Electronic Supporting Information

for

A glutathione-responsive sulfur dioxide polymer prodrug selectively induces ferroptosis in gastric cancer therapy

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Experimental section

Materials

9,10-Anthracenediylbis(methylene) dimalonic acid (AMDA), L-glutathione (GSH) and 2-mercapto-ethanol (ME) were purchased from Sigma–Aldrich. Pyrrole was obtained from Aladdin Reagent Co., Ltd. (Shanghai, China). Trifluoroacetate and L-cysteine (Cys) were purchased from Shanghai Macklin Biochemical Co., Ltd. SO_2 test paper was purchased from Jinghui Co., Ltd. (Wuhu, China). The SO_2 probe 7-diethylaminocoumarin-3-aldehyde (DEACA) was synthesized according to previously published literature[1-3]. Fluorescamine and Cy5-NHS were purchased from Alfa-Aesar (Shanghai, China). mPEG-PLG(DN)) (M_n=18300 *g/mol, Đ* =1.35) was synthesized via click conjugation of methoxy poly(ethylene glycol)-*block*-poly(γ-propargyl-L-glutamate) (mPEG-PPLG) with N-(3-azidopropyl)-2,4-dinitrobenzenesulfonamide (AP-DN), according to our previous reports[3].

Cell Counting Kit-8 (CCK8) was purchased from APExBIO Technology LLC (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$) were obtained from Thermo Fisher Scientific (Shanghai, China).

The GSH Assay Kit and propidium iodide (PI) were purchased from Shanghai Beyotime Co., Ltd. GPX4 rabbit polyclonal antibody was purchased from ABclonal Co.,Ltd. Dihydroethidium, deferoxamine (DFO), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and 4'6-diamidino-2-phenlyindole (DAPI) were also purchased from Sigma–Aldrich. Ferrostatin-1 (Fer-1) was obtained from Med Chem Express LLC (Shanghai, China).

Cell lines and animals

The human gastric mucosa cell line GES-1 and gastric cancer cell line MKN-1 were purchased from BNCC Co., Ltd. (Beijing, China), cultured in DMEM containing 10% FBS and maintained at 37 °C in a 5% CO_2 atmosphere. Female BALB/cA-nu mice (5-6 weeks, 18–22 g) were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). The animals received care in strict conformity with the protocols for animal use, treatment, and euthanasia approved by the institutional animal care and use committee (IACUC) of Northeast Normal University (Reference number: AP2019085).

Preparation of mPEG-PLG(DN) nanoparticles (NP-DNs)

As mentioned in our previous papers[2, 3], the membrane dialysis method was utilized to assemble the mPEG-PLG(DN) micelles (NP-DNs). In brief, 100 mg of mPEG-PLG(DNs) was dissolved in 4 mL of *N*,*N*-dimethylformamide (DMF). The solution was then added to 40 mL of deionized water under stirring in drops. After stirring for 2 h at room temperature, DMF was removed from the mixture by dialysis against deionized water for 12 h (MWCO 3500 Da). The NP-DNs were finally obtained after lyophilization.

Preparation of Cy5-labeled NP-DNs

1 mg Cy5-NHS and 10 mg NP-DNs was dissolved in 3 mL of N,N-dimethylformamide (DMF). After reaction for 72 h at room temperature, DMF was removed from the mixture by dialysis against deionized water for 12 h (MWCO 3500 Da). The Cy5-labeled NP-DNs were finally obtained after lyophilization.

Characterization

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on Bruker AV-300 NMR spectrometer in deuterated dimethyl sulfoxide (DMSO-d_6). A JEOLJEM-1011 transmission electron microscope (Tokyo, Japan) was applied to perform transmission electron microscopy (TEM) measurements with an accelerating voltage of 100 kV. Dynamic light scattering (DLS) was used to measure the size of NP-DNs on a ZEN3600 Zetasizer Ultra (Malvern Panalytical Ltd., U.K.).

Determination of SO_2 production in the thiol-triggered process

DEACA dissolved in ethanol was used as a probe to detect the generation of SO_2. First, 1 mg of NP-DNs was dissolved in 1 mL of PBS (pH=5.8), and then the solution was diluted to different concentrations (0.03, 0.04, 0.05, and 0.06 mg/mL). After treatment with 20 molar equivalents of Cys, the solution was mixed with 5 μM DEACA probe for 5 minutes of reaction. The fluorescence intensity of the mixtures was detected at predetermined time intervals (0.5, 1, 1.5, 2, 3 and 6 h). A Fluorescence Master System (Photon Technology International, Birmingham, NJ, USA) was used to measure fluorescence intensity (λ_{ex}: 390 nm, λ_{em}: 480 nm).

Test paper was also used to detect the generation of SO_2. The NP-DNs samples were diluted to different concentrations (0.5, 0.1 and 0.02 mg/ml). Then, 7.3 mg of Cys was added to the solutions for 5 minutes of reaction. SO_2 test paper was immersed in the mixtures for 30 s at predetermined time intervals (0, 0.5, 1, 2, 3 and 6 h). The

colour of the paper was applied for comparison with a standard chart (darker blue represents less production of SO_2).

Amino group presentation can prove the release of SO_2 from NP-DNs. According to published papers[4], fluorescamine was utilized to test the primary amine generation of NP-DNs with a Fluorescence Master System (PhotonTechnology International, Birmingham, NJ, USA).

Cellular uptake

To investigate the cellular internalization of NP-DNs, Cy5-labeled NP-DNs was employed for fluorescence imaging. GES-1 and MKN-1 cells were seeded on 12-well plates at a density of 3.0×10^5 cells per well and cultured in 1 mL of cell culture medium for 24 h. Then, 200 μg of Cy5-labeled NP-DNs dissolved in 50 μL of PBS was added and incubated for another 6 h. After being washed five times with PBS, the cells were treated with 4% formaldehyde for 10 min. Next, the cells were stained with DAPI for 2 min. After being washed three times with PBS, the cells were imaged by fluorescence microscopy (Lecia DMi8, Germany).

Determination of GSH levels in cells

GES-1 and MKN-1 cells were seeded on 10 cm culture dishes at a density of 5.0×10^6 cells and cultured in 10 mL of cell culture medium. The cells were then treated with or without 200 μg/mL NP-DNs for 12 h. Afterwards, the cells were harvested and counted. After removing the supernatant, protein removal reagent was added to the cell precipitate at 3 times the volume. Then, the samples were frozen and thawed twice in liquid nitrogen and in a 37 °C water bath. After incubating in a 4 °C atmosphere for 5 minutes, the samples were centrifuged again at 4 °C for 10 minutes at 10000 × g. In 96-well plates, 5 μL of the supernatant, 5 μL of protein removal reagent and 150 μL of GSH assay reagent were mixed in the wells for 5 min of reaction time. The optical density (OD) values at an absorbance of 405 nm were measured by a microplate reader (ELX 808, Bio Tek, Winooski, VT, USA). The GSH concentrations of cell precipitate solutions were calculated by GSH standard curves according to the GSH Assay Kit instructions. Then total amount of GSH content was obtained by multiplying the concentrations by the solutions volume. Finally, the intracellular GSH content was obtained by dividing the total amount of GSH content by the number of cells.

Determination of SO_2 and ROS levels in cells

The cells were seeded in a 12-well plate at a density of 3.0×10^5 cells per well and cultured in 1 mL culture medium for 24 h. Then, the cells were treated with NP-DNs at a concentration of 200 μg/mL for another 12 h. A total of 10 μM SO_2 DEACA probe or 10 μM ROS DCFH-DA probe was added to the wells and incubated for another 30 min. Finally, the cells were washed five times with PBS and imaged by fluorescence microscopy (Lecia DMi8, Germany).

Cell viability analysis

The cytotoxicity of NP-DNs was assessed by Cell Counting Kit-8 (CCK8). In brief, GES-1 and MKN-1 cells were cultivated in 96-well plates (10000 cells per well) overnight. Then, the cells were incubated with different concentrations (0,100,200,400 and 800 μg/mL) of NP-DNs for 12 hours. Subsequently, 10 μL of CCK8 solution was added to the wells for 2 h. Cell viability was evaluated by a microplate reader (ELX 808, Bio Tek, Winooski, VT, USA) at an absorbance of 450 nm.

Determination of lipid peroxidation in cells

A fluorescent fatty acid probe C_{11}-BODIPY$^{581/591}$ was used to monitor intracellular lipid peroxidation. In 6-well plates, 3.0×10^5 GES-1 and MKN-1 cells were seeded in 2 mL of culture medium. Concurrently, 200 μg/mL NP-DNs was added to the wells with or without DFO (80 μM) or Fer-1 (10 μM). After incubation for 12 h, 4 μM C_{11}-BODIPY$^{581/591}$ was loaded, and the cell plates were maintained at 37 °C for 30 min. Afterwards, the cells were ready for flow cytometry (FCM) analysis. FCM was performed on a Guava EasyCyte™ 12 Flow Cytometer (Millipore, Billerica, MA, USA).

Cell death analysis

A total of 3.0×10^5 GES-1 and MKN-1 cells were seeded on 6-well plates with 2 mL of culture medium. Concurrently, 200 μg/mL NP-DNs was added to the wells with or without DFO (80 μM) or Fer-1 (10 μM). After incubation for 12 h, the cells were stained with PI for 15 min at room temperature according to the instructions from the kit. Finally, FCM was carried out to determine cell death rates (Millipore, Billerica, MA, USA).

Western blotting

GES-1 and MKN-1 cells (3.0×10^5) were seeded in 6-well plates with 2 mL of culture medium. NP-DNs were added

to the wells at different concentrations (0, 100, 200, and 400 μg/mL). After incubation for 12 h, the cells were harvested to extract protein. Western blotting analysis was used to detect the expression level of GPX4. The immunoblots were assessed by a Bio–Rad ChemiDoc MP image analysis system (California, USA).

Xenograft animal model

MKN-1 cells (1.0×10^6) suspended in 100 μL of PBS were injected into the left axilla of mice to establish an MKN-1 tumour-bearing mouse model. The animals received care in strict conformity with the protocols for animal use, treatment, and euthanasia approved by the institutional animal care and use committee (IACUC) of Northeast Normal University (Reference number: AP2019085)

***In vivo* biodistribution study**

Cy5-labeled NP-DNs were utilized as the probe to trace the *in vivo* biodistribution of NP-DNs. When the volume of tumours grew to approximately 100 mm^3, MKN-1 tumour-bearing mice were intravenously injected with Cy5-labeled NP-DNs (n=3, 10 mg/kg). At predetermined time intervals (1, 2, 4, 6, and 10 h), the mice were visualized by IVIS Lumina LT (PerkinElmer, USA) (excitation light wavelength: 635 nm, filter wavelengths for acquisition: 665-900 nm).

***In vivo* antitumor and biosafety evaluation**

MKN-1 tumour-bearing mice with a tumour volume of approximately 100 mm^3 were randomly assigned to 3 groups (n = 5) and intravenously administered PBS, NP-DNs (10 mg/kg), or NP-DNs (10 mg/kg) with Fer-1 (1 mg/kg) through the tail vein every two days. The tumour volumes and mouse weights were measured following the injection. The tumour volumes were calculated as follows: $V_{tumor} = 0.5 \times a \times b^2$, where a and b represent the length (mm) and width (mm) of the tumour, respectively. At the end of the study, the mice were sacrificed. The tumour tissues and major organs were harvested for further analyses.

Histological analysis

Parts of tumour tissues and major organs were harvested and fixed in 4% paraformaldehyde for 24 h. Then, the tissues were embedded in paraffin and sliced into sections at a thickness of 5 μM. Subsequently, conventional methods were applied for haematoxylin and eosin (H&E), Ki67 and GPX4 staining. The sections were observed by microscopy (Nikon Eclipse Ti, Optical Apparatus Co., Ardmore, USA).

***In vivo* ROS level analysis**

The tumour tissues were harvested and immediately immersed in liquid nitrogen. Then, freezing sectioning procedures were applied to produce frozen sections at a thickness of 5 μM. Subsequently, the sections were stained with dihydroethidium at 37 °C for 30 min. After being washed three times with PBS, the sections were stained with DAPI for another 30 min at room temperature. After being washed three times with PBS, sections were mounted and imaged by fluorescence microscopy (Lecia DMi8, Germany).

Statistical analysis

All the data are expressed as the mean ± standard deviation (SD). The intracellular fluorescence intensity was analysed with NIS-Elements AR (Nikon), ZEN 2.3 SP1 (Carl Zeiss) and Leica Application Suite X 3.6.0.24 (Lecia). Statistical analysis was performed using GraphPad 8.3. *P<0.05 was considered to be statistically significant. **P < 0.01, ***P <0.001 and ****P <0.0001 were regarded as highly significant.

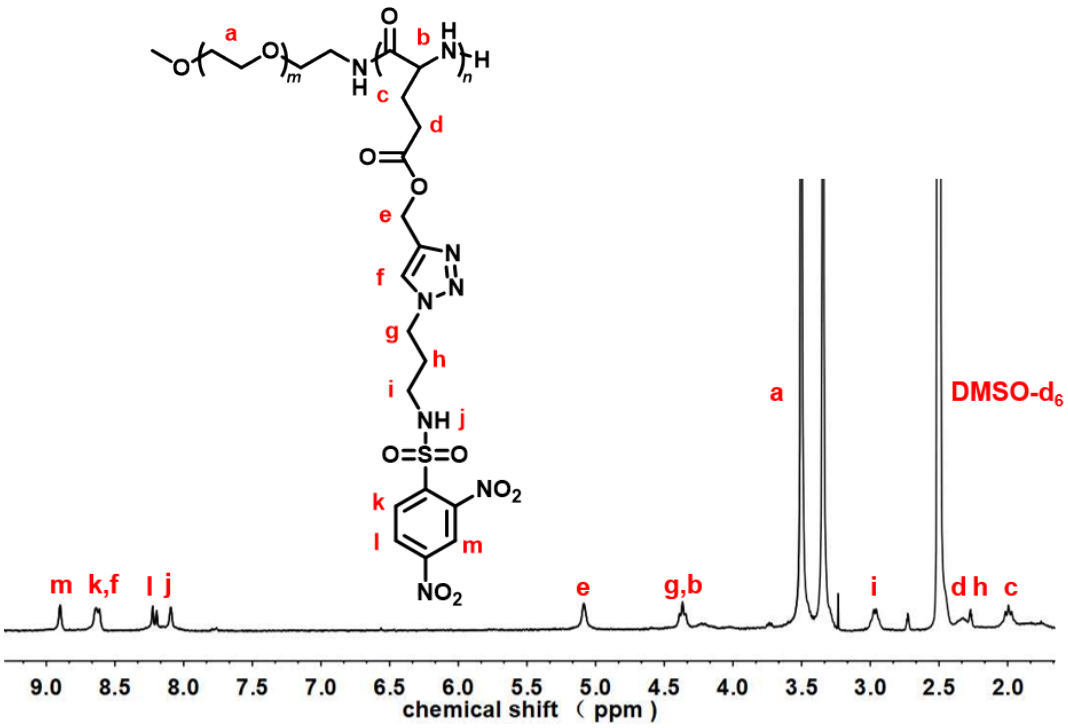

Figure S1 ^{1}H NMR spectra of mPEG-PLG(DNs) in DMSO-d_6

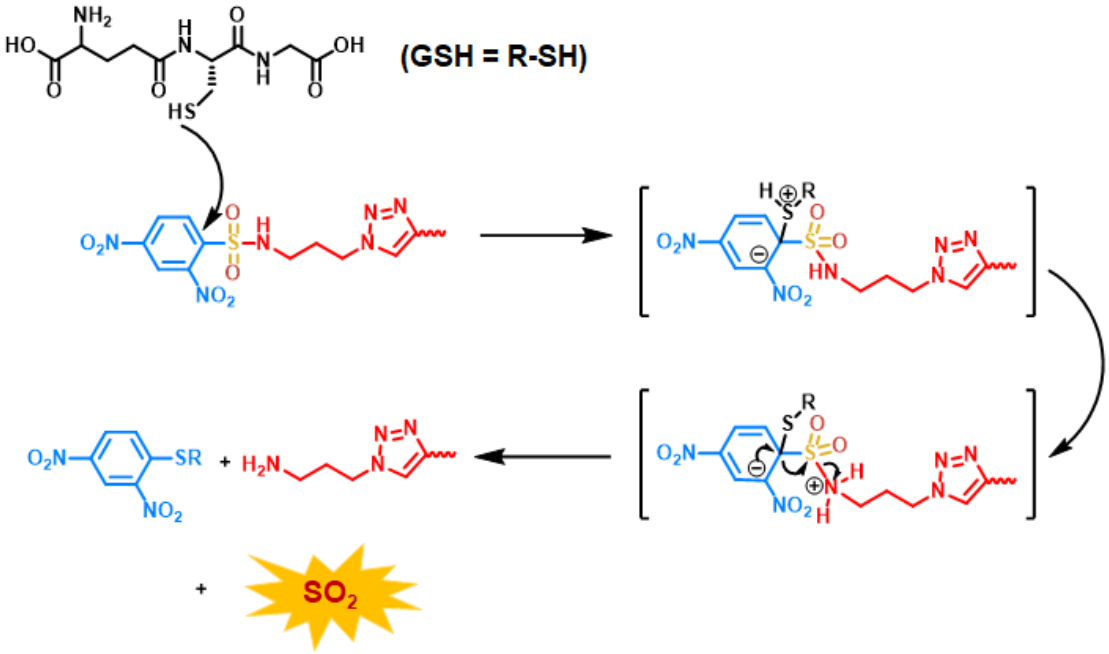

Figure S2 The reaction mechanism of NP-DNs react with thiol compounds to release SO_2.

Notes and references

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