Supplementary Information

A Bioluminescent Probe for NQO1 Overexpressed Cancer Cell Imaging In Vitro and In Vivo

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1. General methods

1.1 Experimental materials and instruments.

All chemicals were obtained from commercial sources and used without further purification except otherwise noted. NAD(P)H: quinone oxidoreductase1(NQO1), tyrosinase (TYR), acetylcholinesterase (AchE), butyrylcholinesterase (BChE) and

Firefly luciferase were purchased from Sigma-Aldrich. Water used for all biological studies was purified with a Milli-Q filtration system. The cell lines were obtained from ShangHai MEIXUAN Biological science and technology Co., LTD.

^{1}HNMR spectra were recorded on Bruker DMX-300 or DMX-400 spectrometer. Electrospray ionization mass spectra (ESI-MS) were carried out with positive mode or negative mode on a Shimadzu LC-MS 2010A instrument (Kyoto, Japan). The absorbance for MTT analysis, Bioluminescence in vitro and in celluar, were measured on BioTek. Bioluminescence images in vivo were taken with IVIS Lumina III (Perkin Elmer) equipped with a cooled charge-coupled device (CCD) camera. The Luminescence spectra were recorded on a Hitachi F-7100 fluorescence spectrophotometer.

1.2 Bioluminescent response of NQO1-Luc to NQO1 in vitro

The Bioluminescence detection of NQO1-Luc *in vitro* was performed in PBS buffer (10 mM, pH 7.4) with 10 mM $MgSO_4$ and 100 μM NADH at 37 °C in 96-well plates. 25 μL of NQO1-Luc and 25 μL of NQO1 solution (or other biomolecules) were first mixed and incubated for 1 h, then 50 μL of firefly luciferase (20 μg/mL) and 50 μL of ATP (2.0 mM) was added at the same time. The bioluminescence intensity of each well was measured by BioTek.

1.3 NQO1 activity inhibition assay

NQO1 (40 μg/mL, 25μL) was pre-treated with different concentrations of dicoumarol (a chemical NQO1 inhibitor, 0-400 μM, 25 μL) and incubated for 1h. Then 25 μL of the above mixture was extracted and incubated with 25 μL NQO1-Luc (20 μM) for another 1h. After that 50 μL firefly luciferase (20 μg/mL) and ATP (2.0 mM) was added. The bioluminescence intensity of each well was measured by BioTek.

1.4 The kinetic assay

The kinetic parameters of NQO1-Luc against NQO1 enzyme catalyzed reaction were further determined according to Michaelis–Menten equation. Different concentration of NQO1-Luc (0-20 μM) was added to NQO1 solutions (40 μg/mL) in PBS buffer and incubated at 37 °C for 15min. Then 20 μg/mL firefly luciferase, 2 mM ATP was added to each well at the same time. The bioluminescence intensity of each well was measured by BioTek. The K_m value was calculated by Lineweaver-Burk plot.

1.5 Bioluminescence imaging in living cells

Cells were plated in a 96-well plate at a density of 2×10^4 cells/well and incubated for 24 h, then 200 μL NQO1-Luc at varied concentrations (0 – 20 μM) dissolved in RPMI 1640 medium were added to each well. After 1 h incubation, the bioluminescence intensity was measured by BioTek. In NQO1 inhibition assay, cells were pre-incubated with dicoumarol (200 μM) and incubated for 1 h, followed by treatment with NQO1-Luc (20 μM), the bioluminescence intensity was measured by BioTek.

1.6 The cytotoxicity assay

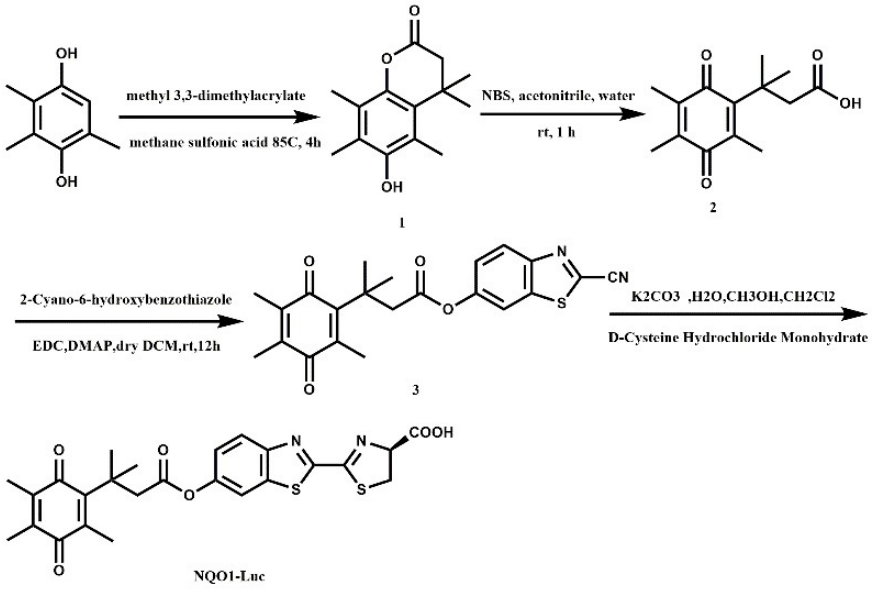
The cytotoxicity of NQO1-Luc was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with A549-Luc cells and MRC5-Luc cells. Cells were seeded in a 96-well plate with RPMI 1640 (10% fetal bovine serum) at a density of 7000 cells/well and incubated (0 – 100 μM) at 37 °C under 5% CO_2 for 24 h, then incubated with varied concentrations of NQO1-Luc (0 – 100 μM) after the removal of medium for another 24 h. 100 μL of MTT solution (0.5 mg/mL in RPMI 1640 medium) was added to each well, and the incubation was continued for 4 h. After that the supernatant was removed then 150 μL DMSO was added to dissolve the formed formazan. The absorbance values were read with BioTek at 490 nm after shaking the plate for 10 min. The viability of cell growth (VR) was calculated according to the

equation: $V_R = A/A_0 \times 100\%$, where A is the absorbance of the treatment group and A_0 is the absorbance of the control group.

1.7 Bioluminescence imaging in Nude Mice

200 μL of cell suspension with 1×10^7 A549-Luc cells were grafted subcutaneously into the right shoulder of each 6-8-week-old BALB/c mouse. Three weeks later, tumor model was established with an average diameter of ca.1.0 cm. The mice were divided into two groups. One group was injected with dicoumarol in saline (200 μM), and another was injected with an equal amount of vehicle. After 1h, all mice were injected intraperitoneally (i. p.) with 100μL NQO1-Luc (2mM, containing 10%DMSO). The bioluminescent signal was recorded by IVIS Kinetic imaging system for 0-100 min.

2. Syntheses and characterizations of NQO1-Luc



Scheme S1 The synthetic route of NQO1-Luc.

2.1 Synthesis of compound 1

Trimethylhydroquinone (32.9 mmol, 5 g) and 3,3-dimethacrylic acid (39.5 mmol, 3.9 g) were dissolved in 30 ml of methanesulfonic acid and the solution was stirred at

85 °C under an nitrogen atmosphere, The reaction solution was stirred for an additional 4-5 h. After the reaction was completed, it was cooled with ice water, the reactant was extracted with ethyl acetate, the organic phase was washed three times with saturated sodium bicarbonate and water, and then dried over anhydrous magnesium sulfate. The crude product was recrystallized with n-hexane/ethyl acetate = 2:1 to give a white solid 1（4.7g，61%）. ^{1}H NMR (300 MHz, DMSO), δ: 4.69 (s, 1H), 2.56 (s, 2H), 2.37 (s, 3H), 2.23 (s, 3H), 2.9 (s, 3H), 1.46 (s, 6H). ESI($C_{14}H_{18}O_3$): $[M\text{-}H^+]^-$ = 233.1.

2.2 Synthesis of compound 2

Compound 1 (4.3 mmol, 1 g) and N-bromosuccinimide (NBS) (6.4 mmol, 1.1 g) were dissolved in 30 ml of acetonitrile, and stirred at room temperature for 1 h. After the reaction was completed, The solvent was removed by reducedpressure, and the crude product was extracted three times with dichloromethane, then extracted with saturated brine, dried over anhydrous magnesium sulfate, and spin-dried to obtain yellow solid 2. (1g, 97%) ^{1}H NMR (300 MHz, DMSO），δ:3.04 (s, 2H), 2.15 (s, 3H), 1.96 (m, 3H), 1.94 (m, 3H), 1.45 (s, 6H). ESI($C_{14}H_{18}O_4$): $[M\text{-}H^+]^-$ = 249.1.

2.3 Synthesis of compound 3

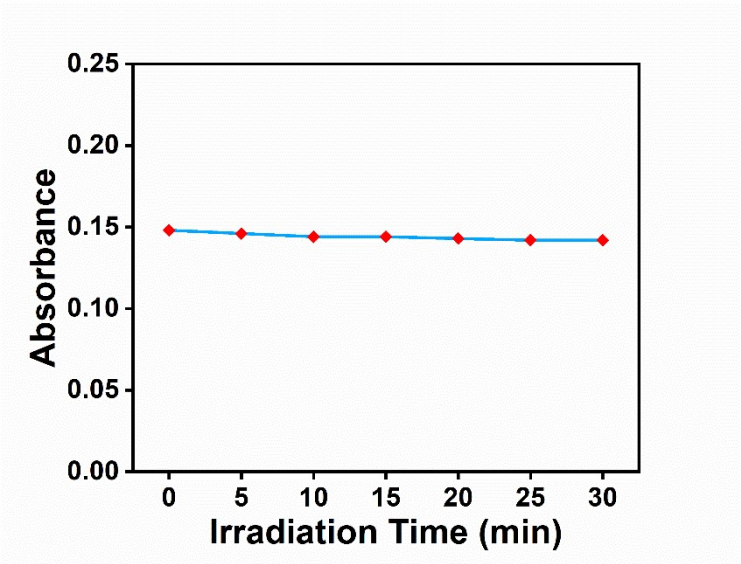
Compound 2 (4.1 mmol, 1 g) ,2-cyano-6-hydroxybenzothiazole (3.4 mmol, 0.6 g), 1-ethyl-3-(3-(dimeth-ylamino) propyl) carbodiimidehydrochloride (EDC 8.4 mmol, 1.3 g), 4-Dimethylaminopyridine (DMAP 9.1 mmol, 0.8 g) were dissolved in dry dichloromethane (20 mL) and stirred at room temperature for 12 h. After the reaction was completed, it was extracted three times with dichloromethane, and the crude product was purified by silica gel chromatography with ethyl acetate/petroleum ether as eluent to obtain a yellow solid 3 (234 mg, 17%). ^{1}H NMR (400 MHz, DMSO), δ: 8.27 (d, J = 8.5 Hz, 1H), 8.10 (s, 1H), 7.40 (d, J = 8.8 Hz, 1H), 3.26 (s, 2H), 2.12 (s, 3H), 1.86 (s, 6H), 1.49 (s, 6H). ESI($C_{22}H_{20}N_2O_4S$):$[M\text{-}H^+]^-$ = 407.1.

2.4 Synthesis of probe NQO1-Luc.

Compound 3 (0.6 mmol, 234 mg) was dissolved in 5 mL of a mixed solvent of methanol and dichloromethane (v/v=1:1), and 5 mL of a mixed solvent of methanol and deionized water (v/v=1:1) was added. of D-cysteine hydrochloride (0.7 mmol, 112 mg) and potassium carbonate (0.7 mmol, 90 mg). The reaction was stirred at r.t. for 20min, and condensed under reduced pressure and the resulting residue was re-dissolved in water. The crude product was obtained by precipitating in the solution when the pH was adjusted to 2-3 with 1 M HCl, then further purified by recrystallized in MeOH to afford the pure product of NQO1-Luc. ^{1}H NMR (300 MHz, DMSO) , δ: 8.16 (d, *J* = 8.9 Hz, 1H), 7.95 (t, *J* = 3.6 Hz, 1H), 7.26 (dd, *J* = 8.9, 2.4 Hz, 1H), 5.40 (dt, *J* = 26.6, 13.3 Hz, 1H), 3.87 – 3.60 (m, 2H), 3.24 (d, *J* = 4.1 Hz, 2H), 2.12 (s, 3H), 1.86 (d, *J* = 4.3 Hz, 6H), 1.49 (s, 6H). ESI ($C_{25}H_{24}N_2O_6S_2$）: $[M\text{-}H^+]^-$ = 511.1.

2.5 The photostability of NQO1-Luc.

The photostability of NQO1-Luc was detected by recording the Time-dependent absorbance spectra of NQO1-Luc (10 μM in DMSO) under irradiation of xenon lamp (5mw/cm^2) for 30 min. The absorbance of 370 nm at every 5 minutes interval were shown below and the kept value indicated the solid photostability of NQO1-Luc.



3. Supplementary figures

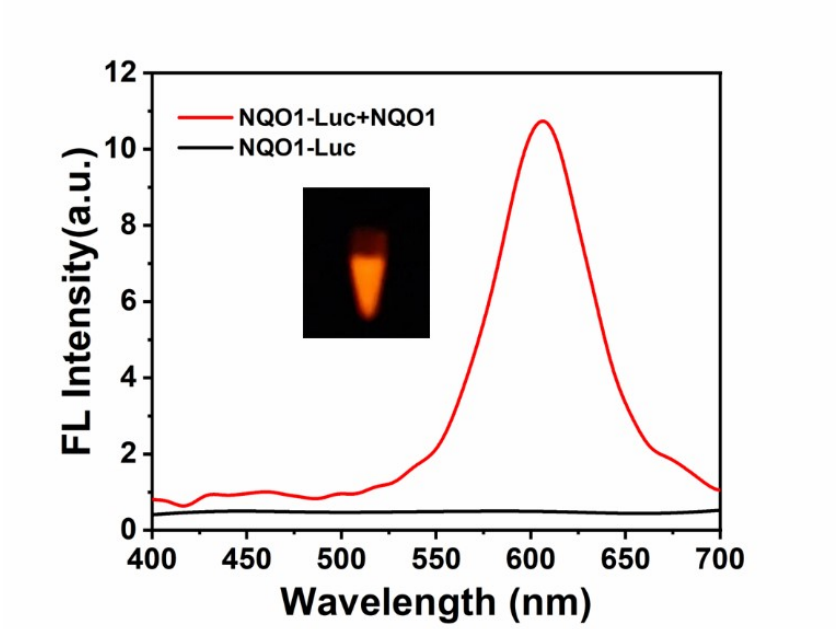


Fig. S1.: Luminescence emission spectra of NQO1-Luc (black), NQO1-Luc with 40 μg /mL NQO1 (red) after 1 h of incubation at 37 ℃ in the PBS buffer then added with luciferase and ATP; the photograph of NQO1-Luc with NQO1 after addition of luciferase and ATP (inset).

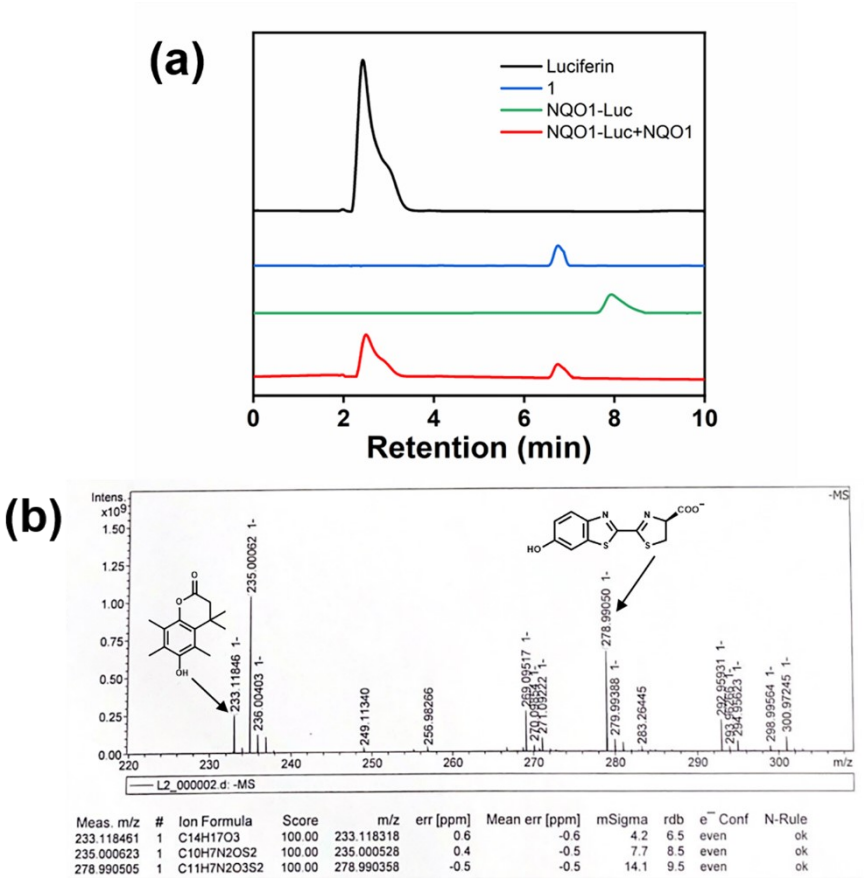


Fig. S2. HPLC-MS analysis results of NQO1-Luc decomposition products induced by NQO1.

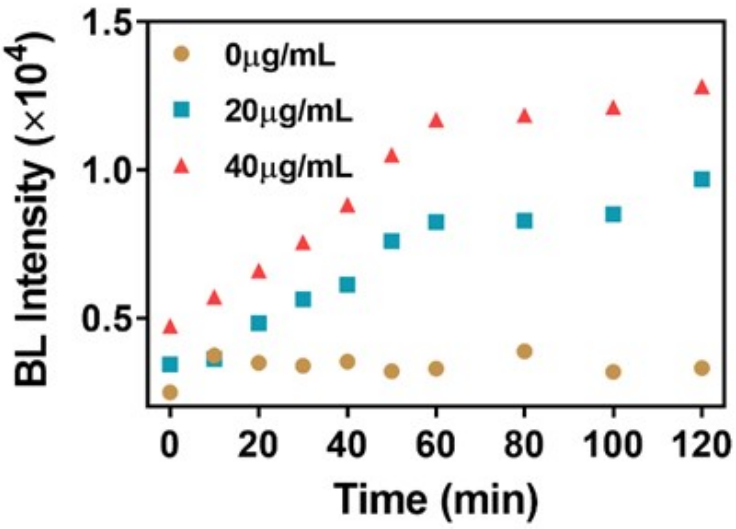


Fig. S3. Bioluminescence intensity versus the incubation time of NQO1-Luc (20 μM) with different concentrations of NQO1.

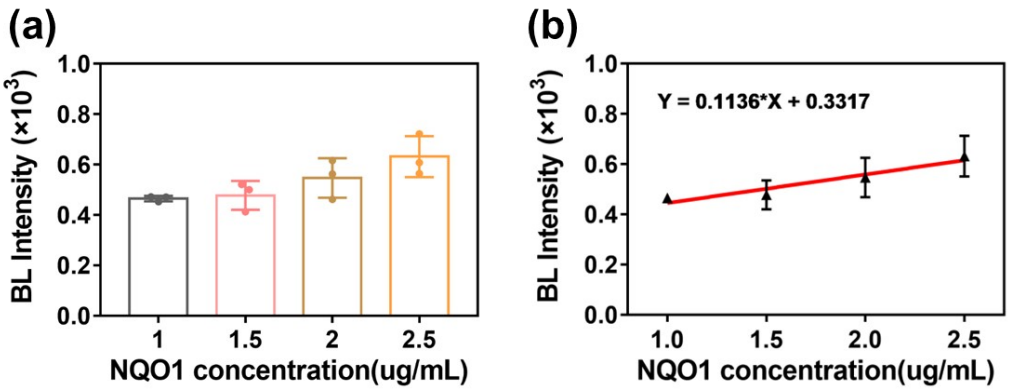


Fig. S4. (a) The relative bioluminescence response of NQO1-Luc to NQO1 at low concentrations. (b) Linear fitting curve of the relative bioluminescence intensity of (a).

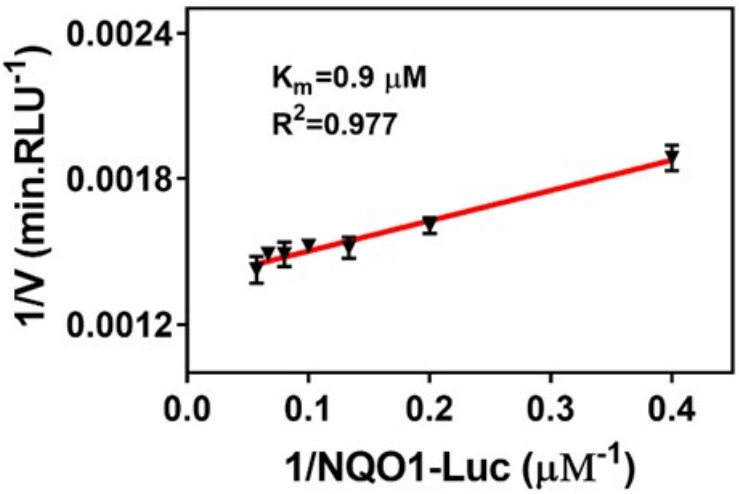


Fig. S5. Lineweaver-Burk plot of NQO1-activated luminescence of NQO1-Luc. *K*m is the Michael constant, correlation coefficient R = 0.977.

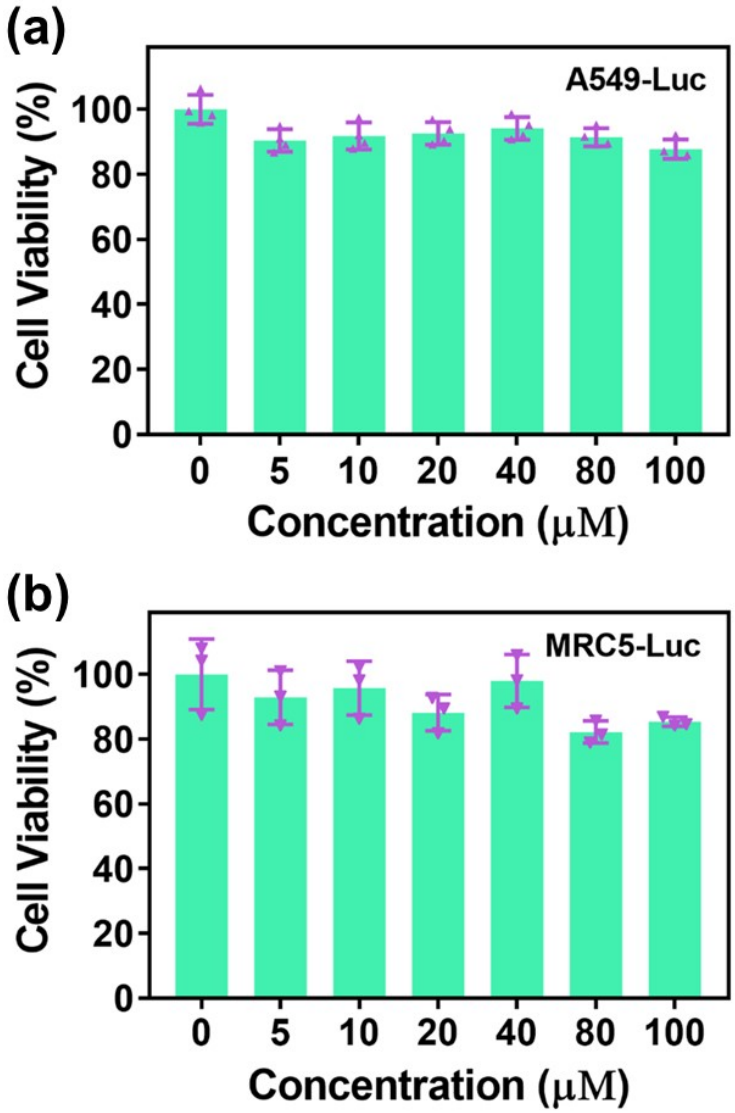


Fig. S6. Cell viability of NQO1-Luc with various concentration towards A549-Luc cells (a) and MRC5-Luc cells (b). Results are representative of three independent experiments. Each error bar represents standard deviations.

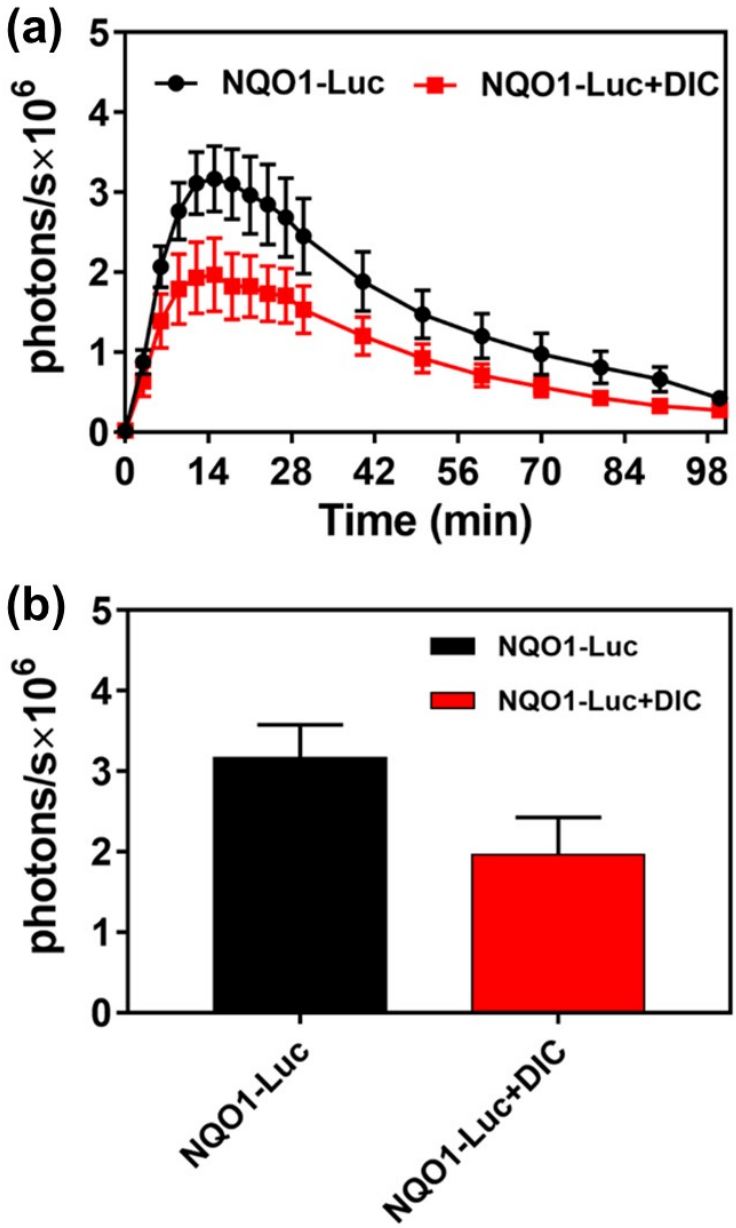


Fig. S7. (a) Fluctuation of time-dependent change of the total flux of the images in Fig. 4 Captured at different time after the i.p. injection of probe (n = 3). (b) Quantification of bioluminescence intensity (without and with DIC) after injecting NQO1-Luc for 15 min in living mice (n = 3).

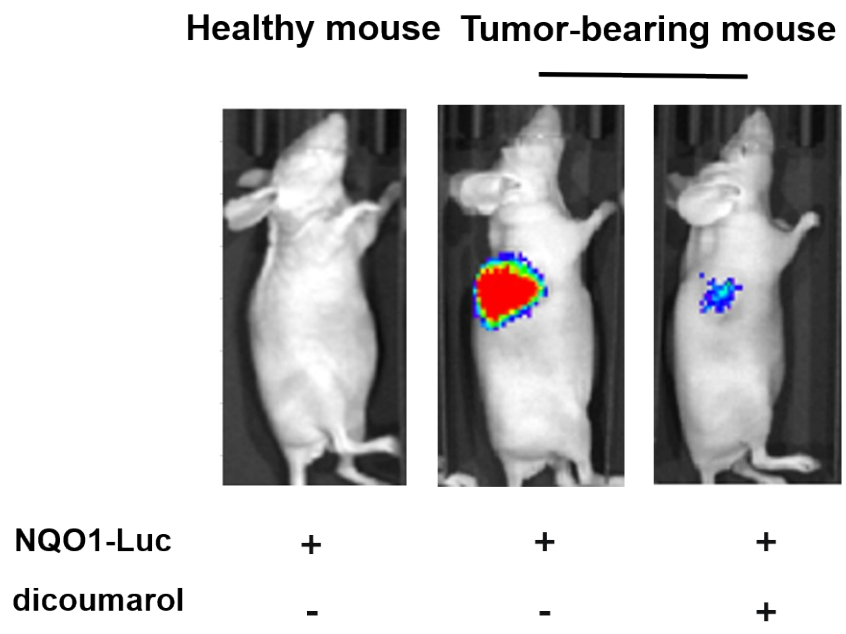
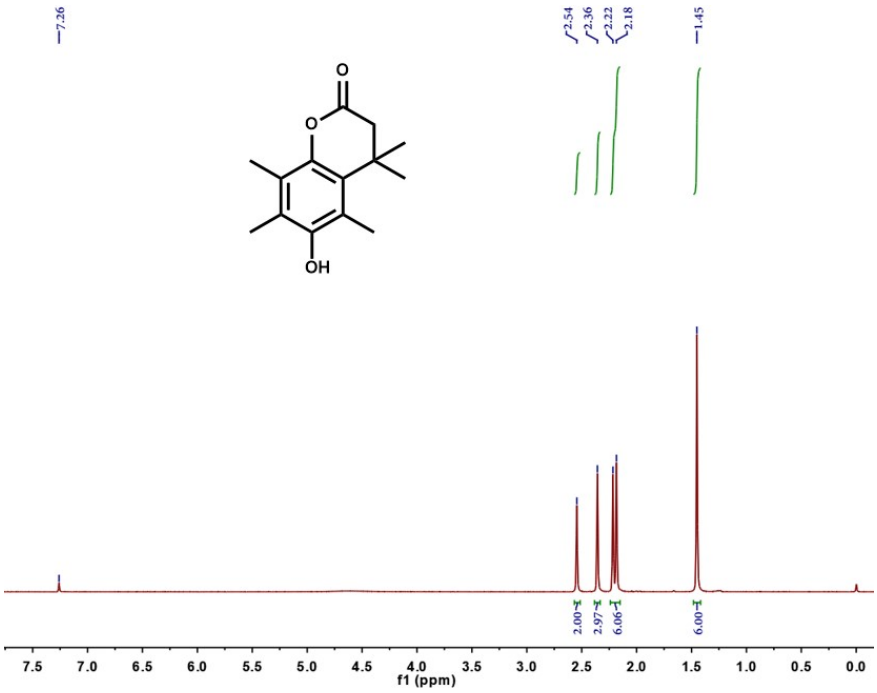
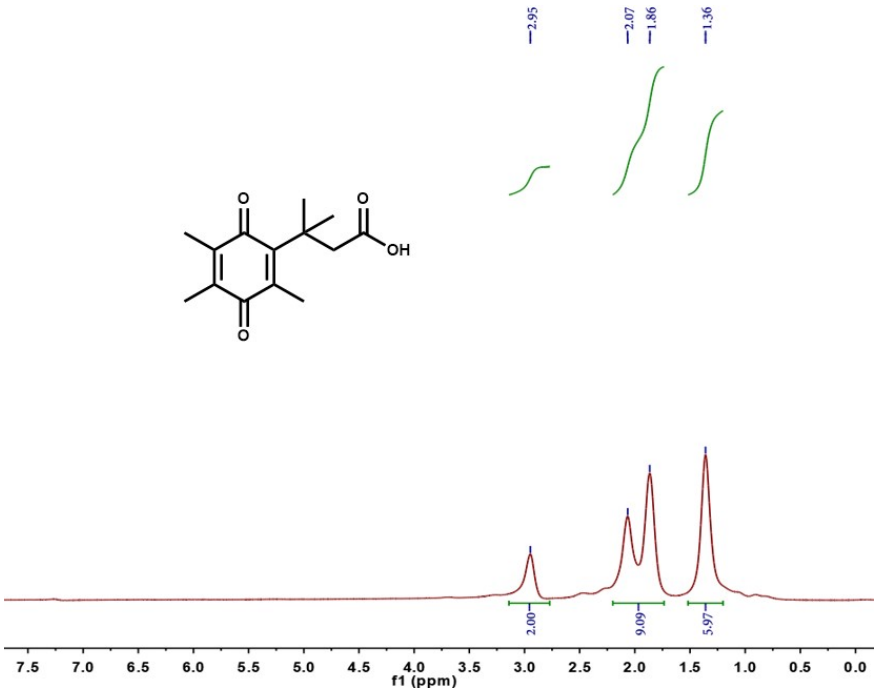


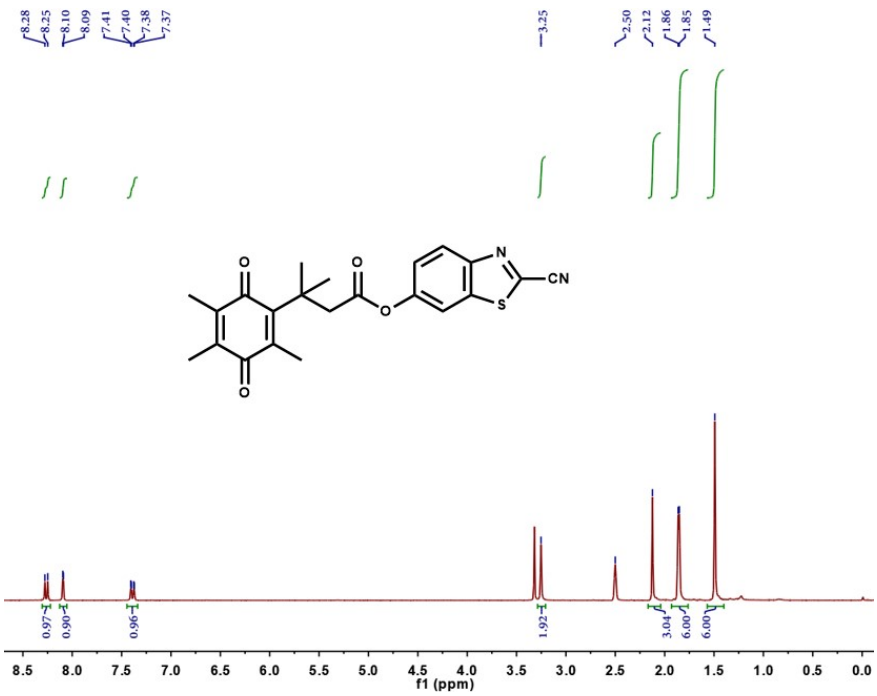
Fig. S8. Bioluminescence images of healthy and tumor-bearing BALB/c nude mice after injection of NQO1-Luc for 15 min. The experimental groups pretreated tumor region with saline and dicoumarol (200 μM, 100 μL) one hour ahead of NQO1-Luc injection respectively.

4. Appendix

4.1 The [1]HNMR spectra of compounds 1、2、3 and NQO1-Luc



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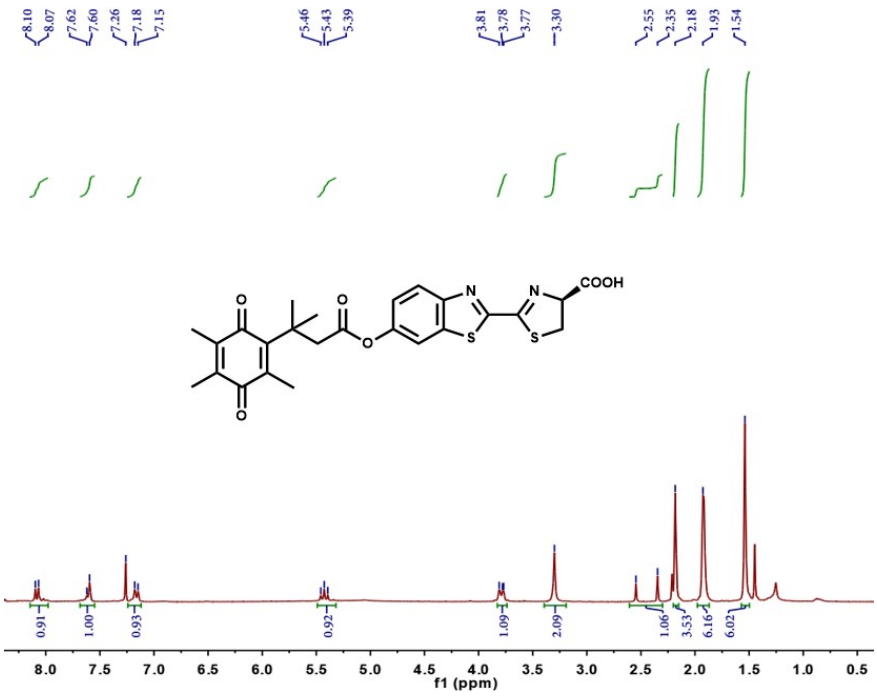
8.10
8.09
7.41
7.40
7.38
7.37
3.25
2.50
2.12
1.86
1.85
1.49
O
O
O
O
N
S
CN
0.97
0.90
0.96
1.92
3.04
6.00
6.00
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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7.26
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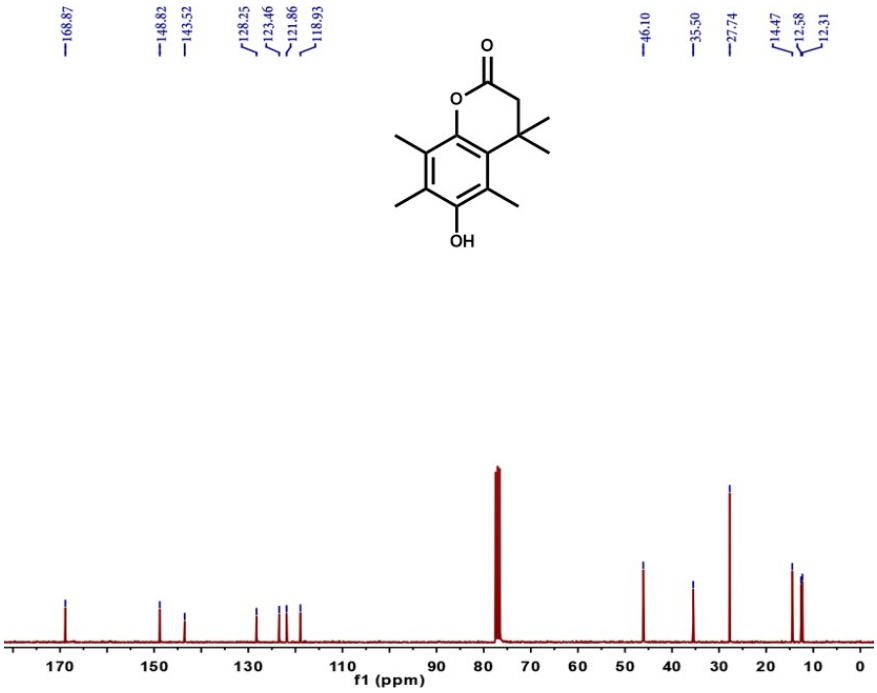
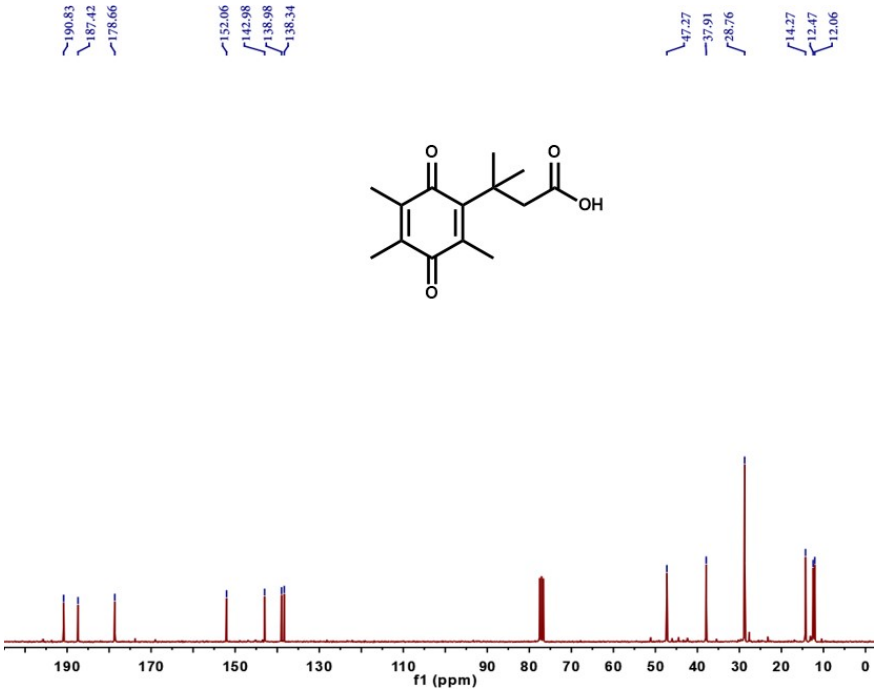
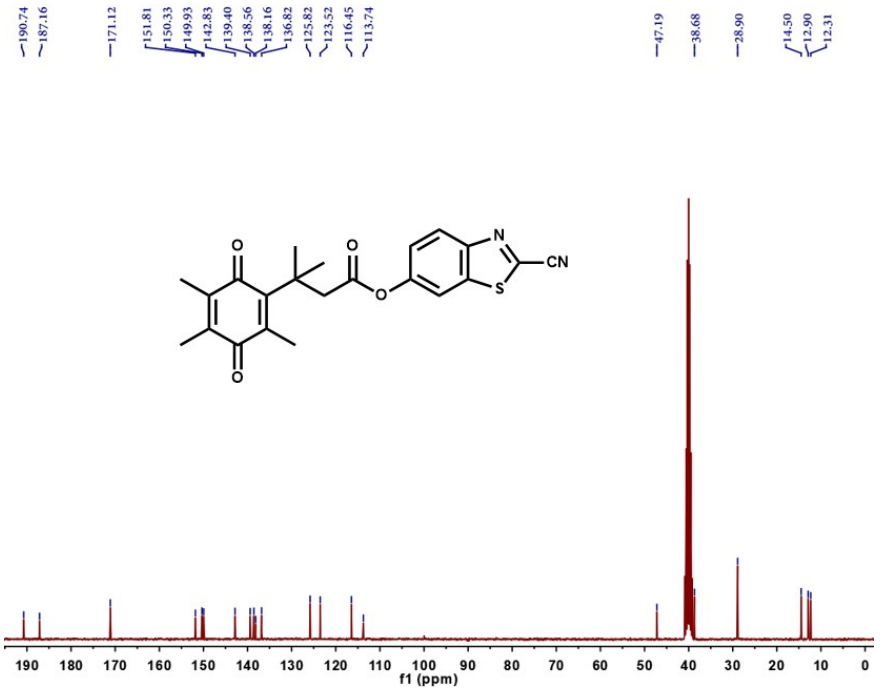
3.30
2.55
2.35
2.18
1.93
1.54
O
O
O
O
N
S
N
S
COOH
0.91
1.00
0.93
0.92
1.09
2.09
1.06
3.53
6.16
6.02
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

4.2 The ^{13}CNMR spectra of compounds 1、2、3 and NQO1-Luc



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151.81
150.33
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138.16
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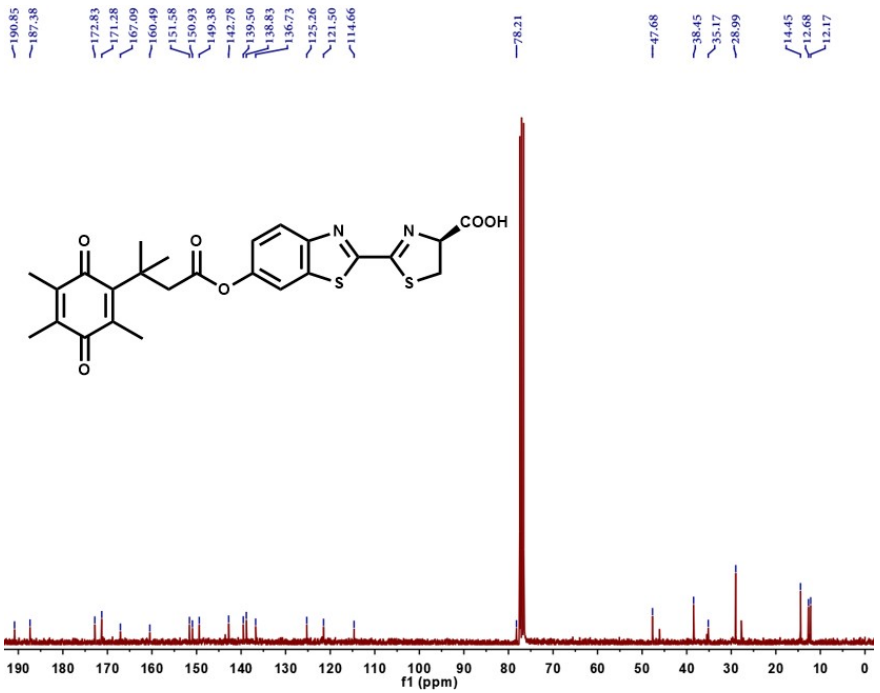
123.52
116.45
113.74
47.19
38.68
28.90
14.50
12.90
12.31
O
O
O
O
N
S
CN
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)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12.17
O
O
O
O
N
S
N
S
COOH
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)