Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

NAD(P)H: quinone oxidoreductase 1 activatable toll-like receptor 7/8 agonist designed for precise immunotherapy

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1. Experimental Section

Materials and Reagents. Dicoumarol was purchased from MedChemExpress. NQO1 was purchased from Sino Biological. Fetal bovine serum (FBS), RPMI-1640 medium and Dulbecco's Modified Eagle Medium (DMEM) were ordered from Gibco. PBS, DAPI, 4% paraformaldehyde (PFA), penicillin/streptomycin and MTT Cell Cytotoxicity Assay Kit were obtained from Beyotime. ATP Assay Kit was purchased from Promega. Anti-Calreticulin antibody, Anti-HMGB1 antibody, Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 568) and Donkey F(ab')2 Anti-Rabbit IgG H&L (Alexa Fluor® 594) were ordered from Abcam. GM-CSF and IL-4 were obtained from PeproTech. Anti-mouse CD16/32, APC anti-mouse CD11c, FITC anti-mouse CD80, PE anti-mouse CD86, FITC anti-mouse CD3, PE anti-mouse CD4, APC anti-mouse CD8a, APC anti-mouse FOXP3, PE anti-mouse CD44 and Pacific Blue™ anti-mouse CD62L were purchased from BioLegend.

Cell Cultures. Mouse breast cancer cells (4T1), human breast cancer cells (MDA-MB-231), human non small cell lung cancer cells (A549), human gastric adenocarcinoma cell line (AGS), human cervical cancer cells (Hela) and human liver cells (HL-7702) were obtained from American Type Culture Collection (ATCC). Except for AGS cells cultured in RPMI-1640 medium, all cells were maintained in DMEM medium supplemented containing 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere at 37 °C with 5% CO_2.

Animals. Balb/c mice were obtained from the Laboratory Animal Center of Nantong University. All animal experiments were performed under the approval of the Institutional Animal Care and Use Committee.

Characterization. ^{1}H NMR spectra were recorded at room temperature on a Bruker Vance III 400MHz NMR system. UV-Vis absorption spectra were measured using UV-1900 (Shimadzu, Japan). Fluorescence measurements were performed on an FS5 fluorescent spectrophotometer (Edinburgh, England). Fluorescence images of cells were captured by a Nikon inverted microscope. The absorbance of cells in 96-well plates was read at 570 nm by a Bio-Rad microplate reader. The secretion of ATP was valued by a BPCL-GP21Q luminescence analyzer. HPLC analyses were conducted on a

system from Hanbon using an NP7005C pump with methanol (MeOH) and water (H_2O) as the eluent. Flow cytometry analyses of cells were carried out on BD FACSAria III flow cytometry analyzer (BD, USA).

Materials Synthesis. The synthetic route of **R848-QPA** was shown in Figure S1.

Synthesis of Compound **1**: The intermediates **1** were carefully prepared according to the literature.[1] Methanesulfonic acid (30 mL) was heated to 85 °C. Then 2,3,5-trimethylbenzene-1,4-diol (2.0 g, 13.2 mmol) and 3-methylbut-2-enoic acid (1.5 g, 15.0 mmol) were added in it under stirring. After a 3 h reaction under nitrogen, 100 g of ice was added to the mixture to quench the reaction. The precipitate was extracted with ethyl acetate for three times. The organic layer was washed with $NaHCO_3$ and brine. The crude product was recrystallized from ethyl acetate and hexane to obtain the white intermediate. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.60 (s, 1H), 2.55 (s, 2H), 2.36 (s, 3H), 2.22 (s, 3H), 2.18 (s, 3H), 1.46 (s, 6H).

Synthesis of Compound **2**: The intermediates **2** were carefully prepared according to the literature.[2] Compound **1** (2.5 g, 10.9 mmol) was suspended in a mixture of acetonitrile (25 mL) and water (5 mL). N-bromosuccinimide (NBS) (2.0 g, 11.5 mmol) was slowly added to the suspension at room temperature with stirring. 1 h later, the reaction mixture was extracted with dichloromethane and the organic layer was combined and dried over Na_2SO_4. The solvent was removed to give a yellow powder solid. **2** was achieved by recrystallizing using dichloromethane and hexane. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.02 (s, 2H), 2.15 (s, 3H), 1.93 (d, 6H), 1.43 (s, 6H).

Synthesis of Compound **QPA-NHS**: The intermediate **QPA-NHS** was synthesized according to the previous work.[3] The chemical structure was characterized by ^{1}H NMR (Figure S5). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.28 (s, 2H), 2.79 (s, 4H), 2.17 (s, 3H), 1.96 (s, 6H), 1.53 (s, 6H).

Synthesis of Compound **R848-QPA**: The **R848-QPA** was synthesized according to the previous work.[4] A mixture of compound **QPA-NHS** (104 mg, 0.3 mmol) and R848 (94 mg, 0.3 mmol) was dispersed in 5 mL anhydrous DMF followed by the addition of DIPEA (250 μL). Subsequently, the mixture was continuously stirred at room temperature for 24 h. After the reaction was completed, the residue was purified by

flash column chromatography (silica gel, CH_2Cl_2:CH_3OH = 15:1, v/v) to obtain **R848-QPA.** ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.06 (d, 1H), 7.83 (d, 1H), 7.52 (t, 1H), 7.33 (t, 1H), 4.84 (d, 4H), 3.66 (q, 2H), 2.16 (m, 8H), 1.28 (m, 18H).

Spectra Responses of R848-QPA to NQO1. R848-QPA (20 μM), NQO1 (2.5 μg/mL), and NADH (100 μM) were incubated in PBS containing 0.1% BSA at 37 °C for 1 h. Changes in absorption spectra and emission spectra were recorded by a UV-vis spectrometer and a fluorescence spectrometer, respectively.

Degradation of R848-QPA. R848-QPA was dissolved in dimethyl sulfoxide (DMSO) to obtain a 10 mM stock solution. Then, **R848-QPA** was incubated with sodium borohydride ($NaBH_4$, 10 equiv) for different durations. Degradation of **R848-QPA** at various time points was monitored by a UV-vis spectrometer.

Release of R848 from R848-QPA. 20 μM **R848-QPA** was incubated with 2.5 μg/mL NQO1 in the presence of 100 μM NADH at 37 °C. After 1 h incubation, the reaction mixture was measured by HPLC to analyze the release of R848.

In Vitro Cytotoxicity Assay. 4T1 cells, MDA-MB-231 cells, A549 cells, AGS cells, Hela cells and HL-7702 cells were cultured in 96-well plates overnight for cell attachment. After discarding the culture medium, cells were treated with gradient concentrations of **R848-QPA** solution (0, 5, 10, 20, 40, 60, 80, 100 □M) for 24 h. Then 10 μL MTT medium (5 mg/mL) was added to 96-well plates. 4 h later, each well was added with 100 μL DMSO to dissolve the purple formazan crystals. The absorbance of each well at 570 nm was measured by a microplate reader. The cell viability was calculated as follows: Cell viability (%) = (absorbance of treatment group/absorbance of the control group) ×100%

Evaluation of DAMPs (ATP, CRT, and HMGB1). 4T1 cells and HL-7702 cells were seeded in cell culture dishes overnight. After cell adhesion, the cells were treated with different formulations for 24 h. Notably, dicoumarol (100 μM) needed to be added 1 h in advance. To measure the secretion of ATP, cell supernatant was collected and extracellular ATP was measured by ATP Assay Kit according to the manufacturer's instructions. To evaluate the exposure of CRT and the migration of HMGB1, cells were fixed with 4 % PFA, permeabilized with 0.1% Triton X-100 and sealed with 3% bovine

serum albumin (BSA) solution. Then the cells were stained with Anti-Calreticulin antibody and Anti-HMGB1 antibody at 4 °C overnight, followed by incubation with Donkey Anti-Rabbit IgG H&L (Alexa Fluor® 568) or Donkey F(ab')2 Anti-Rabbit IgG H&L (Alexa Fluor® 594) for 1 h in the dark. Finally, the cells were stained with DAPI for 15 min and the fluorescent images were observed by an inverted microscope.

In Vitro DCs Maturation. To value the in vitro DCs maturation, bone-marrow-derived dendritic cells (BMDCs) were isolated from 7-8 week-old Balb/c mice and cultured with 10 ng mL^{-1} IL-4 and 20 ng mL^{-1} GM-CSF. 4 days later, these cells were co-cultured with **R848-QPA** (80 μM) pre-treated 4T1 cells for 24 h at a ratio of 2:1. Finally, the harvested non-adherent cells were first blocked with anti-mouse CD16/32 on ice for 10 min to block nonspecific binding and then stained with APC anti-mouse CD11c, FITC anti-mouse CD80 and PE anti-mouse CD86 for 30 min. The maturation of DCs was assessed by flow cytometry.

In Vivo Tumor Model. 1 x 10^6 4T1 cells suspended in DMEM cell culture medium were injected subcutaneously into the right flank of the 3-week-old female Balb/c mice as primary tumors when 5 × 10^5 4T1 cells were subcutaneously inoculated into the left flank of the same mouse as distant tumors. Mice were given different treatments when primary tumor volumes reached about 60 mm^3.

Flow Cytometry Analysis. When primary tumor sizes were around 60 mm^3, 4T1 tumor-bearing mice were randomly divided into 2 groups (n = 3). Then, saline and **R848-QPA** (6 mg kg^{-1}, 200 μL) were administered to these mice. 14 days later, mice in each group were sacrificed and spleens were harvested, cut into pieces, grinded, and filtered through cell strainers (70 μm) to make single-cell suspension for flow cytometry analysis. All collected cells were first pre-incubated with anti-mouse CD16/32 on ice for 10 minutes. To analyze cytotoxic T lymphocytes (CTLs), splenocytes were stained with FITC anti-mouse CD3, PE anti-mouse CD4 and APC anti-mouse CD8a for 30 min. To assess regulatory T cells (Tregs), cells were stained with FITC anti-mouse CD3, PE anti-mouse CD4 and APC anti-mouse FOXP3 for 30 min. To evaluate memory T cells, cells were stained with anti-mouse CD3, APC anti-mouse CD8a, PE anti-mouse

CD44, and Pacific Blue™ anti-mouse CD62L for 30 min. The cells were finally analyzed by flow cytometry.

Histological Analysis. At the end of various treatments, mice in each group were executed. Primary tumors, distant tumors, and other major organs (heart, liver, spleen, lung, and kidney) were collected and fixed in 4% PFA for hematoxylin and eosin (H&E) staining.

2. Supporting Figures

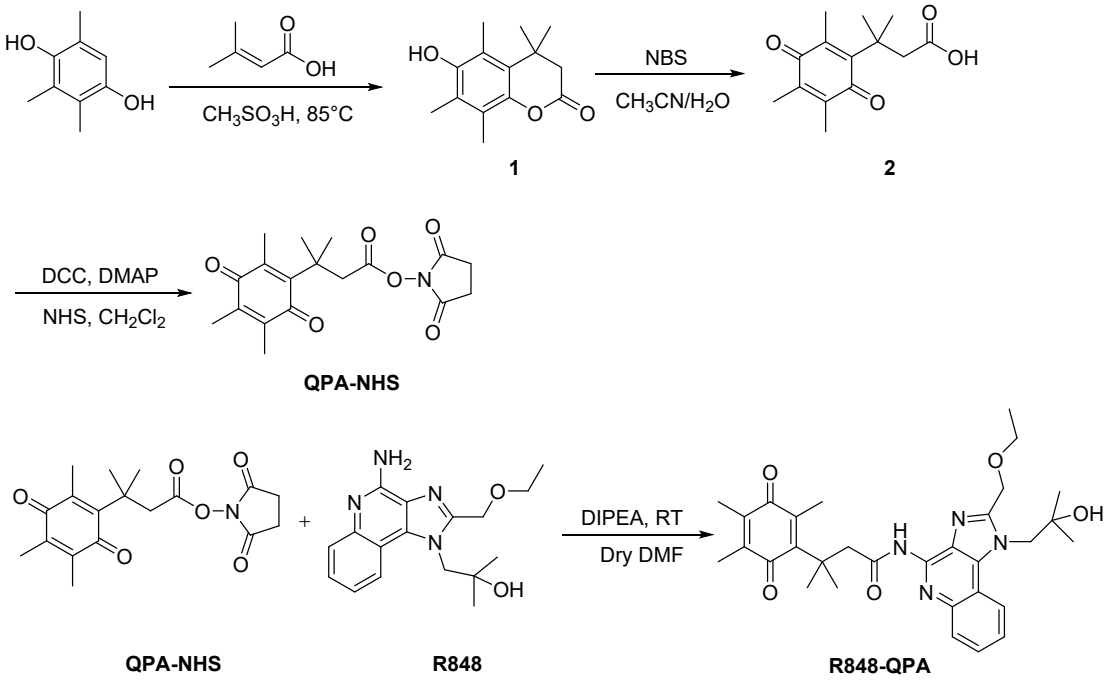

Fig. S1 Synthetic route of **R848-QPA**.

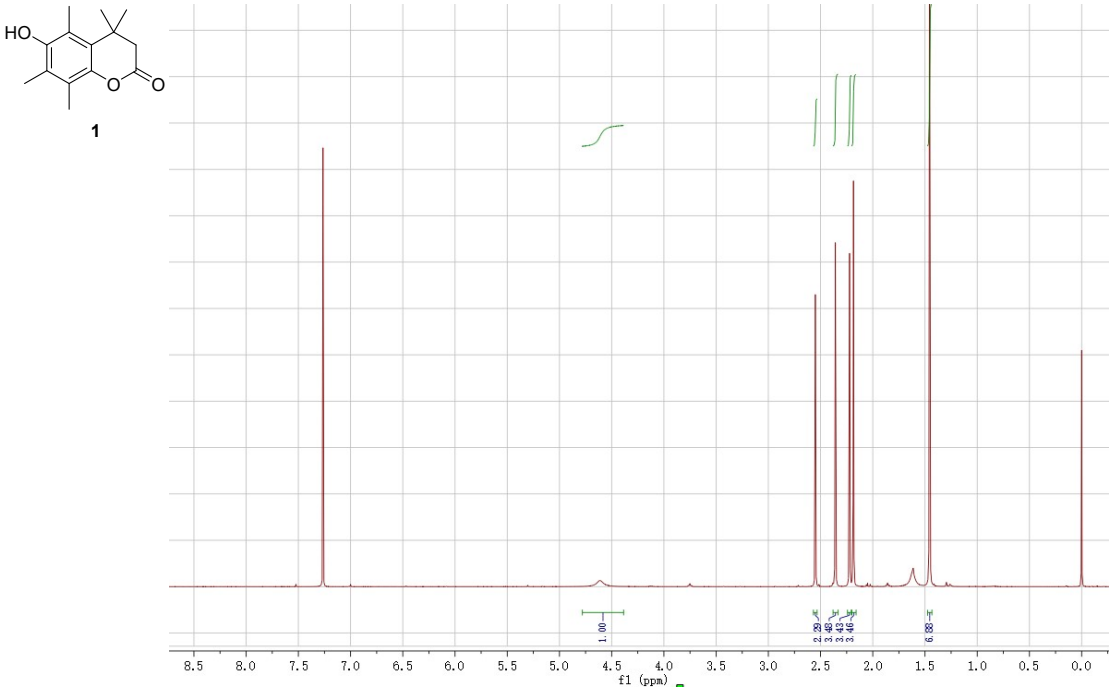

Fig. S2 ^{1}H NMR spectrum of compound **1**.

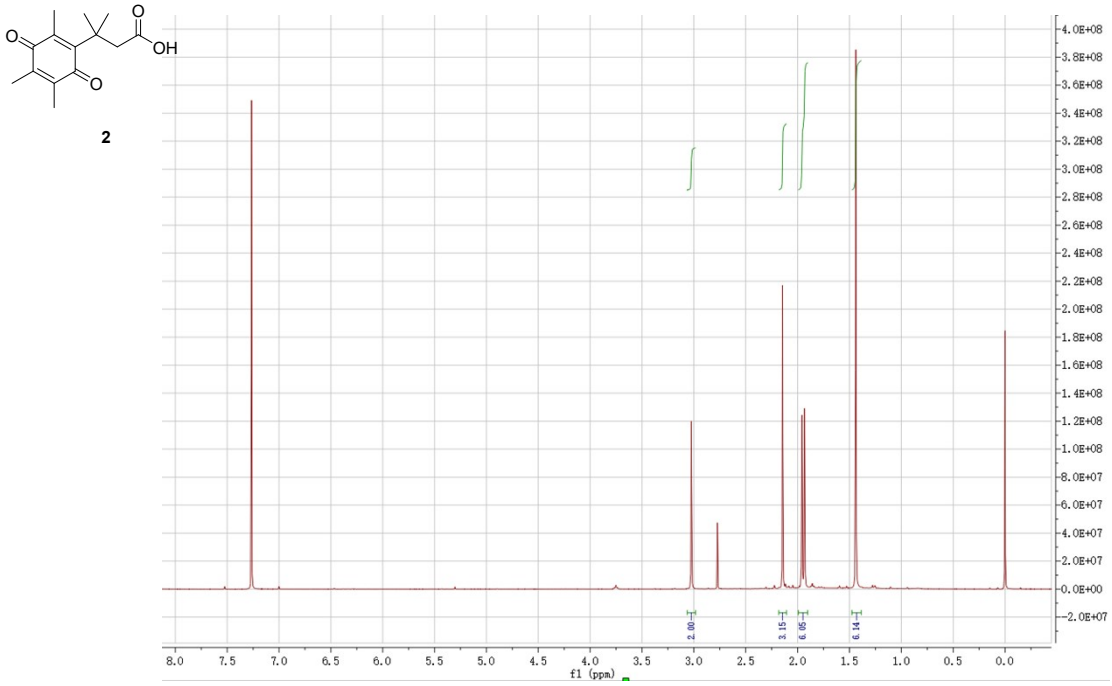

Fig. S3 ^{1}H NMR spectrum of compound **2**.

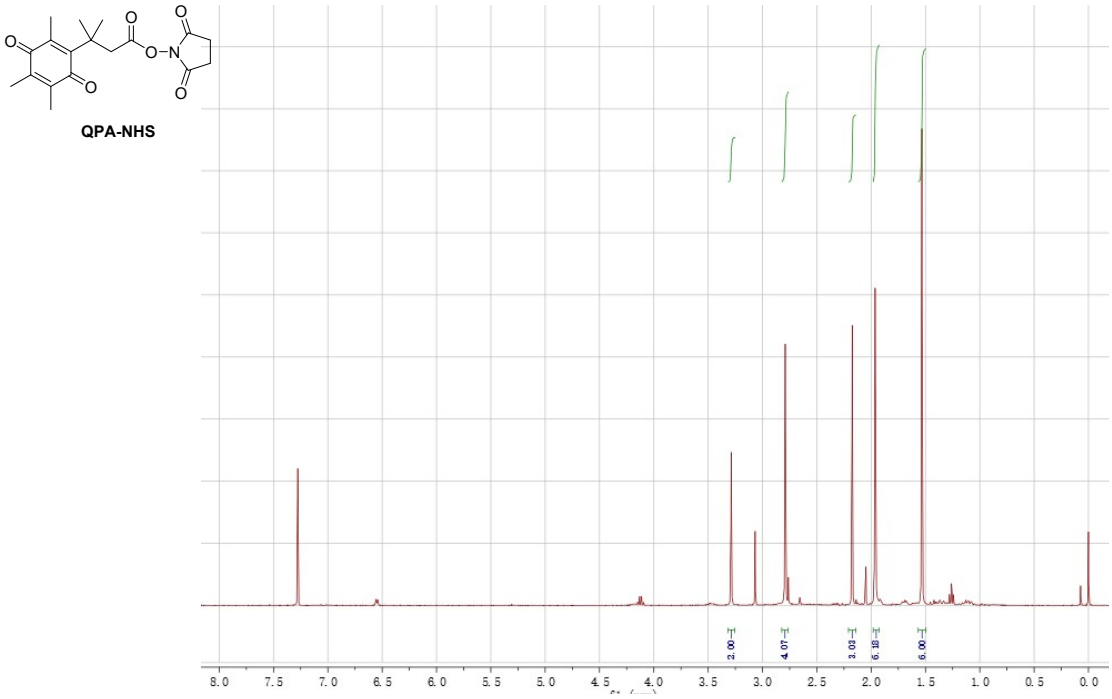

Fig. S4 ^{1}H NMR spectrum of **QPA-NHS**.

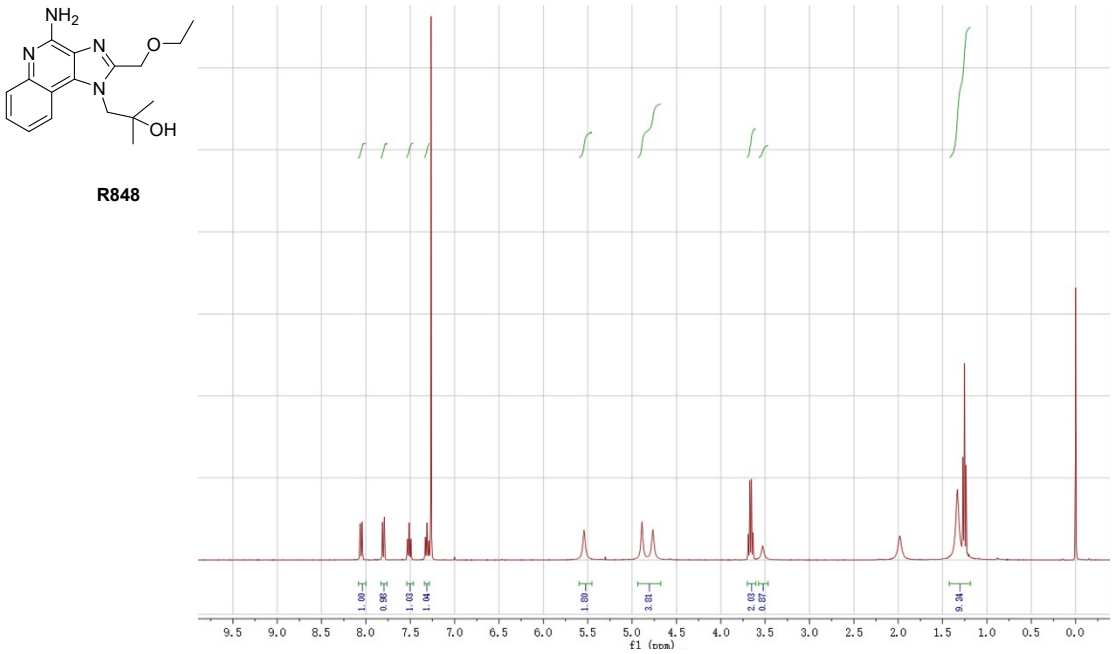

Fig. S5 ^{1}H NMR spectrum of **R848**.

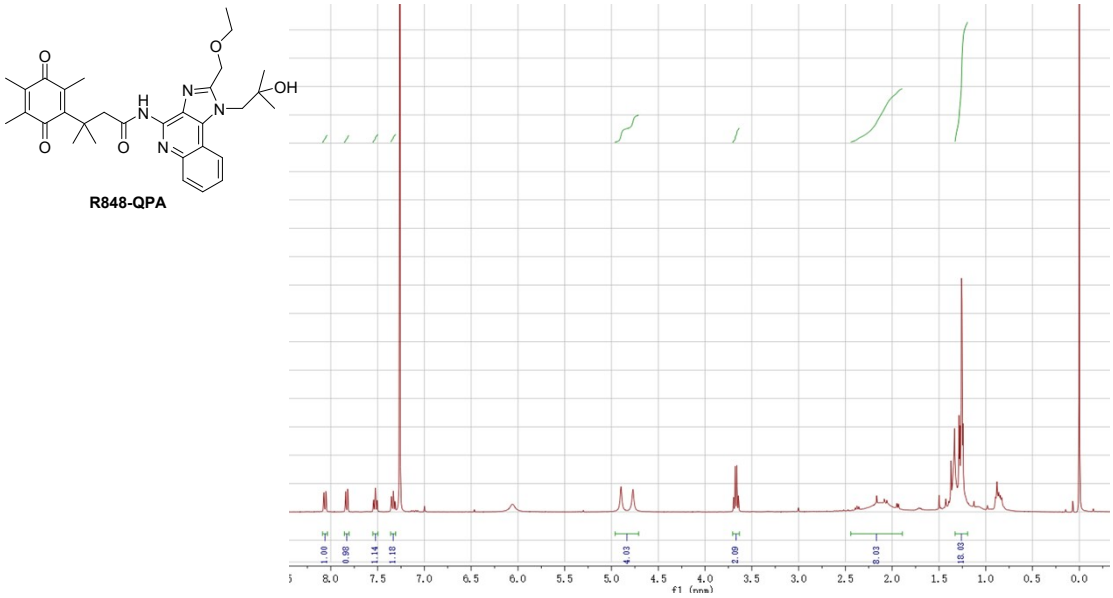

Fig. S6 ^{1}H NMR spectrum of **R848-QPA**.

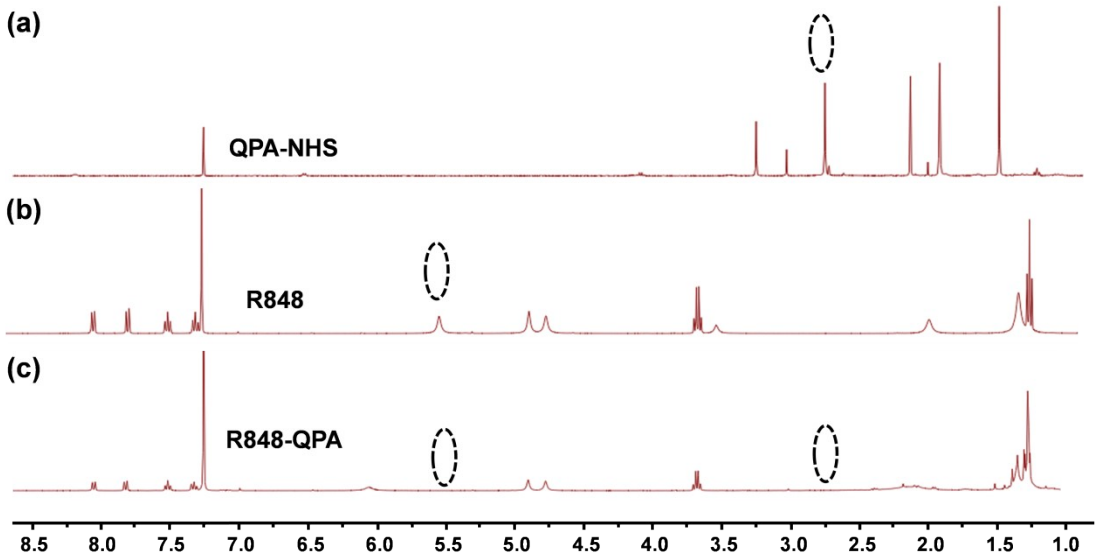

Fig. S7 Partial ^{1}H NMR spectra ($CDCl_3$, 293 K) of (a) QPA-NHS, (b) R848, and (c) **R848-QPA**.

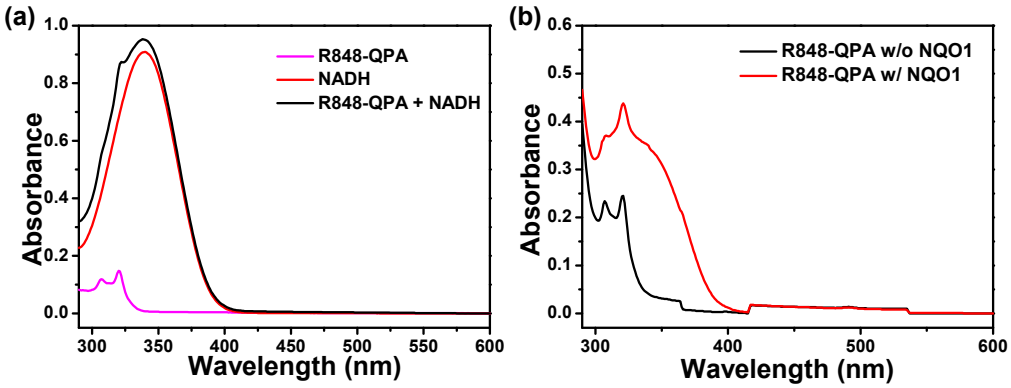

Fig. S8 a) Absorption spectra of different substances. b) Absorption spectra of **R848-QPA** (20 μM) with (red line) and without (black line) the addition of NQO1 (2.5 μg/mL).

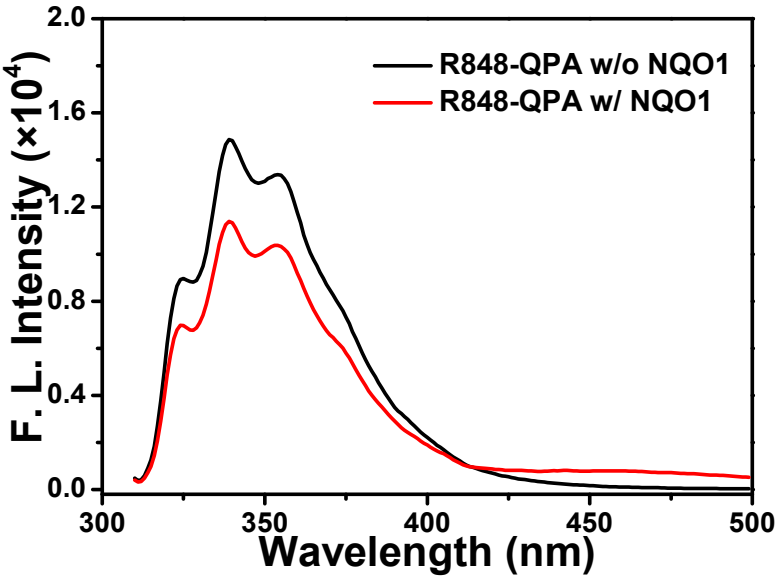

Fig. S9 Fluorescence spectra of **R848-QPA** (20 μM) with (red line) and without (black line) the addition of NQO1 (2.5 μg/mL).

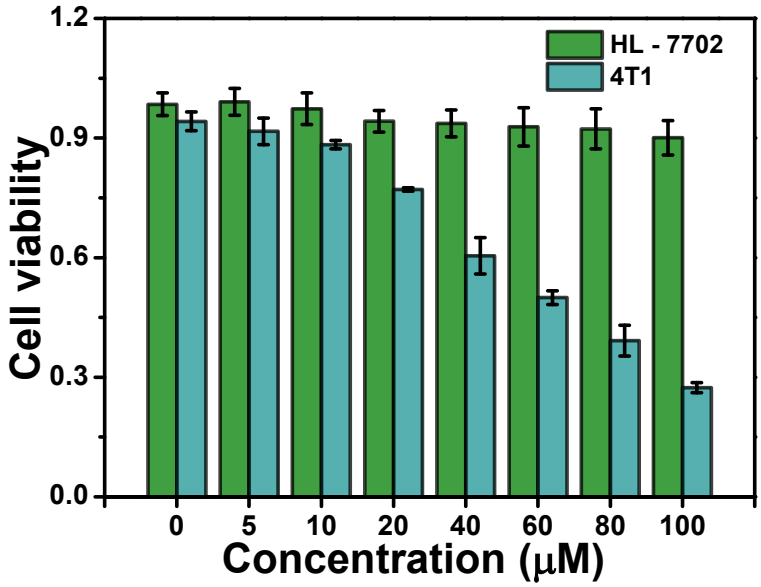

Fig. S10 Cell viability of HL-7702 cells and 4T1 cells after the treatment of R848-QPA at various concentrations (0, 5, 10, 20, 40, 60, 80, 100 μM) for 24 h.

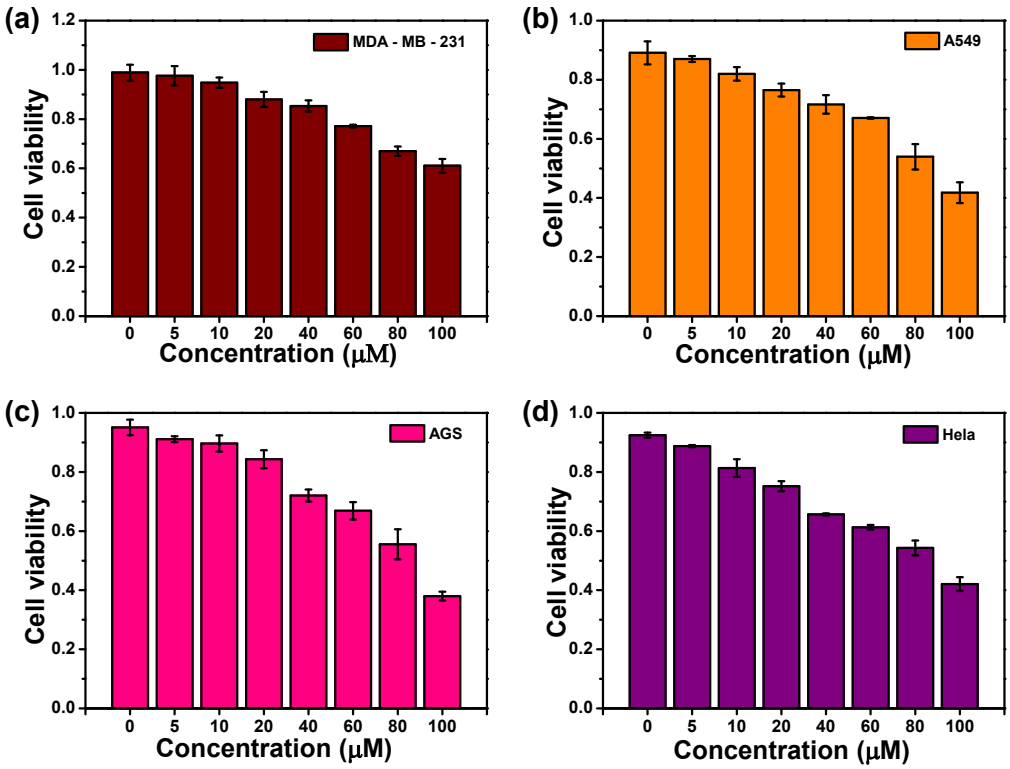

Fig. S11 Cell viability of a) MDA-MB-231 cells, b) A549 cells, c) AGS cells and d) Hela cells after the treatment of R848-QPA at various concentrations (0, 5, 10, 20, 40, 60, 80, 100 μM) for 24 h.

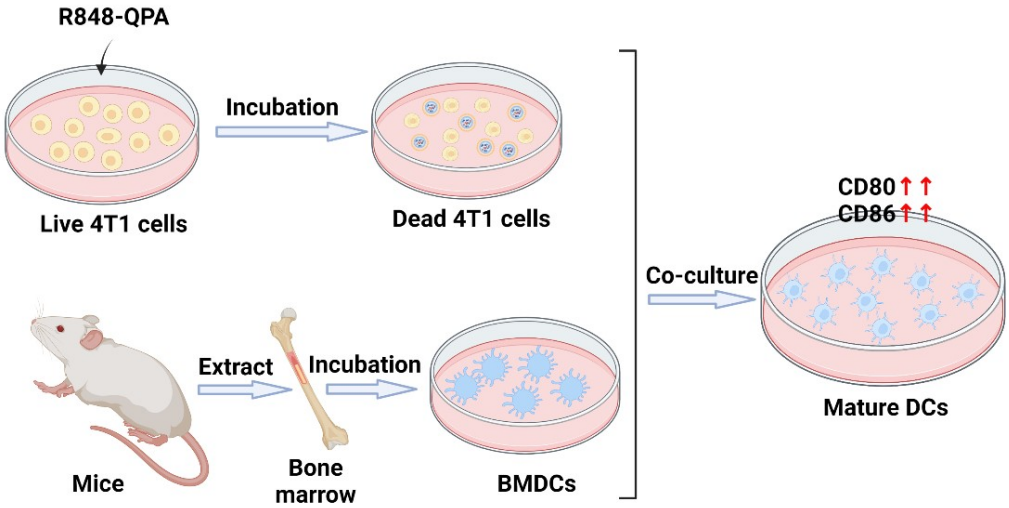

Fig. S12 Experimental design of in vitro stimulation of immature DCs.

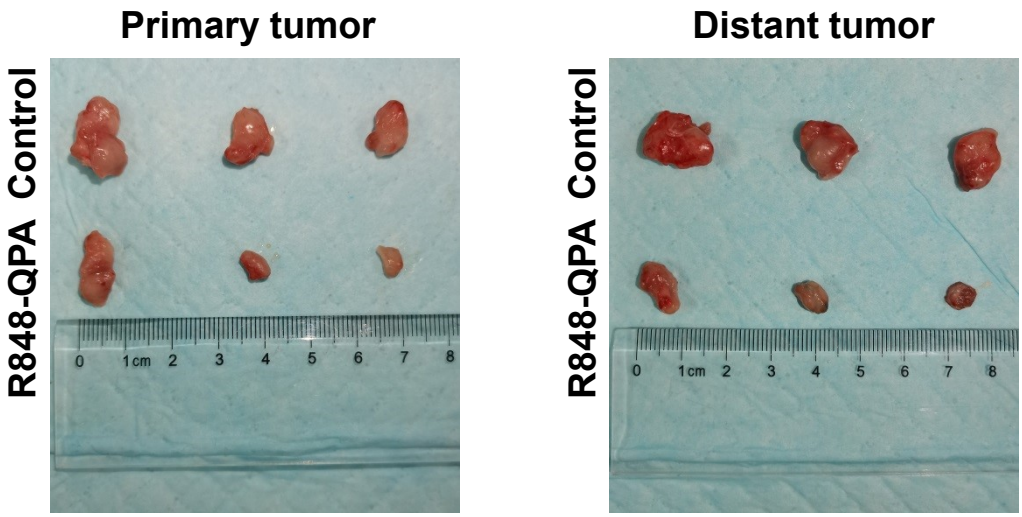

Fig. S13 Photos of primary and distant tumors from 4T1 tumor-bearing mice treated with saline and **R848-QPA** (6 mg kg^{-1}, 200 μL) after therapy.

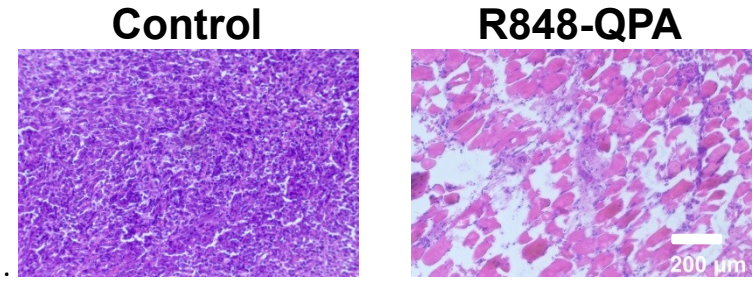

Fig. S14 H&E staining of tumor tissues collected from 4T1 tumor-bearing mice treated with saline and **R848-QPA** (6 mg kg^{-1}, 200 μL) after therapy, respectively. (Scale bar = 200 μm).

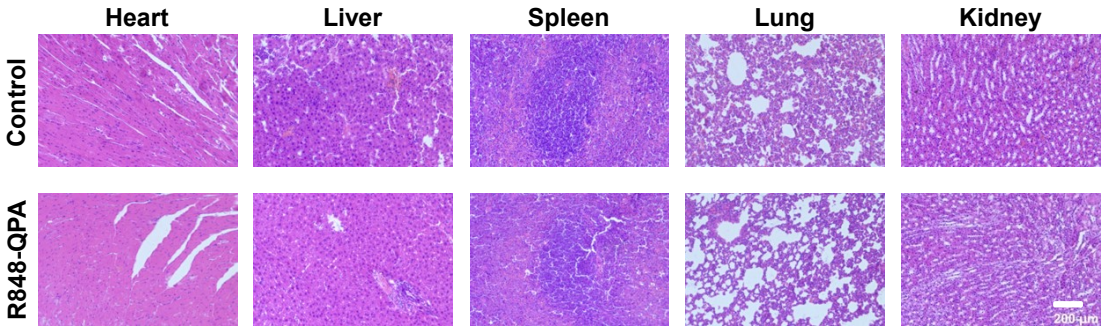

Fig. S15 H&E staining of major organs (heart, liver, spleen, lung, and kidney) collected from 4T1 tumor-bearing mice treated with saline and **R848-QPA** (6 mg kg^{-1}, 200 μL) after therapy, respectively. (Scale bar = 200 μm).

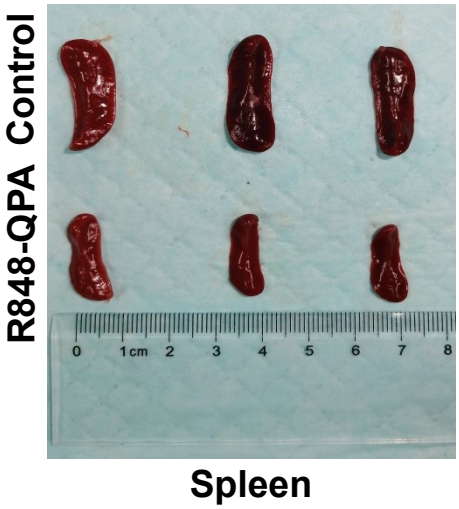

Fig. S16 Photos of spleens from 4T1 tumor-bearing mice treated with saline and **R848-QPA** (6 mg kg^{-1}, 200 μL) after therapy.

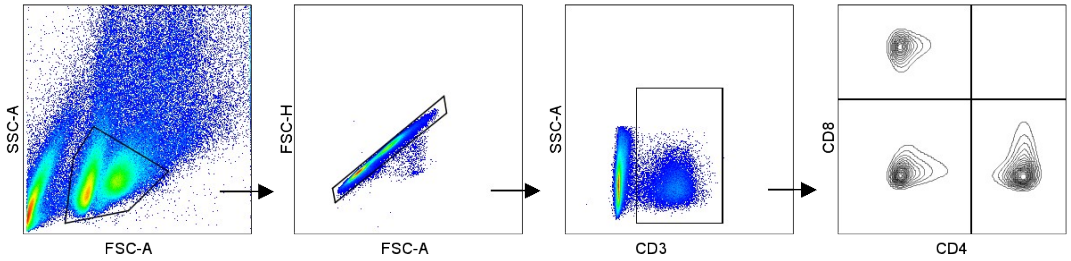

Fig. S17 Representative flow cytometry analysis of cytotoxic T lymphocytes (CTLs) ($CD3^{+}CD4^{-}CD8^{+}$) collected from the spleen.

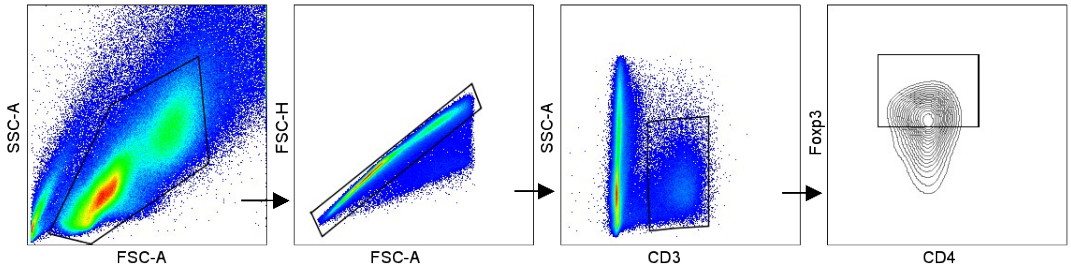

Fig. S18 Representative flow cytometry analysis of regulatory T cells ($CD3^+CD4^+Foxp3^+$) collected from the spleen.

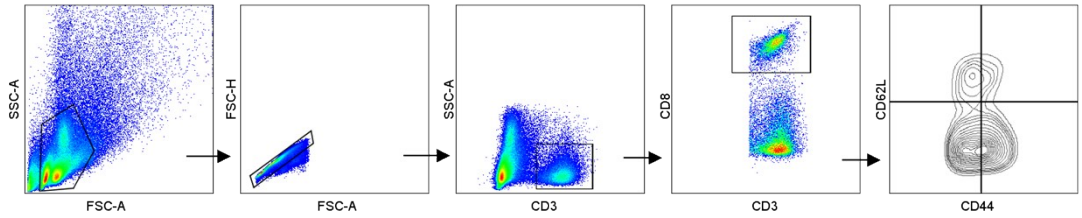

Fig. S19 Representative flow cytometry analysis of memory T cells ($CD3^{+}CD8^{+}CD44^{high}CD62L^{low}$) collected from the spleen.

Table S1 Activity inhibition rate and IC_{50} of R848-QPA toward 4T1 cells, MDA - MB - 231 cells, A549 cells, AGS cells, and Hela cells under the irradiation of light.

	Concentration	Cancer cells					Normal cell
		4T1	MDA - MB - 231	A549	AGS	Hela	HL - 7702
Inhibition rate (1 - viability)	0 μM	5.81%	1.09%	1.11%	4.91%	7.54%	1.04%
	5 μM	8.33%	2.40%	13.01%	8.91%	11.22%	0.92%
	10 μM	11.67%	5.20%	18.02%	10.35%	18.67%	2.67%
	20 μM	22.91%	11.99%	23.50%	15.69%	24.78%	5.78%
	40 μM	39.53%	14.68%	28.35%	27.96%	34.33%	6.33%
	60 μM	50.04%	22.86%	32.94%	33.14%	38.72%	7.21%
	80 μM	60.80%	32.99%	46.07%	44.45%	45.71%	7.71%
	100 μM	72.61%	38.94%	58.22%	62.01%	57.94%	9.94%
IC_{50} (μM)		61.71	86.47	69.64	71.69	66.88	

References

1. X. Zhang, X. Li, X. Wu, Y. Wu, Q. You, and X. Zhang, *Org. Lett*., 2018, **20**, 3635-3638.
2. R. Wang, K. Cai, H. Wang, C. Yin, and J. Cheng, *Chem, Commun*., 2018, **54**, 4878-4881.
3. X. Han, K. Cheng, Y. Xu, Y. Wang, H. Min, Y. Zhang, X. Zhao, R. Zhao, G. Anderson, L. Ren, G. Nie, Y. Li, *J. Am. Chem. Soc.*, 2020, **142**, 2490-2496.
4. H. Kakwere, H. Zhang, E. Ingham, M. Nura-Raie, S. Tumbale, R. Allen, S. Tam, B. Wu, C. Liu, A. Kheirolomoom, B. Fite, A. Ilovitsh, J. Lewis, and K. Ferrara, *Adv. Healthcare Mater*., 2021, 2100008.