Supporting Information

Novel Radiation-Activated N-Oxide Prodrugs for Highly Selective and Synergistic Tumor Therapy to Promote DNA Damage and ATM/ATR Pathway

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1. Materials and methods

1.1 Radiation-activated of N-oxide prodrugs

A 10 mM stock solution of **NOS** and **NOR** were prepared in DMSO and then diluted with anaerobic water to a final concentration of 5 μM. The solutions were irradiated with X-ray at doses ranging from 0 - 40 Gy. The reaction mixture was analyzed by HPLC.

1.2 DFT calculations

DFT calculations were performed using the Gaussian09 software. A single-point energy calculation was conducted at the level of the B3LYP-D3(BJ) functional with the 6-31G* basis set to obtain the wavefunction information and the HOMO/LUMO orbital energies for subsequent analysis.

1.3 Stability analysis in plasma

A mixture of **NOS** (10 mM in DMSO) and fresh mouse plasma was incubated at 37°C to achieve a final concentration of 5 μM. Aliquots were collected at 0, 0.5, 1, 1.5, 2, 4, 8, 12, 24, and 48 h, followed by reaction termination with three volumes of acetonitrile. After centrifugation, the supernatant was analyzed by LC-MS to determine the remaining **NOS** concentration. The data were normalized to 0 h.

1.4 Calculation of radiation chemical yields

The radiation chemical yield (G-value) quantifies the amount of a target chemical product generated per unit of radiation dose. In this work, the G-value is defined as: the nanomoles (nM) of product generated per liter (L) of solution per Gray (Gy) of absorbed radiation dose, with units of nM Gy^{-1}.

$$G\text{-value}=\Delta C/D \quad (1)$$

Where ΔC is the net increase in product concentration (in nM) after irradiation, determined by quantitative **LC-MS** analysis and corrected for the background concentration from non-irradiated controls; D is the absorbed radiation dose (in Gy) delivered to the sample.

1.5 Cell culture

All the cell lines used in this study, including HepG2, A549, HT29 and NCM460, were purchased from Shanghai Institute of Cell Biology (China). The cells were cultured in DMEM or RPMI 1640 media using a cell culture incubator maintained at 37°C with 5% CO_2.

1.6 MTT assay

HepG2, A549, HT29, and NCM460 cells in logarithmic growth phase were trypsinized and resuspended in culture medium containing 10% FBS. The cells were seeded at a density of 1×10^4 per well in 96-well plates with 100 μL of medium and incubated for 24 h. Then, the old culture medium was replaced with fresh medium containing the drugs at a volume of 100 μL per well. The **X-ray** group were incubated with AnaeroPack (D - 07) for 2 h and then exposed to 20 Gy X-ray, then incubated in the normoxic environment for 72 h. The cell viability was measured by MTT assay.

1.7 Immunofluorescence staining of γ-H2AX

HepG2 cells were seeded at a density of 1×10^5 cells/mL in confocal culture dishes and incubated for 24 h. Then, the old culture medium was replaced with 1 ml of fresh medium containing **NOS** (10 μM). Subsequently, the cells in the X-ray groups were incubated with AnaeroPack (D - 07) for 2 h and then exposed to 20 Gy X-ray. The cells were then stained with γ-H2AX rabbit monoclonal antibody, anti-rabbit 488, and DAPI, respectively. The fluorescence images were obtained using a confocal microscopy (λex = 488 nm, λem = 500 - 550 nm).

1.8 Western blot assay

HepG2 cells were seeded at a density of 5×10^4 cells/mL in a 6-well plate and incubated for 24 h. Then, the old culture medium was replaced with 1 ml of fresh medium containing **NOS** (10 μM). Subsequently, the cells in the X-ray groups were incubated with AnaeroPack (D - 07) for 2 h and then exposed to 20 Gy X-ray. Cells were lysed using RIPA lysis buffer containing protease and

phosphatase inhibitors. Protein concentration was measured using the BCA assay, and equal amounts of protein were loaded onto each lane of an SDS-PAGE gel for electrophoresis. Subsequently, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. Antibodies were used to detect their expression levels individually.

1.9 Detection of intracellular ROS by confocal technology

HepG2 cells were seeded at a density of 1×10^5 cells/mL in confocal culture dishes and incubated for 24 h. Then, the old culture medium was replaced with 1 ml of fresh medium containing **NOS** (10 μM). Subsequently, the cells in the X-ray groups were incubated with AnaeroPack (D - 07) for 2 h and then exposed to 20 Gy X-ray. Cells were then incubated with DCFH-DA (10 μM) for 20 min. The luminescence of DCFH-DA was acquired using confocal microscopy (λex = 488 nm, λem = 500 - 550 nm).

1.10 Detection of intracellular ROS by flow cytometry

HepG2 cells were seeded at a density of 1×10^5 cells/mL in confocal culture dishes and incubated for 24 h. Then, the old culture medium was replaced with 1 ml of fresh medium containing **NOS** (10 μM). Subsequently, the cells in the X-ray groups were incubated with AnaeroPack (D - 07) for 2 h and then exposed to 20 Gy X-ray. Cells were incubated with DCFH-DA (10 μM) for 20 min, then digested with trypsin and collected. Cells were analyzed by flow cytometry, and data from 10,000 events were recorded to determine the mean fluorescence intensity.

1.11 Mitochondrial membrane potential (MMP) detection

HepG2 cells were seeded at a density of 1×10^5 cells/mL in confocal culture dishes and incubated for 24 h. Then, the old culture medium was replaced with 1 ml of fresh medium containing **NOS** (10 μM). Subsequently, the cells in the X-ray groups were incubated with AnaeroPack (D - 07) for 2 h and then exposed to 20 Gy X-ray. Cells were then incubated with JC-1 (10 μg) for 20 min. The fluorescence images were obtained using a confocal microscopy. λex = 488 nm, λem = 500 - 550 nm (JC-1 monomers); λex = 535 nm, λem = 580 - 610 nm (JC-1 aggregates).

1.12 Calcein-AM/ PI staining

HepG2 cells were seeded at a density of 1×10^5 cells/mL in confocal culture dishes and incubated for 24 h. Then, the old culture medium was replaced with 1 ml of fresh medium containing **NOS** (10 μM). Subsequently, the cells in the X-ray groups were incubated with AnaeroPack (D - 07) for 2 h and then exposed to 20 Gy X-ray, then incubated in the normoxic environment for 72 h. The cells were then stained with Calcein-AM (2 μM, live cell marker) and PI

(8 μM, dead cell marker) for 30 min. The fluorescence images were obtained using a confocal microscopy. λex = 488 nm, λem = 500 - 530 nm (calcein-AM); λex = 535 nm, λem = 600 - 630 nm (PI).

1.13 Flow cytometry for apoptosis assay

HepG2 cells were seeded at a density of 5×10^4 cells/mL in a 6-well plate and incubated for 24 h. Then, the old culture medium was replaced with 1 ml of fresh medium containing **NOS** (10 μM). Subsequently, the cells in the X-ray groups were incubated with AnaeroPack (D - 07) for 2 h and then exposed to 20 Gy X-ray, then incubated in the normoxic environment for 72 h. The cells were then digested with trypsin, and co-stained with Annexin V-FITC/PI for 30min. Flow cytometry was used to detect cell apoptosis. Cells were analyzed by flow cytometry, and data from 10,000 events were recorded to determine the apoptosis cells.

1.14 Animal models

All animal experiments were performed in compliance with the ARRIVE 2.0 guidelines and were approved by the Animal Ethics Committee of Nantong University (Approval No. S20210925-003). All procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. Nude mice (female mice, 5 - 6 weeks old) were purchased from the Model Animal Research Center Affiliated (MARCA) of the Nanjing University. HepG2 cells (1×10^6) suspended in PBS were subcutaneously injected into flanks of the mice to establish subcutaneous tumor models.

1.15 Enrichment analysis in vivo

HepG2 tumor-bearing mice were administered **NOS** (10 mg/kg) via vein injection. At predetermined time points, the mice were euthanized, and tumor tissues were collected and weighed. The concentration of **NOS** in the tumor tissues was quantified using LC-MS.

1.16 In vivo radiation-activated anti-tumor activity of NOS

Once the tumor volumes reached approximately 80 - 100 mm^3, the mice were randomly divided into four groups (n = 5): PBS, Sorafenib (10 mg/kg), **NOS** (10 mg/kg), and **NOS** + X-ray (10 mg/kg, 4 Gy). The mice received intravenous injections of sorafenib or **NOS** every 3 days for a total of 4 administrations. Two hours post-injection, the X-ray treated groups received X-ray radiation, while the remaining groups did not. Tumor volumes were determined using calipers and the body weights of the mice were monitored throughout the study. At the end of the experiment, the mice were euthanized, and their tumors were dissected out and weighed. Tumor volume was calculated as $V = (\text{length} \times \text{width}^2)/2$.

1.17 Safety and toxicity evaluation of NOS

ICR mice (female, n=6) were intravenously administered the **NOS** (10 mg/kg), Sorafenib (10 mg/kg), or vehicle. On day 14, blood was collected for complete blood count (CBC) and serum biochemistry (AST, ALT, ALP, BUN, CREA). Major organs were harvested for histopathological examination.

1.18 Statistical analysis

Experimental data are presented as Means ± SD. Differences between groups were analyzed using a two-tailed unpaired t-test or one-way ANOVA, with $P < 0.05$ considered statistically significant.

2. Synthesis

2.1 Synthesis of compound NOS

Compound sorafenib (464 mg, 1 mmol) and 3-Chloroperbenzoic acid (5 mmol) were dissolved in 10 mL dichloromethane in a Schlenk flask. The mixture was reacted at room temperature for 72h. Upon completion of the reaction, the solvent was removed by rotary evaporation. The resulting product was purified by silica gel column chromatography (DCM/MeOH = 99/1) to remove impurities, yielding product **NOS** as a white solid (80% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.41 (d, J = 4.9 Hz, 1H, NH), 9.22 (s, 1H, NH), 9.01 (s, 1H, NH), 8.37 (d, J = 7.2 Hz, 1H, ArH), 8.11 (d, J = 2.5 Hz, 1H, ArH), 7.67 – 7.54 (m, 5H, 5ArH), 7.29 – 7.23 (m, 1H, ArH), 7.18 (d, J = 8.6 Hz, 2H, 2ArH), 2.85 (d, J = 4.8 Hz, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ 159.83, 157.10, 152.93, 148.69, 142.89, 140.82, 139.78, 137.57, 132.47, 131.08, 129.29, 128.38, 127.17 (q, $^{2}J_{CF}$ = 30.3 Hz), 123.58, 123.29 (q, $^{1}J_{CF}$ = 272.7 Hz), 122.81, 121.58, 120.98, 117.28, 114.13, 26.32. HRMS (ESI, m/z): calculated for $C_{21}H_{17}ClF_3N_4O_4$ $[M+H]^+$: 481.0885 and 483.0856, found: 481.0880 and 483.0845.

2.2 Synthesis of compound NOR

Compound regorafenib (482 mg, 1 mmol) and 3-Chloroperbenzoic acid (5 mmol) were dissolved in 10 mL dichloromethane in a Schlenk flask. The mixture was reacted at room temperature for 72h. Upon completion of the reaction, the solvent was removed by rotary evaporation. The resulting product was purified by silica gel column chromatography (DCM/MeOH = 99/1) to remove impurities, yielding product **NOR** as a white solid (83% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.38 (q, J = 4.8 Hz, 1H, NH), 9.54 (s, 1H, NH), 8.76 (s, 1H, NH), 8.42 (d, J = 7.2 Hz, 1H, ArH), 8.22 – 8.10 (m, 2H, 2ArH), 7.71 (s, 3H, 3ArH), 7.63 – 7.51 (m, 4H, 4ArH), 7.10 (d, J = 8.9 Hz, 1H, ArH), 2.88 (d, J = 4.9 Hz, 3H, CH3). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.57, 159.78, 156.40,

153.22 (d, $^{1}J_{CF}$ = 243.8 Hz), 152.63, 143.00, 140.92, 139.47, 133.81, 133.20, 132.59, 131.14, 129.30, 128.40, 127.25 (q, $^{2}J_{CF}$ = 30.5 Hz), 125.29 (d, $^{3}J_{CF}$ = 8.4 Hz), 123.26 (d, $^{2}J_{CF}$ = 27.4 Hz), 123.25 (q, $^{1}J_{CF}$ = 271.6 Hz), 117.65, 117.09, 114.74, 109.11 (d, $^{2}J_{CF}$ = 22.0 Hz), 26.36. HRMS (ESI, m/z): calculated for $C_{21}H_{17}ClF_4N_4O_4$ $[M+H]^+$: 499.0791 and 501.0762, found: 499.0784 and 501.0750.

3. Supplementary figures

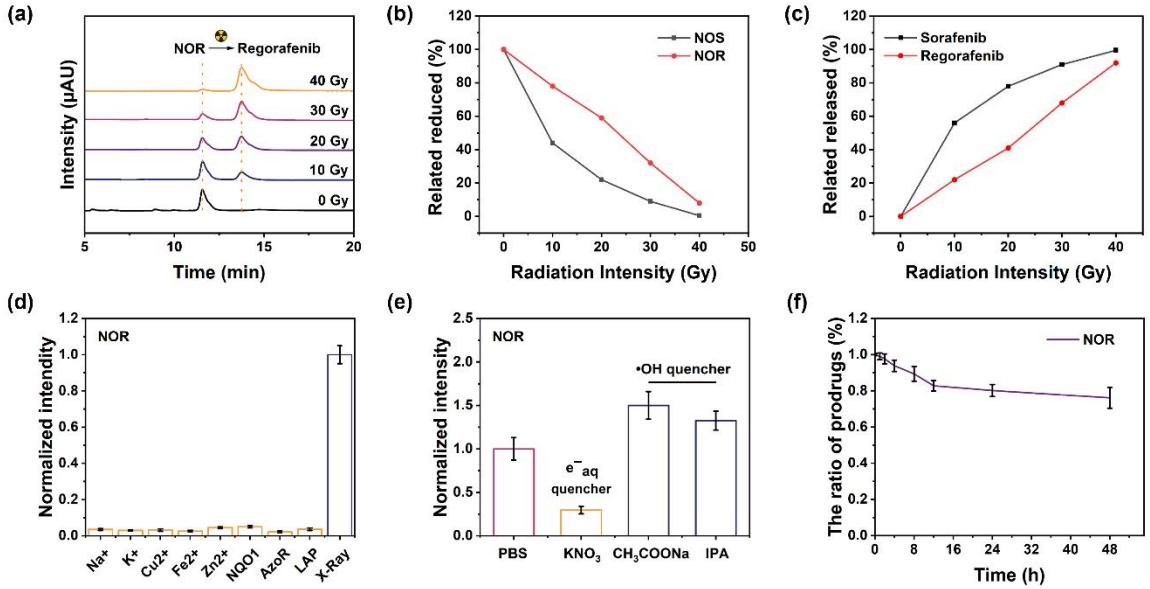

Fig. S1 (a) Radiation dosage-dependent generation of parent drugs from **NOR** determined by HPLC. The related reduce of prodrugs (b) and release of parent drugs (c) after different radiation doses. (d) HPLC analysis of the mixture solutions containing **NOR** and different biologically relevant species. (e) Normalized amount assay of generated regorafenib from **NOR** treated with e^{-}_{aq} and •OH quenchers after radiation. (f) Stability of **NOR** in plasma. Means ± SD, n = 3.

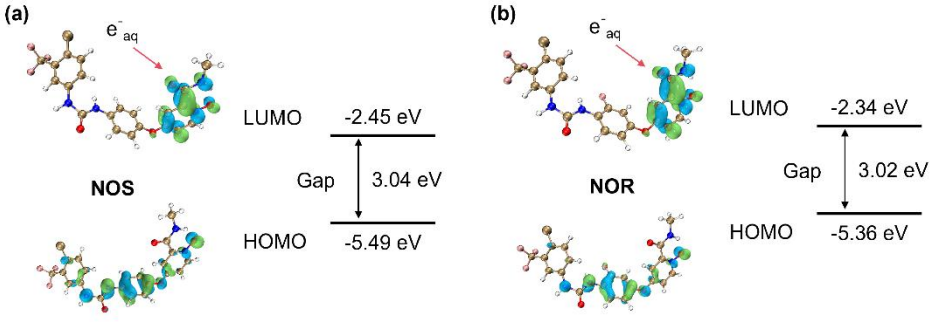

Fig. S2 Calculated HOMO, LUMO and Gap (LUMO-HOMO) of **NOS** (a) and **NOR** (b) from density functional theory (DFT).

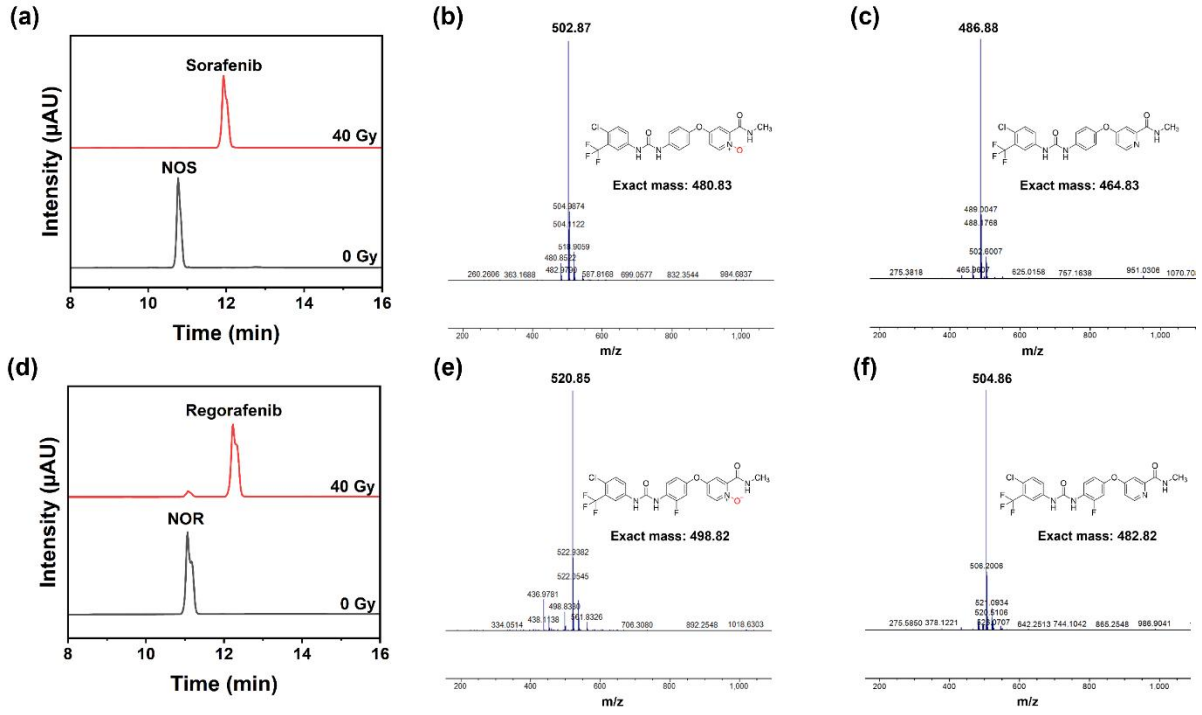

Fig. S3 (a) LC-MS spectra of generated sorafenib from **NOS** after 40 Gy radiation. Positive ion mode mass spectrum is shown for (b) [**NOS**+Na]$^+$ and (c) [sorafenib+Na]$^+$. (d) LC-MS spectra of generated regorafenib from **NOR** after 40 Gy radiation. Positive ion mode mass spectrum is shown for (e) [**NOR**+Na]$^+$ and (f) [regorafenib+Na]$^+$.

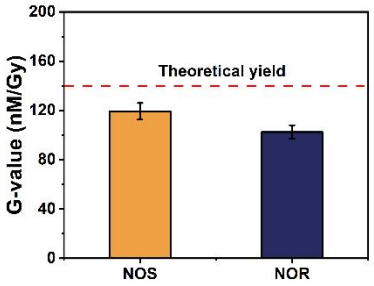

Fig. S4 G-values of generated reduction products from **NOS** and **NOR**. Means ± SD, n = 3.

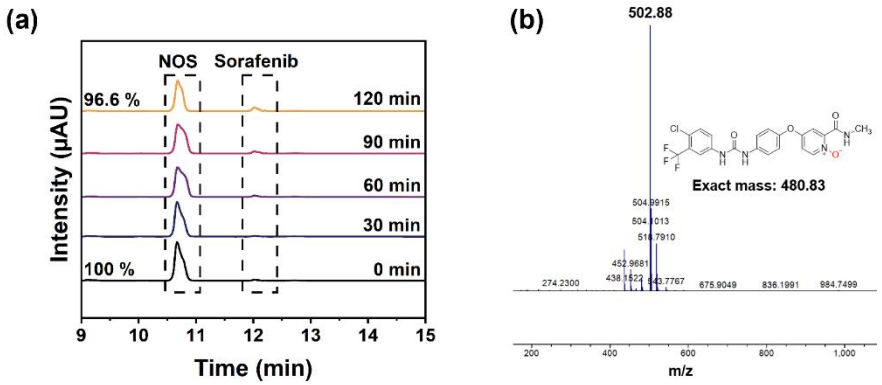

Fig. S5 (a) LC-MS spectra of **NOS** in plasma from 0 - 120 min. (b) Positive ion mode mass spectrum is shown for [**NOS**+Na]$^+$ at 120 min.

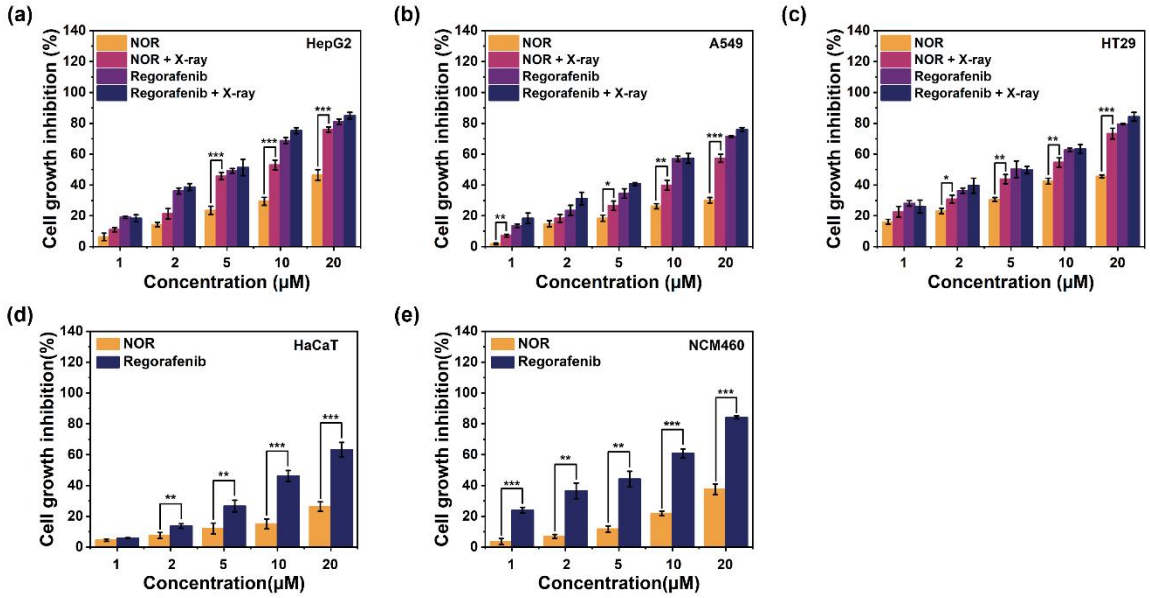

Fig. S6 Cell growth inhibition of **NOR** and regorafenib in (a) HepG2 (b) A549 (c) HT29 cells without X-Ray in normoxia or with X-Ray (20Gy) in hypoxia. Cell growth inhibition of **NOR** and regorafenib in (d) HaCaT and (e) NCM460 cells. Mean ± SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001.

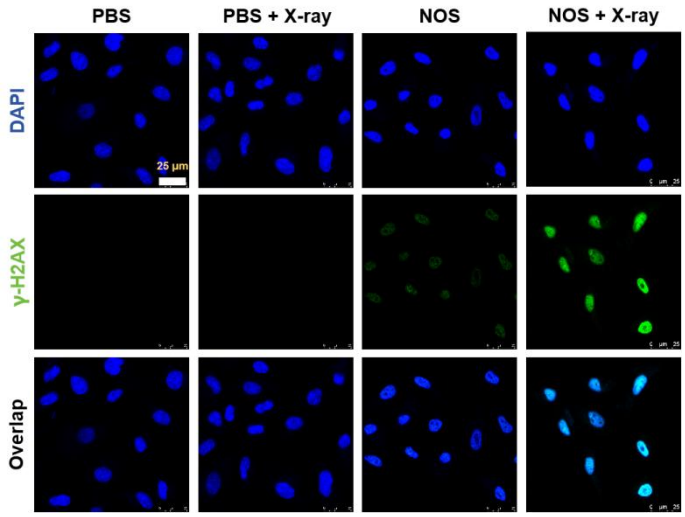

Fig. S7 CLSM imaging of HepG2 cells in different groups after staining with γ-H2AX. Scale bar = 25 μm.

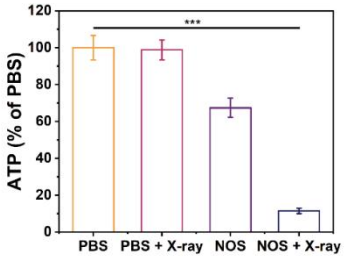

Fig. S8 Quantitative analysis of ATP released into the cell culture supernatant. Mean ± SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001.

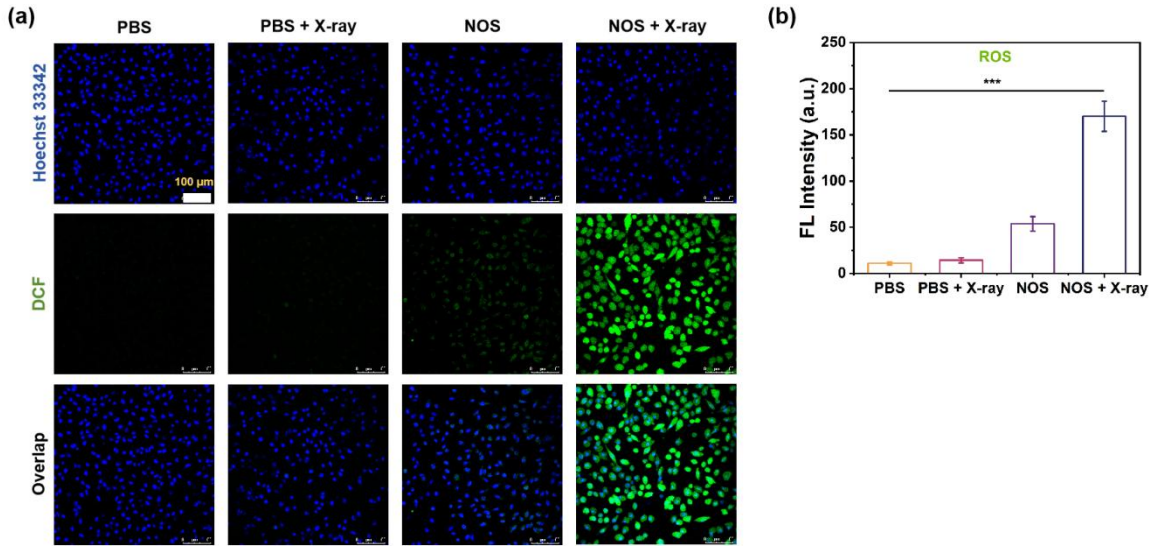

Fig. S9 (a) CLSM imaging of HepG2 cells in different groups after staining with DCFH-DA. Scale bar = 100 μm. (b) Quantitative analysis of DCF fluorescence intensity. Means ± SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001.

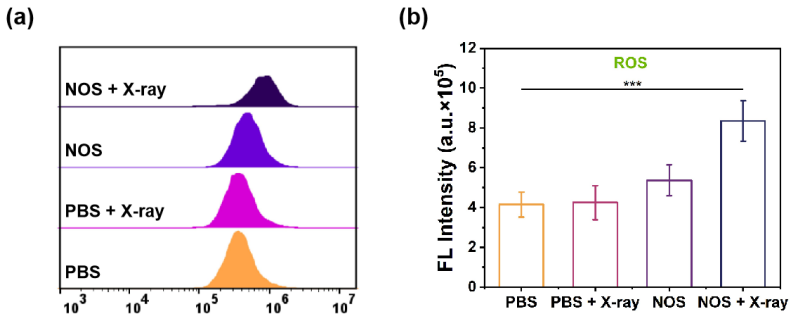

Fig. S10 (a) Flow cytometry analysis of HepG2 cells in different groups after staining with DCFH-DA. (b) Quantitative analysis of DCF fluorescence intensity. Means ± SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001.

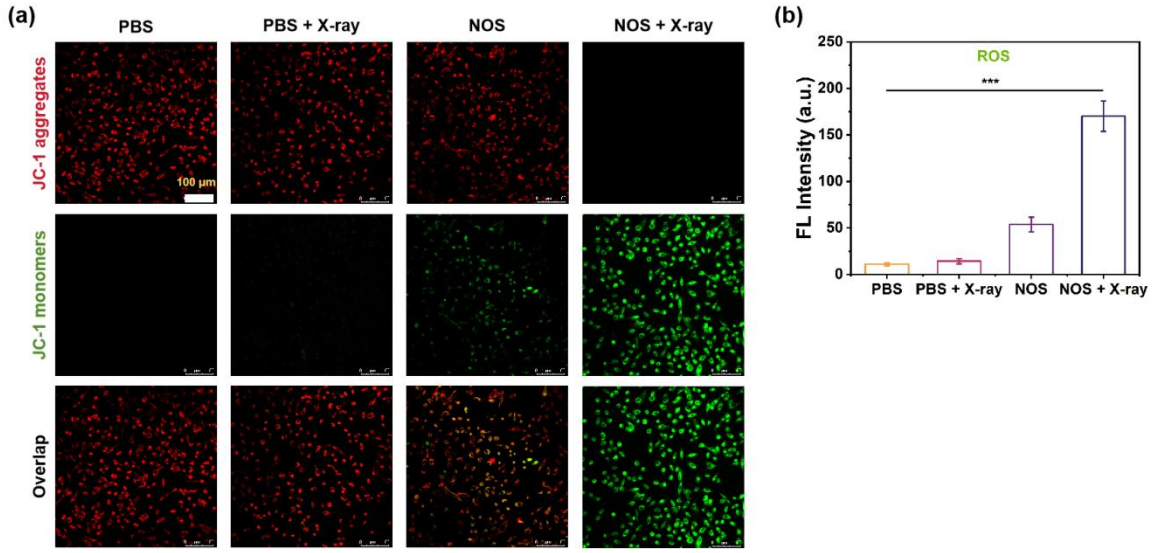

Fig. S11 (a) CLSM imaging of HepG2 cells in different groups after staining with JC-1. Scale bar = 100 μm. (b) Quantitative analysis of red/green fluorescence intensity. Means ± SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001.

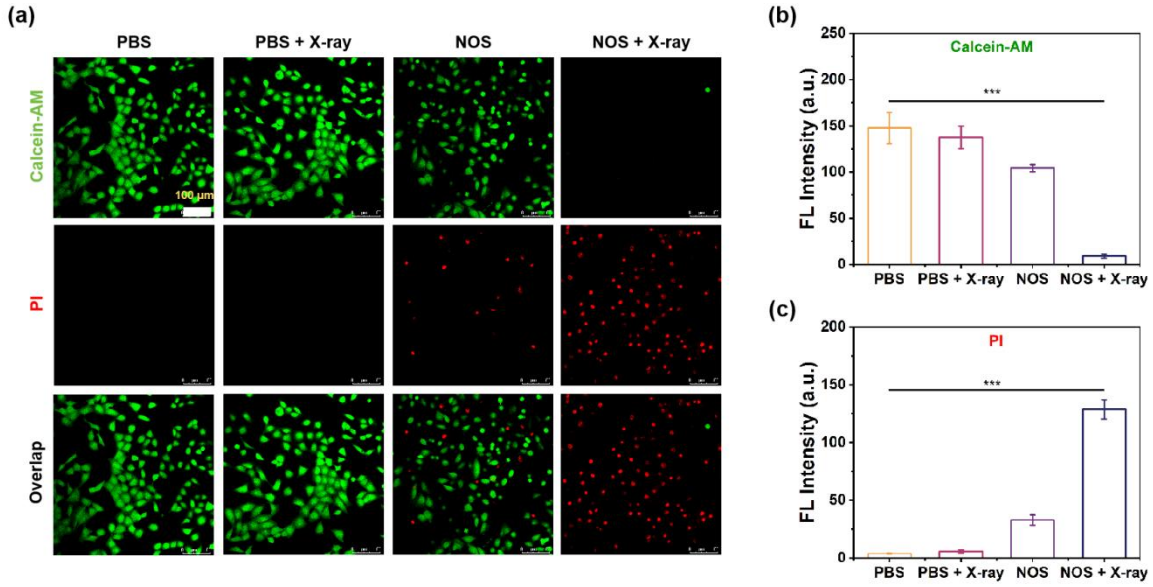

Fig. S12 (a) CLSM imaging of HepG2 cells in different groups after staining with Calcein-AM (live cell marker) and PI (dead cell marker). Quantitative analysis of Calcein-AM (b) and PI (c) fluorescence intensity. Means ± SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001.

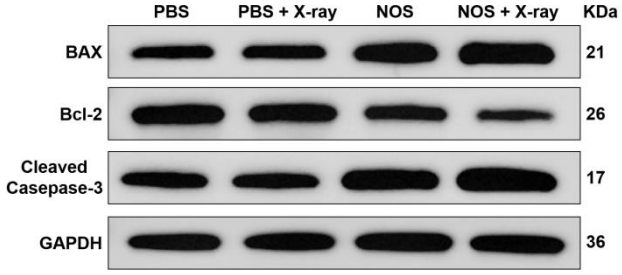

Fig. S13 Western blot results of apoptosis-related proteins.

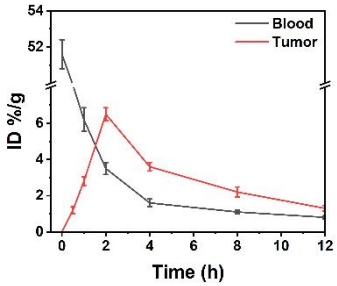

Fig. S14 Time-dependent accumulation of **NOS** in tumor tissue detected by LC-MS. Means ± SD, n = 3.

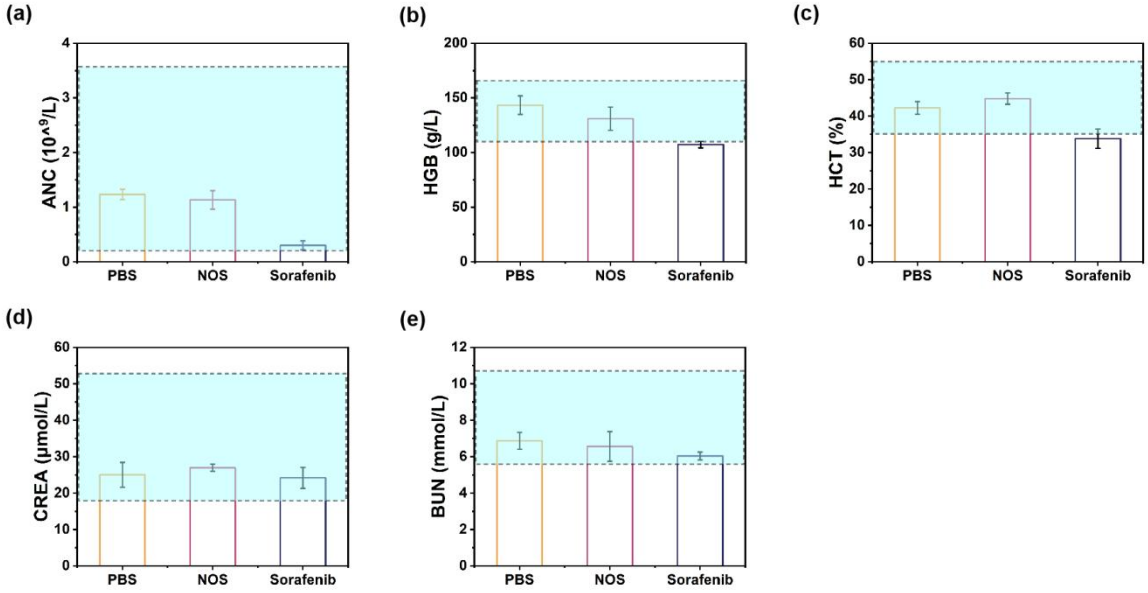

Fig. S15 (a-e) Blood biochemical analysis of mice. Biochemical markers are following: ANC, HGB, HCT, CREA, BUN. Means ± SD, n = 3.

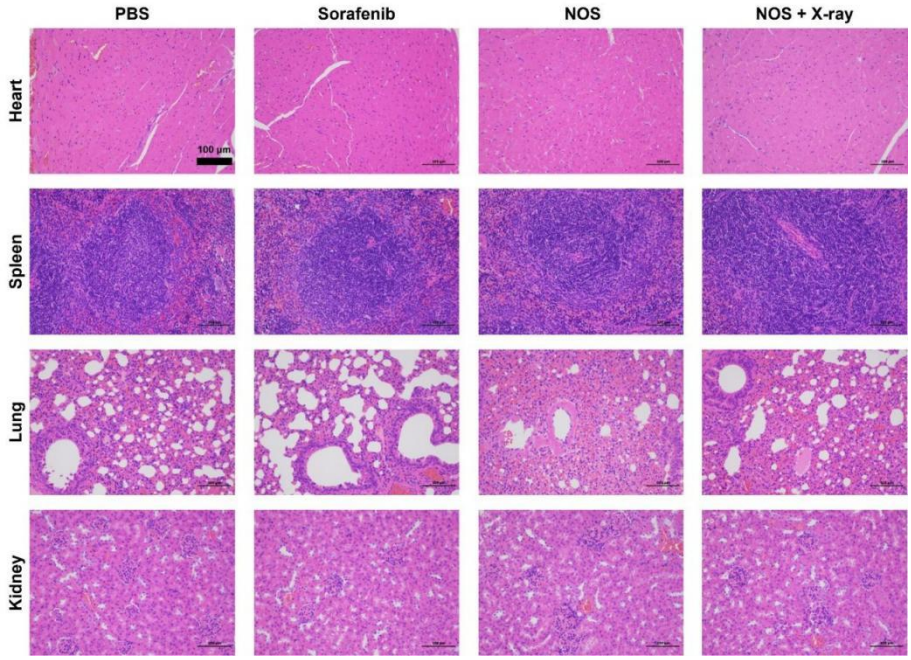

Fig. S16 H&E staining images of different tissues. Scale bar = 100 μm.

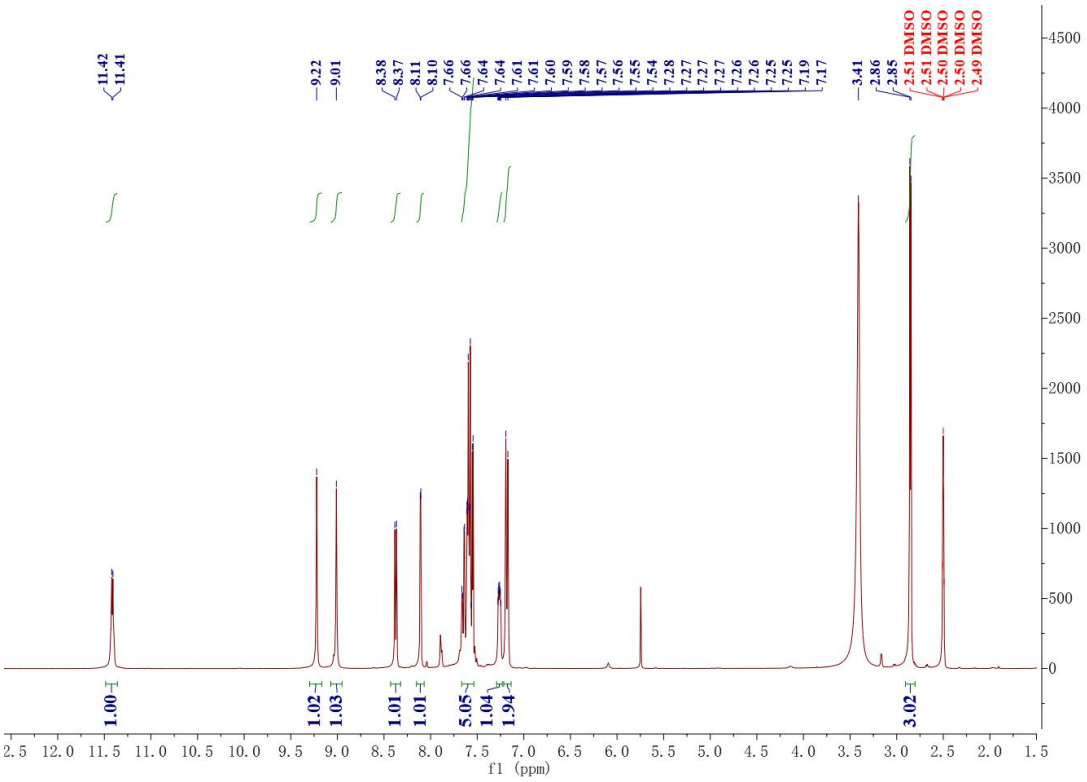

Fig. S17 ^{1}H NMR spectrum of **NOS**

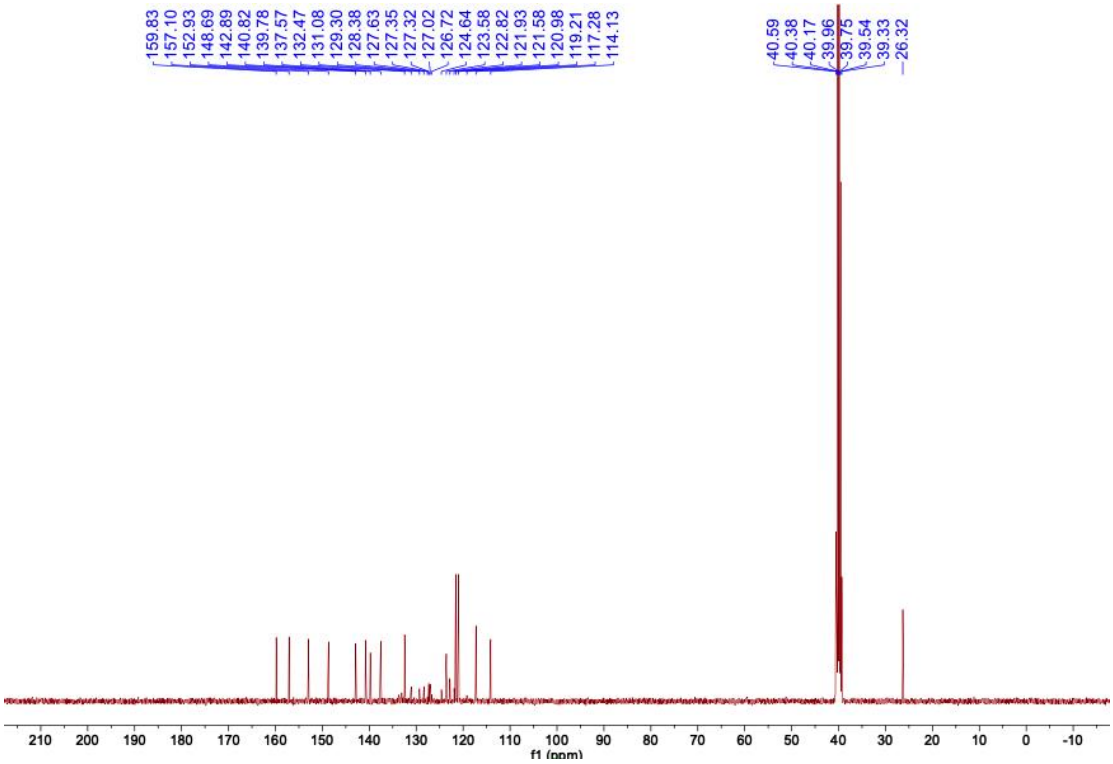

Fig. S18 ^{13}C NMR spectrum of **NOS**

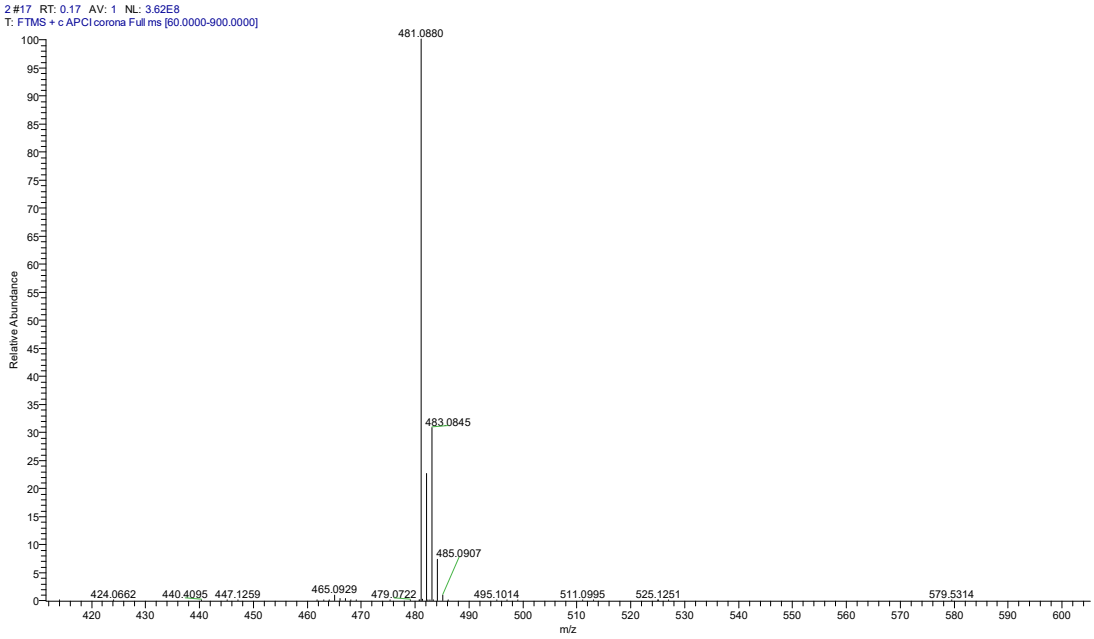

Fig. S19 HRMS spectrum of of **NOS**

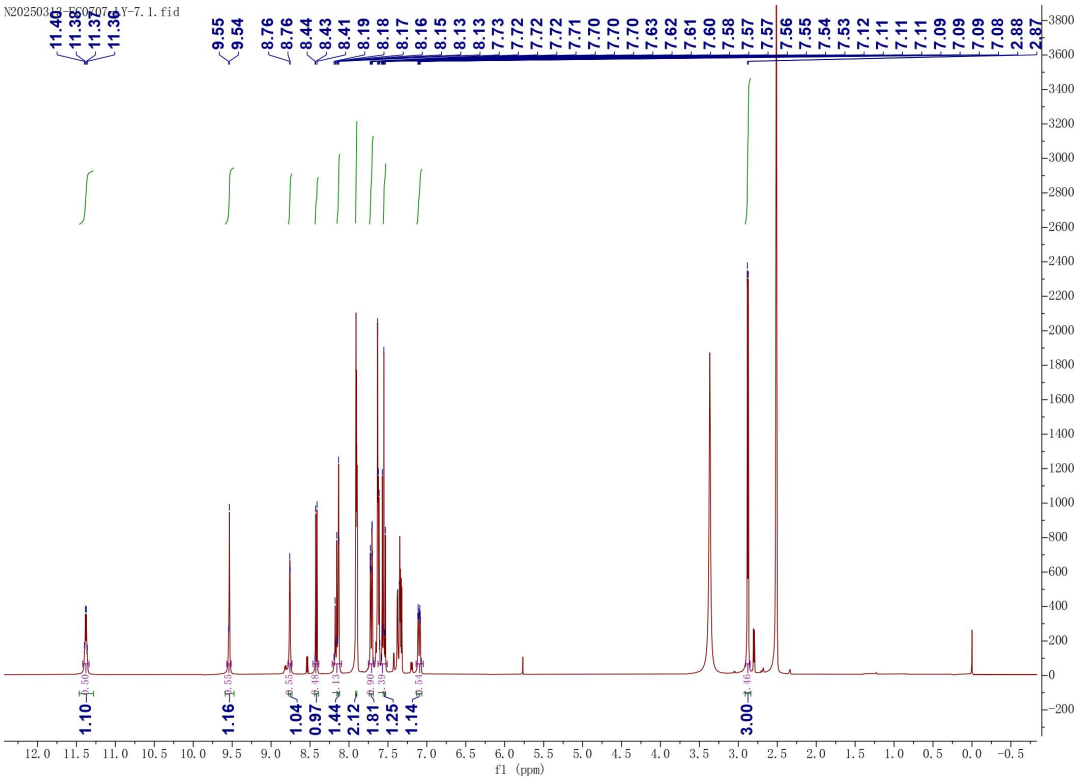

Fig. S20 ^{1}H NMR spectrum of **NOR**

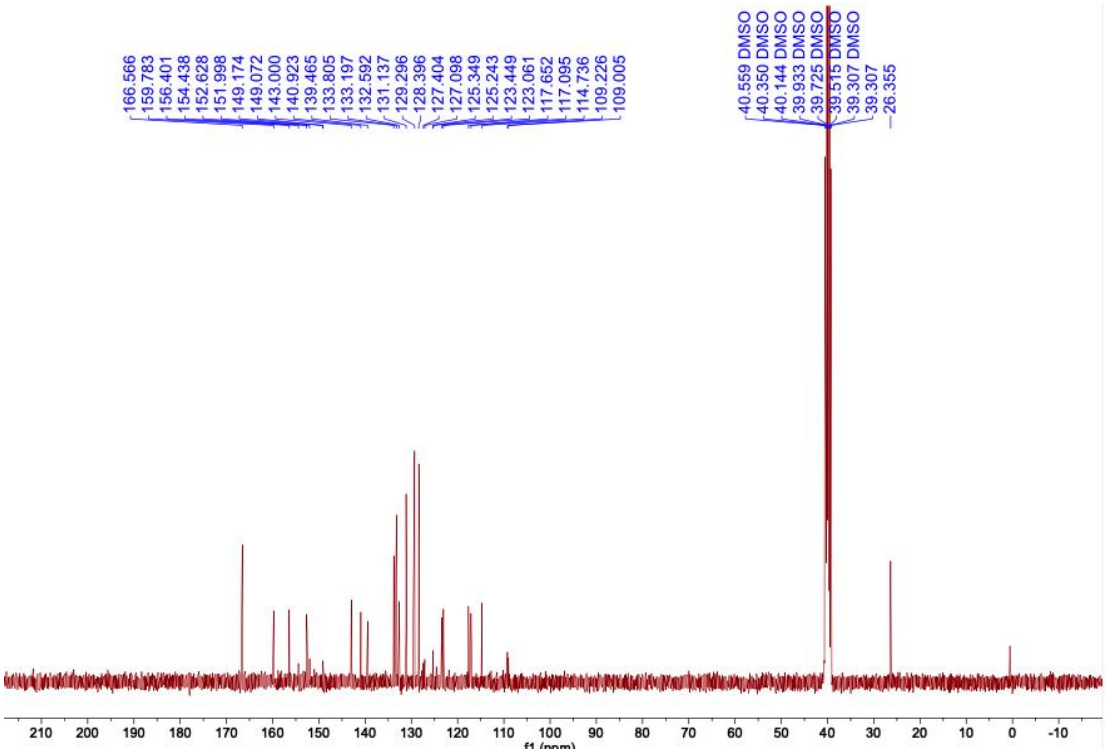

Fig. S21 ^{13}C NMR spectrum of **NOR**

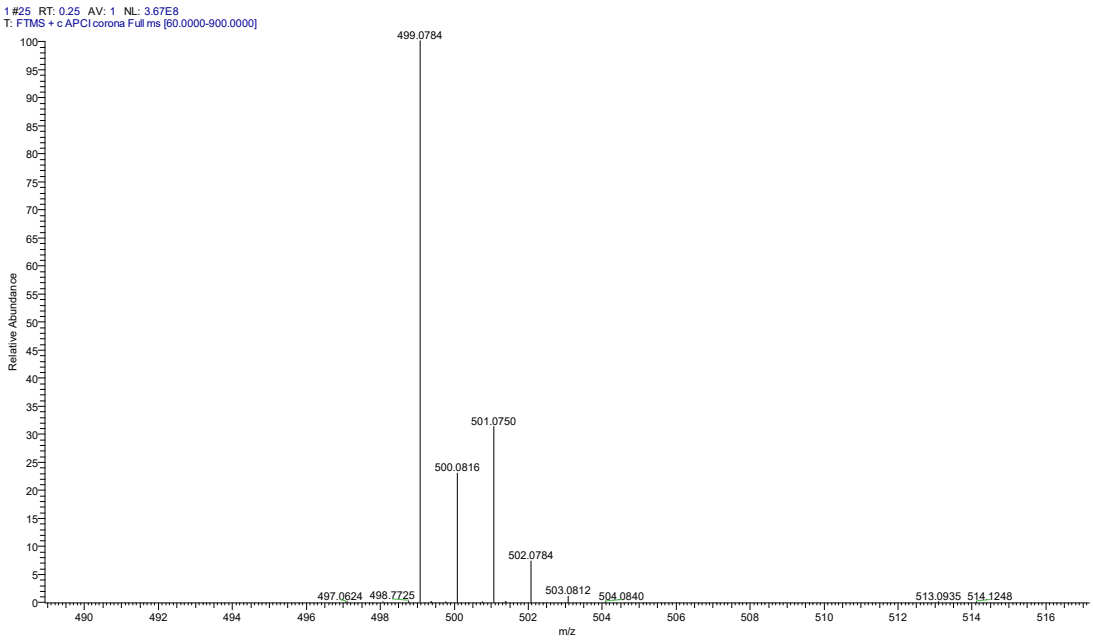

Fig. S22 HRMS spectrum of of **NOR**

4. Supplementary tables

Table S1 *In vitro* chemoradiotherapy activities of N-oxide prodrugs in multiple tumor cells

***In vitro* chemoradiotherapy activities in cancer cells (IC_{50}, μM)**					
Compounds		**NOS**	**Sorafenib**	**NOR**	**Regorafenib**
HepG2	- X-ray[a]	28.6 ± 2.13	2.29 ± 0.11	24.4 ± 1.93	4.40 ± 0.26
	+ X-ray[b]	1.85 ± 0.08	2.07 ± 0.07	7.10 ± 0.31	3.83 ± 0.16
	TI	**15.5**	**1.11**	**3.44**	**1.15**
A549	- X-ray[a]	20.1 ± 1.59	4.01 ± 0.33	33.2 ± 1.88	7.85 ± 0.57
	+ X-ray[b]	3.66 ± 0.19	3.27 ± 0.27	14.9 ± 1.85	6.17 ± 0.22
	TI	**5.49**	**1.23**	**2.22**	**1.27**
HT29	- X-ray[a]	25.9 ± 3.74	5.56 ± 0.41	23.6 ± 1.81	4.13 ± 0.23
	+ X-ray[b]	6.94 ± 0.36	4.46 ± 0.37	6.32 ± 0.49	3.86 ± 0.27
	TI	**3.73**	**1.24**	**3.73**	**1.06**

TI = Therapeutic index defined as [IC_{50}] + X-ray/[IC_{50}] - X-ray. [a]Cytotoxicity of the compounds without X-ray irradiation in normoxia; [b]Cytotoxicity of the compounds with X-ray irradiation in hypoxia. Mean ± SD, n = 3.

Table S2 *In vitro* cytotoxicities of N-oxide prodrugs in normal cell

***In vitro* cytotoxicities in normal cells (IC_{50}, μM)**				
Compounds	**NOS**	**Sorafenib**	**NOR**	**Regorafenib**
NCM460	45.3 ± 4.22	2.86 ± 0.07	37.4 ± 5.59	4.42 ± 0.29
SI	**15.8**		**8.46**	
HaCaT	65.7 ± 2.32	5.05 ± 0.41	123 ± 23.7	12.0 ± 1.01
SI	**13.0**		**10.3**	

SI = Selectivity index defined as [IC_{50}] **NOS** or **NOR** /[IC_{50}] Sorafenib or Regorafenib. Mean ± SD, n = 3.