Supplementary data

Design, docking optimization and evaluation of biotin-PEG4-1,8-naphthalimide as potent and safety antitumor agent with dual targeting of ferroptosis and DNA

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Part 1. General procedures for biological assay and related supplementary results

1.1. Stability of Compound 4d under physiological condition

Tris-HCl buffers provide a relatively stable pH value, but also have similar physiological similarities and biocompatibility with the internal environment, so it is often used in biological experiments to simulate the internal environment for detection. 2 mM compound **4d** was pre-incubated in Tris-HCl (pH = 7.4) solution for 0 h, 24 h, 48 h and 96 h, respectively. Tris-HCl solution containing 2 mM compound **8** was set as the control group. The ability of compound **4d** to release compound **8** after incubation in Tris-HCl (pH = 7.4) solution for different time was detected by HPLC chromatography. The mobile phase was methanol/water (50% : 50%, v/v) solution at the flow rate of 0.1 mL/min. Reversed phase HPLC was performed on a 250×4.5 mm ODS column. The samples were filtered by a 0.45μm filter and analyzed by HPLC. HPLC spectra were recorded at 400 nm for 0h, 24h, 48h and 96h, respectively. It was found that compound **4d** slowly released the original drug **8** after 48 h. The results indicated that compound **4d** should be indeed the prodrug of **8**.

1.2. *In vitro* antipoliferative activity

The cell lines were cultured in a humidified, 5% CO_2 atmosphere at 37°C and maintained in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 mg/mL penicillin. In simple terms, the exponentially growing cells were inoculated in 96-well plates and treated with a specified concentration of the compound for 48 h. Then, 10 mL of MTT (10 mg/mL) was added. After incubation at 37°C for 4 h, the medium was removed, 150 μL of dimethyl sulfoxide (DMSO) was added to each well, and the optical density of the plates was measured at 490 nm using a plate reader (Tecan Infinite M1000). Each process was repeated five times in a 96-well plate, and the same experimental conditions were maintained for all test procedures. The MTT assays were repeated three times for each cell line.

1.3. *In vivo* antipoliferative activity

1×10^6 Tumor cells were injected subcutaneously into the right side of nude mice. When xenograft tumors grew to an average volume of 60–80 mm^3, the mice were randomly divided into solvent control and treatment groups (n = 6 per group). Different doses of the compound (5% *v*/*v* DMSO/saline) were administered intraperitoneally once every 2 days, and the tumor size and weight were recorded. The primary tumor volume was calculated, and length (l) and width (w) were measured. The formula $V = lw^2/2$ was used to calculate the volume, and body weight was used as an indicator of systemic toxicity.

1.4. Spectroscopic studies on DNA interaction

The DNA-binding experiments were performed at room temp-rature, and all spectroscopic experiments were carried out in Tris buffer(pH 7.4). Compound **4d** was prepared as 2.0 mM DMSO stock solutions, and the 2.0 mM ct-DNA stock solution was stored at 4°C for no more than 5 days before use. In PI-DNA-compound ternary competitive binding studies, a 2.5 mL solution containing 2×10^{-4} M DNA and 2×10^{-5} M PI ([DNA]/[PI] = 10 : 1) was prepared. Compound **4d** was added into the above solution with increasing concentrations. Fluorescence emission spectra were recorded with a slit width of 5 nm for Ex and 5 nm for Em, respectively.

1.5. Comet assay

After treatment with 25, 50, and 100μM of **4d** for 24 h, MGC-803 cells (1×10^5) were combined with molten LM Agarose (Trevigen) at a ratio of 1:8 (*v*/*v*) and immediately pipetted onto CometSlide (Trevigen). The slides were incubated at 4°C in the dark for 1 day, immersed in a pre-cooled cracking buffer, and incubated at 4°C for 2 h. The slides were immersed in alkaline unwinding solution, pH > 13 (1mM ethylenediaminetetraacetic acid (EDTA) and 300mM sodium hydroxide (NaOH)) for 45 min at 4°C. Electrophoresis was performed using alkaline electrophoresis solution (1mM EDTA and 200mM NaOH) at 25 V for 30 min. The slides were washed three times with 0.4mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) (pH = 7.5) buffer, and then 20 μL PI was added. The samples were visualized using an EVOS M5000 microscope (Invitrogen). Under these conditions, the formation of a "comet tail" was indicative of DBSs, SSDs, and/or active excision repair of DNA cross-links.

1.6. Gel electrophoresis assay

Briefly, a mixture (20 μL) containing 0.20 μg of pBR322 DNA in relaxation buffer [10mM Tris, 0.1mM EDTA, 5mM magnesium chloride, 50mM potassium chloride, and 0.01% bovine serum albumin (BSA), pH 7.5)] was incubated at 37 °C for 4 h in the absence or presence of different concentrations of compounds. The reaction was terminated by adding 4 μL of 10× loading buffer [0.9% sodium dodecyl sulfate (SDS), 0.05% bromophenol blue and 50% glycerol]. The sample was then analyzed using a 0.8% agarose gel in TAE buffer. The gels were stained with GelRed and visualized in the electrophoresis gel documentation and analysis system.

1.7. Flow cytometry

Prepared MGC-803 cells (1×10^6 cells/mL) were obtained. The cell suspension was prepared with phosphate-buffered saline (PBS), centrifuged at 1000*g* for 10 min, and the supernatant was discarded. The cells were resuspended in 70% ethanol fixative pre-cooled

at –20°C, fixed for 24 h, centrifuged at 1000*g* for 10 min, and then centrifuged twice with PBS and RNase in a water bath at 37℃ for 30 min. Finally, the cells were mixed with 5 μL of PI, incubated for 20 min in the dark, and subsequently analyzed using BD FACSAria III flow cytometer (Becton Dickinson).

1.8. Western blot assay

MGC-803 cells were collected after treatment with the complex (25 and 50μM) for 24 h and then lysed in ice-cold lysis buffer (1% SDS in 25mM, pH 7.5, Tris–HCl, 4mM EDTA, 100mM sodium chloride, 1mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, and 10 mg/mL soybean trypsin inhibitor). Whole-cell lysates were centrifuged at 12,000*g* for 18 min. Thereafter, the protein concentration was determined with a bicinchoninic acid protein assay kit (Beyotime). An aliquot of cell lysate (40–50 μg) was fractionated using sodium dodecyl sulfate - polyacrylamide gel electrophoresis on 10% polyacrylamide gels for 2 h and transferred to polyvinylidene difluoride membranes. The membranes were blocked with skimmed milk for 2 h, incubated with the primary antibody overnight and then with the secondary antibody combined with horseradish peroxidase at room temperature for 1 h. Proteins were detected by electrochemiluminescence (Thermo Fisher Scientific, USA) and analyzed using ImageJ software.

1.9. Transmission electron microscopy(TEM) analysis

The samples were prefixed with 3% glutaraldehyde and refixed with 1% osmium tetroxide. They were dehydrated with acetone step by step, using the concentration gradient of 30% → 50% → 70% → 80% → 90% → 95% → 100% (100% concentration changed three times). The ratios of the dehydrating agent and Epon812 embedding agent were 3:1, 1:1, and 1:3, respectively. The ultrathin slices measuring about 60–90 nm were prepared, the slices were spread, and then the copper mesh was retrieved. It was stained with uranium acetate for 10–15 min, and then with lead citrate for 1–2 min, at room temperature. A JEM-1400 Flash transmission electron microscope produced by Nippon Electric was used to collect images of the copper mesh. Each copper mesh was observed at a magnification of 6000×, and the area to be observed was selected to collect pictures and observe specific lesions.

1.10. Molecular docking

Molecular docking studies were conducted using the SYBYL-X 2.1 software on Windows. The crystal structure of the DNA was retrieved from the RCSB Protein Data Bank (DNA: 144 D, 166 D, and 182 D). The synthesized compound **8** and compounds with

different chain lengths **4a–4g** were selected for virtual screening. The three-dimensional structure of the compound was constructed using Sybyl-X 2.1, followed by energy minimization using MMFF94 force field and Gasteiger–Marsili charge. Before docking, natural ligands were extracted to remove water molecules and increase the number of polar hydrogen atoms. In this study, the method of automatic docking was adopted. The default parameters were set, and the compound was automatically docked to the binding pocket of DNA using the empirical scoring function and patent retrieval in the Surflex docking program. The Surflex–Dock score was used to evaluate the binding ability. The Surflex–Dock total score, expressed in -log10 (Kd) units of binding affinity, was used to estimate ligand–receptor interactions of newly designed molecules. A higher score indicated a stronger binding affinity.

1.11. Molecular dynamics simulations

To examine the binding modes of DNA-**4d**, Molecular dynamics simulations were conducted using the AMBER software. The docking complex with the lowest docking energy was optimized during the simulations. Each DNA base was assigned the AMBER ff99sb force field. Hydrogen atoms were introduced to the initial DNA-4d complex model with the leap module, setting ionizable residues to their default protonation states at a neutral pH. The complex was immersed in a cubic periodic box filled with explicit TIP3P water molecules, ensuring a minimum distance of 10 Å between the solute and the box surface. The particle mesh Ewald (PME) method was employed for handling long-range electrostatic interactions, with a cutoff of 10.0 Å.All bond lengths were constrained using the SHAKE algorithm, and a time step of 2fs was adopted with the Verlet leapfrog algorithm. To alleviate potential clashes between the solute and solvent, a two-step minimization was conducted. Initially, the complex was subjected to minimization while restrained by a harmonic potential. Water molecules and counter ions were optimized through the steepest descent method followed by the conjugate gradient method. Subsequently, the entire system underwent unconstrained optimization using the same method as in the first step. Following the minimization process, an annealing simulation was performed with a weak restraint for the complex, gradually heating the system in the NVT ensemble from 0 to 298K over 500ps.

After the heating phase, a >100ns molecular dynamics (MD) simulation was carried out under 1 atm pressure at a constant temperature of 298K in the NPT ensemble. Temperature stability was maintained using the Langevin thermostat with a collision frequency of 2ps-1, while pressure was regulated using an isotropic position scaling algorithm with a relaxation time of 2ps. Subsequently, based on the final 100ns MD trajectory, 3000 snapshots were extracted from the last 3ns trajectory to derive the average structures of the DNA-4d complex.

1.12. MLM stability studies

(1) Compounds Information

Compounds Information				
Compound Code	Compound ID	Species	MW	Stock Conc. (mM)
Cpd01	**8**	Mouse	352.19	10
Cpd02	**4d**	Mouse	825.41	10

Liver Microsomes Information*				
Species	Cat. No.	Lot. No.	Strain & Gender	Sponsor
Mouse	452701	1313002	Male CD-1	Gentest

*stored at -80°C prior to use

(2) Procedure

Item	**Stock concentration**	**Volume (μL)**	**Final concentration**
Phosphate Buffer Saline	100 mM	348	100 mM
NADPH	10 mM	40	1 mM
Liver Microsomes	20 mg/mL	10	0.5 mg/mL
Test compound	400 μM	2	2 μM
Total		400	

A. Prepare 0.5587 mg/mL of liver microsomes (LM solution) in PBS (100 mM, pH 7.4).

B. Add 358 μL of 0.5587 mg/mL LM solution to a 96 well plate (incubation plate).

C. Add 40 μL of 10 mM NADPH in PBS or 40 μL PBS (for negative control) to the incubation plate, 800 rpm, 10 s, then 37 °C, 10 min.

D. Add 2 μL of test compounds to incubation plate to start the reaction.

E. Transfer 50 μL from the incubation plate at 0.5, 15, 30, 45, 60 minutes to the sample plate containing 200 μL of cold methanol with IS*.

F. Centrifuge the sample plate at 3220 g for 40 minutes.

G. Transfer 100 μL of the supernatant to analysis plate containing a proper volume of H_2O for LC-MS/MS analysis.

3. Data Analysis

a) Calculate the gradient k of the line (ln (remaining % of compound) VS incubation time) using Microsoft Excel.

b) Calculate in vitro t1/2 and in vitro Clint using the equations below:

*IS: 35 ng/mL ketoprofen, 7.5 ng/mL carbamazepine, 5 ng/mL diphenhydramine, 10 ng/mL tolbutamide

1.13. Supplementary Tables

Table S1-1. Metabolic stability of liver microsomes of compounds **4d** and **8**

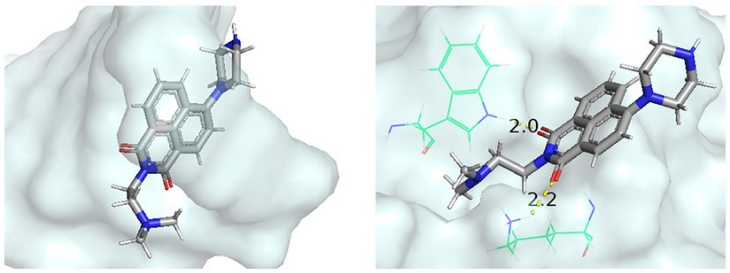
Comp	**Comp**	**Spe**	**Test**	**+/-**	**Remaining (%)**	**$t_{1/2}$**	**CL_{int}**

ound Code	ound ID	cies	Concentration (μM)	Cofactor	0.5 min	15 min	30 min	45 min	60 min	(min)	(μL/min/mg protein)
Cpd01	8	Mouse	2	+ Cofactor	100.00	90.84	92.69	88.27	90.79	>184.79	<7.5
				- Cofactor	100.00	92.42	90.67	108.86	90.12		
Cpd02	4d	Mouse	2	+ Cofactor	100.00	72.68	51.35	38.38	33.51	36.57	37.90
				- Cofactor	100.00	83.48	55.91	34.95	32.21		
§Remaining < 1% were not used to calculate $t_{1/2}$ and CL_{int}.											
*Note $t_{1/2}$ ">184.79" and CL_{int} "<7.50" were reported for the cpds remaining % > 80 at 60 minutes.											

Table S1-2. Cell cycle staging table by flow cytometry

Percentage of cell (%)	**4d**			
	Control	25μM	50μM	100μM
G0G1	59.09	53.99	33.17	39.71
G2M	12.91	23.3	26.25	18.57
S-Phase	28.00	22.70	40.58	41.72

1.14. Supplementary Figures



DNA(Score:5.89) **GPX4(Score:4.48)**

Fig. S1-1. Docking results for compound **8** in the DNA:182D-model protein and the GPX4:6HKQ protein.

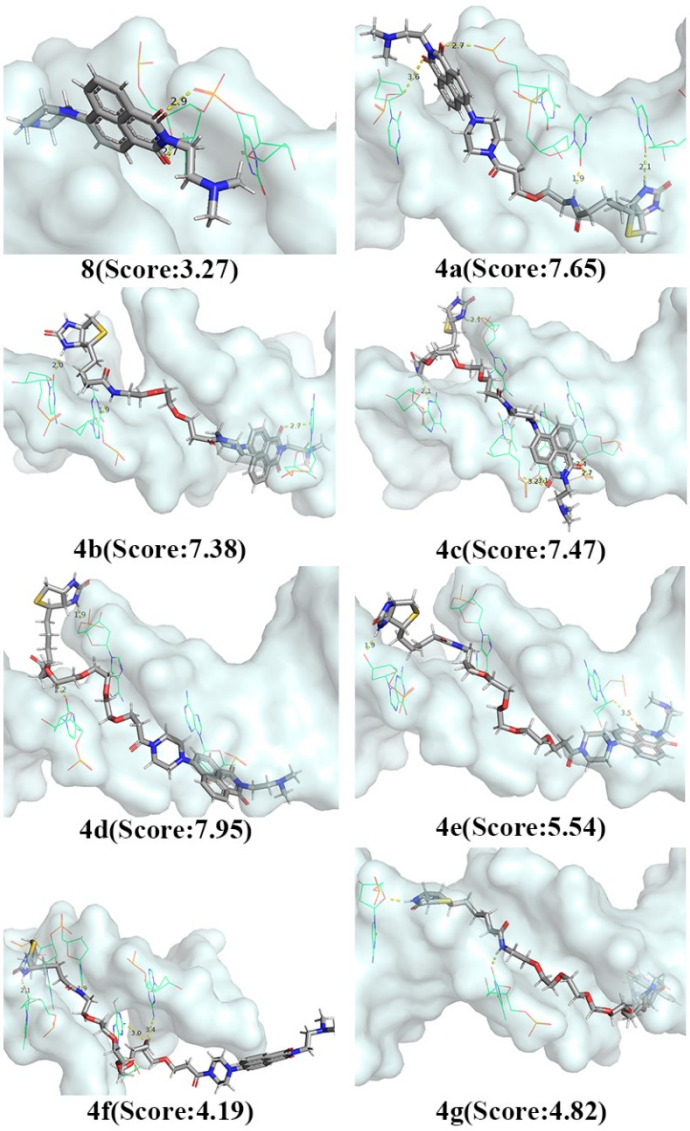


Fig. S1-2. Docking results for compound **8** and **4a-4g** in the DNA:144D-model protein.

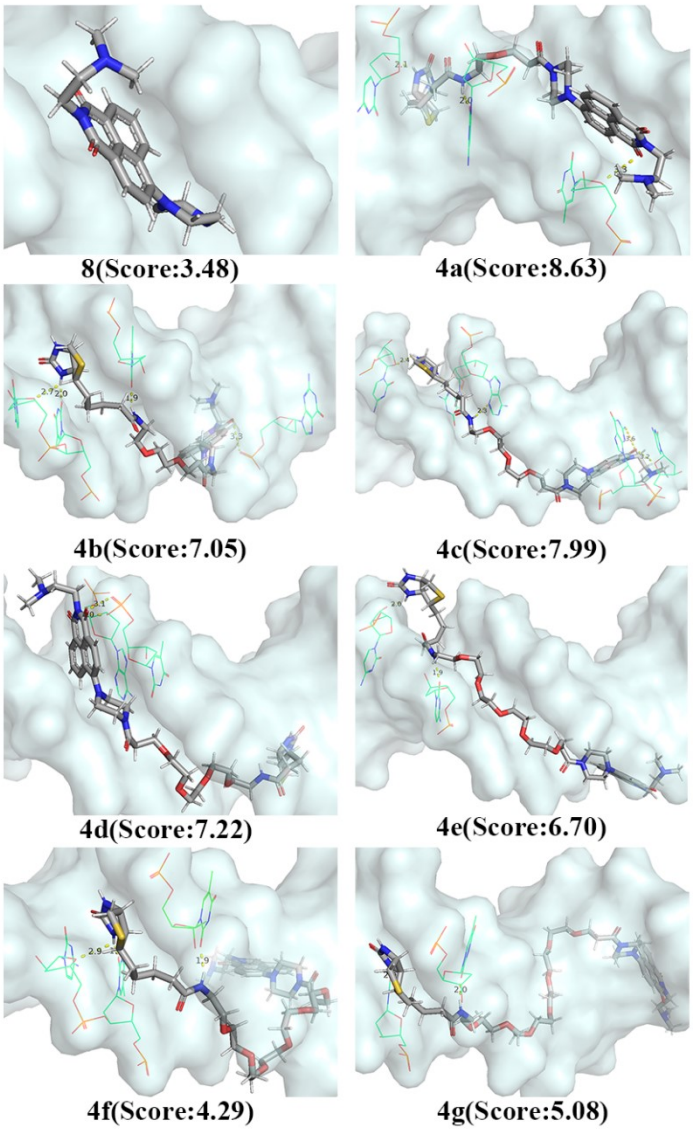


Fig. S1-3. Docking results for compound **8** and **4a-4g** in the DNA:166D-model protein.

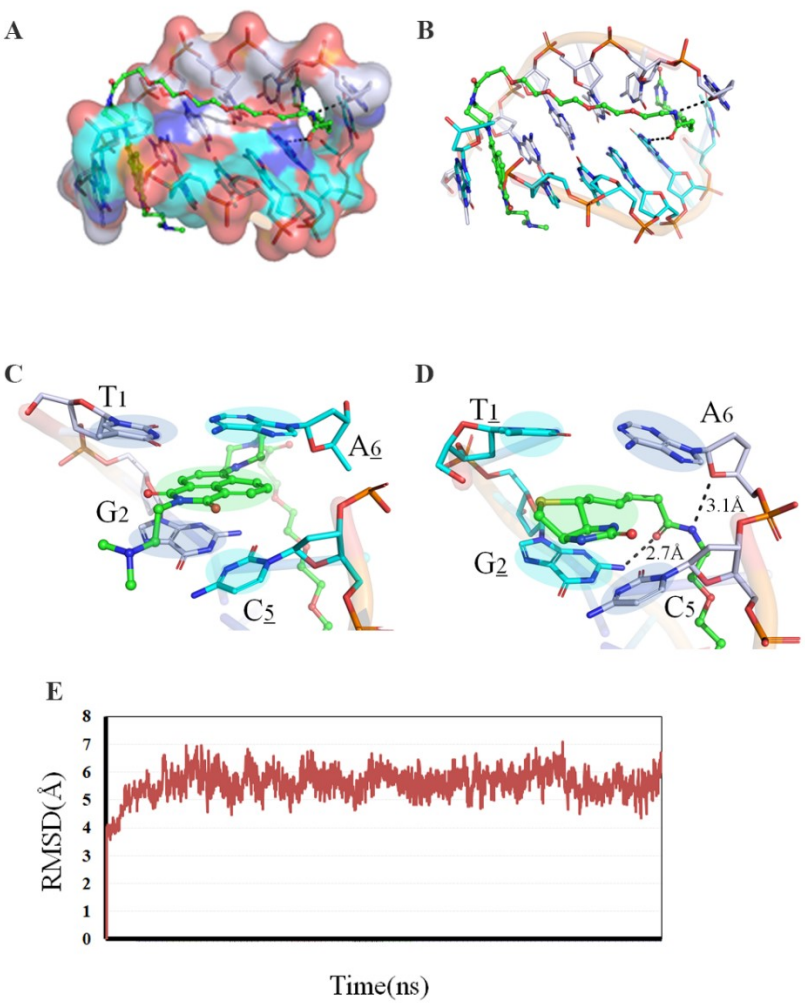


Fig. S1-4. Binding modes between DNA and **4d.** (A/B) Binding interface between DNA and 4**d**. The DNA was shown as cartoon. Two chains forming the DNA were colored by white and cyan, respectively. The ligand was colored as green. (C/D) Key residues of the binding interface that forming aromatic stacking π-π effect. (E) Root-mean-square deviation (RMSD) value change within 180ns.

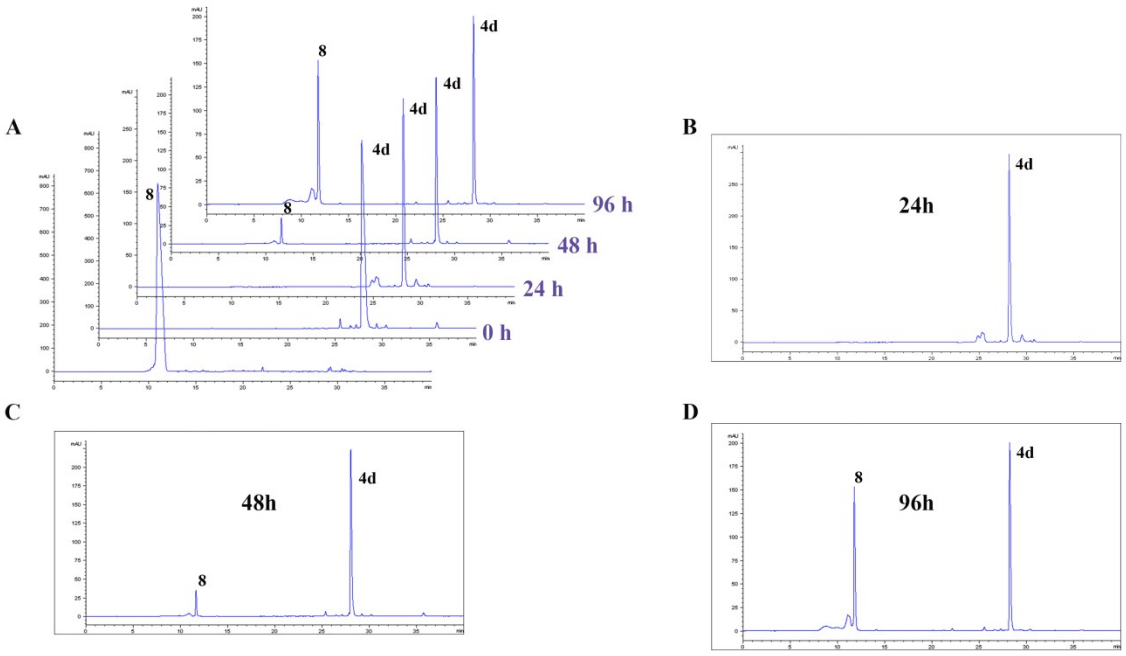


Fig. S1-5. Release profile of compound **4d** in Tris-HCl (pH 7.4). (A) Overlay of liquid chromatography profiles at different time points for compound **8** and compound **4d** incubation. (B) Liquid chromatography profile after 24 hours of compound **4d** incubation. (C) Liquid chromatography profile after 48 hours of compound **4d** incubation. (D) Liquid chromatography profile after 96 hours of compound **4d** incubation.

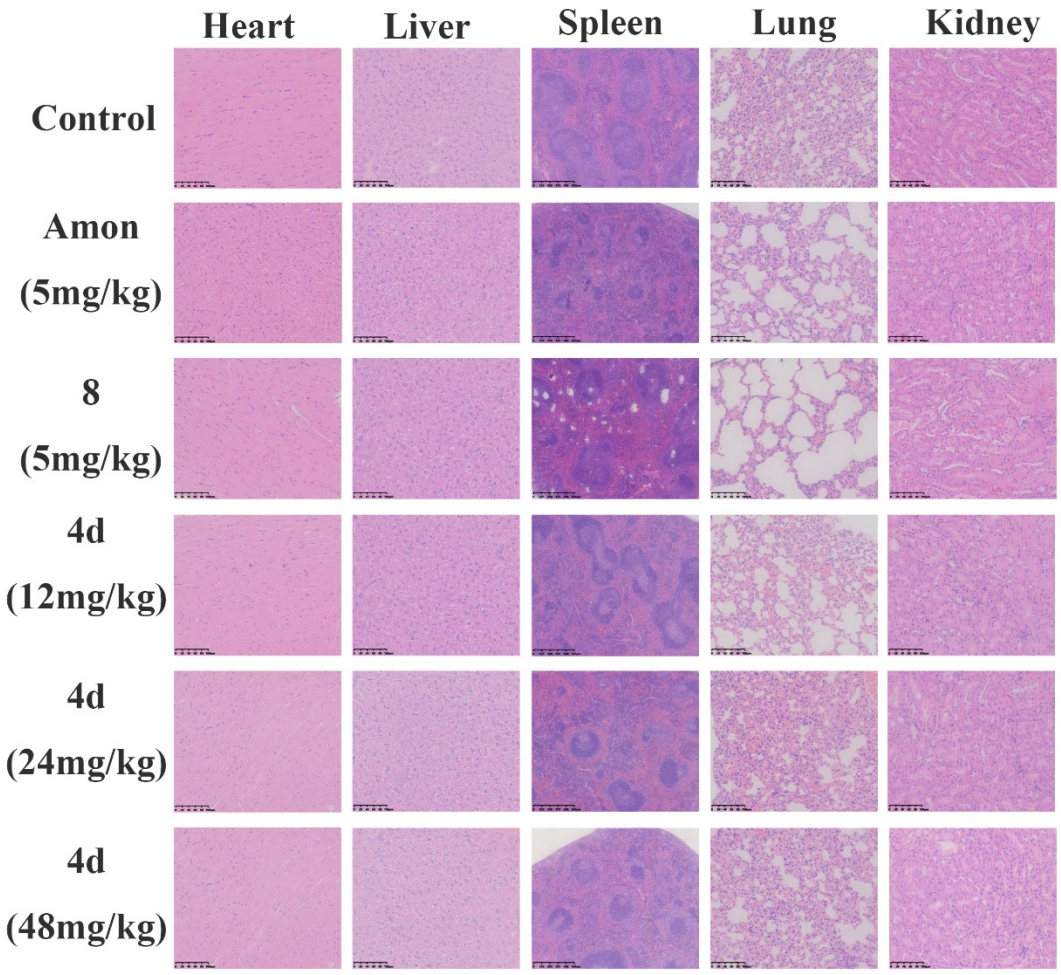


Fig. S1-6. HE staining of the major organs of MGC-803 xenograft nude mice treated with **8** and **4d** (200 ×).

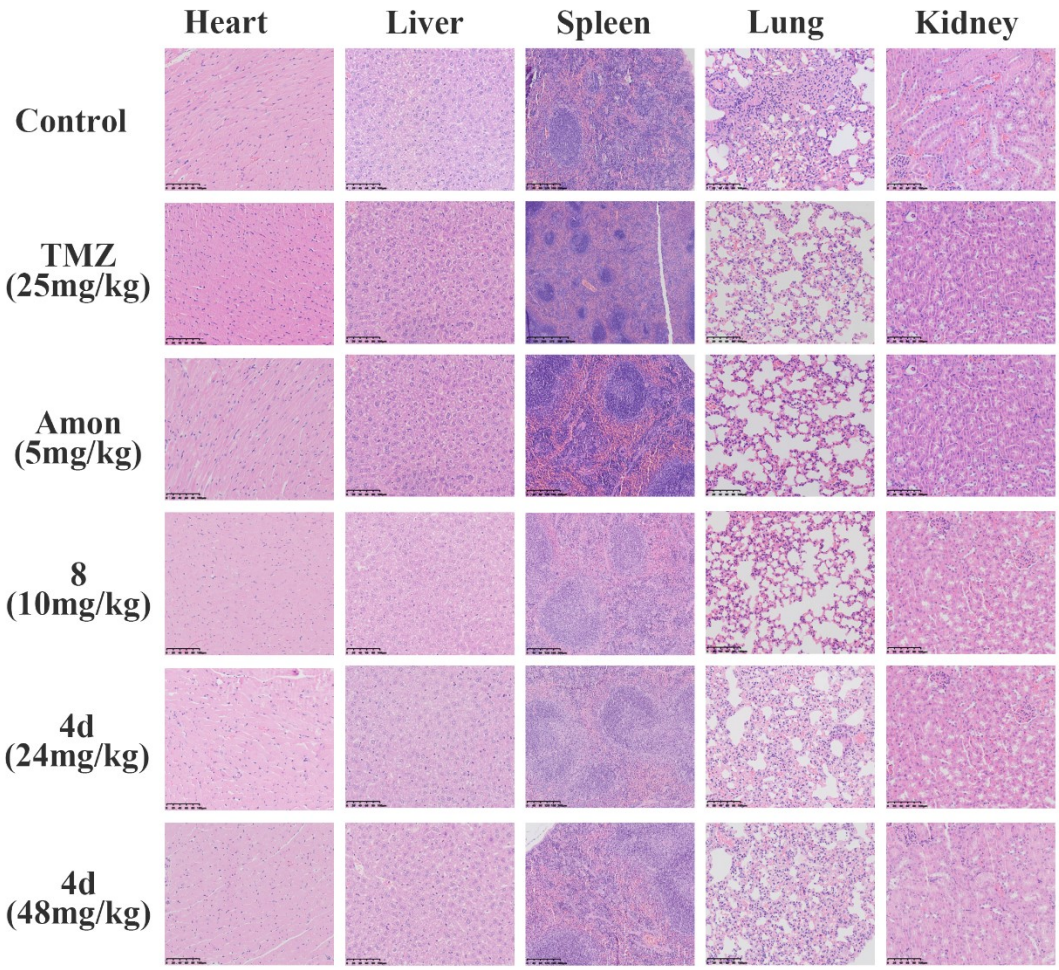


Fig. S1-7. HE staining of the major organs of U251 xenograft nude mice treated with **8** and **4d** (200 ×).

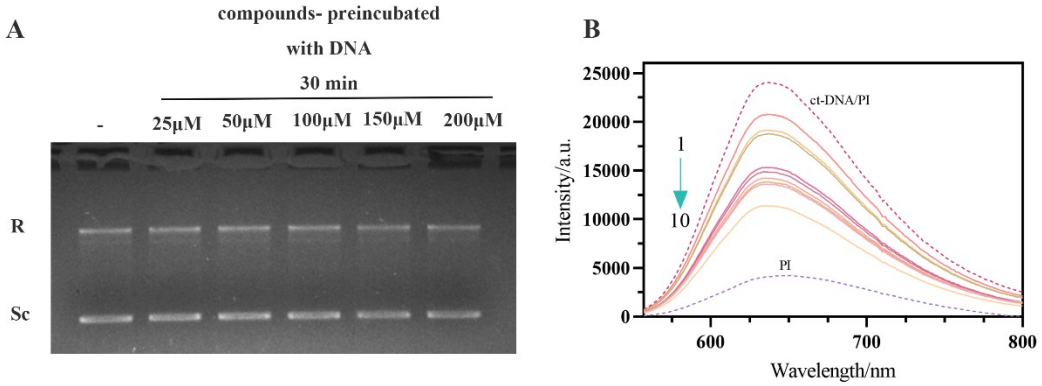
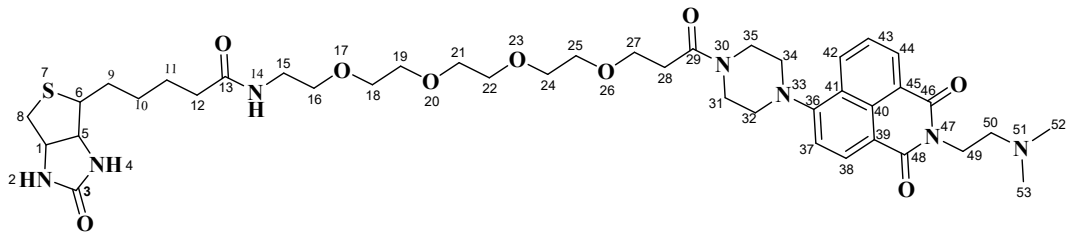


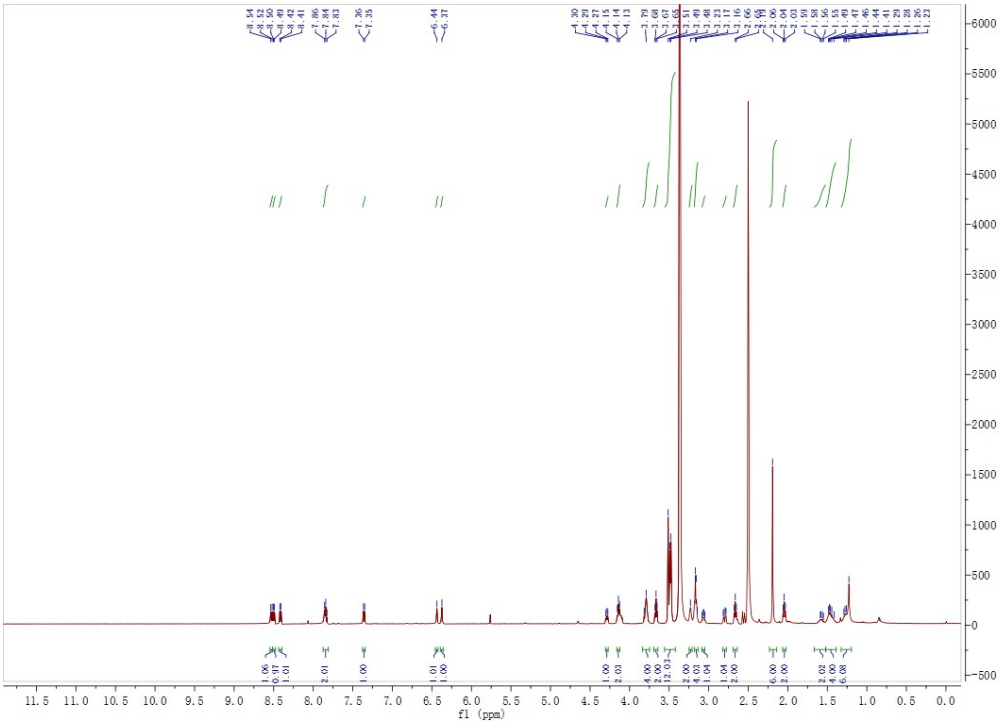
Fig. S1-8. (A) Gel electrophoresis assay results of pBR322 DNA treated with compounds **4d**. R: linear form DNA, Sc: supercoiled form DNA. (B) Fluorescence spectra of competitive bonding of PI-CtDNA / **4d** system (Arrow 1→10 indicates **4d**:PI=1:1 to 10:1): Propidium Iodide (PI) and Ethidium Bromide (EtBr, EB) are both DNA fluorescent dyes. Both PI and EB function by intercalating between the base pairs of DNA and interacting with the double helical structure of DNA. This interaction leads to enhanced fluorescence of DNA, enabling their use in DNA staining and detection. We chose PI as an environmentally safe and ultrasensitively fluorescent probe for DNA embedding to investigate the binding ability of compound 4d to ct-DNA. As shown in Fig.S1-7, compound 4d can effectively quench the fluorescence emission of PI. When the excitation wavelength is Ex=536 nm and the Slit Width is 5 nm/5 nm, PI-ctDNA [1:10] shows a strong fluorescence emission characteristic peak near 636 nm (I_0=24046), which indicates that PI has been fully embedded between the DNA base pairs. The results showed that at a lower concentration (PI/ctDNA/4d=1:10:1), I=20785, the color reduction rate was 13.56%; When PI/ctDNA/4d =1:10:5, I=14867, the color reduction rate was 38.17%; When PI/ctDNA/4d =1:10:10, I_{min}= 11362, the color reduction rate was 52.75%, the degree of color reduction was more obvious, and did not reach saturation. These results strongly suggest that compound 4d competes with PI for binding DNA.

Part 2. ^{1}H NMR, ^{13}CNMR，HRMS，and UHPLC/HPLC of 4d and 8.

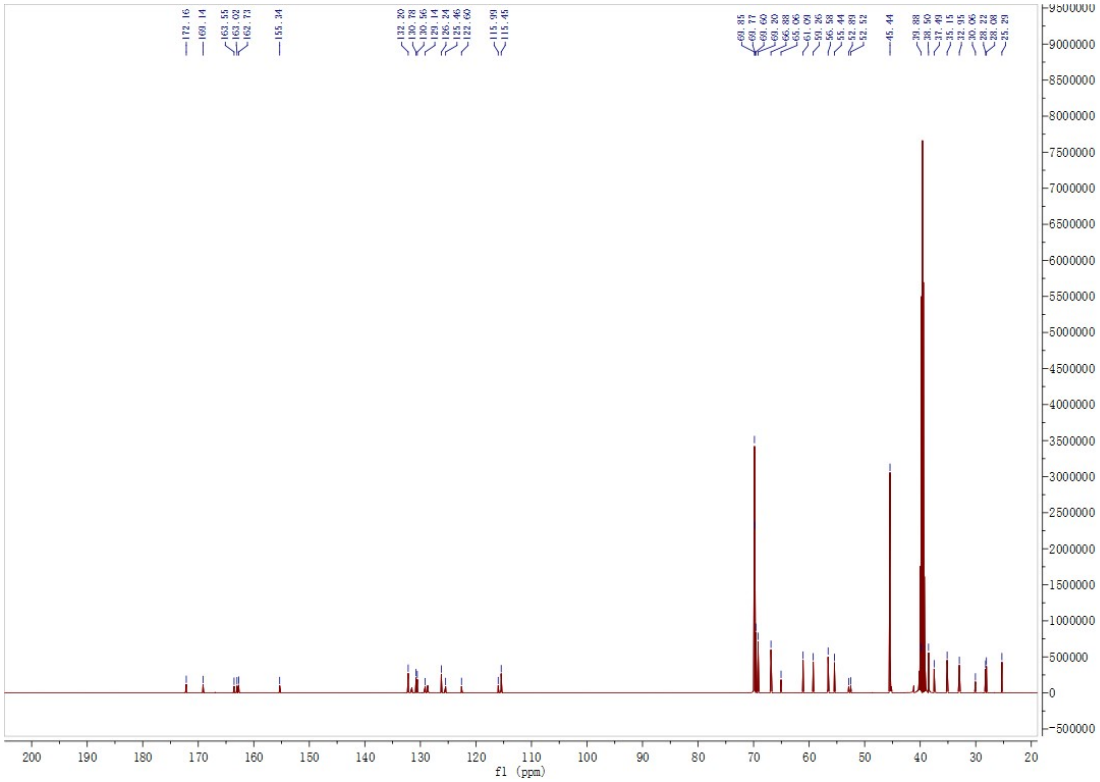


4d

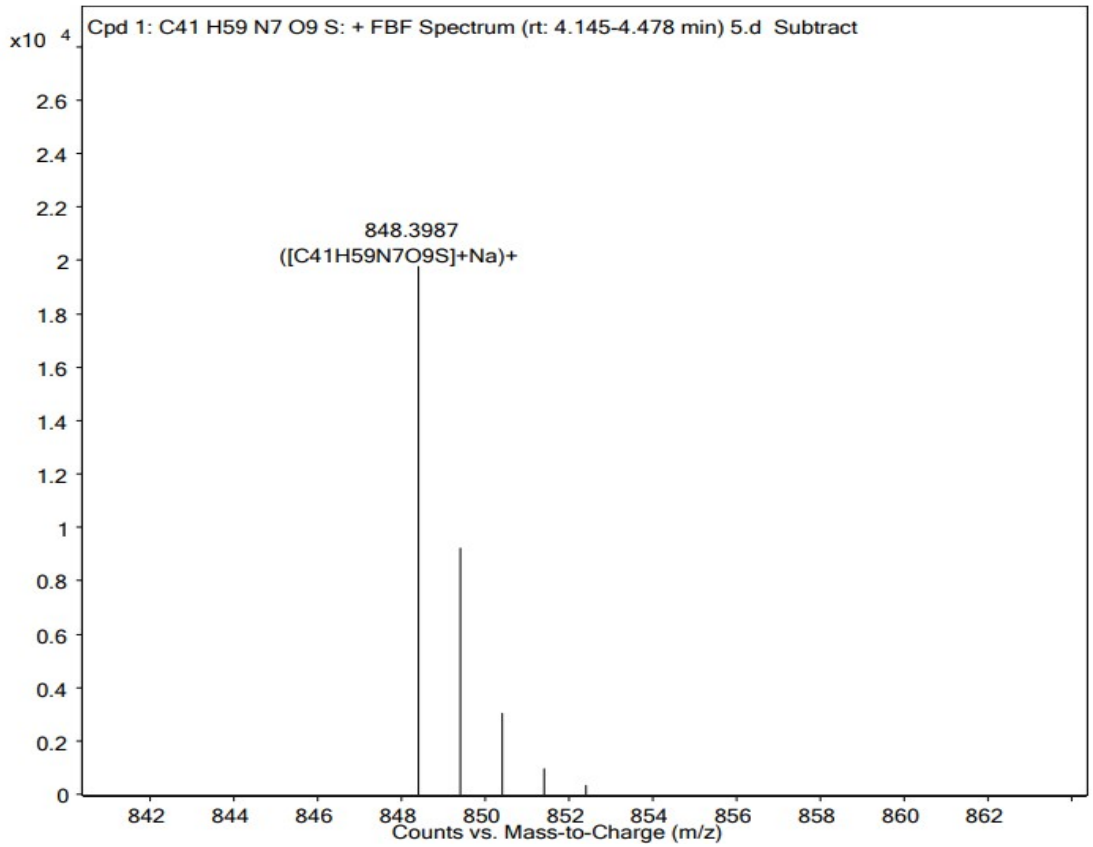
N-(15-(4-(2-(2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-6-yl)piperazin-1-yl)-15-oxo-3,6,9,12-tetraoxapentadecyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide(4d). Yellow solid, yield 63.4%. m.p. = 95–96 ℃. IR (KBr,cm^{-1}) v: 3426, 2924, 2854, 1644, 1025, 997, 827, 765, 667,633; ^{1}H NMR (500 MHz, *d*-DMSO) 8.53 (d, *J* = 8.5 Hz, 1H, H-42), 8.50 (d, *J* = 7.3 Hz, 1H, H-44), 8.41 (d, *J* = 8.1 Hz, 1H, H-38), 7.8 (t, *J* = 7.6Hz, 2H, H-14, 43), 7.36 (d, *J* = 8.1 Hz, 1H, H-37), 6.44 (s, 1H, H-4), 6.37 (s, 1H, H-2), 4.31–4.27 (m, 1H, H-6), 4.14 (t, *J* = 7.0 Hz, 2H, H-1, 5), 3.79 (s, 4H, H-31, 35), 3.67 (t, *J* = 6.5 Hz, 2H, H-27), 3.56–3.42 (m, 12H, H-18, 19, 21, 22, 24, 25), 3.23 (s, 2H, H-16), 3.18–3.14 (m, 4H, H-32, 34), 3.09–3.05 (m, 1H, H-8), 2.80 (dd, *J* = 12.5, 5.0 Hz, 1H, H-8), 2.66 (t, *J* = 6.6 Hz, 2H, H-28), 2.19 (s, 6H, H-52, 53), 2.04 (t, *J* = 7.3 Hz, 2H, H-12), 1.57 (m, 4.8 Hz, 2H, H-15), 1.52–1.39 (m,4H,H-49,50), 1.33–1.20 (m, 6H, H-9,10,11).^{13}C NMR (126 MHz, *d*-DMSO) δ 172.16 (s, C-13), 169.14 (s, C-29), 163.55 (s, C-46),163.02 (s, C-48), 162.73 (s, C-3), 155.34 (s, C-36), 132.20 (s, C-38), 130.78 (s, C-44), 130.56 (s, C-42), 129.14 (s, C-41), 126.24 (s, C-43), 125.46 (s, C-45), 122.60 (s, C-40), 115.99 (s, C-39), 115.45 (s, C-37), 69.85 (s, C-16,18,19), 69.77 (s, C-21,22), 69.60 (s, C-24), 69.20 (s, C-25), 66.88 (s, C-27), 65.06 (s, C-50), 61.09 (s, C-5), 59.26 (s, C-1), 56.58 (s, C-6), 55.44 (s, C-49), 52.89 (s, C-32), 52.52 (s, C-34), 45.44 (s, C-31,35), 39.88 (s, C-52), 38.50 (s, C-53), 37.49 (s, C-8), 35.15 (s, C-12), 32.95 (s, C-28), 30.06 (s, C-15), 28.22 (s, C-10), 28.08 (s, C-11), 25.29 (s, C-9). HRMS (m/z) (ESI): ($C_{41}H_{59}N_7O_9S$) [M+Na]+ calcd for: 848.3992, found: 848.3987.



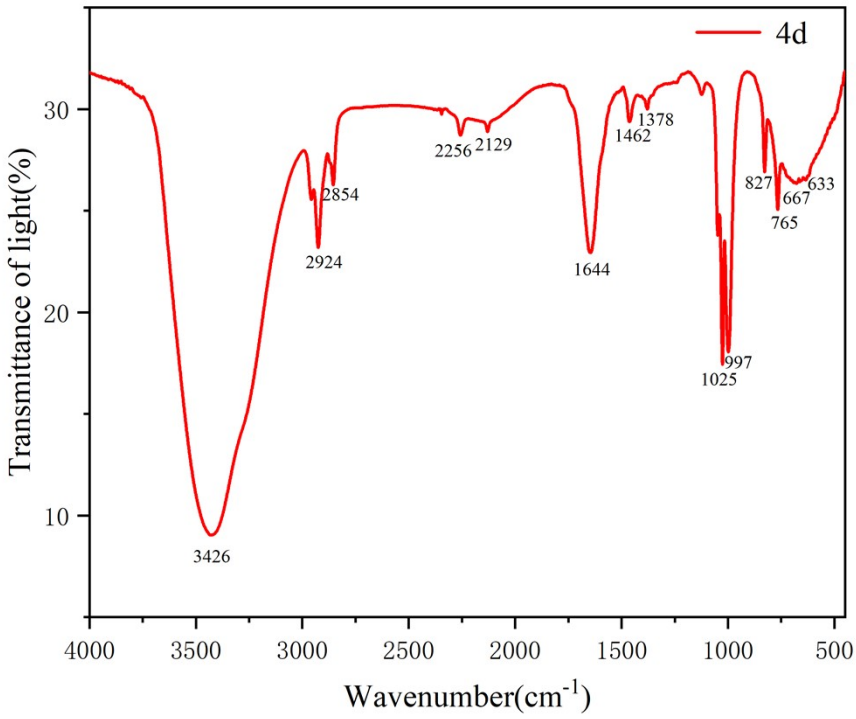
^{1}H NMR of compound **4d.**



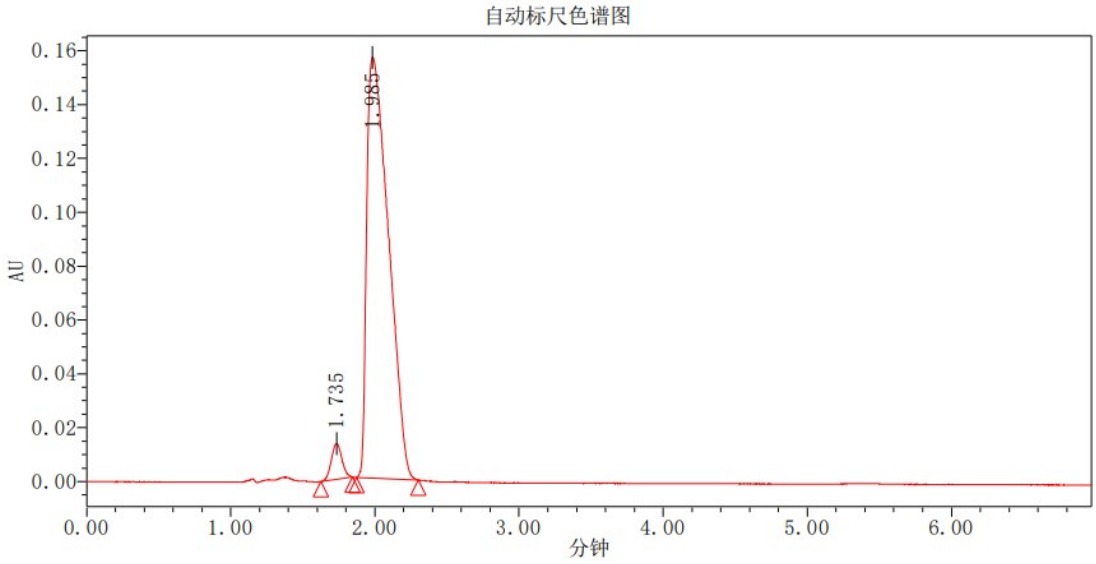
^{13}C NMR of compound **4d.**



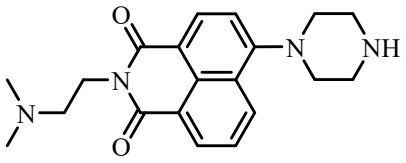
HR-MS of compound **4d.**



IR of compound **4d.**

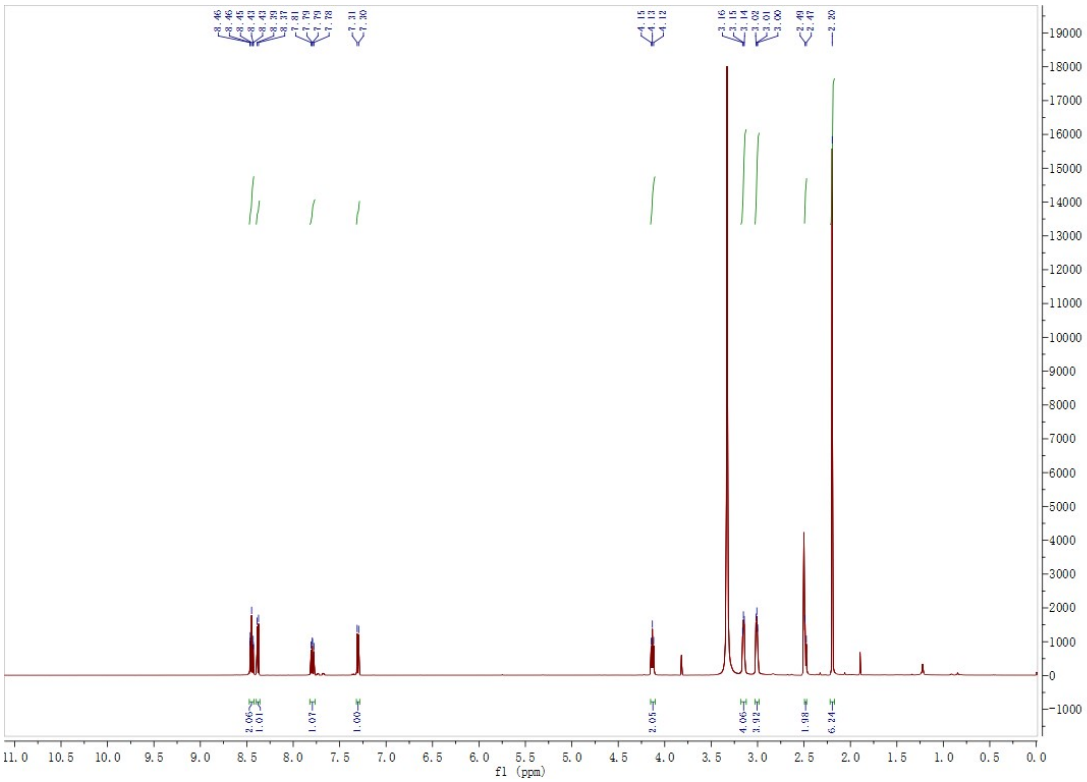


UPLC of compound **4d.**

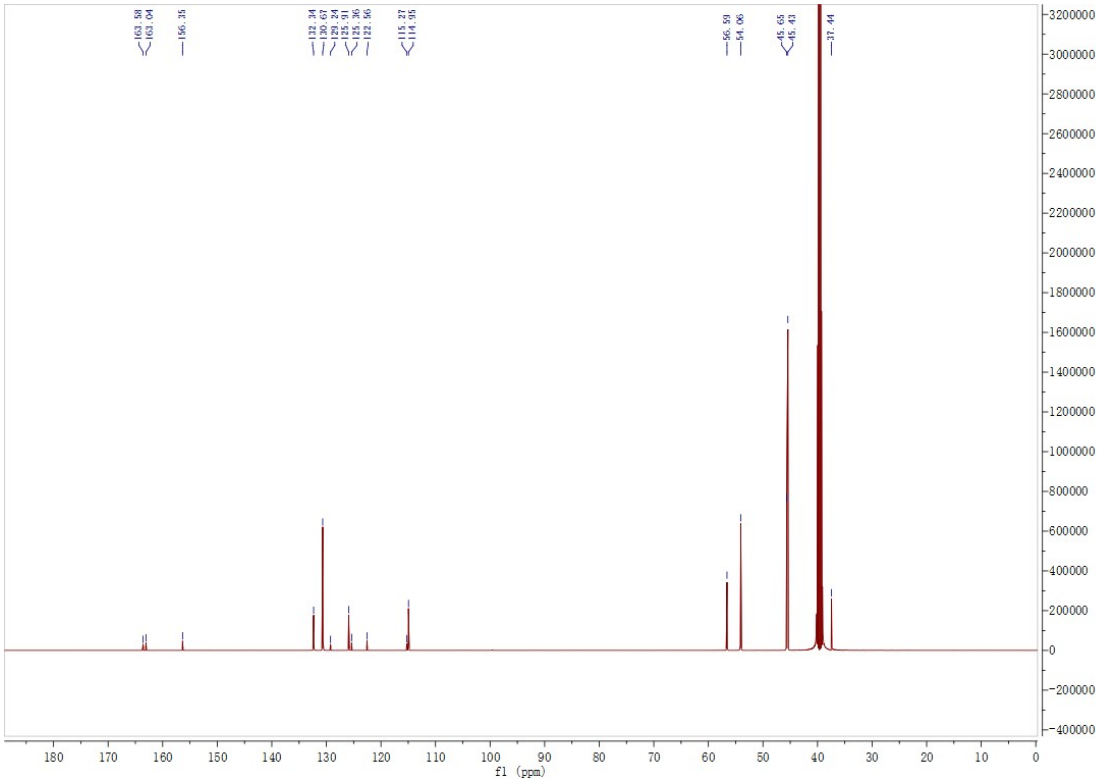


8

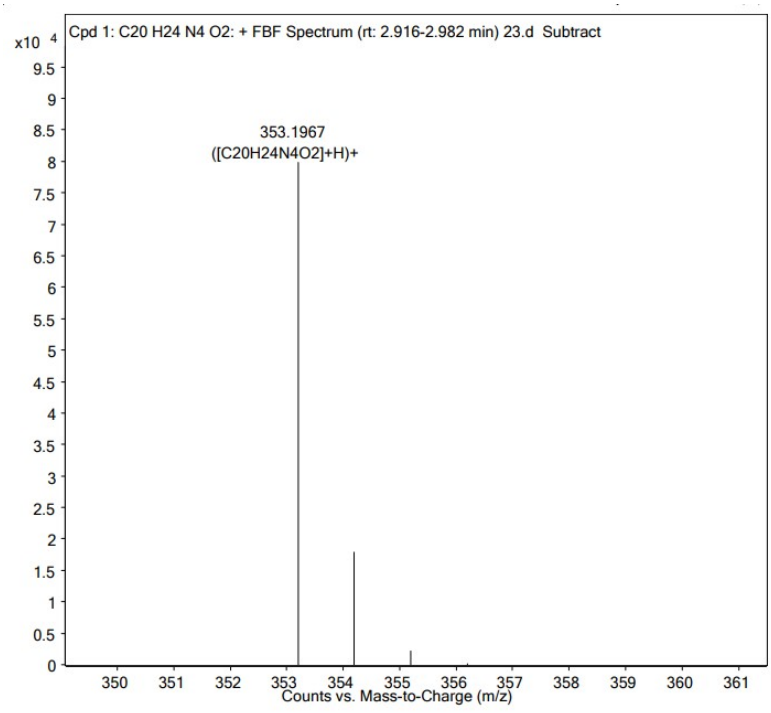
2-(2-(dimethylamino)ethyl)-6-(piperazin-1-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione Yellow solid, yield 82.5%. m.p. = 212–213℃. IR (KBr,cm^{-1}) v: 3414,2925,1647,1460,1383,758,721, ^{1}H NMR (500 MHz, DMSO) δ 8.47 – 8.42 (m, 2H), 8.40 – 8.36 (m, 1H), 7.79 (dd, *J* = 8.4, 7.3 Hz, 1H), 7.30 (d, *J* = 8.2 Hz, 1H), 4.13 (t, *J* = 6.9 Hz, 2H), 3.18 – 3.12 (m, 4H), 3.03 – 2.98 (m, 4H), 2.49 – 2.47 (m, 2H), 2.20 (s, 6H).^{13}C NMR (126 MHz, DMSO) δ 163.58 (s), 163.04 (s), 156.35 (s), 132.34 (s), 130.67 (s), 129.24 (s), 125.91 (s), 125.36 (s), 122.56 (s), 115.27 (s), 114.95 (s), 56.59 (s), 54.06 (s), 45.65 (s), 45.43 (s), 37.44 (s). HRMS (m/z) (ESI): ($C_{20}H_{24}N_4O_2$) $[M+H]^+$ calcd for: 353.1977, found: 353.1967.



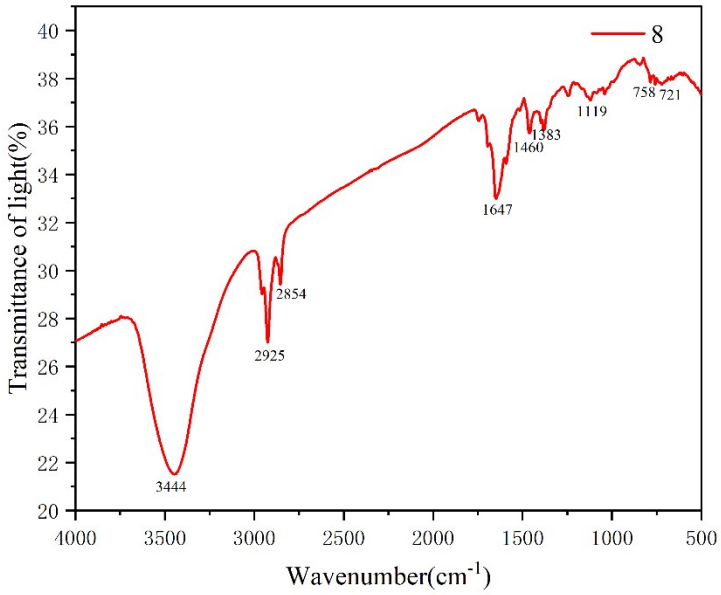
^{1}H NMR of compound **8.**



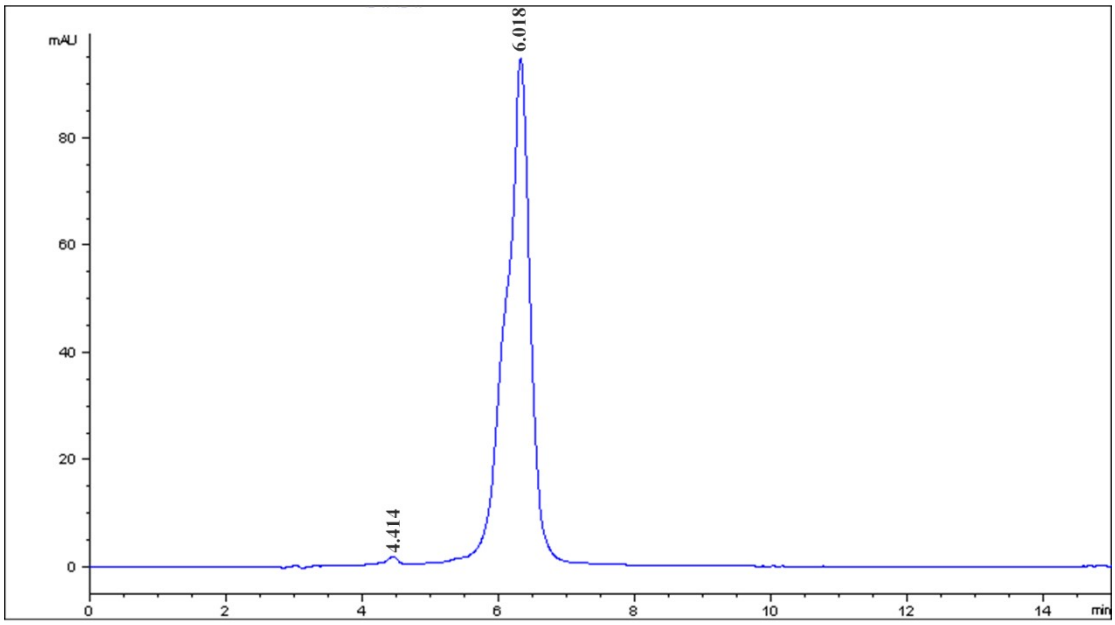
^{13}C NMR of compound **8.**



HR-MS of compound **8.**



IR of compound **8.**



HPLC of compound **8.**