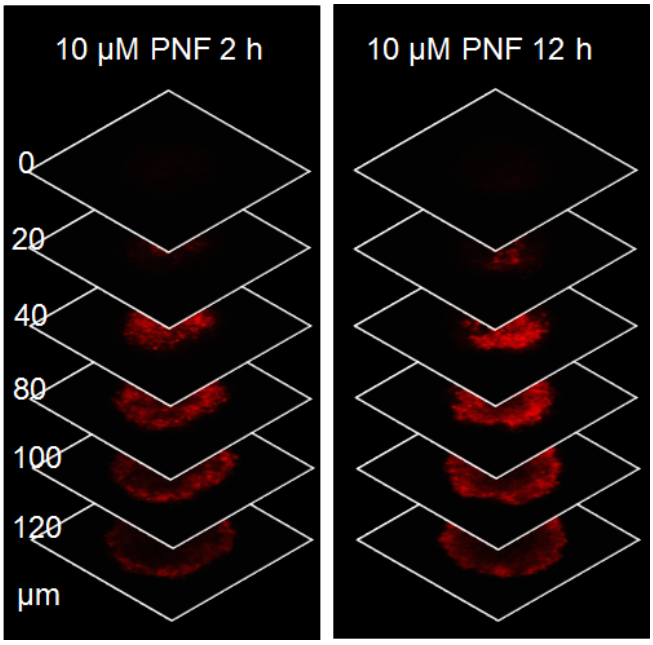


Figure S17 Fluorescence images of A549R MCTSs at different depths after treatment with PNF (10 μM) for 2 h or 12 h, respectively.

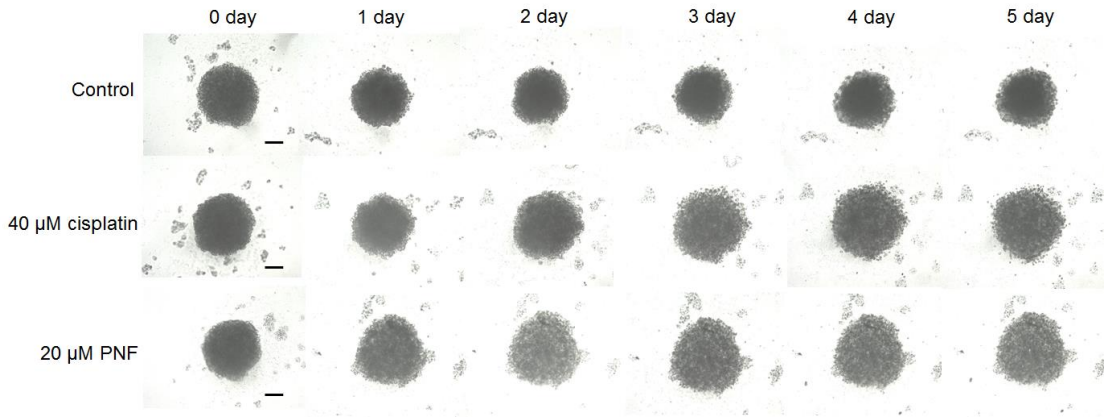


Figure S18 Representative pictures of 3D A549R tumor spheroids treated with PNF (20 μM) or cisplatin (40 μM) at different times. Scale bar: 100 μm.