

Electronic supplementary information for **New Journal of Chemistry**  
**Target Exploration of Rhein as Small-Molecule Necrosis Avid Agents**  
**by Post-treatment Click Modification**

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## **Content**

### **1. Materials and Methods**

1.1 General

1.2 Chemicals

1.3 *In Vitro* Cell Cytotoxicity

1.4 Radiochemistry and *in Vitro* Stability

1.5 DNA Binding Studies

1.6 *In Vitro* Cell Uptake Assay

1.7 Animal Models of Necrosis

1.8 Biodistribution Studies and Blocking Experiment in Mice

1.9 Ex Vivo Autoradiography and Histochemical Staining

1.10 Cell Treatment and Post-treatment Click Modification

1.11 Confocal Microscopy

### **2. Supplementary Figures: Figure S1-9**

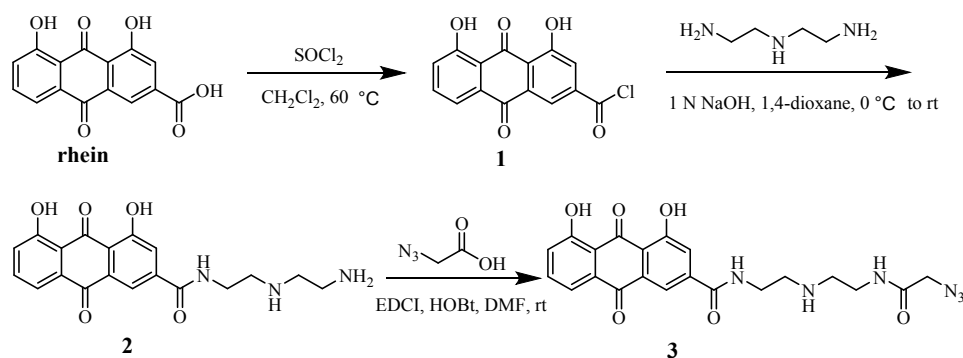
## **1. Materials and Methods**

### **1.1 General**

All reagents were used as obtained from commercial sources without further purification unless indicated otherwise.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were carried out on a Bruker 400 MHz NMR (Switzerland) at 303 K using DMSO- $d_6$  as an internal standard. Chemical shifts ( $\delta$ ) are given in parts per million (ppm) relative to internal standard tetramethylsilane (TMS). Electrospray-ionization mass spectrometry (ESI-MS) was carried out on an HP1100 mass spectrometer (Agilent, Santa Clara, CA, USA). Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Waters Alliance 2695 Separations module equipped with Waters 2998 UV/visible detector (Waters Cooperation, Milford, MA, USA) and Berthold HERM LB500 radiometric detector (Millipore, Billerica, MA, USA). The RP-HPLC column was GRACE Alltima C18 analytical column (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ). All reactions were monitored by TLC (Merck Kieselgel GF254) and spots were visualized with UV light or iodine. Sodium iodide's ( $\text{Na}^{131}\text{I}$ ) radionuclidic purity was >99% and specific activity was 370 MBq/mL, which was supplied by HTA Co., Ltd. (Beijing, China). Calf thymus DNA (Ct-DNA) and ethidium bromide (EB) were purchased from Sigma Aldrich, USA. Plasmid DNA (pUC19, 2686 bp) was purchased from Thermo Fisher Scientific Co., USA. The human lung cancer A549 cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) incubated in 5%  $\text{CO}_2$  humidified atmosphere at 37  $^\circ\text{C}$ . Kunming mice (male, 24–28 g) and Sprague-Dawley rats (male, 260–280 g) were provided by Sippr-BK laboratory animal Co. Ltd (Shanghai, China) [Certificate No. SCXK (HU) 2017-0005]. Adequate measures were taken to minimize pain of the experimental animals.

### **1.2 chemicals**

Synthetic Procedures and Product Characterization of Compound 3



To a solution of rhein (0.5 g, 1.76 mmol) dissolved in dry  $\text{CH}_2\text{Cl}_2$  (50 mL) was added  $\text{SOCl}_2$  (10.0 mL) dropwise at  $0\text{ }^\circ\text{C}$ , Then the mixture stirred at  $60\text{ }^\circ\text{C}$  overnight. After evaporation, the obtained acyl chloride **1** was directly used in the next reaction. To a solution of diethylenetriamine (1.8 g, 17.6 mmol) and  $1\text{ N NaOH}$  (5.0 mL) in 1,4-dioxane (50 mL). Then temperature of the reaction system was cooled to  $0\text{ }^\circ\text{C}$  and was added acyl chloride **1** (dissolved in 30 mL 1,4-dioxane) dropwise. The resulting solution was stirred at room temperature for 5 h. The solvent was evaporated to give purple oil amide **2**.

To a cooled solution ( $-10\text{ }^\circ\text{C}$ ) of triazidoacetic acid (0.20 g, 1.98 mmol) dissolved in anhydrous DMF (50 mL) was added EDCI (0.76 g, 3.96 mmol) and HOBT (0.40 g, 2.97 mmol). After 15 min, amide **2** (0.88 g, 2.38 mmol) was added. The mixture stirred at  $-10\text{ }^\circ\text{C}$  for 0.5 h and at room temperature for 10 h. The mixture was added water (100 mL) and extracted with  $\text{CH}_2\text{Cl}_2$  (20 mL  $\times$  3 times). The organic layer was dried over  $\text{MgSO}_4$ , filtered, and concentrated under vacuum. The residue was purified by chromatography ( $\text{CH}_2\text{Cl}_2/\text{MeOH} = 30:1$ ) to afford compound **3** (100 mg, 5% yield), yellow solid.  $^1\text{H NMR}$  (400 MHz,  $\text{DMSO-d}_6$ )  $\delta$  14.18 (s, 1H, -OH), 9.61 (s, 1H, -OH), 8.76 (s, 1H, -NHCO-), 8.50 (s, 1H, -NHCO-), 8.28-8.18 (m, 1H, ArH), 7.80-7.35 (m, 3H, ArH), 7.30-6.90 (m, 2H, ArH), 3.99-3.74 (m, 4H,  $2\times\text{-CH}_2\text{-}$ ), 3.23-3.15 (m, 4H,  $2\times\text{-}$

CH<sub>2</sub>-), 2.68-2.56 (m, 2H, -CH<sub>2</sub>-). <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>) δ 169.37, 169.12, 168.90, 165.50, 161.87, 160.29, 154.39, 141.11, 141.08, 135.87, 127.63, 126.36, 122.17, 119.67, 119.48, 66.62, 63.50, 52.88, 52.71, 49.87, 48.80. HRMS calcd for C<sub>21</sub>H<sub>21</sub>N<sub>6</sub>O<sub>6</sub> [M+1]<sup>+</sup> 453.1523, found 453.1533.

### 1.3 *In Vitro* Cell Cytotoxicity

The human liver LO2 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 mg/mL) maintained at 37 °C, 5% CO<sub>2</sub>. The medium was replaced every 2 days, and cells were split into a 96-well plate (1 × 10<sup>4</sup> cells/well). Various compound **3** concentrations (0.025-1.0 mM) of the contrast agent were added into the culture serum free media followed by incubation for 24 h. Then 10 μL of stock MTT solution (5 mg/mL, Sigma) was added to each well, and cells were incubated for 4 h at 37 °C in a 5% CO<sub>2</sub> environment. The medium was aspirated from the well without disturbing the formazan crystals. DMSO (100 μL) was added to each well. The plates were shaken for 5 min on a plate shaker, and the absorbance at 570 nm was measured by ELISA reader.

### 1.4 Radiochemistry and *in Vitro* Stability

The radiochemical purity was determined by HPLC using methanol/0.1% phosphoric acid in water (80:20, v/v) at a flow rate of 1 mL/min under 30 °C.

Iodogen (1, 2, 4, 6-tetrachloro-3a, 6a-diphenylglycouracil; Sigma) was dissolved in dichloromethane and deposited on the wall of tubes as a thin film. Radioiodination was initiated by adding dimethylsulfoxide (DMSO) solutions of rhein and rhein-N<sub>3</sub> (1 mg/mL) and Na<sup>131</sup>I solutions (4:1, v/v) into Iodogen-coated tube, adjusting pH with

phosphate buffered saline (PBS, pH 6.8).

### 1.5 DNA Binding Studies

Ct-DNA was prepared freshly in Tris-HCl buffer (50mM, pH 7.4). The purity of the DNA was checked by observing the ratio of the absorbance at 260/280 nm. The solution gave the  $A_{260}/A_{280}$  in the range of 1.8–1.9:1, which indicated that DNA was sufficiently free of protein. The concentration of CT DNA was determined spectrophotometrically by employing an extinction coefficient of  $6600 \text{ M}^{-1} \text{ cm}^{-1}$  at 260 nm. DNA solutions were stored at  $4^\circ\text{C}$  and used after no more than 4 days. Rhein and rhein- $\text{N}_3$  were dissolved in DMSO.

The UV spectra were recorded with a Cary 60 UV-visible spectrophotometer (Agilent Technologies Inc., California, USA) using a  $1 \text{ cm} \times 1 \text{ cm}$  quartz cuvettes. The spectra of rhein, rhein- $\text{N}_3$  and Ct-DNA complex were recorded in the wavelength range of 200 – 800 nm. Experiment was carried out in the presence of fixed concentration of rhein and compound 3 in a total volume of 2 mL and titrated with varying concentration of DNA. The binding constant ( $K_b$ ) was determined from the spectroscopic titration data using the following equation<sup>1</sup>:

$$[\text{DNA}] / (\epsilon_a - \epsilon_f) = [\text{DNA}] / (\epsilon_b - \epsilon_f) + 1/K_b (\epsilon_b - \epsilon_f)$$

where  $[\text{DNA}]$  is the concentration of DNA in base pairs, the apparent absorption coefficient ( $\epsilon_a$ ) was obtained by calculating  $A_{\text{obsd}}/[\text{compound}]$ . The terms  $\epsilon_f$  and  $\epsilon_b$  correspond to the extinction coefficient of free (unbound) and the fully bound compounds, respectively.

Ethidium bromide (EB) is a well-known DNA intercalator. DNA-EB fluorescence

quenching experiments were employed to further investigate the interaction mode and the number of binding sites ( $n$ ) between compound **3** and DNA. The fluorescence emission spectra were carried out with Cary Eclipse Fluorescence (Agilent Technologies Inc., USA) in the wavelength range of 530-750 nm. Different concentrations (0, 0.60, 1.20, 1.80, 2.40,  $3.00 \times 10^{-5}$  M) of compound **3** were added directly into a quartz cell containing  $0.60 \times 10^{-5}$  M ethidium bromide (EB) and  $2.40 \times 10^{-5}$  M Ct-DNA (total volume 3 mL), and the reaction was performed at 25°C. The synchronous fluorescence spectra were recorded by scanning at excitation and emission wavelengths simultaneously. The emission spectrum of DNA-EB was taken in the region of 540-700 nm using an excitation wavelength at 530 nm.

As shown in **Figure S7**, the addition of increasing compound **3** to DNA-EB mixture resulted in the significant quenching of the fluorescence intensity. The DNA binding ability  $K_{SV}$  value (Stern-Volmer quenching constant) and the number of binding sites ( $n$ ) for compound **3** were  $1.02 \times 10^4 \text{ M}^{-1}$  and 0.9, respectively. As reported in our previous study,  $K_{SV}$  value of rhein was determined to be  $1.01 \times 10^4 \text{ M}^{-1}$ , which indicated that azide group shows minimal effect on DNA-binding affinity  $K_{SV}$  value of rhein compounds.

The  $K_{SV}$  value of compound **3** was determined by means of classical Stern-Volmer equation<sup>2</sup>:  $F_0 / F = 1 + K_{SV} [Q]$ ; where  $F$  and  $F_0$  are the fluorescence intensity in presence and absence of the quencher, and  $[Q]$  is the concentration of the compound,  $K_{SV}$  is the Stern-Volmer quenching constant. And the number of binding sites ( $n$ ) was calculated from the Scatchard equation<sup>3</sup>:  $\lg[(F_0 - F)/F] = \lg[K] + n \lg[Q]$ , where  $K$  and  $n$  are the binding constant and the number of binding sites, respectively, and  $[Q]$  is the concentration of the compound,  $F_0$  and  $F$  are the fluorescence intensities in the absence and presence of the quencher, respectively.

## 1.6 *In Vitro* Cell Uptake Assay

The necrosis affinity of  $^{131}\text{I}$ -rhein and  $^{131}\text{I}$ -rhein- $\text{N}_3$  were assessed *in vitro* with Human lung carcinoma (A549) cells and red blood cells. Necrosis was induced after incubation of the cells for 1 h under intense hyperthermia at 57 °C according to Perek

et al. Briefly, A549 cells and red cells were seeded onto one 6-well plate with a density of  $5 \times 10^5$  cells/well 1 d before the experiment. Then the A549 cells and red cells were induced with necrosis after incubation for 1 h under intense hyperthermia at 57 °C. Nontreated cells were used as a control. The percentage of necrotic cells reached a maximum of 75.77% as determined by flow cytometry (**Figure S5**).

The two types of cells in duplicate were incubated with  $^{131}\text{I}$ -rhein or  $^{131}\text{I}$ -rhein- $\text{N}_3$  (37 KBq/mL) for 15 min and washed twice with PBS. The culture supernatant and cells were collected by centrifuging at 12000 rpm for 15 min, respectively. The radioactivity was counted using an automated gamma counter. The data were expressed as the percentage uptake per  $10^5$  cells (%uptake/ $10^5$  cells). Experiments were performed in triplicate.

### 1.7 Animal Models of Necrosis

**Muscular necrosis model.** All animals were given 0.12% potassium iodide in drinking water from 3 days before the experiment till the end of experiment to protect the thyroid gland from taking up free  $^{131}\text{I}$ . Each mouse was intramuscularly injected with 0.1 mL absolute alcohol in the left hind limb to induce muscular necrosis.

**Liver infarction necrosis model.** Simply, male SD rats were anesthetized with intraperitoneal injection of sodium pentobarbital at a dose of 40 mg/kg. Under laparotomy, the hilum of the right liver lobes was ligated with a suture to generate ischemia for 3 h. Then we released the hepatic inflow obstruction, massaged the liver lobe for reperfusion, and closed the abdominal cavity with two-layered sutures. The coexisting normal liver lobes were used as control for intraindividual comparison.

### 1.8 Biodistribution Studies and Blocking Experiment in Mice

For biodistribution studies, Kunming mice of MN (n = 4/group) were intravenously administered with  $^{131}\text{I}$ -rhein or  $^{131}\text{I}$ -rhein- $\text{N}_3$  under anesthesia and sacrificed at 6 h and 12 h p.i., respectively. Organs of interest were removed, weighed, and counted for radioactivity using a  $\gamma$  counter. Uptake of the tracers was calculated as the percentage of the injected dose per gram of tissue (% ID/g) with the values expressed as mean  $\pm$

standard deviation (SD). The decay-corrected activity per mass of tissue was calculated.

To verify whether the specificity of  $^{131}\text{I}$ -rhein- $\text{N}_3$  binding and the azide-containing linker has effect on necrosis affinity of rhein, blocking experiment were also performed in necrotic-muscle bearing mice. For the blocking studies, mice ( $n = 4$ ) were injected with excess rhein (10 mg/kg) 1 h prior to injection of 0.74 MBq of  $^{131}\text{I}$ -rhein- $\text{N}_3$  and sacrificed at 6 h p.i. of tracer. Biodistribution studies were then performed as described for other mice groups.

### **1.9 Ex Vivo Autoradiography and Histochemical Staining**

For ex vivo autoradiography studies, representative tissues of necrotic muscle and normal muscle at each time point were removed and washed thoroughly with 0.9% saline (4°C) to remove blood pool activity. Sections of 10 and 30  $\mu\text{m}$  were cut using a cryostat microtome (Shandon Cryotome FSE; Thermo Fisher Scientific Co., MA) at  $-20\text{ }^\circ\text{C}$  and were thawmounted on glass slides. Autoradiographs of these slides were obtained by exposing the sections for 6–24 h to a high performance phosphor screen (Cyclone; Canberra-Packard, Ontario, Canada). After the exposure, the screen was read using a Phosphor Imager scanner and analyzed using Optiquant software (Cyclone; Canberra-Packard, Meriden, CT). Relative tracer concentration was estimated by regions of interest (ROI) analysis for the necrotic and viable tissues on all autoradiographs. The slides were stained with hematoxylin–eosin (H&E) using a conventional procedure and digitally photographed to confirm the presence or absence of necrosis. Photomicrographs were obtained from an optical microscope (Axioskop; Zeiss, Oberkochen, Germany) with magnification at  $\times 200$ .

### **1.10 Cell Treatment and Post-treatment Click Modification**

A549 cells were seeded into poly-D-lysine coated glass bottom Petri dishes (MatTeck Corporation, Ashland, MD, USA) with  $2 \times 10^5$  cells $\cdot\text{mL}^{-1}$  suspended in 2 mL of medium per dish, incubated overnight and divided into necrosis and normal A549 groups. Necrosis A549 groups were induced after incubation of the cells for 1 h under intense hyperthermia at  $57\text{ }^\circ\text{C}$ . Then all cells were treated with rhein- $\text{N}_3$  (50  $\mu\text{M}$ )



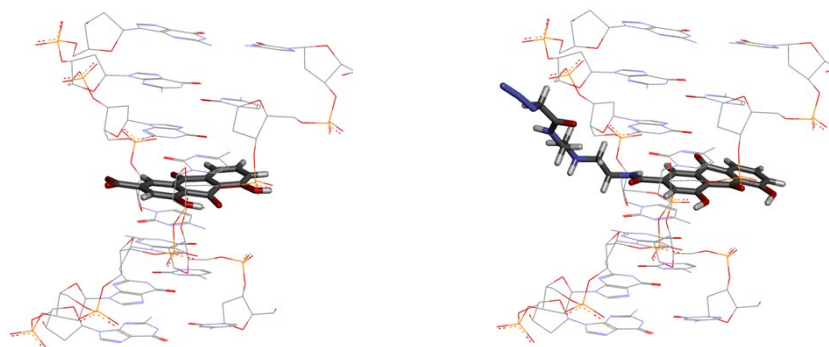
(or media for controls) for 3 h.

After rinsing with chilled PBS (3×), cells were fixed by treatment with 3.7% formaldehyde solution (in PBS, pH 7.4) for 15 min at room temperature and subsequently washed with a 3% solution of bovine serum albumin (BSA; Sigma) in PBS (pH 7.4) twice for 10 min. Cells were then permeabilized by treatment with 0.5% Triton X-100 (in PBS, pH 7.4) at room temperature for 20 min. Permeabilization buffer was quenched with 3% BSA in PBS (2 × 10 min) and the cells were incubated with 250 μL of click reaction mixture (1 mM CuSO<sub>4</sub>; 0.5 μM Alexa Fluor 488-alkyne (Thermo Fisher Scientific Co., USA); 10 mM sodium ascorbate; 50 mM Tris-HCl, pH 7.4) at room temperature for 30 min. For the no-copper control, the copper solution in the click buffer was replaced with water. Cells were subjected to extensive washes with gentle agitation: 1) 3% BSA in PBS (5 min); 2) 0.5% Triton X-100 in PBS (2 × 10 min); 3) PBS for (3 × 10 min). Nuclei were stained by 1 μg·mL<sup>-1</sup> DAPI (Sigma) in PBS for 15 min. Three final PBS washes were performed immediately prior to image capture.

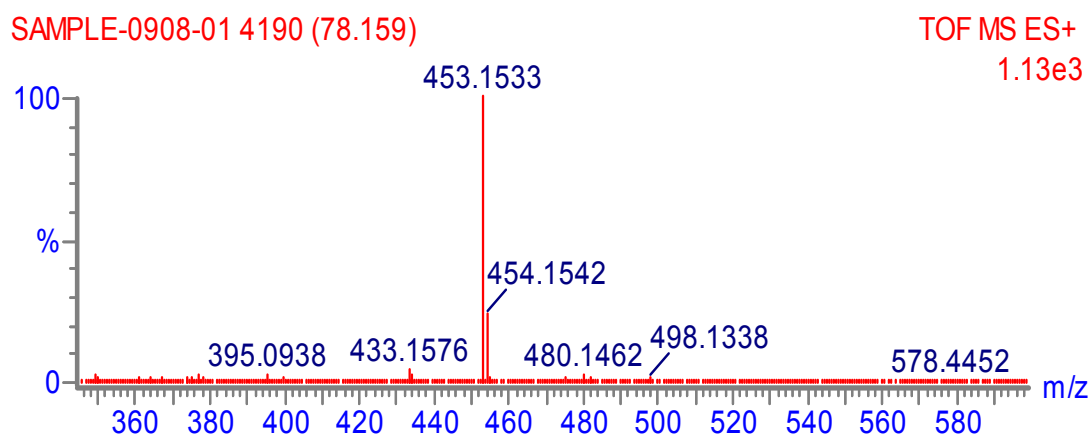
### **1.11 Confocal Microscopy**

Images were collected using a Zeiss LSM 710 confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) using either a 40x (PLAN APO, 0.95 NA) or a 63x (PLAN APO, 1.2 NA) objective lens. All images were acquired in multi-track configuration mode to minimize excitation cross talk and emission bleed-through. We utilized a 405 nm laser line (for DAPI) with an emission range of 424-466 nm and a 488 nm laser line (for Alexa Fluor 488) with an emission range of 489-553 nm.

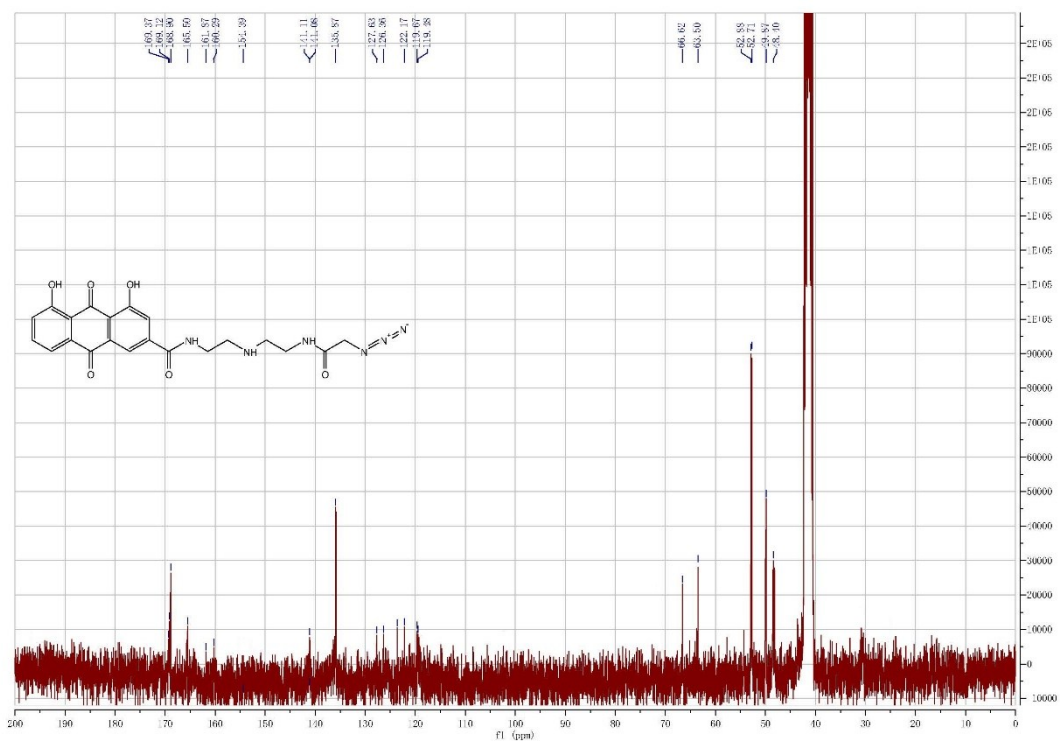
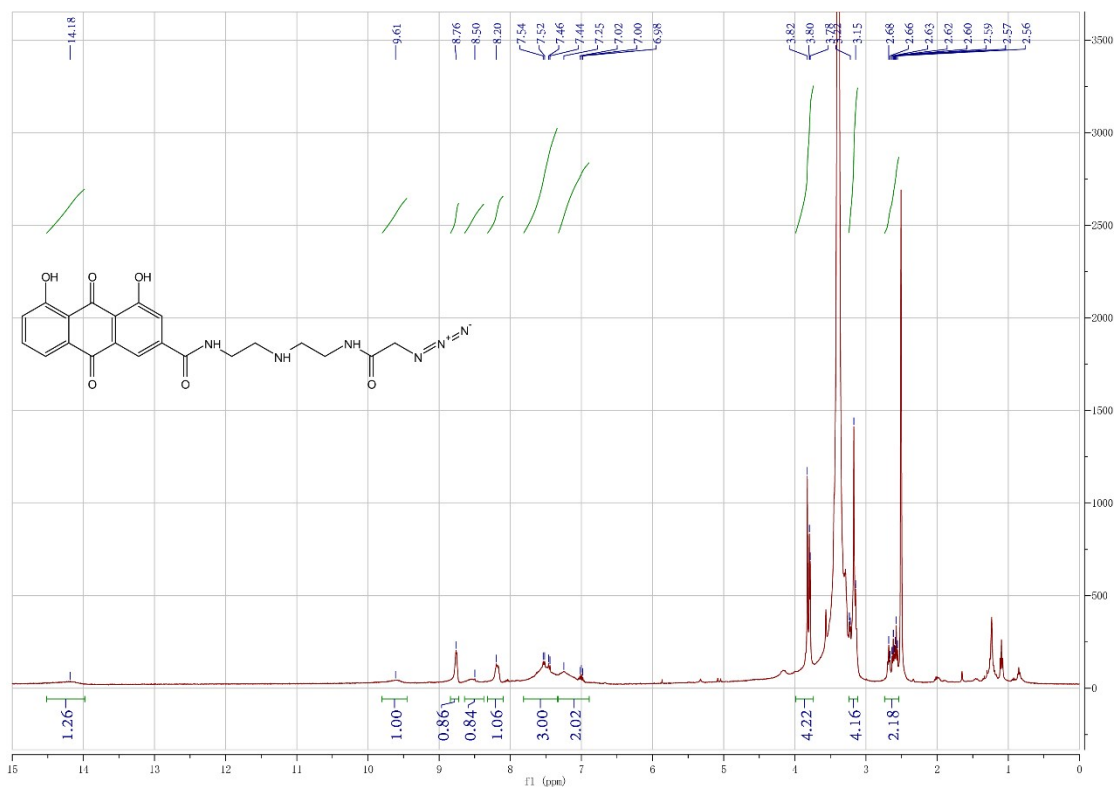
## **2. Supplementary Figures**

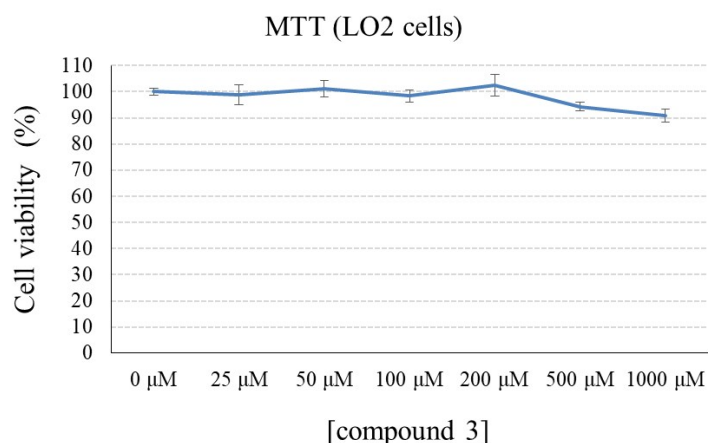


**Figure S1.** Molecular models of the monofunctional intercalative adducts at the DNA major groove formed by rhein (left) and compound **3** (right) based on the NMR solution structure of the hybrid adduct (PDB 282D).

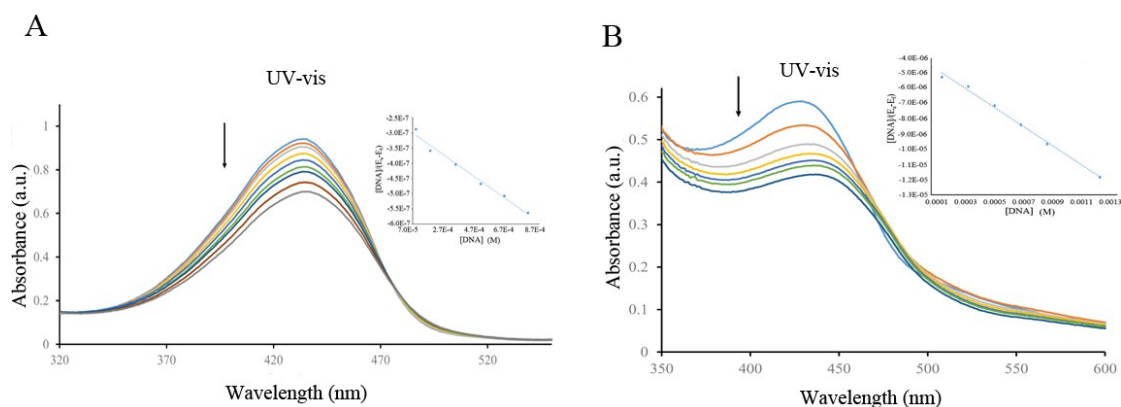


**Figure S2** HRMS of compound **3**

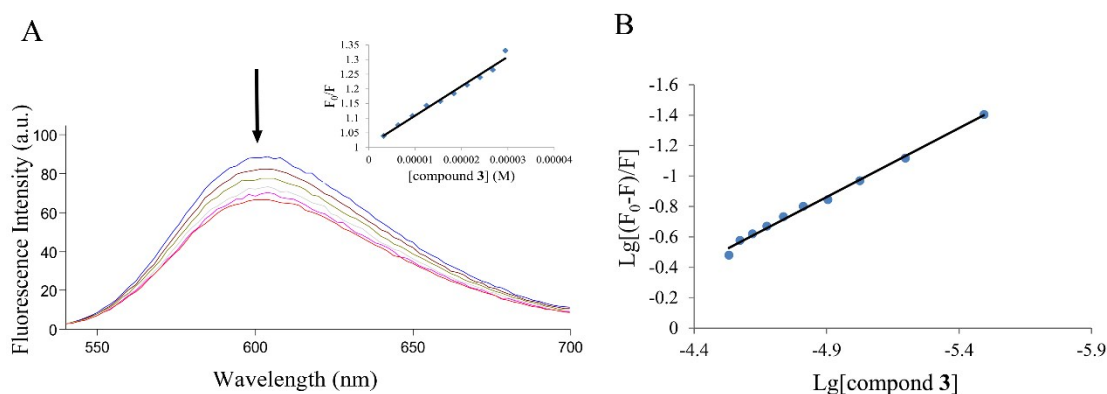




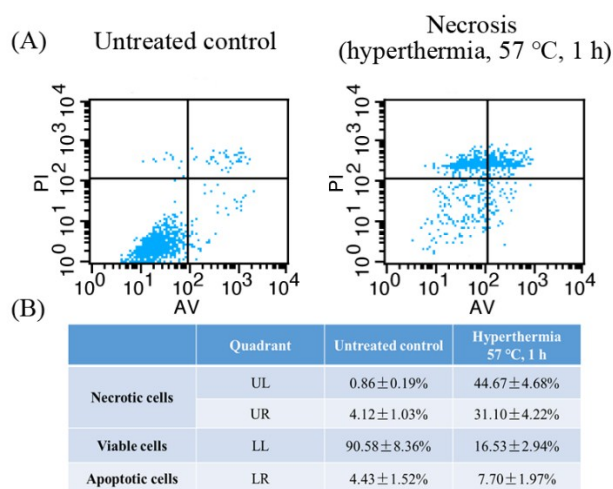
**Figure S5.** Relative cell cytotoxicity (%) of human liver LO2 cells obtained by compound **3**. The standard deviations ((SD) were obtained on a triplicate analysis (n = 3).



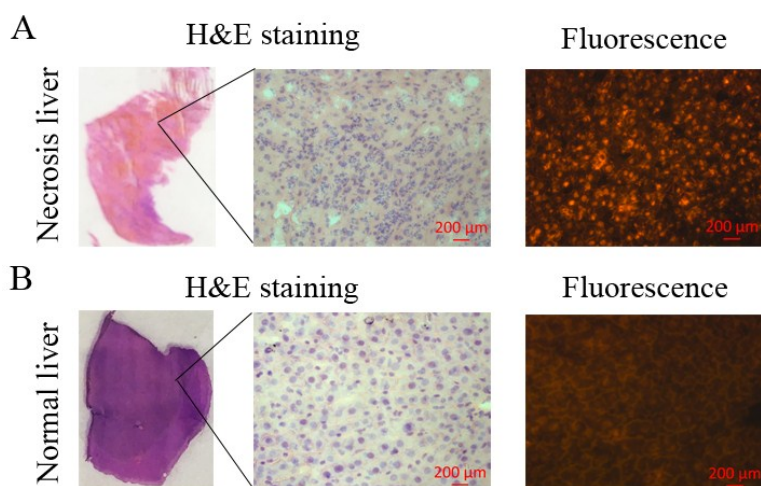
**Figure S6.** (A) Absorption spectra of rhein ( $1.0 \times 10^{-4}$  M) varying with concentrations of ct-DNA (up to down: 0,  $1.0 \times 10^{-4}$ ,  $1.8 \times 10^{-4}$ ,  $3.4 \times 10^{-4}$ ,  $5.0 \times 10^{-4}$ ,  $6.5 \times 10^{-4}$ ,  $8.0 \times 10^{-4}$ ,  $1.06 \times 10^{-3}$  and  $1.31 \times 10^{-3}$  M, respectively). (B) Absorption spectra of compound **3** ( $1.7 \times 10^{-3}$  M) varying with concentrations of ct-DNA (up to down: 0,  $1.7 \times 10^{-4}$ ,  $3.4 \times 10^{-4}$ ,  $5.1 \times 10^{-4}$ ,  $6.8 \times 10^{-4}$ ,  $8.5 \times 10^{-4}$  and  $1.2 \times 10^{-3}$  M, respectively). The arrow shows the absorbance change upon increasing the ct-DNA concentration. Inset: plot of  $[DNA]/(\epsilon_a - \epsilon_f)$  versus  $[DNA]$ .



**Figure S7.** (A) Fluorescence spectra of EB bound to Ct-DNA in the absence and presence of compound **3** ( $[DNA] = 2.40 \times 10^{-5}$  M,  $[EB] = 0.60 \times 10^{-5}$  M,  $[compound\ 3] = 0, 0.60, 1.20, 1.80, 2.40, 3.00 \times 10^{-5}$  M), the arrows show the intensity changes upon the addition of increasing concentrations of compound **3**, respectively. Inset: plot of  $[F_0]/F$  versus  $[compound\ 3]$ . (B) Scatchard plot of  $\lg[(F_0 - F)/F]$  vs.  $\lg[compound\ 3]$  for compound **3**.



**Figure S8** (A) Flow cytometry obtained with untreated A549 cells and those treated with intense hyperthermia at 57 °C for 1 h. Apoptotic cells (annexin-V-positive, PI-negative) were on the LR (lower right) quadrant, necrotic cells (annexin-V-positive or -negative, PI-positive) were on the UR (upper right) and UL (upper left) gate and viable cells (annexin-V-negative, PI-negative) were on the LL (lower left) gate. (B) In vitro determination of the percentage of viable, apoptotic and necrotic cells by flow cytometry after the treatment of A549 cells. The results are expressed as the percentage relative to the mean of fluorescence.



**Figure S9** Images of H&E staining and fluorescence imaging from 10 μm frozen sections of PI (10 mg/kg) at 3 h p.i. in rat models of Liver infarction necrosis. (A) PI in necrosis liver, (B) PI in normal liver.

**References:**

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