Supporting Information

Carrier-free single-molecule hypoxia-activated nanoprodrug of SN38 with ultrahigh drug loading for pancreatic cancer treatment

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Materials and Methods

Materials

4-Nitrobenzyl alcohol, zinc powder, sodium hydroxide, thionyl chloride, potassium carbonate, phosphate-buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and silica gel (spherical, 100 μm) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Rat liver microsome (RLM), SN38, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from EMD Millipore Corp. (Billerica, MA, USA). Gibco Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco^{TM}), fetal bovine serum (FBS), penicillin-streptomycin (PS), trypsin-EDTA, and Hoechst 33342 were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Deuterated chloroform was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All of the solvents used in this research were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Synthesis of compound 1

Zinc powder (2.6 g, 40 mmol) was added to a solution of 4-nitrobenzyl alcohol (1.5 g, 10 mmol) dissolved in 30 mL of aqueous NaOH (20%). The reaction mixture was stirred for 12 h at 70 ℃. After cooling to room temperature, the mixture was filtered and washed with DI water. The residue was recrystallized in methanol, and an orange solid was obtained as the product (0.92 g, 76%). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 7.87 (d, J = 7.8 Hz, 4H), 7.54 (d, J = 7.8 Hz, 4H), 5.38 (t, J = 5.3 Hz, 2H), 4.61 (d, J = 5.3 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 151.33, 146.74, 127.60, 122.83, 62.92.

Synthesis of compound 2

To compound **1** (242 mg, 1 mmol) stirring in anhydrous DCM (6 mL), $SOCl_2$ (365 μL, 5 mmol) was added slowly. The reaction was stirred at room temperature for 6 h and then condensed. After washing with DI water, the residue was recrystallized in methanol. The product was obtained as an orange solid (268 mg, 96%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.91 (d, J = 7.4 Hz, 4H), 7.54 (d, J = 7.4 Hz, 4H), 4.66 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 152.38, 140.40, 129.41, 123.30, 45.65.

Synthesis of SN38-Azo1

A mixture of compound **2** (56 mg, 0.2 mmol), SN38 (235 mg, 0.6 mmol), and K_2CO_3 (69 mg, 0.5 mmol) in 5 mL of DMF was stirred at 80 ℃ for 4 h. After condensation, the residue was washed with DI water. The crude product was purified by silica gel column chromatography using a mixed eluent of methanol and DCM to afford the product as an orange solid (78 mg, 39%). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.12 (d, J = 9.4 Hz, 2H), 7.96 (d, J = 8.1 Hz, 4H), 7.79 (d, J = 8.1 Hz, 4H), 7.64 (d, J = 7.9 Hz, 4H), 7.28 (s, 2H), 6.50 (s, 2H), 5.49 (s, 2H), 5.43 (s, 4H), 5.29 (s, 4H), 3.19 (d, J = 7.0 Hz, 4H), 1.86 (q, J = 7.2 Hz, 4H), 1.28 (t, J = 7.0 Hz, 6H), 0.89 (t, J = 7.2 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 173.01, 157.41, 157.29, 151.91, 150.55, 150.04, 146.65, 144.91, 144.39, 140.72, 131.92, 129.07, 128.70, 128.17, 127.62, 123.19, 123.10, 122.93, 118.71, 104.16, 96.63, 72.86, 69.62, 65.69, 49.92, 30.73, 22.71, 13.84, 8.19.

Fabrication of SN38-Azo1-NPD

SN38-Azo1-NPD was prepared through a nano-assembling procedure, as described in our previous study.[1] The SN38-Azo1 prodrug (5 mg) dissolved in 10 mL of DMSO was dispersed into 120 mL of DI water under robust stirring (1000 rpm) and was kept stirring overnight. The prodrug molecules were self-assembled into nanoparticles spontaneously, which were subsequently dialyzed in Spectra/Por® dialysis tube (MWCO: 6-8 kDa) for 24 h. The collected nanoparticles were filtered through a 0.45 μm syringe filter and stored at 4 °C for future use. The particle size, size distribution (polydispersity index, PDI), and surface charge were measured by DLS and zeta potential measurement using a Zetasizer (Nano ZS, Malvern Instruments Ltd., Malvern, UK). The spherical morphology of the nanostructures was observed using Hitachi HT7800 transmission electron microscopy (TEM, Hitachi High-Technologies Corporation, Tokyo, Japan).

To measure the drug-loading efficiency of the nano-assembling process, all the collected NPDs were completely dried through lyophilization. The resulting solid was weighed, and the drug-loading efficiency of SN38-Azo1-NPD was calculated by the following equation.

$$\text{Drug loading efficiency (\%)} = \frac{\text{weight of the prodrug in the NPDs}}{\text{weight of the prodrug fed}} \times 100\%$$

As SN38-Azo1-NPD is a single-molecule NPD, its drug-loading content was calculated based on its chemical composition using the following equation.

$$\text{Drug loading content (\%)} = \frac{\text{weight of the drug in the NPDs}}{\text{weight of the NPDs}} \times 100\%$$

Colloidal stability

The colloidal stability of SN38-Azo1-NPD was obtained by DLS analysis. NPDs dispersed in PBS buffer (pH 7.4) or PBS buffer containing 10% FBS (pH 7.4) were incubated at 37 °C. The hydrodynamic size of the NPDs was monitored with DLS after predetermined time intervals.

Drug release

The release profiles of SN38 from SN38-Azo1-NPD were studied in degassed PBS buffer solutions (pH 7.4) containing 20% (v/v) ethanol as the release media. The NPDs (equivalent to 200 nmol of SN38) were incubated in the release media with and without FBS, NADPH, RLM, and NADPH+RLM in the dark. At preselected time points, 1 mL of the release media was sampled and immediately analyzed by high-performance liquid chromatography (HPLC). The chemical species generated in the final release media were analyzed by high-resolution mass spectrometry (HRMS).

Cell culture

Human pancreatic cancer cells (AsPC-1, BxPC-3, and PANC-1) were cultured in RPMI 1640 cell culture medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in 100 mm cell culture dishes at 37 °C under a humidified atmosphere of 5% CO_2. Cells were subcultured when the cell confluence attained 70-80%.

Cell viability assay

The cytotoxicity of SN38-Azo1-NPD was evaluated by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were cultured at a density of 5,000 cells/well in 96-well plates at 37 °C with 5% CO_2 for 24 h prior to the test. In the tests, cells were incubated in a fresh cell culture medium containing SN38 or the NPD at varied concentrations for 24 h under normoxic (5% CO_2 + 20% O_2 + 75% N_2) or hypoxic (5% CO_2 + 1% O_2 + 94% N_2) conditions. Cells in the control group were incubated in a fresh medium with no drug treatment. Then, the medium was replaced with a solution of the MTT reagent (1 mg/mL) in a fresh medium. After incubation for another 4 h, the cell culture medium was removed carefully, and DMSO (100 μL) was added to each well of the plate. The plate was gently shaken to dissolve the purple crystals generated by the MTT reagent. The optical density of the plates was recorded at 570 nm on a microplate reader (BioTek Synergy H1 multimode microplate reader).

Cell apoptosis assay

Cell apoptosis induced by SN38-Azo1-NPD was studied by live & dead cell staining. Cells were cultured at a density of 5,000 cells/well in 96-well plates at 37 °C with 5% CO_2 for 24 h prior to the test. In the tests, cells were incubated in a fresh cell culture medium containing the NPD under hypoxic (5% CO_2 + 1% O_2 + 94% N_2) conditions. Cells in the control group were incubated in a fresh medium. After incubation for 12 h, cells were stained with calcein acetoxyl methyl ester (Calcein-AM) and propidium iodide (PI) and then imaged by a fluorescence microscope.

Confocal fluorescence imaging

Fluorescence images of cells incubated with SN38-Azo1-NPD were recorded by confocal laser scanning microscopy (CLSM). Cells were seeded at a density of 200,000 cells/dish in a 35 mm^2 Petri dish with a glass window at the bottom with 5% CO_2 at 37 °C for 24 h. After washing with PBS (pH 7.4), cells were separately incubated with Hoechst 33342 and SN38-Azo1-NPD for 1 h. Cells cultured in fresh cell culture medium were used as a control. Then, all cells were washed with PBS three times and fixed with paraformaldehyde (4% in PBS) for 10 min at room temperature. After the removal of paraformaldehyde/PBS, cells were further washed with fresh PBS. At last, cells were imaged by a confocal microscope (Olympus FV3000 Laser Scanning Confocal Microscope).

Animal model

All animal experiments were conducted following NIH regulations and approved by the Institutional Animal Care and Use Committee of the University of North Dakota (IACUC2306-0044). A xenograft tumor model of BxPC-3 cancer was established in athymic BALB/c mice (8-10 weeks old, ~20 g, Jackson Laboratories). In brief, BxPC-3 cells suspended in RPMI 1640 culture medium (5,000,000 cells/100 μL) were inoculated subcutaneously to mice. The tumor volume was measured by a digital caliper and calculated using the following formula: Tumor volume = 0.5 × (tumor length) × (tumor width) × (tumor width). Tumor growth was monitored every other day. The animals were observed for body weight change and any sign of pain every other day throughout the duration of the experiments.

In vivo biodistribution

To study the biodistribution of SN38-Azo1-NPD in the body, tumor-bearing mice were divided randomly into two groups (n = 5 for each group), including an NPD-treated group and a control group. Mice were intravenously injected with SN38-Azo1-NPD contained in PBS at a dose of 1 mg/kg in the experimental group and PBS alone in the control group. The mice were sacrificed at 6 h post-injection, and their tumors and major organs (heart, liver, spleen, lungs, and kidneys) were excised for ex vivo imaging. The fluorescence emission of organ and tumor tissues was recorded by a noninvasive Lago X whole-body imaging system (Spectral Instruments Imaging Inc.).

In vivo therapeutic efficacy

When the tumor volume reached 100 mm^3, tumor-bearing mice were randomly assigned into three groups (n = 5 for each group), including a control group and two experimental groups, with each mouse individually marked and weighed prior to treatment. Mice were intravenously administered with varying treatments, including PBS buffer (the control group), SN38 at a dose of 5 mg/kg, and SN38-Azo1-NPD (5 mg/kg equivalent to SN38). The volume of tumors in mice was measured every three days until the end of the experiment. The treatments were given to mice twice per week. After three weeks, all mice were euthanized, and the tumor tissues and main organs of the mice were collected for further analysis.

Histological analysis

The collected main organs (heart, liver, spleen, lungs, and kidneys) and tumors were fixed in a paraformaldehyde solution (4% in PBS) and then embedded in optimal cutting temperature (OCT) gel. The OCT gels were frozen and sectioned into 5 μm thickness, followed by staining with hematoxylin and eosin (H&E) and imaging under a light microscope. The histological analysis was carried out in a blinded fashion by professional personnel at the University of North Dakota.

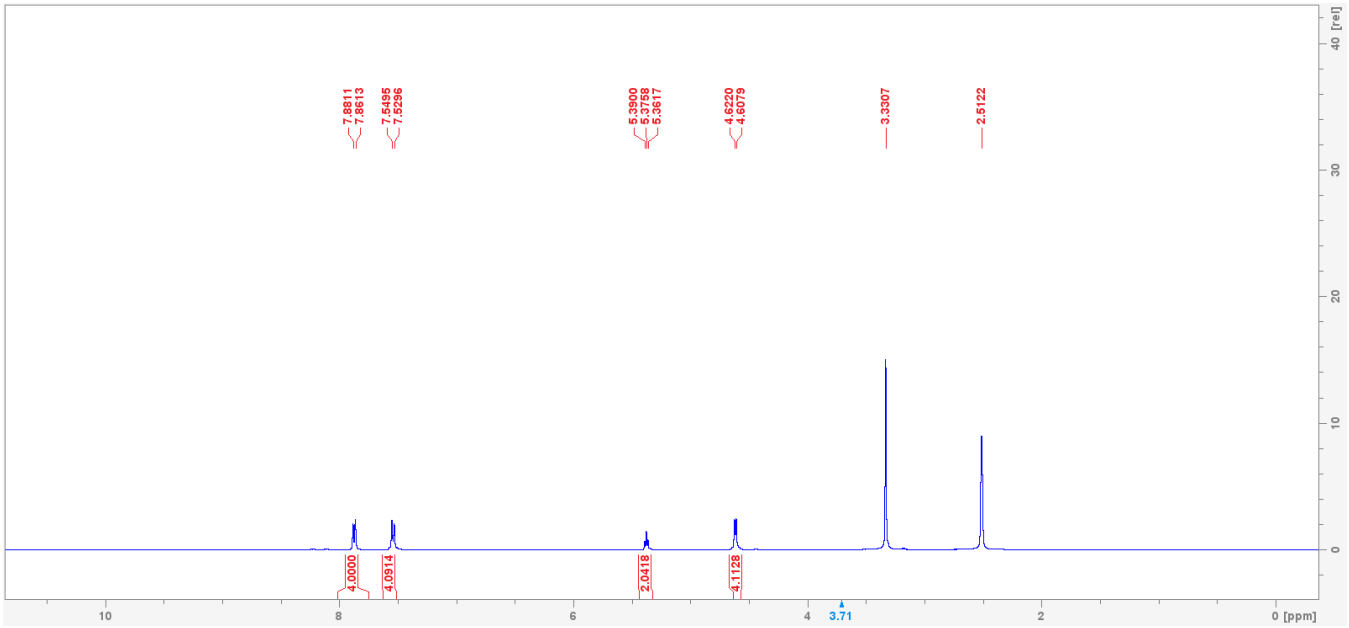

Figure S1. ^{1}H NMR spectrum of compound **1**.

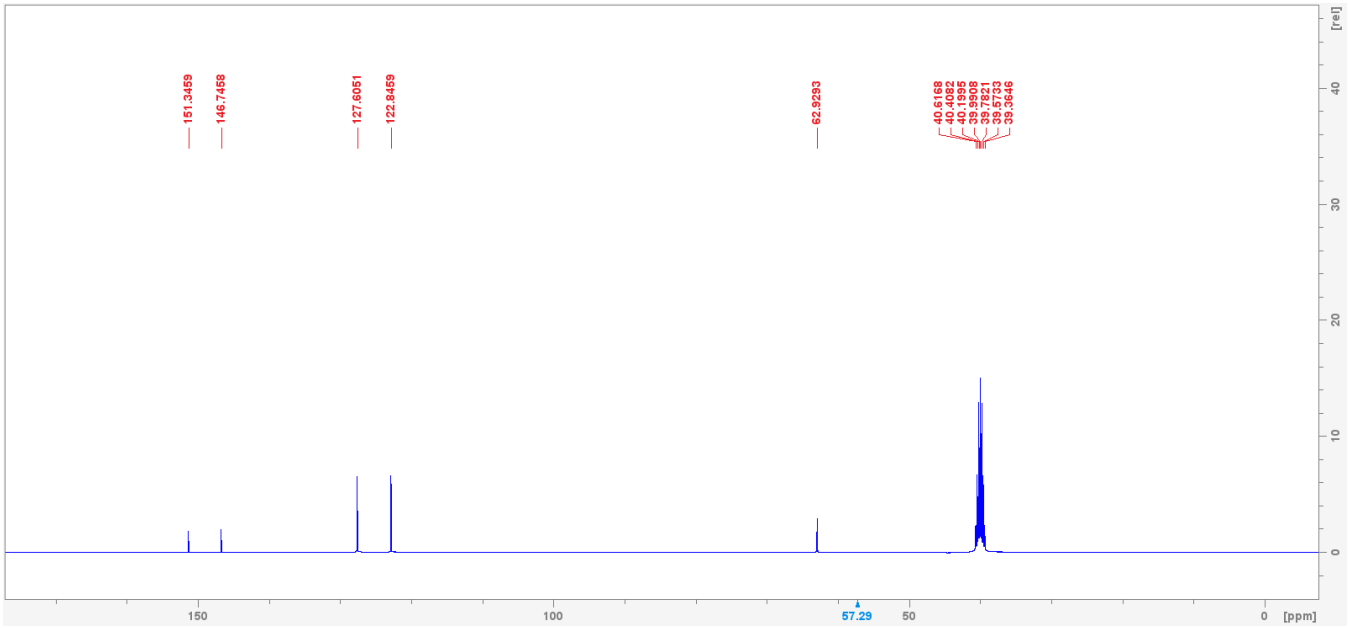

Figure S2. ^{13}C NMR spectrum of compound **1**.

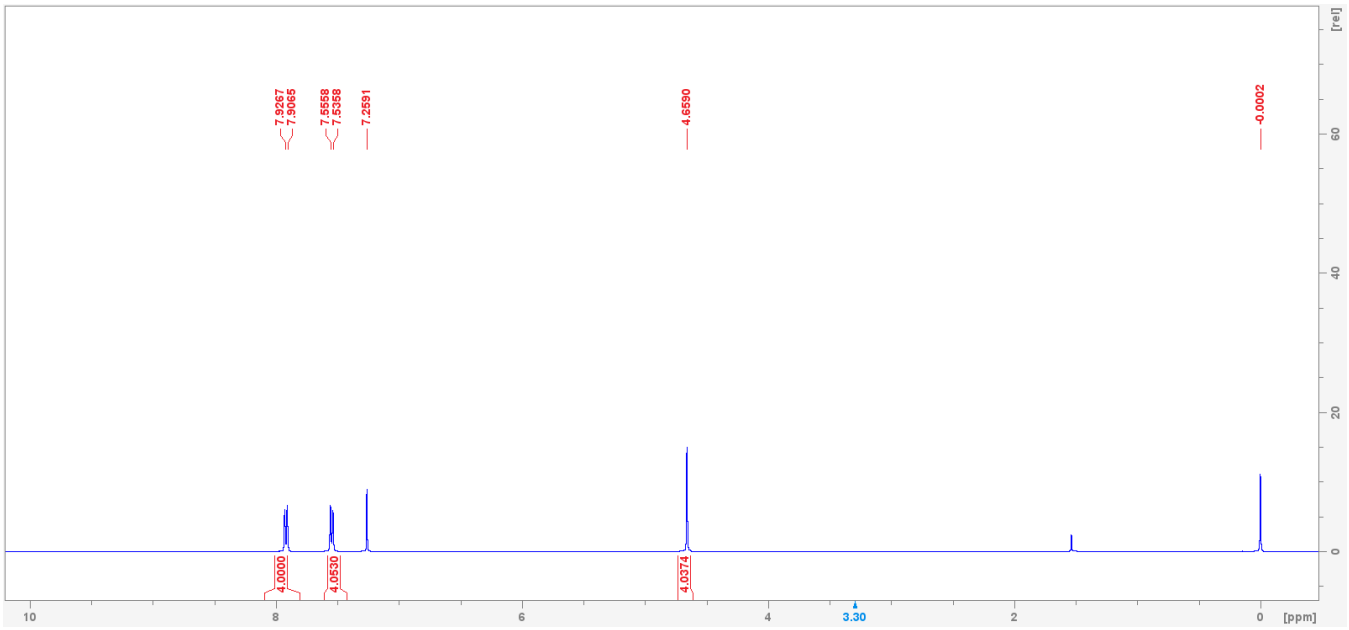

Figure S3. ^{1}H NMR spectrum of compound **2**.

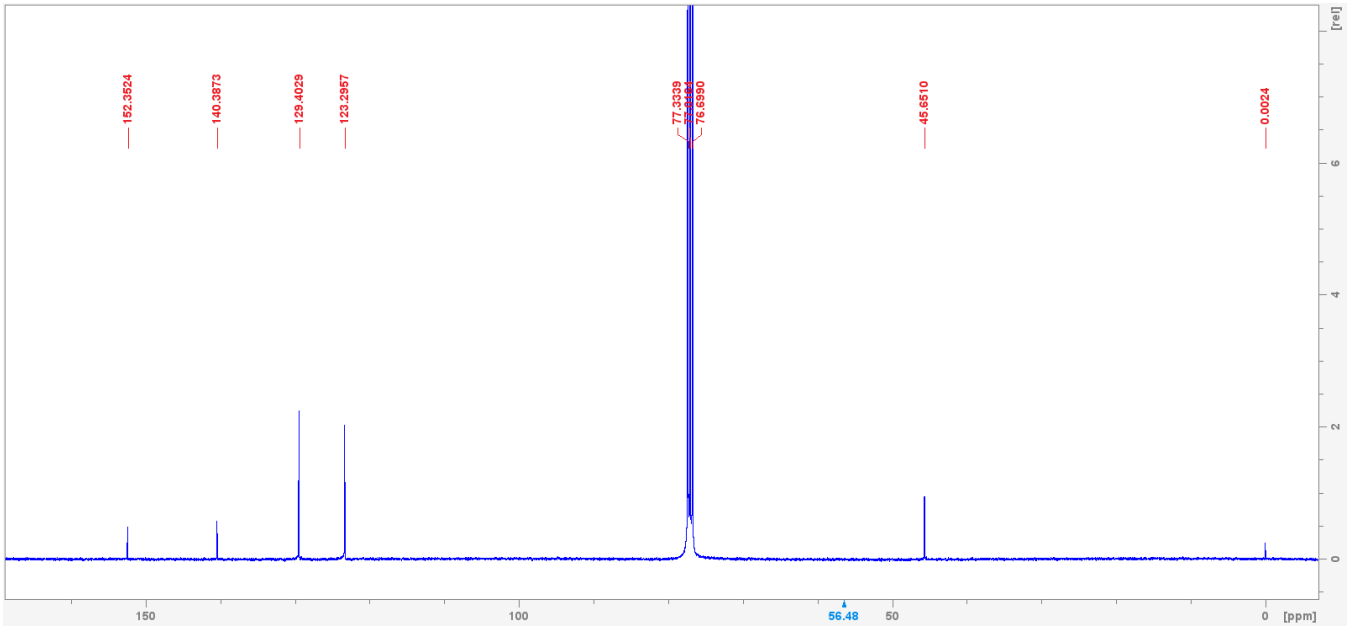

Figure S4. ^{13}C NMR spectrum of compound **2**.

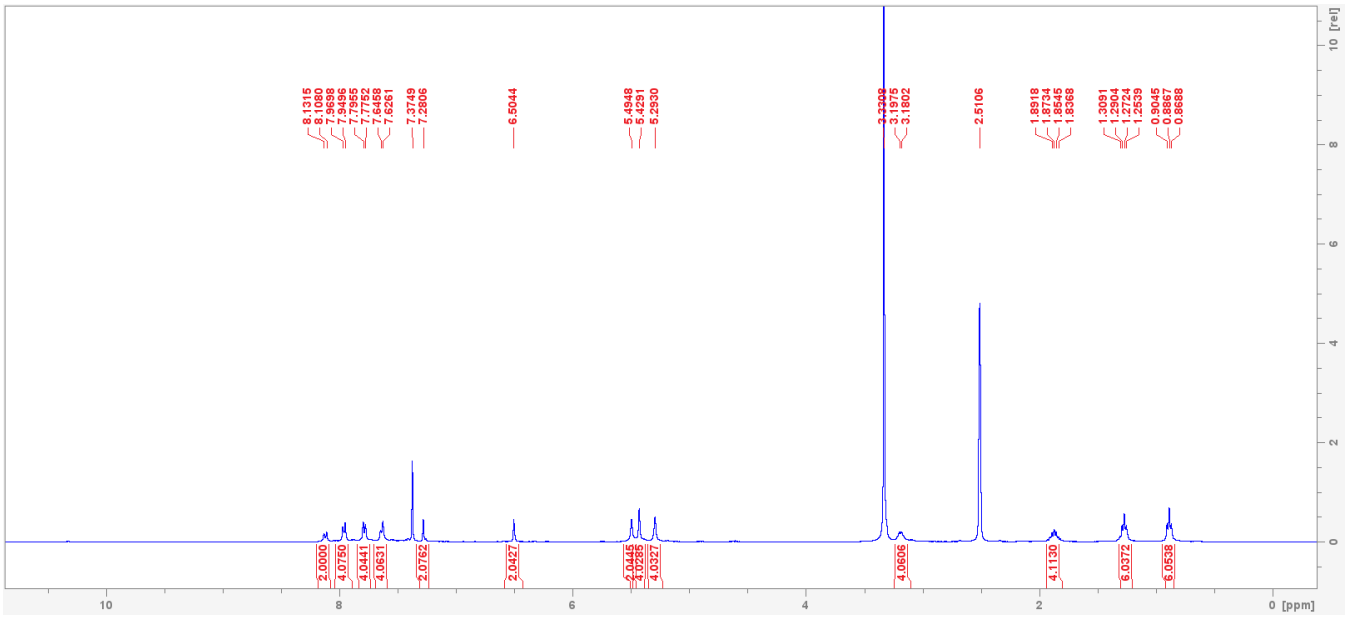

Figure S5. ^{1}H NMR spectrum of SN38-Azo1.

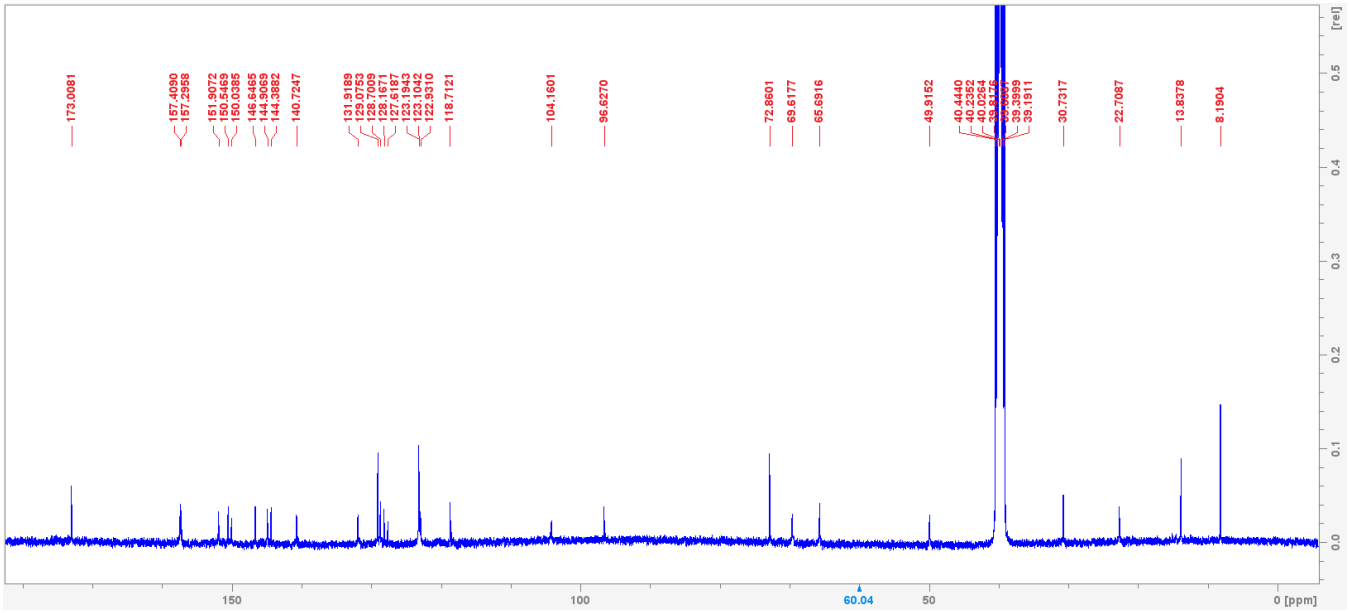

Figure S6. ^{13}C NMR spectrum of SN38-Azo1.

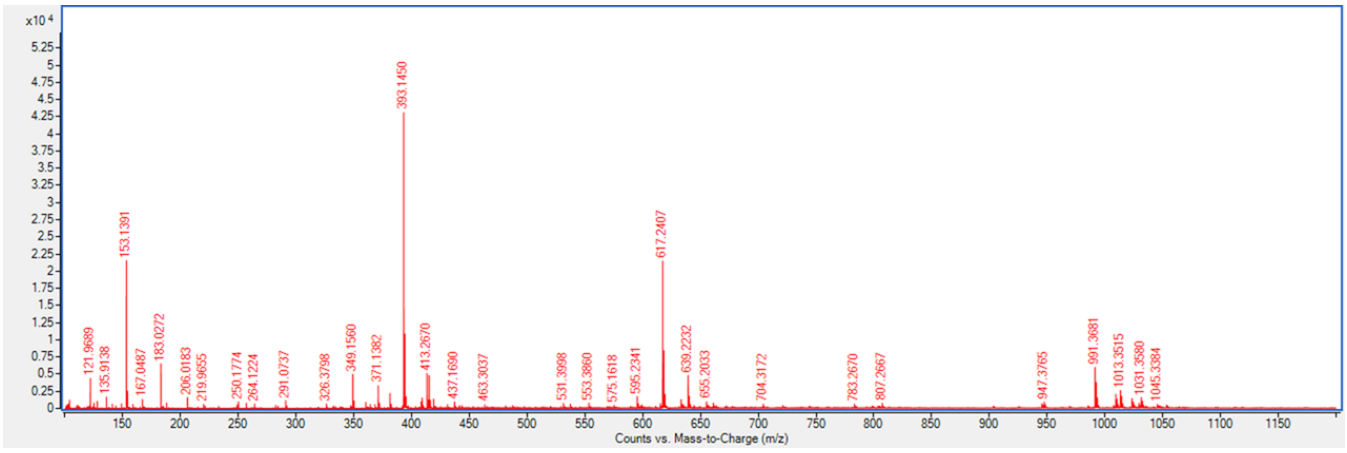

Figure S7. HRMS (ESI, m/z) spectrum of SN38-Azo1. $C_{58}H_{50}N_6O_{10}$ ($[M+H]^+$): calcd 991.3661; found 991.3681.

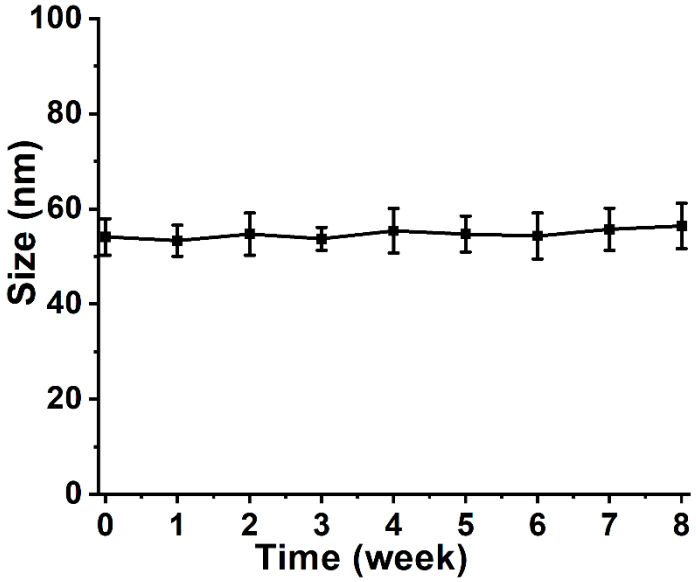

Figure S8. The hydrodynamic size of SN38-Azo1-NPD in DI water over two months.

References

1. Safiya Nisar, Elisabeth Starosta, Mouhmad Elayyan, Amrit Regmi, and Binglin Sui. Photoinduced Electron Transfer-Based Glutathione-Sensing Theranostic Nanoprodrug with Self-Tracking and Real-Time Drug Release Monitoring for Cancer Treatment. *ACS Appl. Mater. Interfaces.* **2024**, *16*, 6859-6867.