Supporting Information

A self-immolated fluorogenic agent triggered by H_2S exhibiting potential anti-glioblastoma activity

Chao Ge, Ji Li, Lu Liu, Hong-Ke Liu* and Yong Qian*

Jiangsu Key Laboratory of Biofunctional Materials, School of Chemistry and Materials Science, Nanjing Normal University, Nanjing, 210023, P. R. China

Email: hongkeliu@njnu.edu.cn; yongqian@njnu.edu.cn

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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. UV/Vis spectra were recorded on a LAMBDA 365 UV-vis spectrophotometer. ESI-MS was determined on an LCQ electrospray mass spectrometer. Fluorescence measurements were performed on a FS5 Spectrophotometer. 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.

Sodium hydrosulfide (NaHS) was firstly prepared as a stock solution (20 mM) for further testing, other analytes including DL- homocysteine, lysine, cysteine, methionine, glycine, proline, valine, glutathione, NaCl, KCl, KF, KBr, $FeCl_2$, $FeCl_3$, $CuCl_2$, $ZnSO_4$, $MgCl_2$, $AlCl_3$, $CaCl_2$, Na_2CO_3, $NaHCO_3$, $NaNO_3$, NH_4Cl, Na_2SO_4, $NaHSO_4$, NaH_2PO_4, Na_2HPO_4, NaH_2PO_2, Na_3PO_4, NaOAc, $Na_2S_2O_3$, $Na_2S_2O_5$, $Na_2S_2O_4$, Na_2SO_3, $NaNO_2$, HClO were dissolved by dilution. SNF was dissolved in DMSO to get a 10 mM stock solution. The final concentration of SNF 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, and both excitation and emission slit widths were 5 nm, excitation wavelength = 415 nm.

DMSO (dimethyl sulfoxide, Sigma Aldrich), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, HEOWNS), MitoGreen (Mito Tracker Green, KeyGEN BioTECH, China), LysoGreen (KeyGEN BioTECH, China), anti-cytochrome C, anti-LC3, and anti-p53 (Proteintech, USA), anti-γH2AX, and anti-PINK1 (Cell Signaling Technology, USA), RIPA lysis buffer (Beyotime Biotechnology, China), BCA Protein Quantitation Assay (KeyGEN BioTECH, China), Cell Cycle Detection Kit (KeyGEN BioTECH, China).

Synthesis and characterization

Synthesis of 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).

Synthesis of 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).

Synthesis of 4-azidobenzyl alcohol (2). 4-aminobenzyl alcohol (600 mg, 4.88 mmol) was dissolved in 10% HCl (10 mL). Subsequently, it was cooled to 0 °C, and then a solution of sodium nitrite (402 mg, 5.84 mmol) in 6 mL of water was added dropwise to the above mixture, followed by the addition of 5 mL sodium azide (380 mg, 5.84 mmol) solution in 1 h at 0 °C. After stirring overnight, the mixture was warmed to room temperature and the reaction was quenched with brine. The organic layer was extracted with CH_2Cl_2, washed with brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure. The resulting product was purified by column chromatography (hexanes/ethyl acetate = 4:1, v/v) to give a yellow oil of 2 (420 mg, yield, 70.0%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (d, J = 8.2 Hz, 2H), 7.08 – 7.00 (m, 2H), 4.69 (s, 2H).

Synthesis of SNF. ANF (286 mg, 1.00 mmol) and anhydrous triethylamine (0.28 mL, 2 mmol) in anhydrous CH_2Cl_2 (15 mL), a solution of triphosgene (0.30 g, 1 mmol) in dry CH_2Cl_2 (5 mL) was added dropwise at -10 °C under argon atmosphere. The mixture was stirred for 3 h at -10 °C, and then a solution of 2 (0.23 g, 1.5 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise, followed by stirring at room temperature overnight. After that, the reaction mixture was quenched with water (30 mL) and then added with aqua ammonia to get a basic solution. The organic phase was extracted by CH_2Cl_2, washed with brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure. Then, column chromatography (hexanes/ethyl acetate/trimethylamine = 50:50:2, v/v) was used to purify the crude product and give SNF (71 mg, yield, 25.0%). ^{1}H NMR (400 MHz, DMF–d_7) δ 8.96 (d, J = 2.2 Hz, 1H), 8.86 – 8.76 (m, 1H), 8.66 – 8.49 (m, 2H), 8.10 – 7.95 (m, 1H), 7.85 – 7.72 (m, 2H), 7.50 – 7.31 (m, 2H), 5.49 (d, J = 13.6 Hz, 2H), 4.43 (t, J = 6.9 Hz, 2H), 2.78 (t, J = 6.9 Hz, 2H), 2.45 (s, 6H). ^{13}C NMR (101 MHz, DMF –d_7) δ 163.78, 163.52, 153.94, 139.79, 138.66, 133.85, 133.57, 132.76, 130.12, 128.87, 127.68, 124.23, 123.38, 122.41, 119.69, 119.29, 65.92, 56.87, 45.25, 37.98. ESI-MS: $C_{24}H_{23}N_6O_4$ $[M + H]^+$, Calculated: 459.2, Found: 459.1.

Determination of the fluorescence quantum yield

The fluorescence quantum yield Φ_u was estimated by the participation ratio method, where using the ethanol solution of rhodamine B (10 μM, $\Phi_s = 0.69$, $\lambda_{ex} = 365$ nm) as a standard according to a published method using the following equation:

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

Φ_s is the quantum yield of the reference substance, A_s and A_u represent 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 determination and reference, respectively. The absorbance should be controlled to be less than 0.05 in the detection process.

SNF: $\Phi = 0.012$; ANF: $\Phi = 0.44$

The detection limit of the probe

The emission spectrum of 10 μM SNF in PBS (10 mM, 5% DMSO, 1mM CTAB, pH 7.4) was collected for 20 times, and the background noise σ was determined. The linear regression curve was then fitted according to the data of NaHS in the range of 0-10 equivalents to obtain the slope of the curve, then the detection limit was calculated using the following equation:

The limit of detection (LOD) = 3σ/k

TLC analysis

TLC analysis of SNF after incubation without or with NaHS at 37 °C was performed on a TLC plate. The unfolding agent system used for the separation was as follows: MeOH/CH_2Cl_2: 10/90 (v/v).

Measurement of lipophilicity

Lipophilicity was expressed as log $P_{o/w}$ values, which was determined by the shaking flask 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 200 rpm to allow partitioning. After the sample was centrifuged at 8000 rpm for 10 min, the probe content in 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 cells). 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%), which was considered to be 100% viable.

Cell cycle analysis

U87MG cells were seeded in 6-well cell plates and incubated at 37 °C for 18 h in an atmosphere of 5% CO_2 and 95% air, and then added the desired concentration of SNF. After treatment with SNF for 24 h at 37 °C, cells were harvested, washed twice with cold PBS, and fixed overnight at 4 °C with 70% ethanol. 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 twice with PBS, and subjected to flow cytometry analysis.

Western blot analysis

U87MG cells were treated with the desired concentration of SNF. After 24 h, cells were collected and washed twice with ice-cold PBS. Cell pellets were lysed in 50-100 μL RIPA lysis buffer containing 1 mM PMSF for several minutes on ice. Cell debris was removed by centrifugation at 13,000 rpm for 20 min at 4 °C. The protein-containing supernatant was collected, and the total protein content of each sample was quantified by using the BCA Protein Assay Kit (Beyotime). Approximately 30-40 micrograms of proteins from each sample were mixed in the loading buffer [100 mM DTT, 1×protein loading dye] and heated at 95 °C for 5 min. Electrophoretic analysis of protein samples 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 in the buffer (5% skim milk/0.1% Tween-20/PBS) for 1 h at room temperature. The membranes were incubated with the primary antibodies of interest overnight at 4 °C. Then, 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 the amount of internal reference GAPDH and Tublin expression. The Western blotting signal was enhanced with Tanon High-sig ECL Western Blotting Substrate and visualized by Tanon 5200 Multi.

Viability assay of three-dimensional (3D) tumor spheroids

Spheroids were prepared from U87MG cells by seeding 1,500 cells/well in Ultra-Low Attachment 96-well plates (Corning). The 3-day spheroids were incubated in a normal medium with the desired concentration of SNF and cisplatin. The spheroid growth was monitored using a live-cell phase-contrast microscope (Axio Observer, Zeiss).

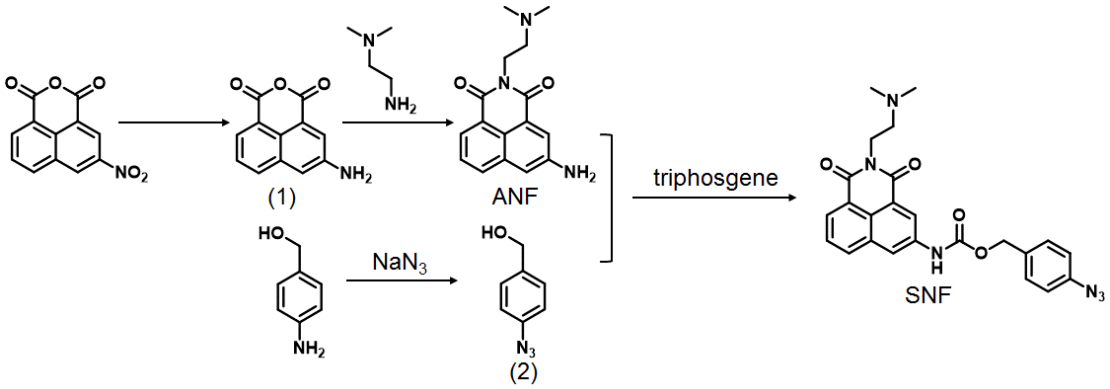
Imaging of 3D tumor spheroids

Spheroids were prepared from U87MG cells by seeding 1,500 cells/well in Ultra-Low Attachment 96-well plates (Corning). For imaging, the 3-day spheroids were incubated in a normal medium with the desired concentration of SNF for 48 h. Spheroids were then washed twice with PBS, stained with AO/EB according to the manufacturer's instructions, and fixed in 4% paraformaldehyde. The spheroids were placed in a glass-bottom dish and imaged at different depths (z-stacking) with confocal scanning microscopy (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 SNF against U87MG, with cisplatin as the control.

	IC_{50}/ μM		
Time	ANF	SNF	cisplatin
24 h	57.9 ± 3.0	7.9 ± 0.1	87.6 ± 4.7
48 h	10.7 ± 0.1	5.3 ± 0.1	10.9 ± 0.5
72 h	8.0 ± 0.1	4.8 ± 0.1	4.9 ± 0.1



Scheme S1. Chemical synthesis of SNF.

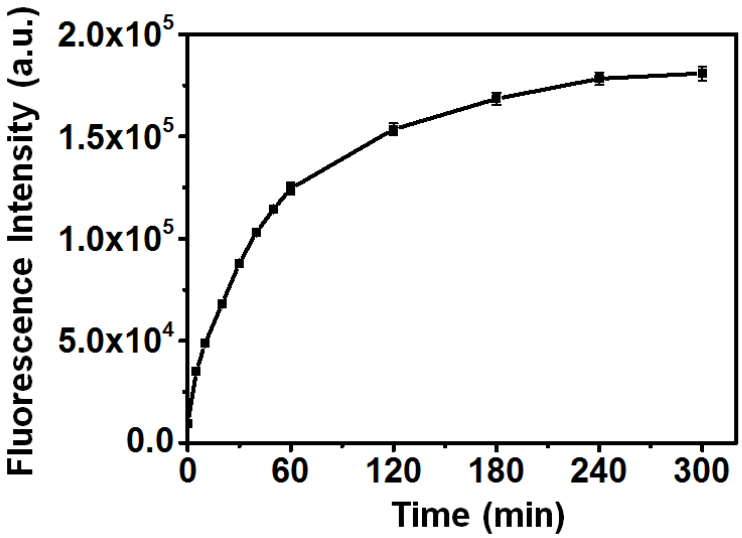


Fig. S1. Time-dependent fluorescence intensity of SNF (10 μM) incubated with NaHS (100 μM) in PBS buffer (10 mM, 5% DMSO, 1mM CTAB, pH 7.4) at 37 °C. All the data represent the average of three independent experiments. The changes of fluorescence intensity were recorded at 598 nm. Excitation: 415 nm, slit/slit: 5 nm/5 nm.

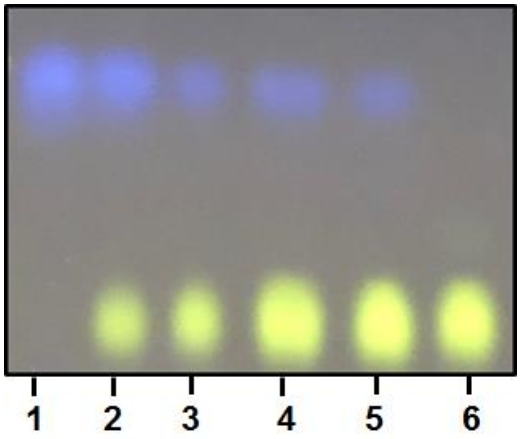


Fig. S2. TLC analysis of SNF (10 μM) after incubation with NaHS (0 μM, **1**; 50 μM, **2**; 100 μM, **3**; 150 μM, **4**; 200 μM, **5**) at 310 K for 1 h, with ANF (10 μM, **6**) as reference under a hand-held 365 nm lamp.

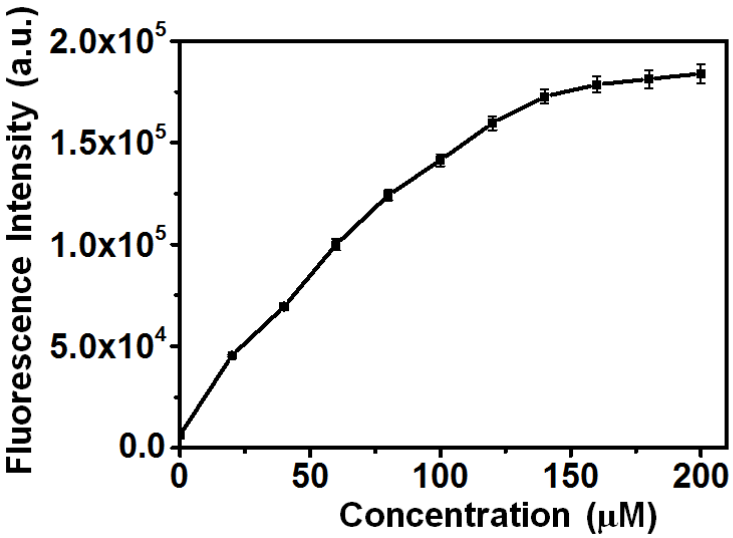


Fig. S3. Fluorescence responses of SNF (10 μM) towards increasing concentrations of NaHS (0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 μM) in PBS buffer (10 mM, 5% DMSO, 1mM CTAB, pH 7.4) for 1 h. All the data represent the average of three independent experiments. The changes of fluorescence intensity were recorded at 598 nm. Excitation: 415 nm, slit/slit: 5 nm/5 nm.

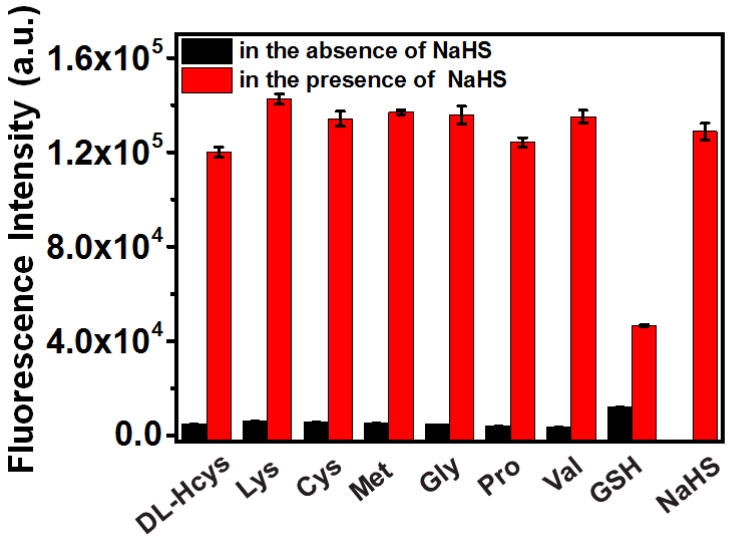


Fig. S4. Fluorescence intensity of SNF (10 μM) in the absence/presence of NaHS (100 μM) under the condition of various amino acids in PBS buffer (10 mM, pH 7.4, 5% DMSO) at 37 °C for 1 h. In the absence of NaHS: DL-Hcys (100 μM); Lys (100 μM); Cys (100 μM); Met (100 μM); Gly (100 μM); Pro (100 μM); Val (100 μM)); GSH (1 mM); In the present of NaHS: DL-Hcys (1 mM); Lys(1 mM); Cys (1 mM); Met (1 mM); Gly (1 mM); Pro (1 mM); Val (1 mM); GSH (1 mM). All the data represent the average of three independent experiments. The changes of fluorescence intensity were recorded at 598 nm. Excitation: 415 nm, slit/slit: 5 nm/5 nm.

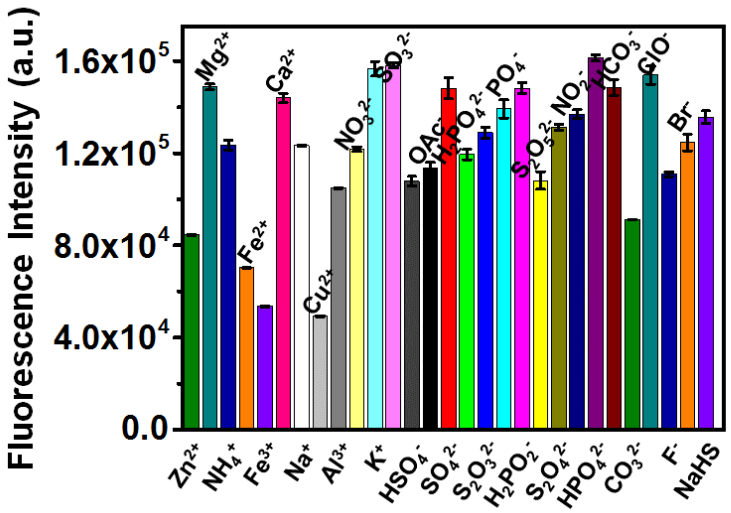


Fig. S5. Fluorescence intensity of SNF (10 μM) towards NaHS (100 μM) in the presence of reactive oxygen species (200 μM ClO^-), reactive nitrogen species (200 μM NO_2^-), sulfur-containing inorganic ions (200 μM SO_3^{2-}, $S_2O_3^{2-}$, $S_2O_5^{2-}$, $S_2O_4^{2-}$) and other inorganic salts (1 mM NaCl, 1 mM KCl, 200 μM KF, 200 μM KBr, 100 μM $FeCl_2$, 100 μM $FeCl_3$, 100 μM $CuCl_2$, 100 μM $ZnSO_4$, 1 mM $MgCl_2$, 1 mM $AlCl_3$, 1 mM $CaCl_2$, 200 μM Na_2CO_3, 200 μM $NaHCO_3$, 200 μM $NaNO_3$, 200 μM NH_4Cl, 200 μM Na_2SO_4, 200 μM $NaHSO_4$, 200 μM NaH_2PO_4, 200 μM Na_2HPO_4, 200 μM NaH_2PO_2, 200 μM Na_3PO_4, 100 μM NaOAc). All the data represent the average of three independent experiments. The changes of fluorescence intensity were recorded at 598 nm. Excitation: 415 nm, slit/slit: 5 nm/5 nm.

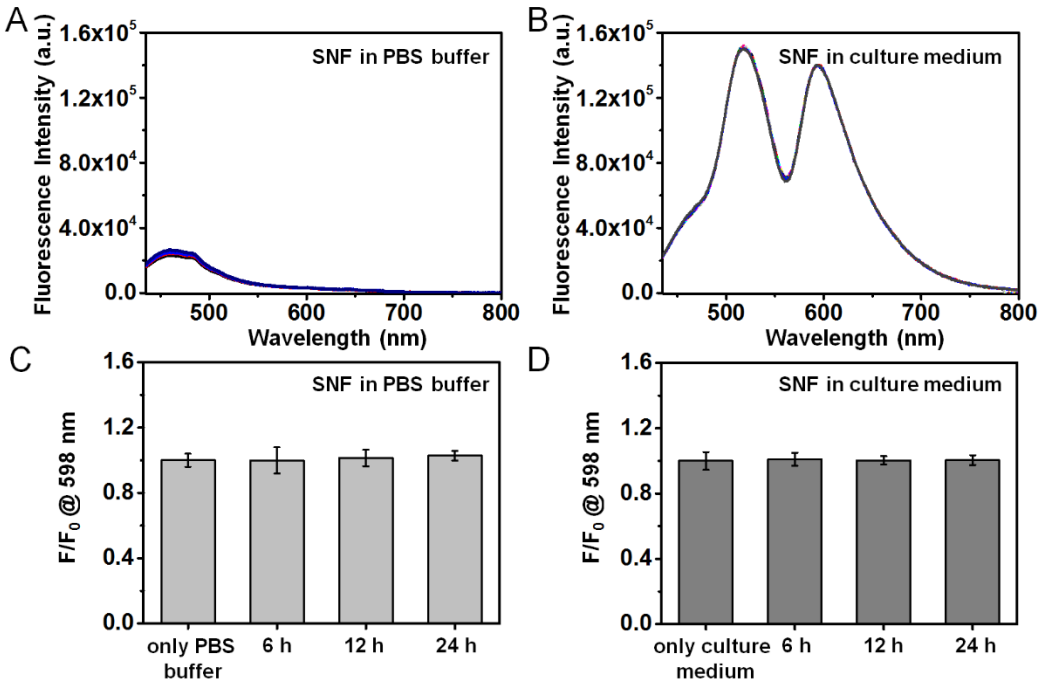


Fig. S6. (A-B) Stability studies of SNF (10 μM) in PBS buffer (10 mM, 5% DMSO, 1mM CTAB, pH 7.4) and culture medium (10% fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin) after incubation for 6, 12, 24 h, respectively. (C-D) The ratio of F (fluorescence intensity of different time for incubation) to F_0 (fluorescence intensity of only PBS buffer or culture medium). All the data represent the average of three independent experiments. The changes of fluorescence intensity were recorded at 598 nm. Excitation: 415 nm, slit/slit: 5 nm/5 nm.

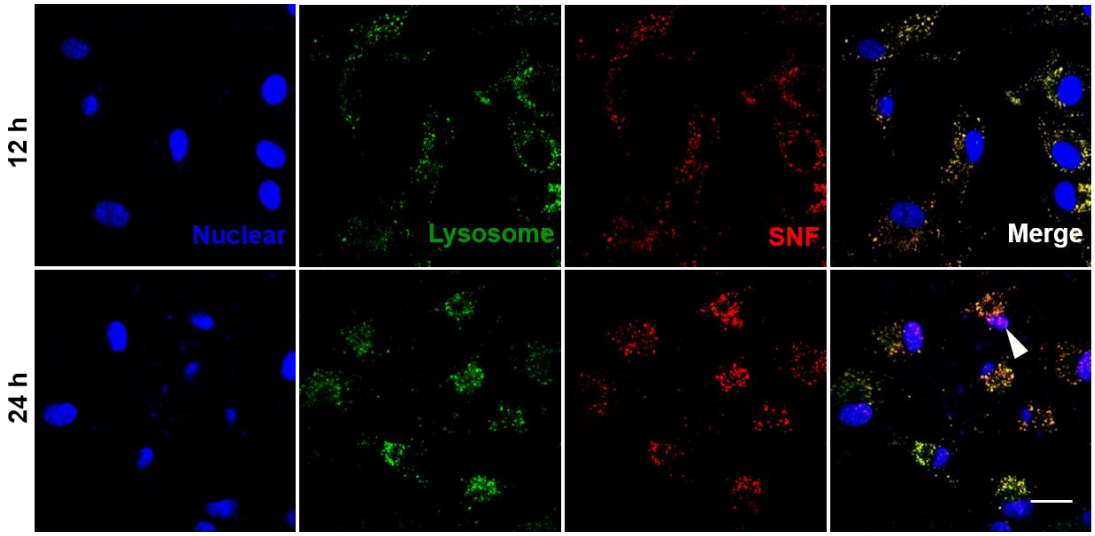


Fig. S7. Monitoring of the location and release of chemotherapeutic drug in living U87MG cells. Cells were treated with 2.5 μM SNF (Excitation: 405 nm, collected at 570-620 nm) for 12 h or 24 h. All the tested cells were stained with 2 μM LysoTracker Green (Excitation: 488 nm, collected at 500-550 nm) and 1X Hoechst33324 (Excitation: 405 nm, collected at 425-475 nm). The white arrow represents the transportation of activated SNF from lysosomes to the nucleus. Scale bar: 20 μm.

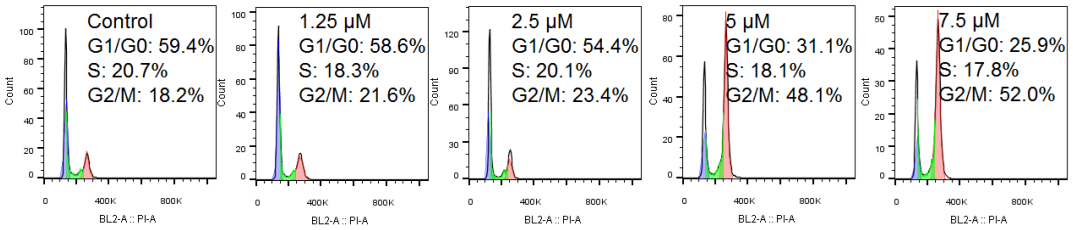


Fig. S8. Flow cytometry analysis of cell cycle changes. Flow cytometry data of cell cycle distribution of U87MG cells after treatment with different concentrations of SNF (0-7.5 μM) for 24 h.

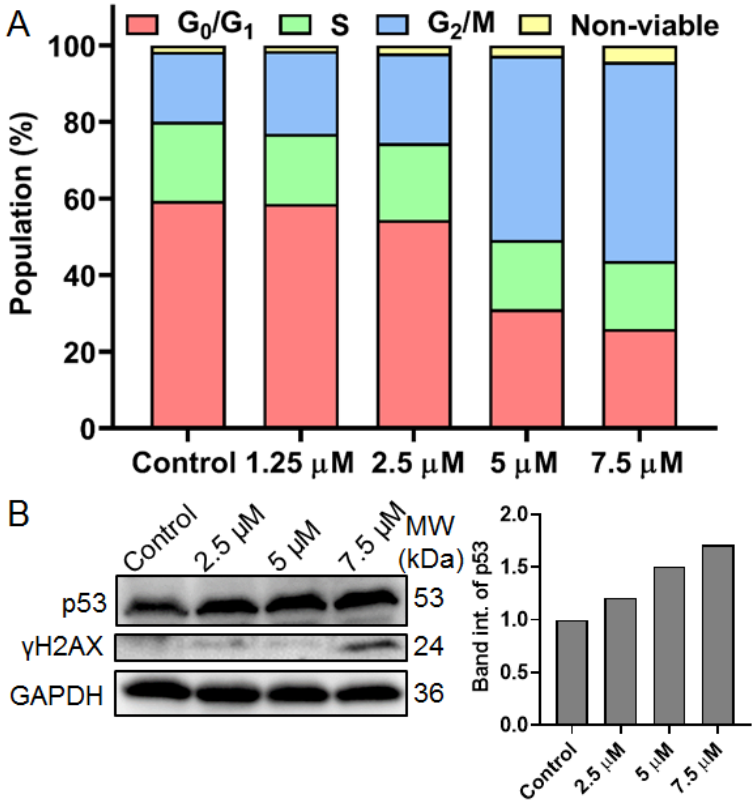


Fig. S9. (A) Effect of SNF on the cell cycle of U87MG cells after incubation for 24 h. (B) U87MG cells were treated with the indicated doses of SNF for 24 h. The expression levels of p53 and γH2AX in U87MG cells were determined by Western blot.

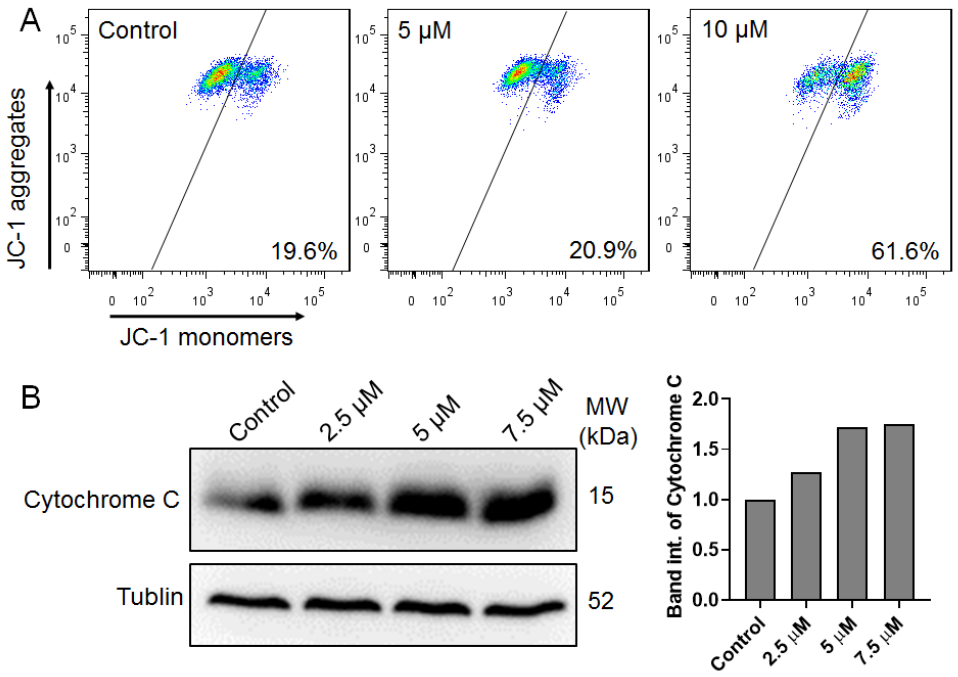


Fig. S10. (A) Flow cytometry quantification of JC-1-labeled U87MG cells treated with SNF (5 μM and 10 μM) for 12 h. (B) Immunoblotting of cytochrome C expression levels in U87MG cells treated with SNF for 24 h.

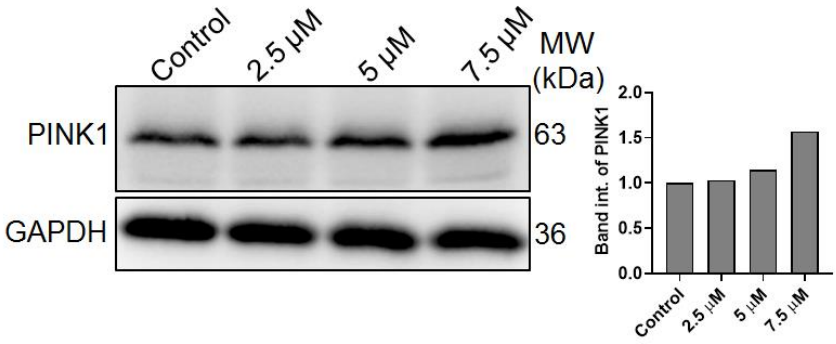


Fig. S11. Immunoblotting of PINK1 expression levels in U87MG cells treated with SNF for 24 h.

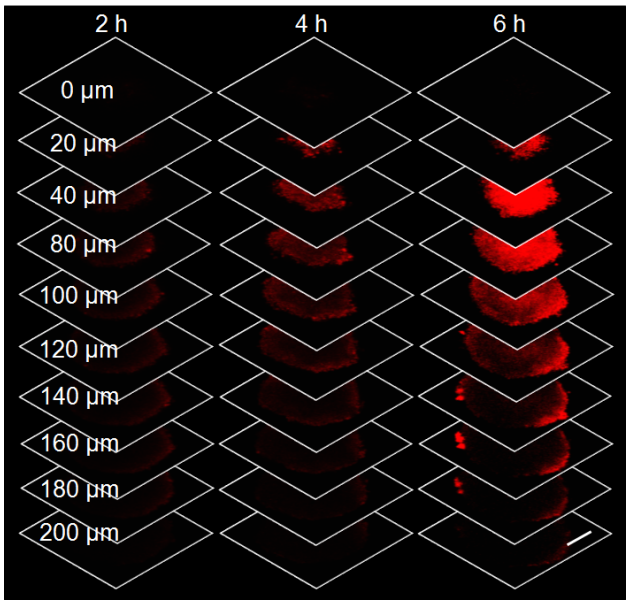


Fig. S12. Imaging U87MG tumor spheroids at different depths. Tumor spheroids were treated with SNF (20 μM) and then incubated for different times. After incubation, spheroids were fixed with 4% paraformaldehyde and then observed with confocal microscopy (Excitation: 405 nm, collected at 570-620 nm). Scale bar = 100 μm.

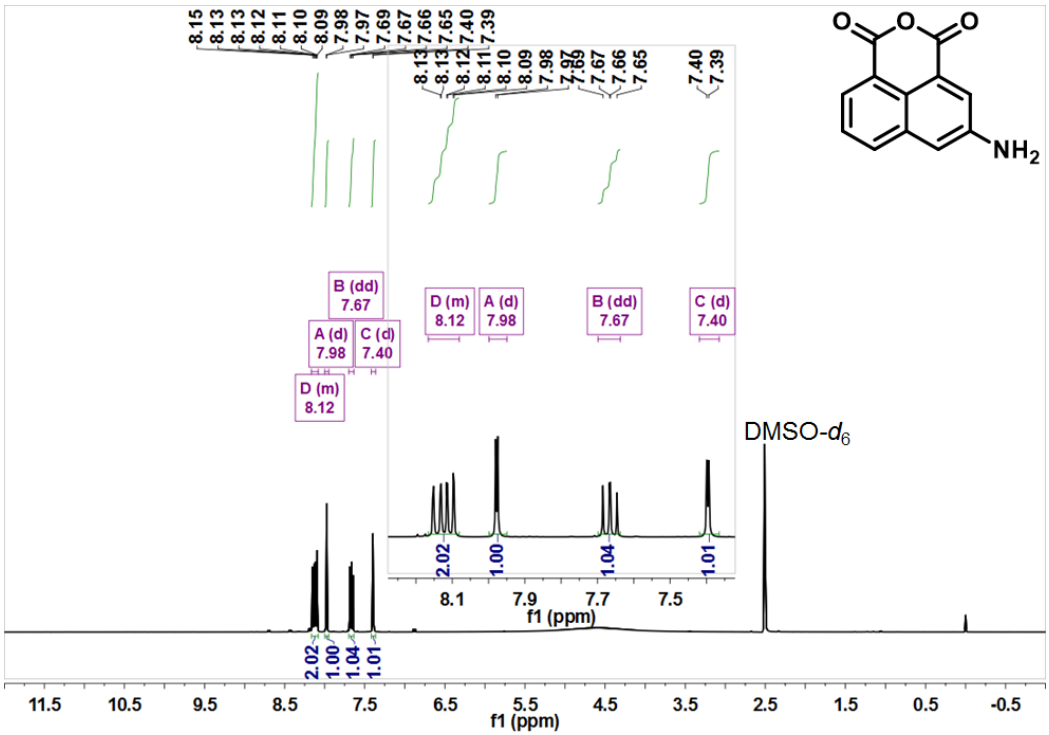


Fig. S13. ^{1}H NMR spectrum of 3-amino-1,8-naphthalic anhydride (1).

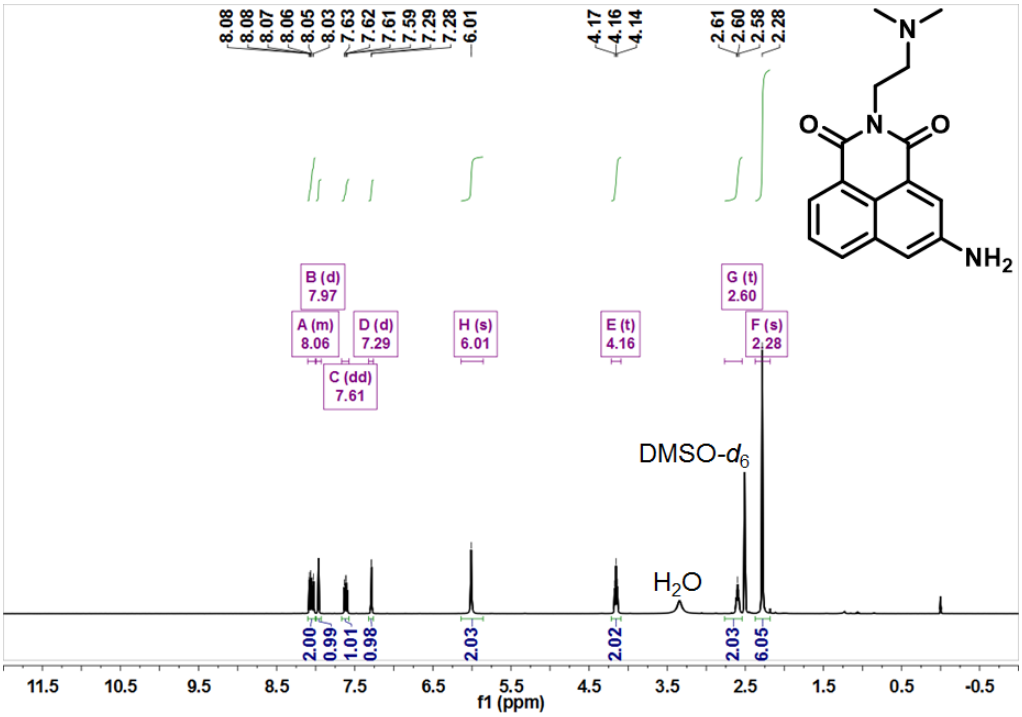


Fig. S14. ^{1}H NMR spectrum of amonafide (ANF).

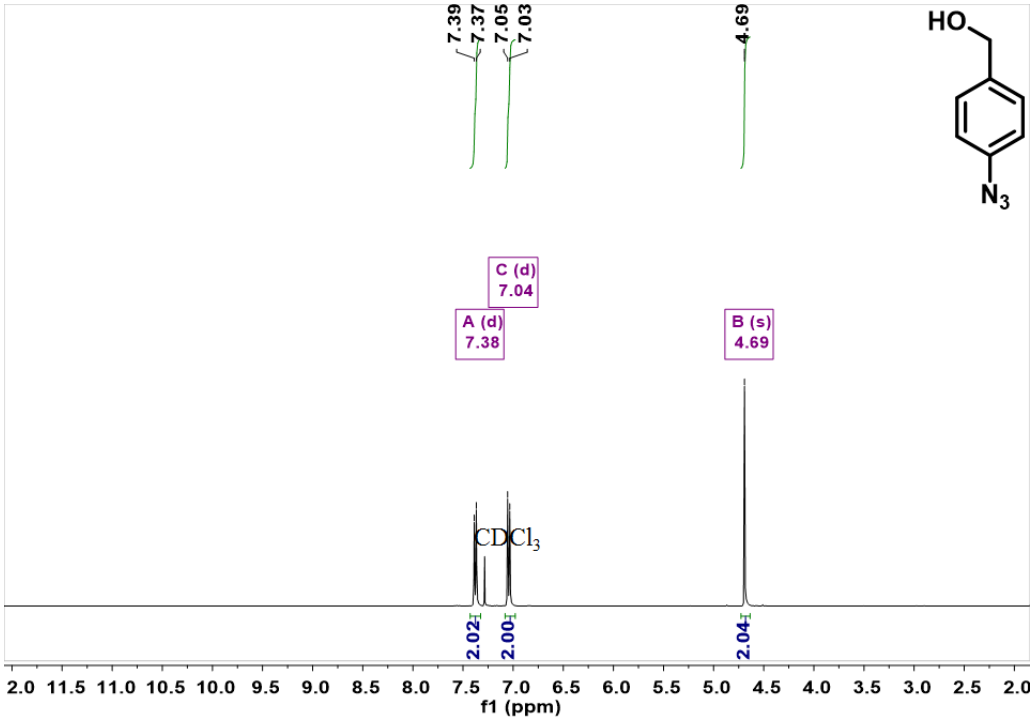


Fig. S15. ^{1}H NMR spectrum of 4-azidobenzyl alcohol (2).

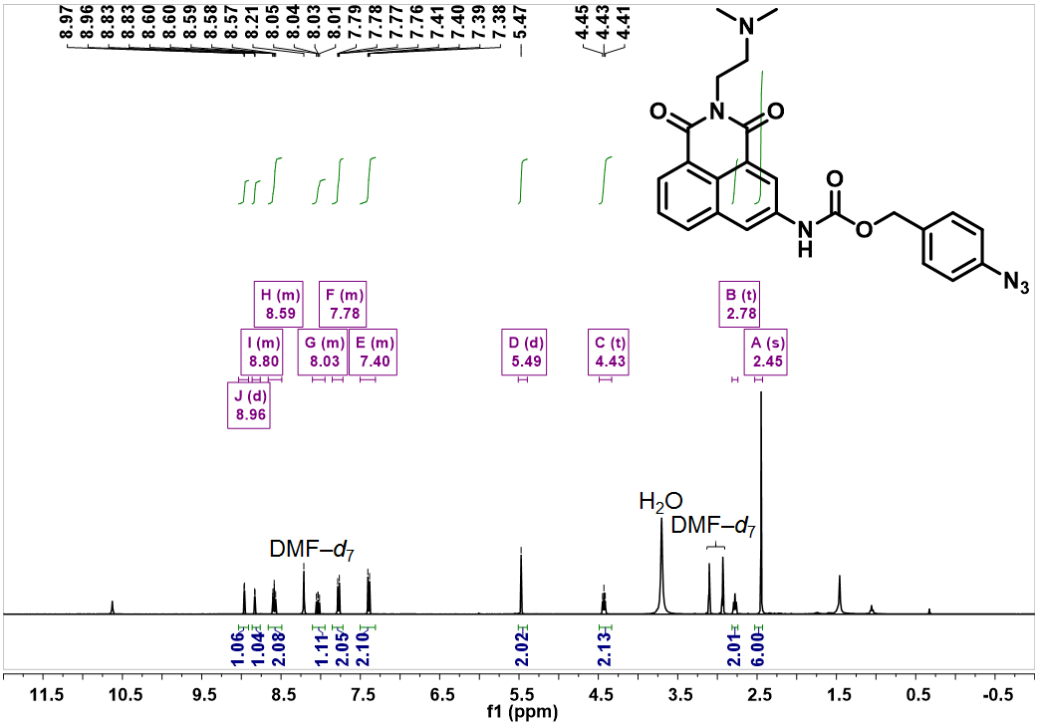


Fig. S16. ^{1}H NMR spectrum of SNF.

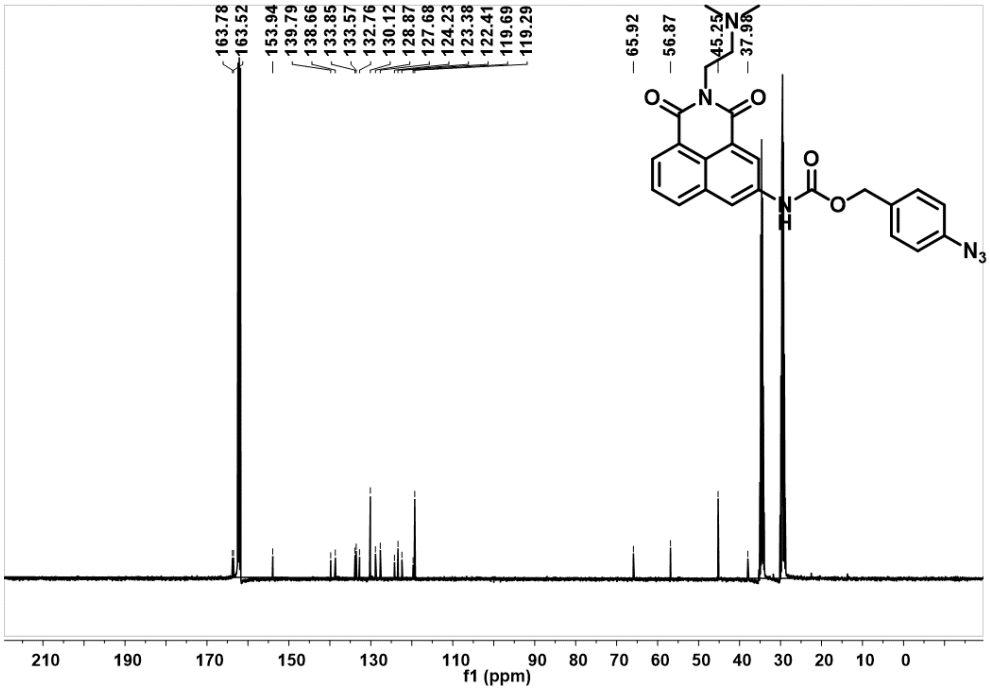


Fig. S17. ^{13}C NMR spectrum of SNF.

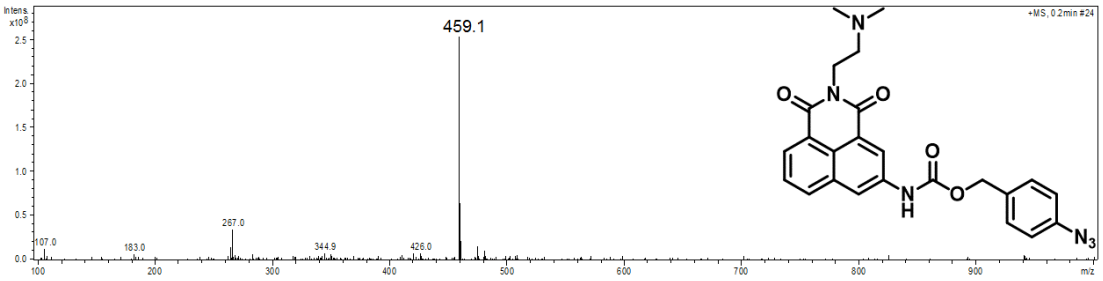


Fig. S18. ESI-MS spectrum of SNF.