Supporting Information

An ultrasonic small-molecule chimera for sono-controllable proteolysis

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Experimental procedures

Materials

All chemicals and solvents were purchased from J&K Chemicals or Energy Chemical (Shanghai, China), and all chemical reagents were used as purchased without further purification. JQ1 was purchased from Bide Pharmatech (Shanghai, China). Protoporphyrin IX (PPIX) was purchased from Heowns Biochem Technologies (Tianjin, China). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Sigma-Aldrich (Shanghai, China). The DNA damage assay kit (γ-H2AX immunofluorescence staining) was purchased from Beyotime Biotechnology (Shanghai, China). Trolox was obtained from Yeasen Biotechnology (Shanghai, China). L-histidine and chloroquine were purchased from MedChemExpress (MCE, Shanghai, China). Carfilzomib (PR-171) was obtained from CSNpharm (Chicago, USA). Singlet oxygen sensor green (SOSG) was obtained from Meilunbio (Shanghai, China). Penicillin-Streptomycin-Amphotericin B Solution, Cell Counting Kit-8 (CCK-8), and Annexin V-FITC Apoptosis Detection Kit were obtained from Keygen Biotechnology (Jiangsu, China). MCF-7 cells were purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). BRD4 antibody (13440)and NF-κB p65 (D14E12) was purchased from Cell Signaling Technology (Shanghai, China). EGFR antibody (EP38Y) and Nucleolin antibody (ab129200) was from Abcam. BCL-2 antibody (EPR17509) was from Jiangsu Real-gen Biotechnology Co., Ltd. Vinculin antibody (sc-73614) and GAPDH antibody (sc-32233) were purchased from Santa Cruz Biotechnology (Shanghai, China). The DMEM (High-glucose Dulbecco's modified Eagle's medium) and FBS (fetal bovine serum) were bought from ThermoFisher (Shanghai, China). Dulbecco's PBS, and glucose were from Solarbio Science & Technology Co. (Beijing, China).

Characterization

^{1}H NMR spectra and ^{13}C NMR spectra were obtained on 400 MHz Bruker Avance III 400 spectrometers. High-performance liquid chromatography (HPLC) was carried out on Thermo Scientific Dionex Ultimate 3000 with CH_3CN/H_2O (0.1% CF_3COOH) as eluents (the column type: Venusil XBP C18A from Agela Technologies). ESI-HRMS was done on Q Exactive (Thermo Fisher, Germany). The light source was a CEL-PE300-3A lamp purchased from CeauLight (Beijing, China). The Portable ultrasound instrument was purchased from Chattanooga (model No. CHA2776, USA). The UV-Vis spectra

were measured with Shimadzu UV-3600. The fluorescence spectra were measured with HITACHI F-7000. ESR spectra were obtained using a Bruker EMX-10/12 X-band variable-temperature apparatus and were simulated with the software of WINEPR SimFonia. Flow cytomtry data were acquired on CytoFLEX (Beckman Coulter Life Sciences). Biolayer interferometry was performed on Octet K2 instrument (Fortebio, Sartorius). Fluorescence images of cells were acquired with an Olympus SpinSR10 spinning disk confocal super resolution microscope.

Synthesis and characterization

Compound 2: JQ1 ($C_{19}H_{17}ClN_4O_2S$, 100 mg, 0.2495 mmol, 1 eq.), HATU ($C_{10}H_{15}OF_6N_6P$, 142 mg, 0.3737 mmol, 1.5 eq.) and DIEA ($C_8H_{19}N$, 130 μL, 0.7474 mmol, 3 eq.) were fully dissolved in DCM (CH_2Cl_2) and mixed in a round-bottomed flask at room temperature. Then, *tert*-Butyl (2-(2-aminoethoxy) ethyl)carbamate ($C_9H_{20}N_2O_3$, 62 mg, 0.2942 mmol, 1.2 eq) was added dropwise to the mixture, and the product was purified by column chromatography (DCM/MeOH = 30/1) after overnight reaction. The collected product was treated with 30% TFA ($C_2HF_3O_2$)/DCM (v/v, 5 mL) solution for 3 h at room temperature. After the solvent was evaporated, the mixture was subjected to column chromatography purification to afford compound **2** (81.5 mg, 67.1%).^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.11 (s, 2 H), 8.43 (s, 1 H), 8.05 (s, 2 H), 7.42 (d, *J* = 8.3 Hz, 2 H), 7.36 (d, *J* = 8.2 Hz, 2 H), 4.90 - 4.75 (m, 1 H), 3.68 - 3.56 (m, 4 H), 3.44 (t, *J* = 8.7 Hz, 2 H), 3.22 (d, *J* = 12.7 Hz, 2 H), 2.77 (s, 3 H), 2.43 (s, 3 H), 1.68 (s, 3 H). ESI-HRMS Calc'd $[M+H]^+$ 487.16000; found 487.16592.

Compound 3 (USC): PPIX ($C_{34}H_{34}N_4O_4$, 100 mg, 0.1779 mmol, 1 eq), HATU (101.4 m, 0.2669 mmol, 1.5 eq), and DIEA (92.8 μL, 0.5338 mmol, 3 eq) were completely dissolved in a round-bottomed flask using anhydrous DMF (C_3H_7NO) at room temperature. Then, compound **2** (86.65 mg. 0.1779 mmol, 1 eq) was added and reacted overnight. The residue obtained after removal of solvent on the next day was purified by HPLC with CH_3CN/H_2O (0.1% CF_3COOH) as eluents (the CH_3CN from 40% to 95% in 30 min, the flow rate was 15mL/min) to give the desired dark brown solid product USC (compound **3**) (35.5 mg, 19.4%).^{1}H NMR (400 MHz, DMSO-d_6) δ 10.28 (s, 1 H), 10.25 - 10.18 (m, 3 H), 8.55 - 8.44 (m, 2 H), 7.98 (t, *J* = 5.5 Hz, 1 H), 7.93 (q, *J* = 3.4 Hz, 1 H), 7.40 (d, *J* = 8.4 Hz, 2 H), 7.33 (d, *J* = 8.5 Hz, 2 H), 6.49 - 6.41 (m, 2 H), 6.26 - 6.20 (m, 2 H), 4.43 (t, *J* = 7.1 Hz, 1 H), 4.33 (t, *J* = 7.7 Hz, 4 H), 3.71 (d, *J* = 3.9 Hz, 6 H), 3.20 (t, *J* = 7.5 Hz, 3 H), 3.14 (q, *J* = 6.2, 4.9 Hz, 6 H), 3.04 (q, *J* = 7.3, 5.9 Hz, 4 H), 2.25 (s, 3 H), 1.51 (s, 3 H), 1.26 - 1.21 (m, 11 H), 0.88 - 0.81 (m, 2 H). ESI-HRMS Calc'd $[M+2H]^{2+}$ 516.20500; found 516.21100.

Measurement of $^1O_2$ generation

To measure the ability of USC to produce $^1O_2$, we used two approaches. First, electron spin resonance (ESR) experiments were performed as 2,2,6,6-tetramethylpiperidine (TEMP) can specifically trap $^1O_2$. TEMP (100 mM) was added to a 1 μM USC or PPIX solution, and the solution was then irradiated with 1 MHz ultrasound (1 W/cm^2, 50% duty cycle) for 1 min. ESR spectra were then obtained using an electron paramagnetic resonance spectrometer (EMX-10/12, Bruker, Germany). An alternative method is to use SOSG as a fluorescent indicator, which reacts rapidly with $^1O_2$ to emit green fluorescence. Typically, 1 μM USC or PPIX in PBS was added by SOSG (5 μM), and the solution was irradiated with light (670 nm, 115 mW/cm^2) or ultrasound (1 MHz, 1 W/cm^2, 50% duty cycle) for different time points. The fluorescence intensity of SOSG at 520 nm was collected under excitation at 488 nm.

Detection of superoxide anion and hydroxyl radical

Dihydroethidium (DHE), Terephthalic acid (TA) were used to detect USC ROS generation. 1 μM USC was mixed with DHE (10 μM) or TA (2.5 μM), respectively, and irradiated for 1 min using ultrasound (1 MHz, 1 W/cm^2, 50% duty cycle). Afterwards, the fluorescence changes of the two indicators in solution were tested by fluorescence spectroscopy.

Biolayer interferometry

To validate the affinity of USC or JQ1 with the BRD4 protein. The Fortebio Octet K2 instrument was used at 30°C. His1K Anti-Penta-His probes (Fortebio, Cat# 185120) were used in this assay. Recombinant Human BRD4 protein and USC or JQ1 were diluted with equilibrating buffer (PBS, 0.05% Tween-20, 0.1% BSA). The BLI assays were performed as described below: (1) Sensor check: sensors were immersed in equilibrating buffer for 600 s with shaking at 800 rpm. (2) Protein loading: sensors immersed with BRD4 at 20 μg/mL for 600 s with shaking at 1000 rpm. (3) Baseline: sensors immersed in equilibrating buffer for 180 s with shaking at 800 rpm. (4) Association: sensors immersed with samples at different concentration respectively for 60 s with shaking at 600 rpm. (5) Dissociation: sensors immersed in equilibrating buffer for 90 s with shaking at 800 rpm. Curve fitting was performed by Fortebio Octet Data analysis software.

Cell culture

MCF-7 cells were cultured in DMEM containing 10% FBS and 1% Penicillin/Streptomycin/Amphotericin B Solution, and maintained in 5% CO_2 at 37 °C.

Flow cytometric analysis

To assess cellular uptake, MCF-7 cells (2×10^5) were cultured 1 day before the experiment. MCF-7 cells were incubated with 1 μM USC for 0, 1, 3, 6, 9, or 12 h, and then washed 3 times with cold PBS, followed by analysis on CytoFLEX (Beckman Coulter Life Sciences). Data were analyzed using FlowJo software (V 10.0.8r1). To assess apoptosis, MCF-7 cells were cultured 1 day prior to the experiment. After reaching approximately 70% growth density, MCF-7 cells were incubated with 1 μM USC, and cells were sonicated (1 MHz, 1 W/cm^2, 50% duty cycle) for 1 min at the time point of 3 h post incubation. After 24 h of incubation, the supernatant and cells were collected according to the instructions of the Annexin V-FITC Apoptosis Detection Kit (Shanghai Biyuntian Biotechnology Co., Ltd.) and then gently resuspended in 200 μL of binding solution. Then 5 μL of Annexin V-FITC and 5 μL of propidium iodide staining solution were added, mixed for 10 min, and analyzed by CytoFLEX (Beckman Coulter Life Sciences).

Measurement of Intracellular $^1O_2$ generation

MCF-7 cells (1.5×10^5) were plated in glass-bottomed petri dishes. After reaching 70% confuency, USC/PPIX (1 μM) was added to the MCF-7 cells and incubated for 3 h at 37 °C. Cells were washed twice with cold PBS (1×). Afterwards, cells were washed carefully with cold PBS (1 ×) for three times and incubated with Hoechst 33342 for 10 min. Then, DCFH-DA (5 μM) was added and incubated with the cells for 30 min. Cells were then exposure to ultrasound radiation (1 MHz, 1 W/cm^2, 50% duty cycle) for 1 min. Fluorescence images were taken by an Olympus SpinSR10 rotating disc confocal super-resolution microscope.

Cell viability assay

MCF-7 cells were seeded in 96-well plates at a density of 10,000 cells per well. 12 hours later, cells were treated with PPIX or USC at indicated concentrations. After 3 hours of incubation, cells were irradiated with US or NIR light for 1 minute, respectively. After 24 h of incubation, cells were washed with cold PBS and CCK-8 solution (10 μL)

was added. After 1.5 h of incubation at 37 °C, the absorbance (OD) at 450 nm was recorded in each well using a microplate reader. The absorbance (OD-control) of cells without any treatment was used as a control. The percentage of cell viability was then calculated for each treatment.

Western blot analysis

MCF-7 cells were seeded into 12-well plates. After reaching 70% confluency, the cells were incubated with 1 μM USC/PPIX/JQ1 for 3 h. Cells were irradiated using ultrasonication (1 MHz, 1 W/cm^{2,} 50% duty cycle) for 1 min. After 12 h from the start of drug administration, the cells were rinsed with PBS and lysed with cell lysis buffer, and the extracted protein content was quantified by BCA protein assay kit. The lysate was then loaded onto an 6% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was sealed with skimmed milk powder solution for 2 h and then washed thoroughly with TBST. Immunoblotting was performed by incubating the proteins with anti-BRD4, anti-EGFR, anti-Bcl-2, anti-GADPH, or anti-Vinculin at 4°C overnight. The membranes were then treated with anti-goat/mouse HRP secondary antibody for 1 h and then detected by chemiluminescence HRP substrate. In the experiments simulating tissue penetration, chicken breast tissues of different thicknesses (0/0.5/1/2 cm) were placed between the cells and the radiation source, and the proteins were extracted by the method described above after the application of NIR light or US radiation.

Wound healing experiment

To test the inhibitory effect of USC on the migration of MCF-7 cells, MCF-7 cells were inoculated in 6-well plates at a density of 10^6 cells per well. After reaching 70% confluency, 1 μM USC was added to co-incubate with the cells for 3 h. Then cold PBS was washed three times before applying 1 min of ultrasonic radiation (1 MHz, 1 W/cm^2, 50% Duty cycle). The monolayers were manually scraped using a 200 uL pipette tip to form a wound, and then the peri wound cell migration was observed with a confocal microscope at 0 and 24 h, and the data were analysis on ImageJ.

Statistical analysis

Statistical comparisons between groups were evaluated by Student's t-test. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 were statistically significant.

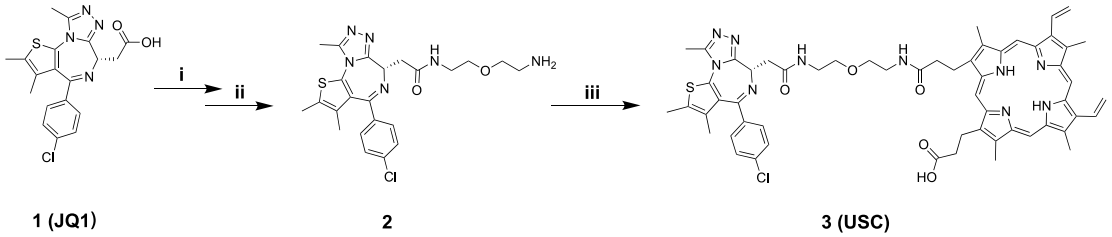


Figure S1. Synthesis of USC. i: *tert*-butyl (2-(2-aminoethoxy)ethyl)carbamate, DCM, HATU, DIEA, RT, overnight; ii: DCM, TFA, RT, 3 h; iii: PPIX, DMF, HATU, DIEA, RT, overnight.

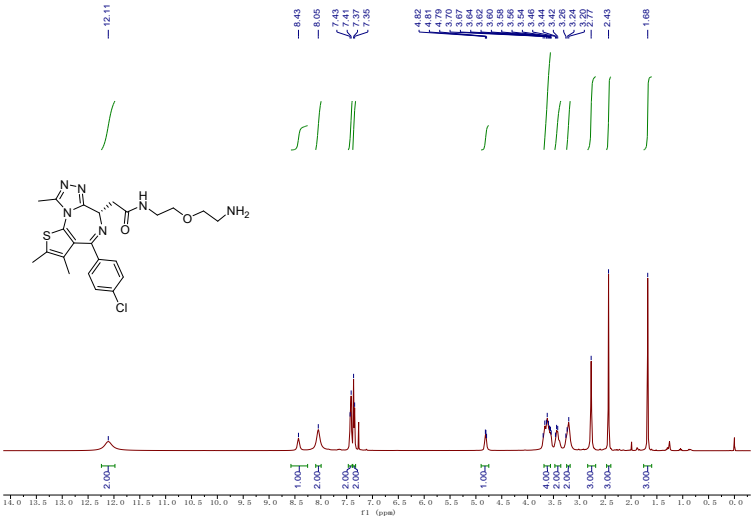


Figure S2. ^{1}H NMR spectrum of compound ***2.***

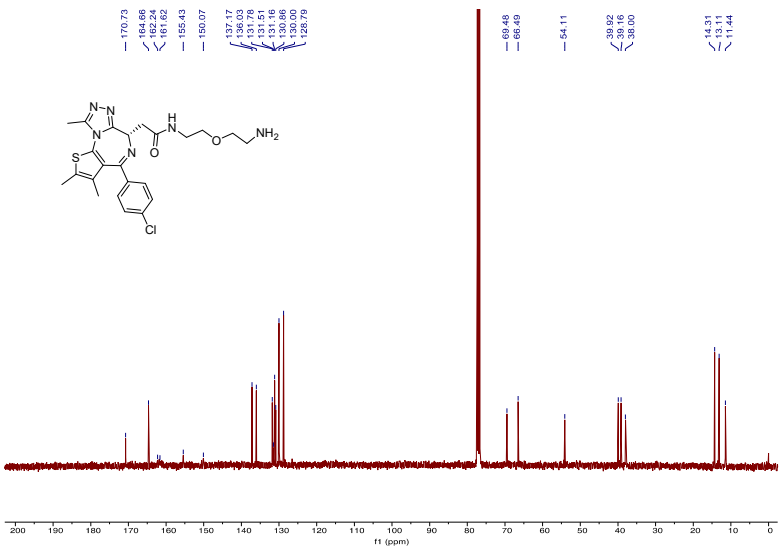


Figure S3. ^{13}C NMR spectrum of compound ***2***.

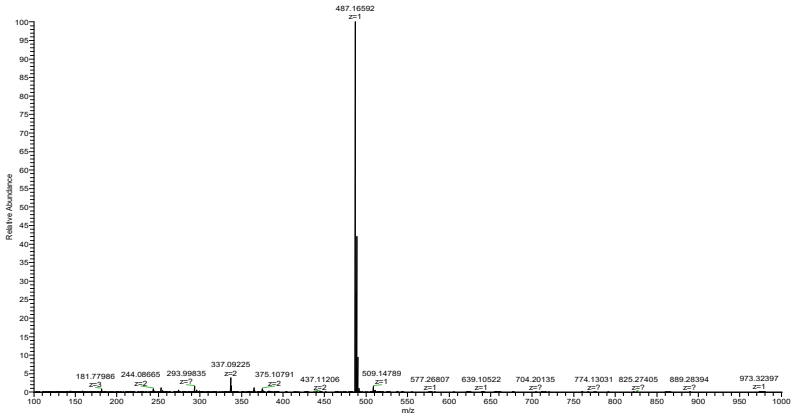


Figure S4. ESI-HRMS of compound **2**. Calcd $[M+H]^+$ 487.16000; found 487.16592.

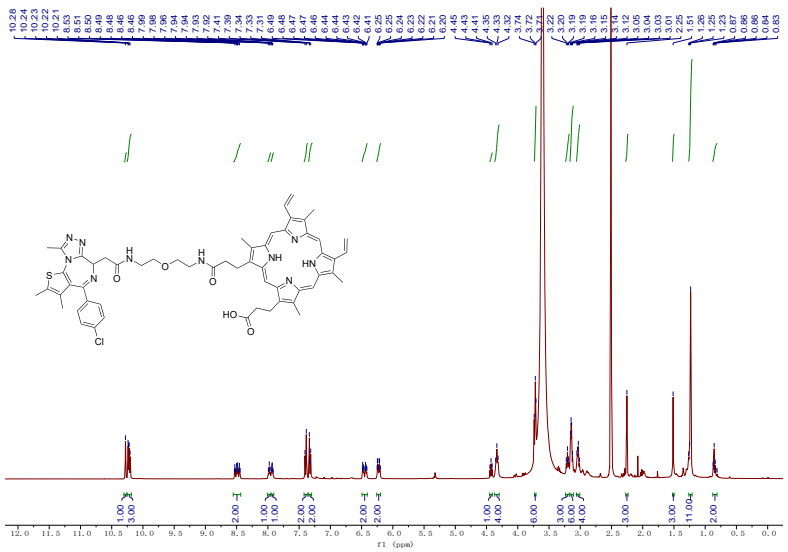


Figure S5. ^{1}H NMR spectrum of compound **3** (USC).

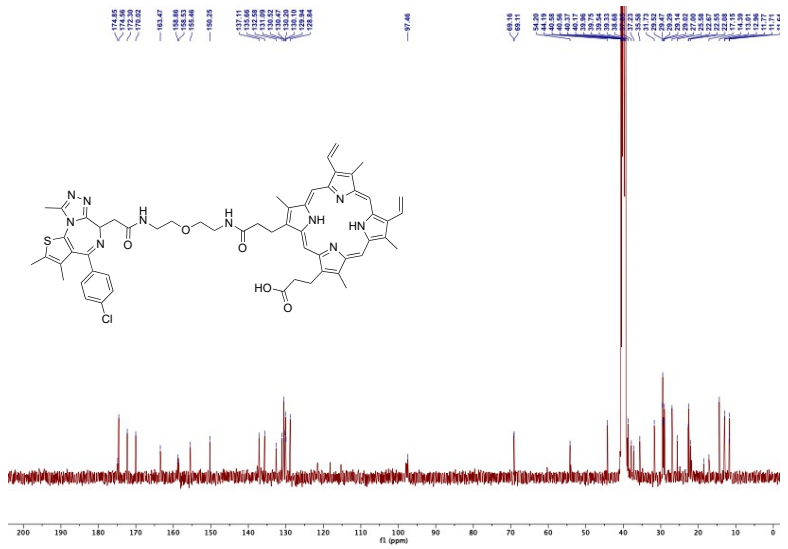


Figure S6. ^{13}C NMR spectrum of compound **3** (USC).

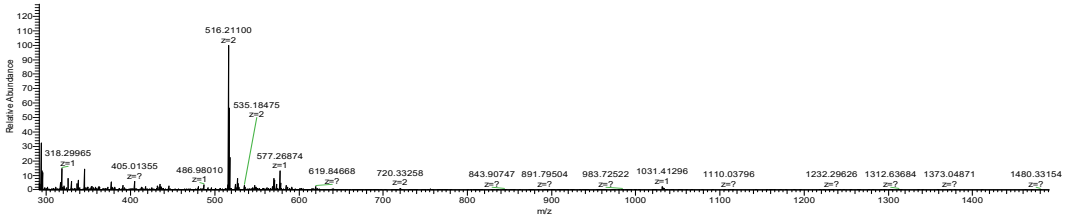


Figure S7. ESI-HRMS of compound **3** (USC). Calc'd $[M+2H]^{2+}$ 516.20500; found 516.21100.

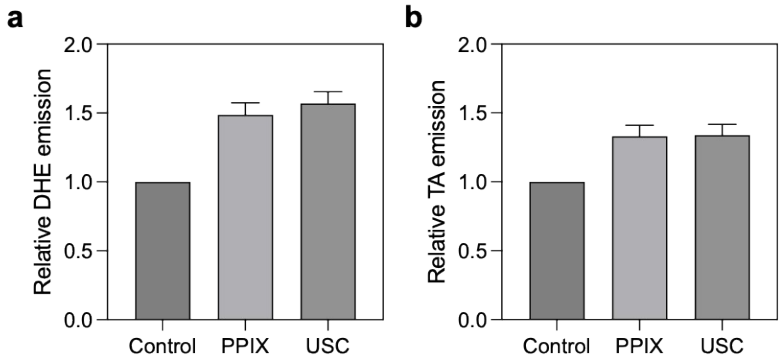


Figure S8. Relative (**a**) DHE (superoxide anion) and (**b**) TA (hydroxyl radical) emissions in 1 μM PPIX or USC solutions that were subjected to sonoirradiation (1 MHz, 1 W/cm^2, 50% duty cycle, 1 min). For DHE, $\lambda_{ex}/\lambda_{em}$ = 520/610 nm; for TA, $\lambda_{ex}/\lambda_{em}$ = 315/425 nm. Data are shown as mean ± SEM (n=3).

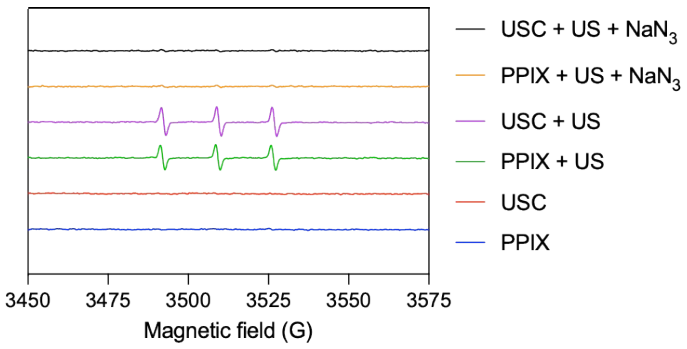


Figure S9. ESR spectra analysis of $^1O_2$ produced by USC (1 μM), PPIX (1 μM), USC/PPIX (1 μM) with NaN_3 (40 μM) under sonoirradiation (1 MHz, 1 W/cm^2, 50% duty cycle, 1 min).

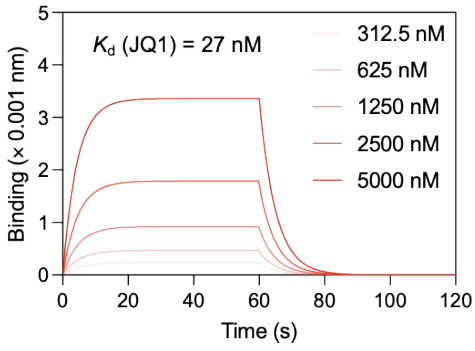


Figure S10. BLI analysis of JQ1 (312.5-5000 nM) binding to recombinant BRD4 protein (300 nM).

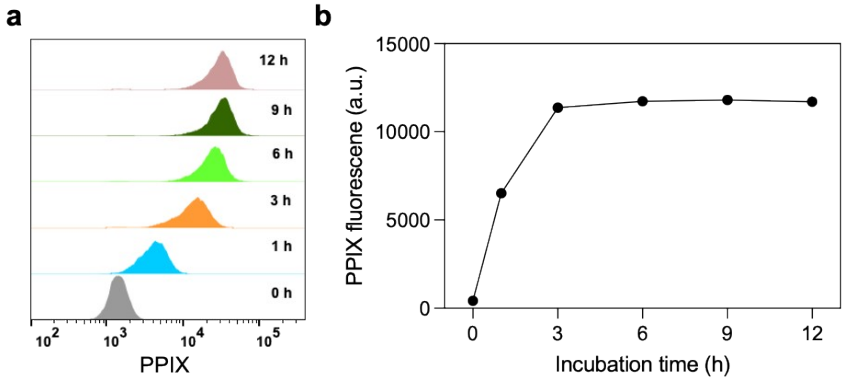


Figure S11. (**a**) Representative flow cytometry data of MCF-7 cells treated with USC (1 μM) for 0, 3, 6, 9, or 12h. (**b**) Quantification data. Data are shown as mean ± SEM (n=3).

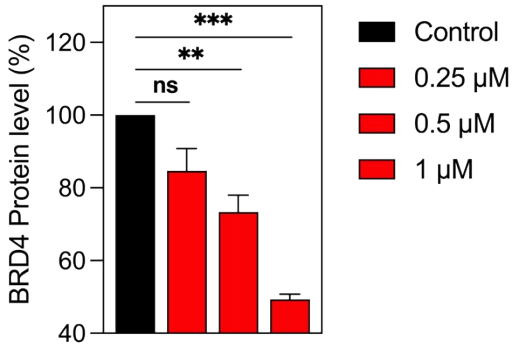


Figure S12. Quantitative analysis of the Western blot results presented in Figure 2a of the manuscript. Data are shown as mean ± SEM (n=3). ***P* < 0.01, ****P* < 0.001.

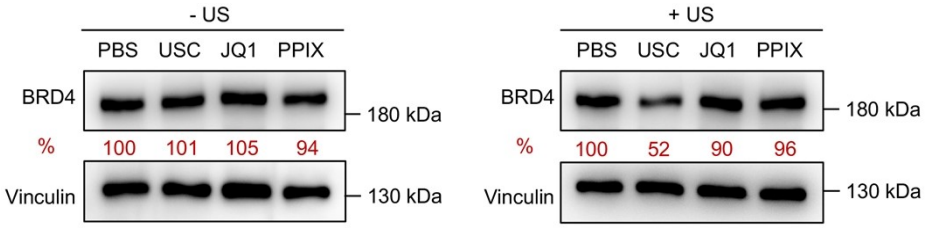


Figure S13. Western blot analysis of BRD4 protein levels in MCF-7 cells treated with USC (1 μM), JQ1 (1 μM), or PPIX (1 μM) for 12 h. Vinculin served as the internal control.

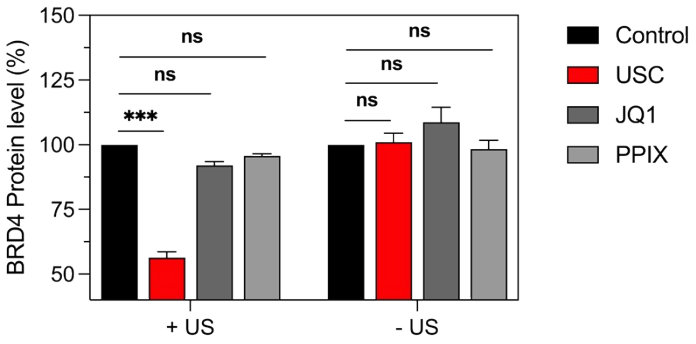


Figure S14. Quantitative analysis of the Western blot results presented in Figure S13 of the supporting information. Data are shown as mean ± SEM (n=3). ****P* < 0.001.

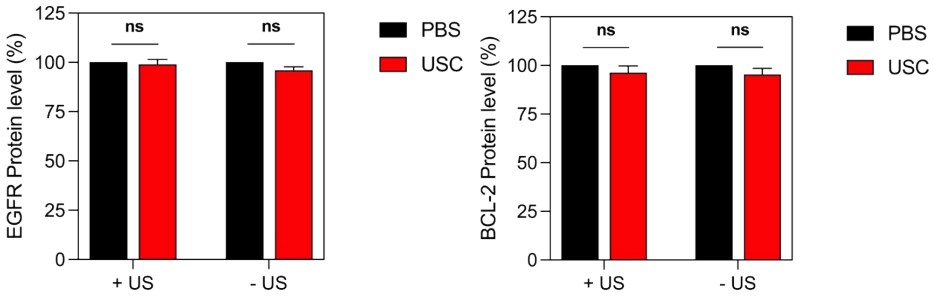


Figure S15. Quantitative analysis of the Western blot results presented in Figure 2b of the main text. Data are shown as mean ± SEM (n=3).

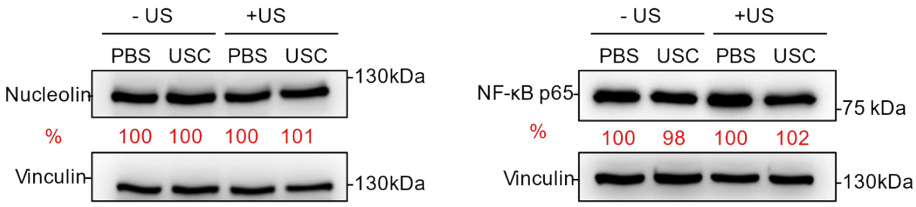


Figure S16. Western blot analysis of the expression levels of Nucleolin and NF-κB p65 in MCF-7 cells treated with USC under. For + US groups, cells were subjected to sonoirradiation (1 MHz, 1 W/cm^2, 50% duty cycle, 1 min) at 3 h post incubation. Vinculin served as the internal controls.

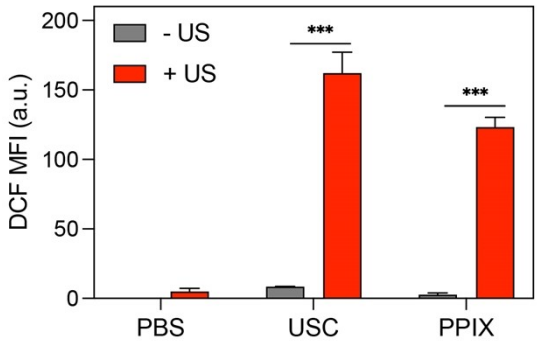


Figure S17. Mean fluorescence intensity (MFI) of confocal fluorescence images shown in Figure 2c in the main text. Data are shown as mean ± SEM. ***P < 0.001.

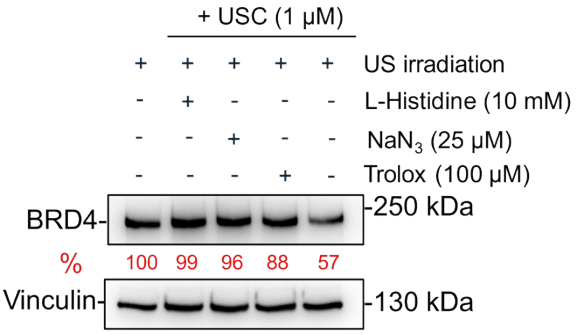


Figure S18. Western blot analysis of BRD4 protein levels in MCF-7 cells treated with USC (1 μM) and sonoirradiation (1 MHz, 1 W/cm^2, 50% duty cycle) in the presence or absence of ROS scavengers.

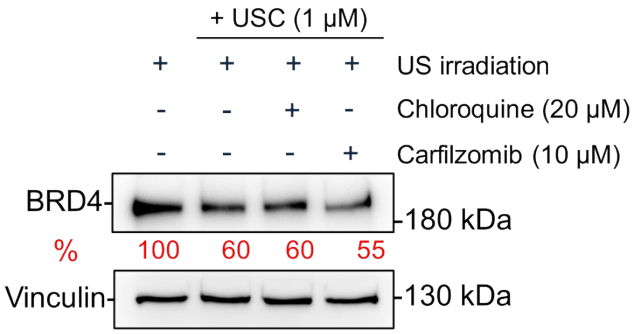


Figure S19. Western blot analysis of the effect of chloroquine (20 μM) and carfilzomib (10 μM) on BRD4 degradation in MCF-7 cells induced by ultrasound-activated USC. Vinculin served as the internal controls.

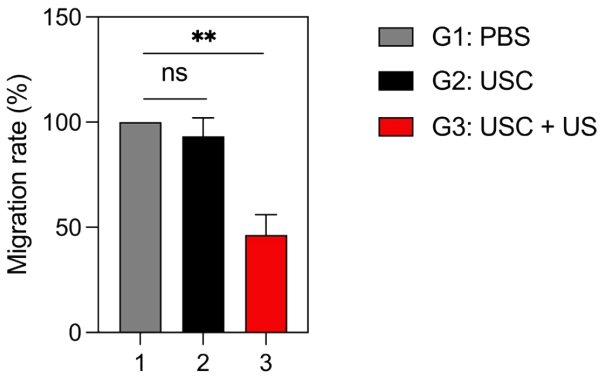


Figure S20. Migration rates of MCF-7 cells treated with USC and US (shown in Figure 3b in the main text). Data are shown as mean ± SEM (n = 3). ***P* < 0.01.

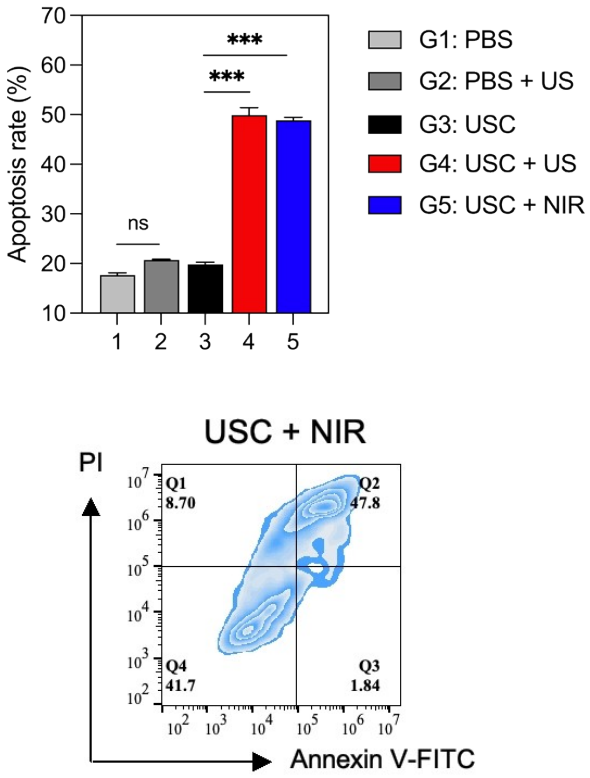


Figure S21. Apoptotic rates of MCF-7 cells treated with USC and US/NIR (shown in Figure 3c in the main text). Data are shown as mean ± SEM (n = 3). ****P* < 0.001.

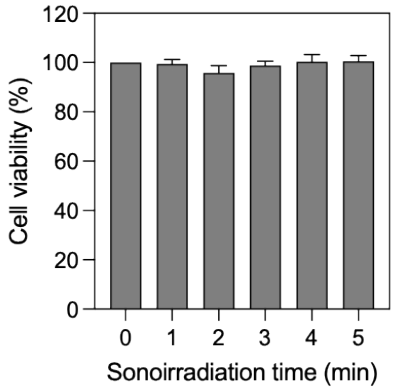


Figure S22. Viabilities of MCF-7 cells subjected to sonoirradiation (1 MHz, 1 W/cm^2, 50% duty cycle) for 0-5 min. Data are shown as mean ± SEM (n=4).

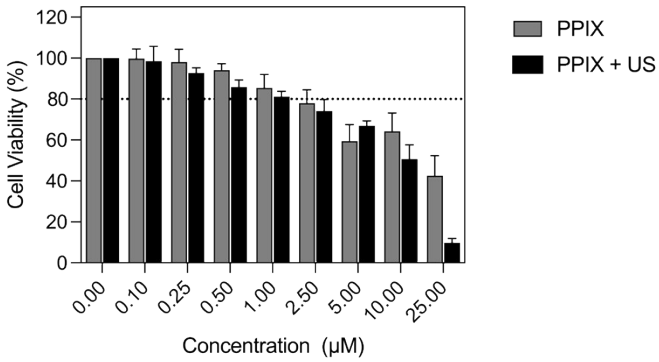


Figure S23. Viabilities of MCF-7 cells treated with PPIX (0-1 μM) for 12 h. Cells were subjected to sonoirradiation (1 MHz, 1 W/cm^2, 50% duty cycle, 1 min) at 3 h post incubaiton. Data are shown as mean ± SEM (n=4).

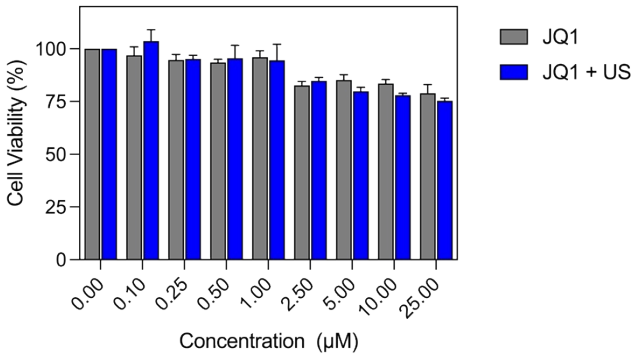
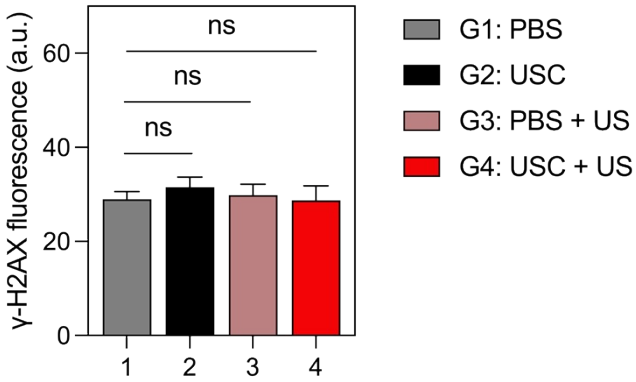


Figure S24. Viabilities of MCF-7 cells treated with JQ1 (0-25 μM) for 12 h. Cells were subjected to sonoirradiation (1 MHz, 1 W/cm^2, 50% duty cycle, 1 min) at 3 h post incubation. Data are shown as mean ± SEM (n=4).



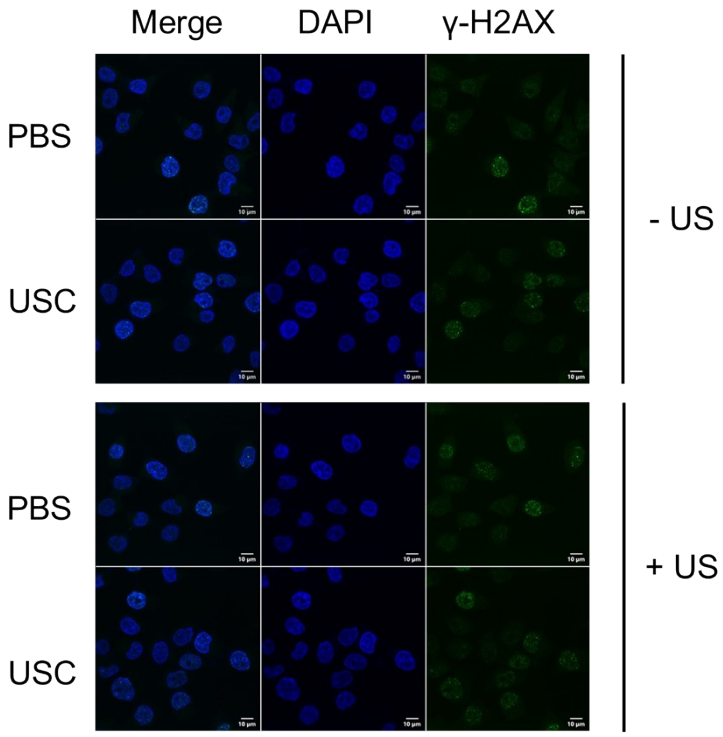


Figure S25. Representative confocal fluorescence images of γ-H2AX staining in MCF-7 cells after treatment with USC (1 μM), For + US groups, cells were subjected to sonoirradiation (1 MHz, 1 W/cm^2, 50% duty cycle, 1 min). Immunofluorescence staining of γ-H2AX (green) was performed to evaluate DNA damage. DAPI (blue) was used for nuclear staining.

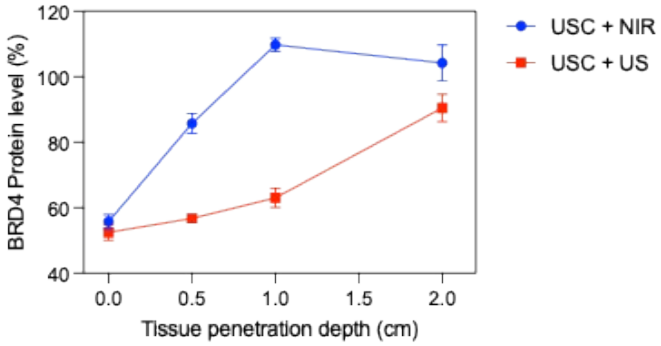


Figure S26. Quantification of BRD4 protein levels in MCF-7 cells treated with USC (1 μM) for 12 h. US or NIR light irradiations were implemented through chicken breast tissues (0-2 cm) at 3 h post incubation. Data are shown as mean ± SEM (n=4).

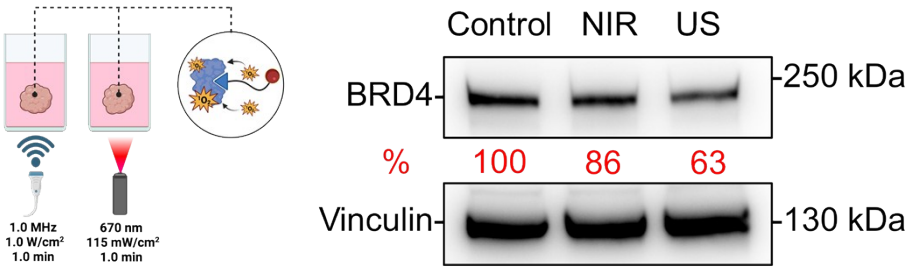


Figure S27. Western blot analysis of BRD4 protein levels in 3D tumor spheroids after treatment with USC (1 μM) and US or NIR light irradiations. For US and NIR groups, 3D tumor spheroids were subjected to sonoirradiation (1 MHz, 1 W/cm^2, 50% duty cycle, 1 min) and NIR photoirradiation (670 nm, 115 mW/cm^2, 1 min) at 3 h post incubation.

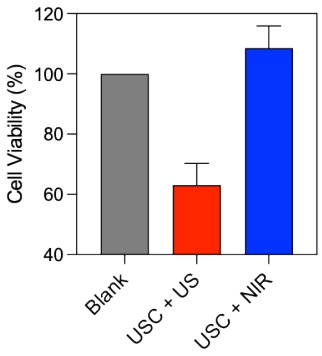


Figure S28. Viabilities of MCF-7 cells treated with USC (1 μM) for 24 h. US or NIR light irradiations were implemented through chicken breast tissues (1 cm) at 3 h post incubation. Data are shown as mean ± SEM (n=4).

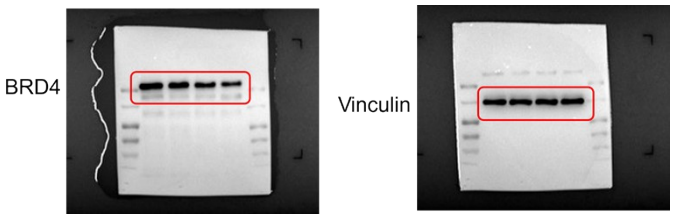


Figure S29. Source western blotting images of Figure 2a.

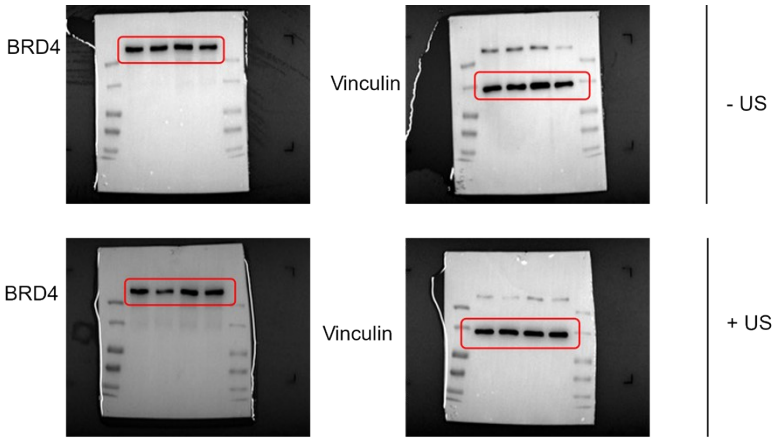


Figure S30. Source western blotting images of Figure S13.

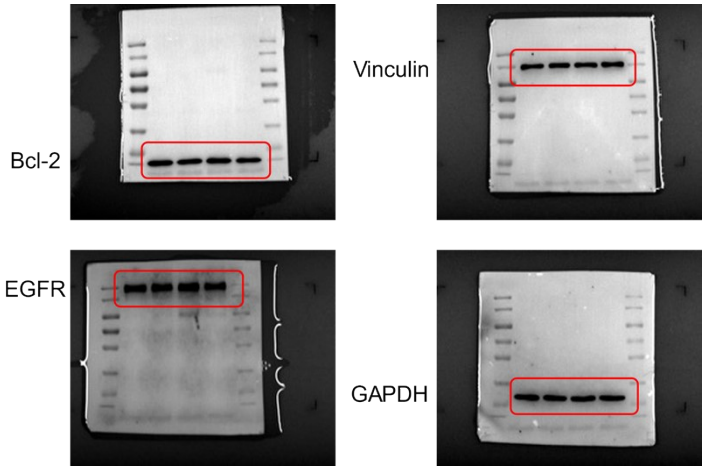


Figure S31. Source western blotting images of Figure 2b.

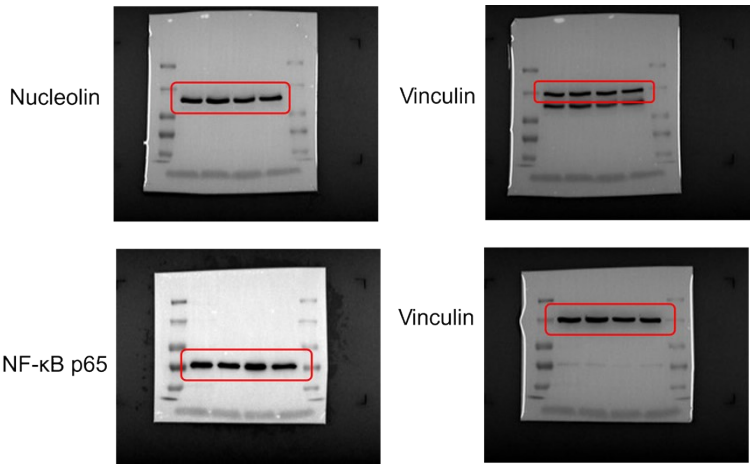


Figure S32. Source western blotting images of Figure S16.

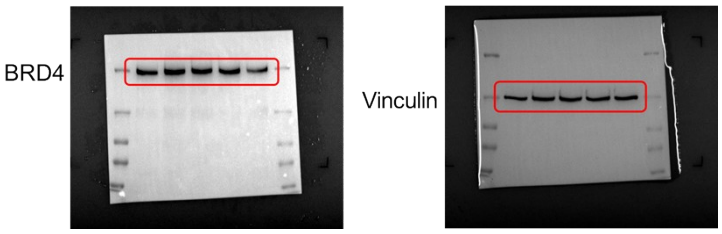


Figure S33. Source western blotting images of Figure S18.

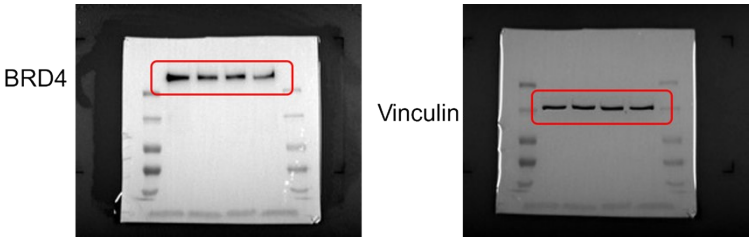


Figure S34. Source western blotting images of Figure S19.

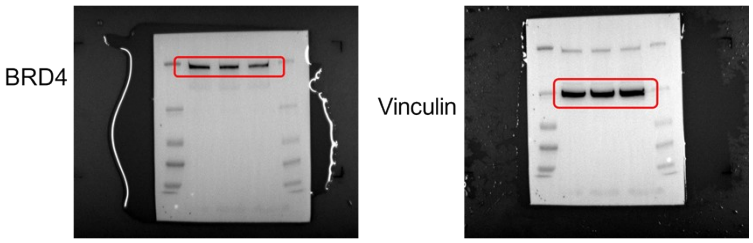


Figure S35. Source western blotting images of Figure S27.

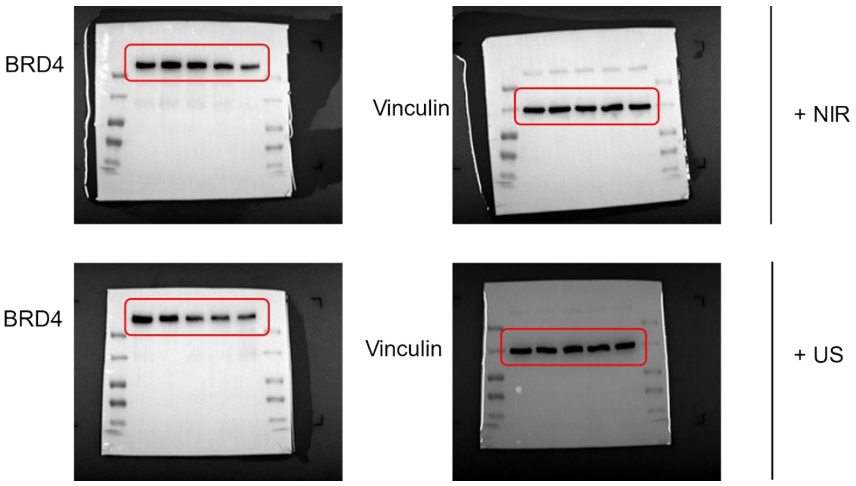


Figure S36. Source western blotting images of Figure 4d.