Electronic Supplementary Information

Mesoporous Nanocarriers with Stimulus-Responsive Cyclodextrin Gatekeeper for Targeting Tumor Hypoxia

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Materials and instruments. Cetyltrimethylammonium bromide (CTAB), tetraethylorthosilicate (TEOS), 3-aminopropyltriethoxysilane, copper(II) sulfate, doxorubicin (DOX), propargyl amine, methoxy poly(ethylene glycol) (MW 2000), hexamethylene diisocyanate, ferric acetylacetonate, NQO1, NADPH, ninhydrin, and sodium ascorbate from Aldrich were used as received. Azobenzene-4,4'-dicarbonyl dichloride from TCI and mono-6-azido-6-deoxy-β-cyclodextrin (N_3-β-CD) from CycloLab were used as received. TEM images were obtained using a Philips CM 200 instrument operated at an acceleration voltage of 120 kV. For the preparation of TEM samples, a drop of dispersed sample in distilled water (100 $mg \cdot L^{-1}$) was placed onto a 300-mesh copper grid coated with carbon film. About 2 min after deposition, the grid was touched with filter paper to remove surface water. The samples were dried before measurement. All fluorescence measurements were performed using a Shimadzu RF-5301PC spectrofluorophotometer with an excitation wavelength of 485 nm (absorption maximum wavelength of DOX). Emission and excitation slit widths were set at 3 nm. FT-IR spectra were obtained using a VERTEX 80V vacuum spectrometer. All UV/Vis absorption spectra were obtained using an Agilent 8453E UV/Vis spectrophotometer in a quartz cuvette with a path length of 1 cm. Zeta-potential values of dispersed samples in distilled water were obtained using an OTSUKA ELS-Z2 Particle Size Analyzer. Brunauer-Emmett-Teller (BET) nitrogen adsorption/desorption isotherms and Barrett-Joyner-Halenda (BJH) pore size distribution analysis were performed at 77 K on a Quantachrome instrument (ASAP 2020).

Synthesis of MCM-41. MCM-41 type MSNs with a pore size of ~2.5 nm were prepared according to a previously described procedure.[1] Briefly, an aqueous solution (192 mL) of CTAB (0.43 g) was added to a 2 M NaOH solution (1.4 mL). After stirring at 80°C for 5 min, TEOS (2 mL) was added and the mixture was stirred for 2 h. The resulting solid was washed thoroughly with water by centrifugation and dried in vacuo.

Synthesis of Si-NH_2. 3-Aminopropyltriethoxysilane (3.5 mL) was added to an ethanol solution (20 mL) of MCM-41 (50 mg), which was then allowed to react overnight at 60°C. The resulting solid was washed thoroughly with ethanol and dried in vacuo.

Synthesis of Azo-alkyne. A DMF solution of azobenzene-4,4'-dicarbonyl dichloride was allowed to react with propargyl amine for 3 h at room temperature. The resulting product was used without purification to modify silica surfaces.

Synthesis of Si-Azo-alkyne. Si-NH_2 was dispersed in DMF, after which Azo-alkyne was added and stirred overnight at room temperature. The resulting solid was washed thoroughly with DMF and ethanol by centrifugation to yield Si-Azo-alkyne.

Synthesis of Si-Azo-CD. Surfactant was removed from silica nanoparticles by stirring 60 mg of ammonium nitrate into a solution of Si-Azo-alkyne (50 mg in 25 mL of EtOH) at 60°C for 30 min. The resulting solid was washed thoroughly with ethanol and dried in vacuo. For DOX loading, surfactant-free Si-Azo-alkyne (50 mg) was soaked in a 1-mL solution of DOX (5 mg) in DMF and stirred overnight. Then, N_3-β-CD (50 mg) in 1 mL of DMF, copper (II) sulfate (11.24 mg) in 100 μL of water, sodium ascorbate (17.83 mg) in 100 μL of water, and 500 μL of t-butanol were added. The click reaction of silica nanoparticles was performed by stirring

under a microwave (150 W) at 90°C for 10 min. The resulting solid was washed thoroughly with DMF and distilled water, then dried in vacuo.

Synthesis of Si-Azo-CD-PEG. PEG was introduced onto the surface of Si-Azo-CD by allowing a DMF solution of methoxy poly(ethylene glycol) (MW 2000, 1 g, 0.5 mmol) to react with hexamethylene diisocyanate (0.5 mmol) containing ferric acetylacetonate (10 μmol) for 3 h at 50°C. The resulting mixture was added to a DMF solution of Si-Azo-CD (50 mg) and stirred for 2 days. The resulting product was washed thoroughly with DMF and distilled water by centrifugation to yield Si-Azo-CD-PEG.

Cell lines and culture conditions. A549 human lung adenocarcinoma epithelial cells and MDA-MB-231 human breast cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and RPMI medium, respectively. Cells were incubated at 37°C in a humidified 5% CO_2/95% air incubator. Hypoxic conditions were created by incubation of cells in an Invivo$_2$ 500 hypoxia workstation (The Baker Company, Sanford, ME, USA). Oxygen concentrations were fixed using a gas mixture containing 5% carbon dioxide and a balance of nitrogen.

Antibodies. The primary antibodies included those against PARP, cleaved caspase-3 and β-actin, obtained from Cell Signaling Technology (Beverly, MA, USA), and NQO1, from Invitrogen (Carlsbad, CA, USA). The secondary antibodies used for immunoblotting included horseradish peroxidase (HRP)-conjugated anti-mouse (Cell Signaling Technology) and HRP-conjugated anti-rabbit (Santa Cruz Biotechnology, Dallas, TX, USA).

Immunoblot analysis. After lysing cells, proteins in cell lysates were separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to

nitrocellulose membranes. The membranes were blocked with 1% (v/v) nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20, then incubated first with primary antibodies against the proteins of interest and then with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies, as appropriate. Immunoreactive proteins were visualized using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

Plasmids. To construct pCDNA3.1-myc-his$_6$-NQO1 (pNQO1) and pCDNA3.1-myc-his$_6$-NQO1 C609T mutant (pNQO1(C609T)), total RNA was obtained from A549 cells using the TRIzol reagent (Invitrogen) and cDNA was generated using SuperScript^{TM}III Reverse Transcriptase (Invitrogen). The open reading frame (ORFs) of NQO1 was PCR-amplified using the appropriate primers. The primers sequences used were as follows: 5'-GGG GTA CCA TGG TCG GCA GAA GAG CAC-3' (forward), 5'-CCG CTC GAG TTT TCT AGC TTT GAT CTG G-3' (reverse), 5'-TCT TAG AAT CTC AAC TGA CA-3' (internal C609T forward) and 5'-TGT CAG TTG AGA ATT CTT AAG A-3' (internal C609T reverse). The PCR products were digested with restriction enzymes and directly ligated into the pCDNA3.1-myc-His$_6$ (Invitrogen). All constructs were confirmed by DNA sequencing (Bionics, Seoul, Republic of Korea).

Construction of stable cell lines. Stable cell lines were constructed by first seeding cells at 5 × 10^4 cells per well in 24-well plates and then transfecting cells with 50 μL of a mixture containing 1 μg of pCDNA3.1-myc-His$_6$-NQO1, pCDNA3.1-myc-His$_6$-NQO1(C609T) or pshNQO1 (Qiagen, Valencia, CA, USA), together with TurboFect *In Vitro* Transfection Reagent (Fermentas, Hanover, MD, USA). pCDNA3.1-myc-His$_6$ and pshCont (Qiagen, Valencia, CA, USA) were used as controls. Transfected cells were selected by culturing in the presence of 1 mg/mL G418 (DuchefaBiochemie, Haarlem, Netherlands) for 1 week and were maintained in DMEM or RPMI-1640 containing 0.5 mg/mL G418 during experiments.

Assessment of Dox release by confocal laser-scanning microscopy. The intracellular release of DOX from Si-Azo-CD-PEG was assessed by first seeding cells in 8-well chamber slides (Nunc, Roskilde, Denmark), and then culturing for 24 h under normoxic or hypoxic conditions in the presence of Si-Azo-CD-PEG loaded with 5 μM DOX. Thereafter, DOX fluorescence was imaged by obtaining single-plane confocal image sequences every 2 h using a Nikon C1-Plus laser-scanning TE2000E confocal microscope (Nikon, Tokyo, Japan).

Assessment of localization of DOX by confocal laser scanning microscopy. Intracellular release of DOX from Si-Azo-CD-PEG and the accumulation of DOX in nuclei were assessed microscopically in cells seeded on coverslips coated with 0.2% gelatin. After treating with Si-Azo-CD-PEG loaded with 5 μM DOX for 24 h, coverslip-mounted cells were fixed with 3.7% paraformaldehyde (PFA) for 5 min. The coverslips were washed three times with PBS, and cell nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 2 min. Coverslips were washed three times with PBS and mounted onto slides using a mounting reagent (Invitrogen, Carlsbad, CA, USA). The fluorescence intensities of DOX and DAPI-stained nuclei were analyzed using a laser-scanning TE2000E confocal microscope (Nikon, Tokyo, Japan).

Clonogenic cell survival. Cells were seeded in 6-well plates and incubated overnight in a humidified 5% CO_2/95% air incubator at 37°C. Cells were treated with 5 μM DOX, Si-Azo-CD-PEG without DOX or Si-Azo-CD-PEG loaded with 5 μM DOX, and then exposed to 20% or 0.1% O_2. After treating for 6 h, cells were gently washed three times with PBS and cultured for an additional 14 days. Colonies formed were fixed with methanol and stained with crystal violet (0.1% in methanol), and the number of colonies containing more than 50 cells was

counted. The surviving cell fractions of treated groups were calculated by expressing the plating efficiency of treated cells relative to that of untreated control cells.

TUNEL assay. Cells were first seeded in 8-well chamber slides and incubated overnight in a humidified 5% CO_2/95% air incubator at 37°C. Cells were then treated with 5 μM DOX, Si-Azo-CD-PEG without DOX or Si-Azo-CD-PEG loaded with 5 μM DOX, and exposed to 20% or 0.1% O_2. After treating for 10 h, cells were fixed with 4% (v/v) paraformaldehyde (PFA) for 15 min, washed with PBS containing 1% (w/v) bovine serum albumin, permeabilized with 0.1% (v/v) Triton-X100, washed with PBS, and incubated for 1 h at 37°C in the dark with an apoptosis detection solution (Apoptosis Detection System kit; Roche Molecular Biochemicals, Mannheim, Germany). *In situ*-labeled nuclei were observed and photographed using a Nikon C1-Plus laser-scanning TE2000E confocal microscope.

NQO1 enzymatic activity. Cells were washed twice with phenol-red–free Hank's balanced salt solution, resuspended in PBS (pH 7.2) containing 10 mg/mL aprotinin, sonicated four times using 10-s pulses on ice, and centrifuged at 14,000 × g for 20 minutes. The supernatants were collected, transferred to microcentrifuge tubes, and stored at -80°C until NQO1 enzymatic activity could be assessed. NQO1 enzymatic activity was measured using an NQO1 activity assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's recommendations.

Determination of cell proliferation. The proliferation rates of NQO1-null MDA-MB-231 cells and NQO1-overexpressing MDA-MB-231/pNQO1 cells were determined by counting cells at 24, 48, and 72 h after seeding. Cell viability was assessed by trypan blue staining, and cells were counted with a hemocytometer.

Tumor xenograft model. All animal studies were carried out according to an Institutional Animal Care and Use Committee protocol (INHA 151008-383) approved for this study by Inha University. Eight-week-old, female nude mice (BALB/c-nu) were purchased from Orient Bio Laboratory Animal Inc. (Seoul, Korea) and maintained under specific-pathogen–free conditions, with water and food provided ad libitum. The antitumor efficacy of free DOX, Si-Azo-CD-PEG without DOX, and DOX-loaded Azo-Si-CD-PEG was investigated in an 8-wk BALB/c nude mice model. For this, 2×10^6 MDA-MB-231 or MDA-MB-231/pNQO1 cells were injected into the right flank of mice. Ten days after tumor implantation, MDA-MB-231 and MDA-MB-231/pNQO1 tumor-bearing mice were each randomly divided into the following three experimental groups (n = 7 mice/group): free DOX, Si-Azo-CD-PEG without DOX, and DOX-loaded Azo-Si-CD-PEG. Each group received intravenous administration of 180 mg/kg silica (equivalent to a DOX dose of 7.5 mg/kg) twice weekly for 3 weeks; the free DOX group received the same drug dose (7.5 mg/kg) at the same interval. Body weight was recorded every week. Tumor dimensions were measured by calipers, and volume was calculated according to the formula, Tumor volume (mm^3) = (length in mm) × (width in $mm)^2/2$.

Measurement of DOX in mouse tumors and organs. DOX concentration in mouse tumors and organs (liver, kidney, lung and spleen) was measured by DOX fluorescence as previously described.[2] Briefly, 400 μL of nuclear homogenates was added to 1.5 mL of acidified isopropanol, 200 μL of 10% Triton X-100, and 200 μL of water. Each sample was extracted overnight and centrifuged at 15,000 × g for 20 min, and DOX fluorescence was measured at excitation and emission wavelengths of 480 and 560 nm, respectively, using a Perkin Elmer LS55 fluorescence spectrometer.

Immunofluorescence staining. Xenograft tumor tissues harvested from mice in each treatment group were fixed in 4% buffered formalin for 24 h and then paraffin embedded using conventional methods. After antigen retrieval with citrate buffer, tissue sections were incubated overnight with a primary antibody against human NQO1 (Novus Biologicals) diluted 1:100 in antibody diluent (IHC). After incubation with primary antibody, tissue sections were washed three times with Tris-HCl buffer (pH 7.8) for 10 min each, and incubated for 3 h with AlexaFluor 488-conjugated, affinity-purified goat-anti-rabbit IgG (1∶200; Molecular Probes) at room temperature. DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride) was used to counterstain nuclei. Cell death in tumor xenografts was determined by assessing apoptotic cells using a TUNEL assay (In Situ Cell Death Detection Kit; Roche Applied Science) according to the manufacturer's instructions. Confocal images were captured on a Zeiss LSM 510 Confocal System (Carl Zeiss Inc.) using Zeiss AIM software. Sections were examined microscopically, and representative field were photographed under ×400 magnifications. Pimonidazole was used to generate hypoxic adducts by intraperitoneal injection at 60 mg/kg (Hydroxyprobe) 60 min prior to sacrifice. Hypoxia detection and pimonidazole staining was performed using a FITC conjugated primary antibody overnight at 4°C.

Statistical Analysis. All grouped data are presented as means ± standard error of the mean (SEM). Differences among/between groups were analyzed by analysis of variance (ANOVA) or Student’s t-test, as appropriate, using GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA, USA). All experiments were repeated in at least duplicate with triplicate technical replicates.

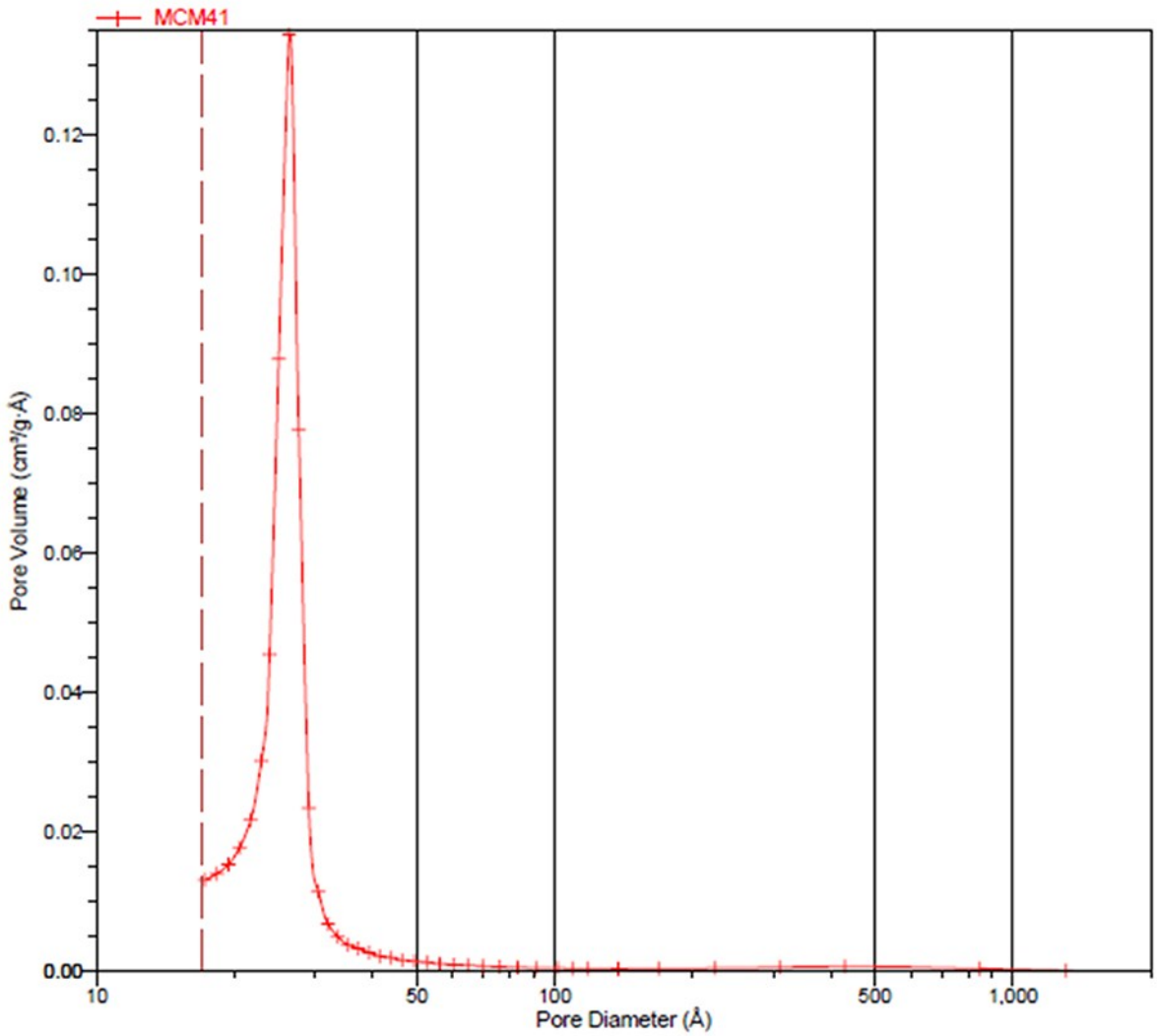

Fig. S1. BJH pore size distribution analysis of MCM-41.

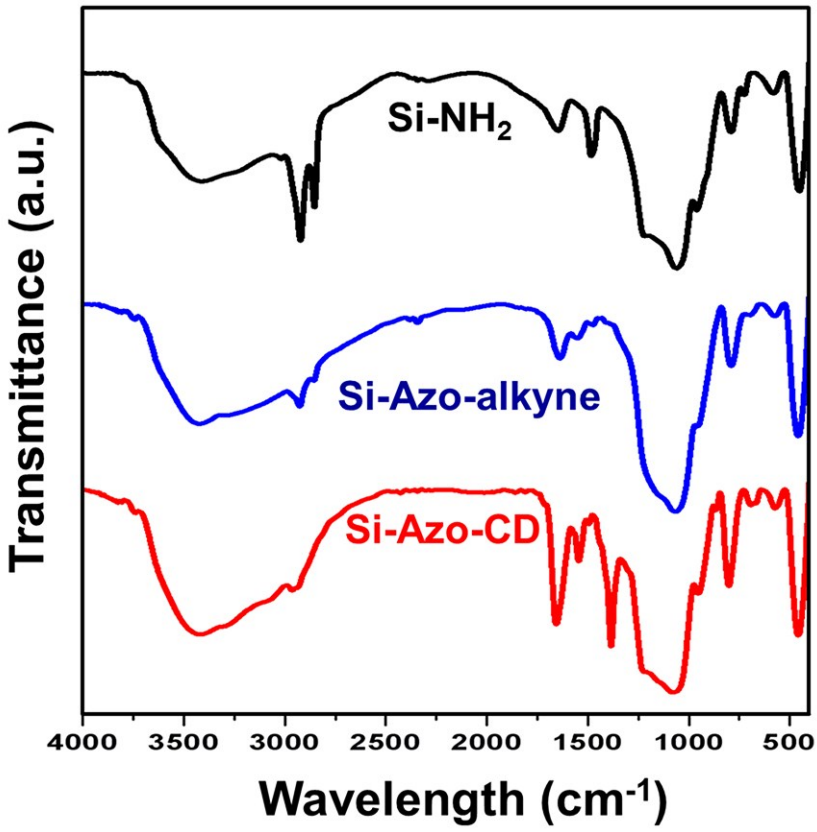

Fig. S2. FT-IR spectra of Si-NH_2, Si-Azo-alkyne and Si-Azo-CD.

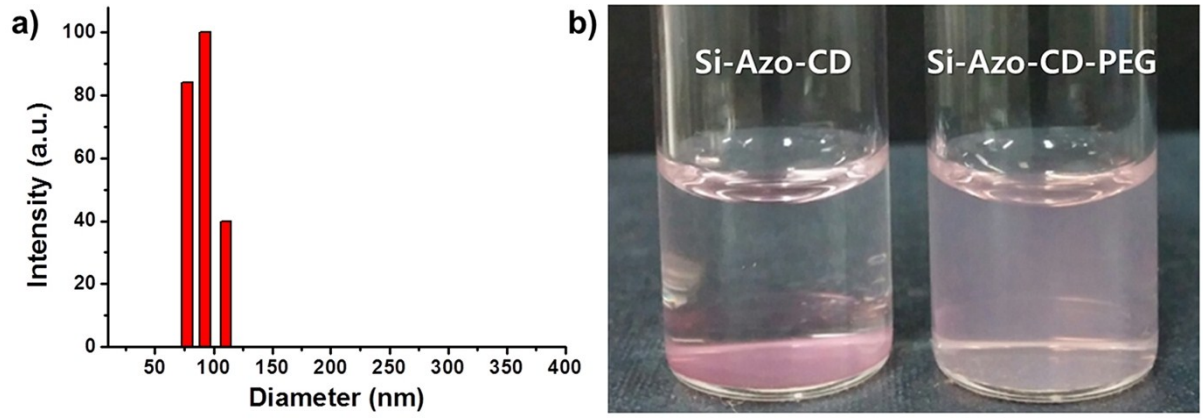

Fig. S3. a) DLS data of Si-Azo-CD-PEG. b) Images of Si-Azo-CD and Si-Azo-CD-PEG dispersed in PBS buffer after 1 week.

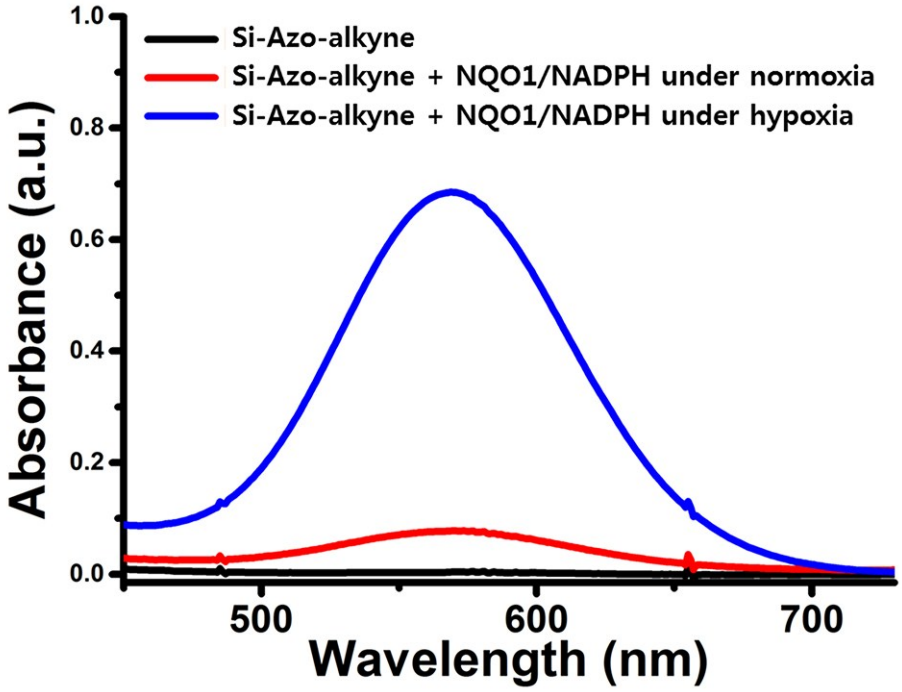

Fig. S4. UV/Vis absorption spectra of a ninhydrin test solution of Si-Azo-alkyne before and after treatment with NQO1 and NADPH under normoxic or hypoxic conditions.

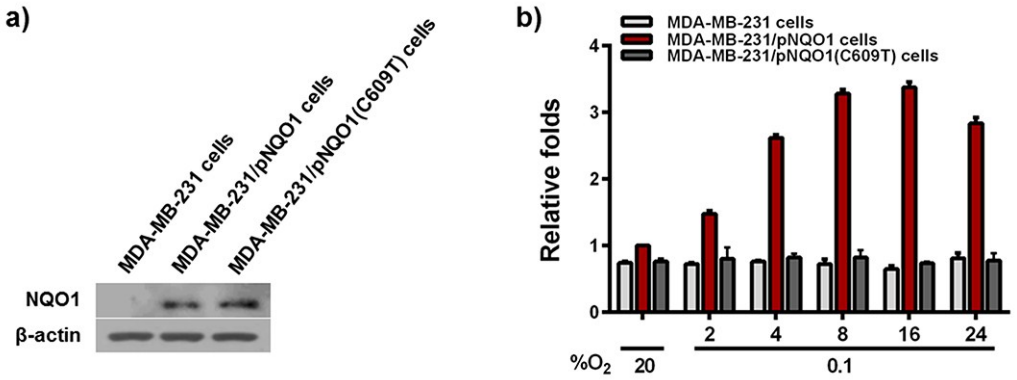

Fig. S5. Effect of hypoxia on NQO1 activity in MDA-MB-231 cells with NQO1 gain-of-function. (a) Expression of NQO1 in MDA-MB-231, MDA-MB-231/pNQO1, and MDA-MB-231/pNQO1(C609T) cells under normoxia. (b) NQO1 activity in MDA-MB-231, MDA-MB-231/pNQO1, and MDA-MB-231/pNQO1(C609T) cells exposed to hypoxia for the indicated times. The results from three independent experiments are expressed as means ± SEM.

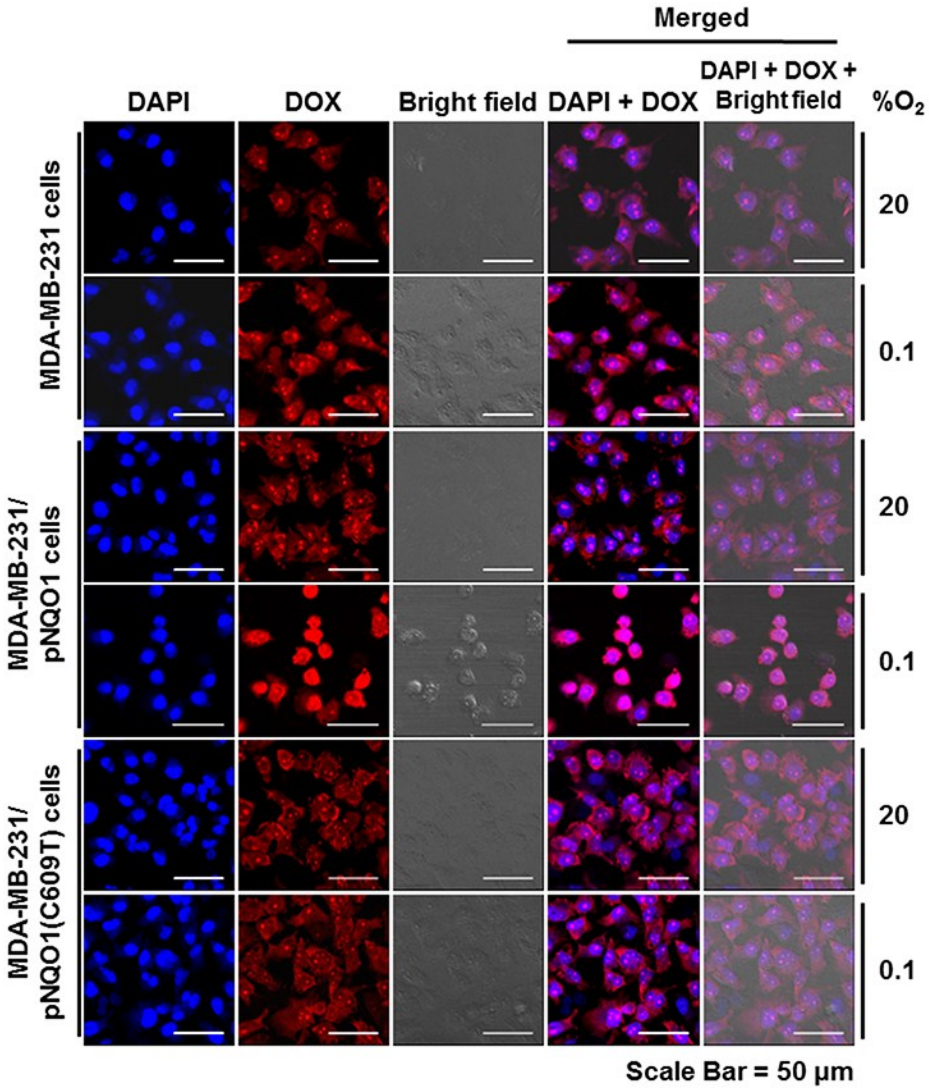

Fig. S6. The NQO1-induced release of DOX from Si-Azo-CD-PEG in cancer cells depends on hypoxia. MDA-MB-231, MDA-MB-231/pNQO1, and MDA-MB-231/pNQO1(C609T) cells were treated with Si-Azo-CD-PEG loaded with 5 μM DOX for 12 h. The cells were fixed with 3.7% PFA, washed three times with PBS, and stained with DAPI. The fluorescence intensities of DOX and DAPI-stained nuclei were examined using a TE2000E laser-scanning confocal microscope (Nikon, Tokyo, Japan).

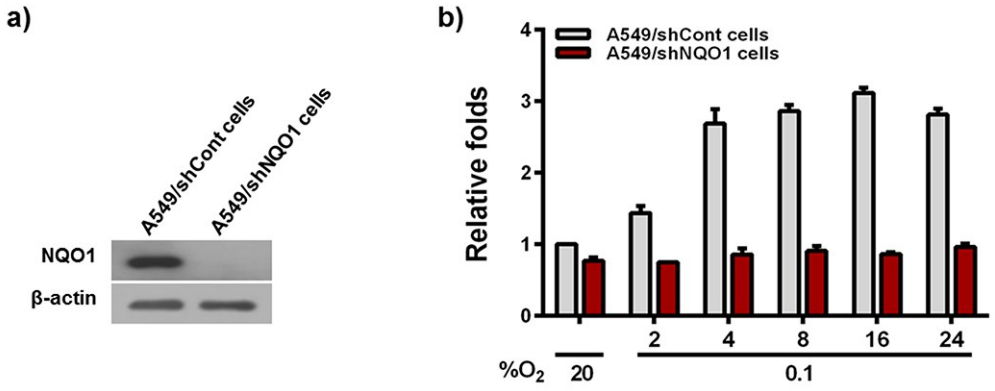

Fig. S7. Effect of hypoxia on NQO1 activity in A549 cells with NQO1 loss-of-function. (a) Expression of NQO1 in A549/shCont and A549/shNQO1 cells under normoxia. (b) NQO1 activity in A549/shCont and A549/shNQO1 cells exposed to hypoxia for the indicated times. The results from three independent experiments are expressed as means ± SEM.

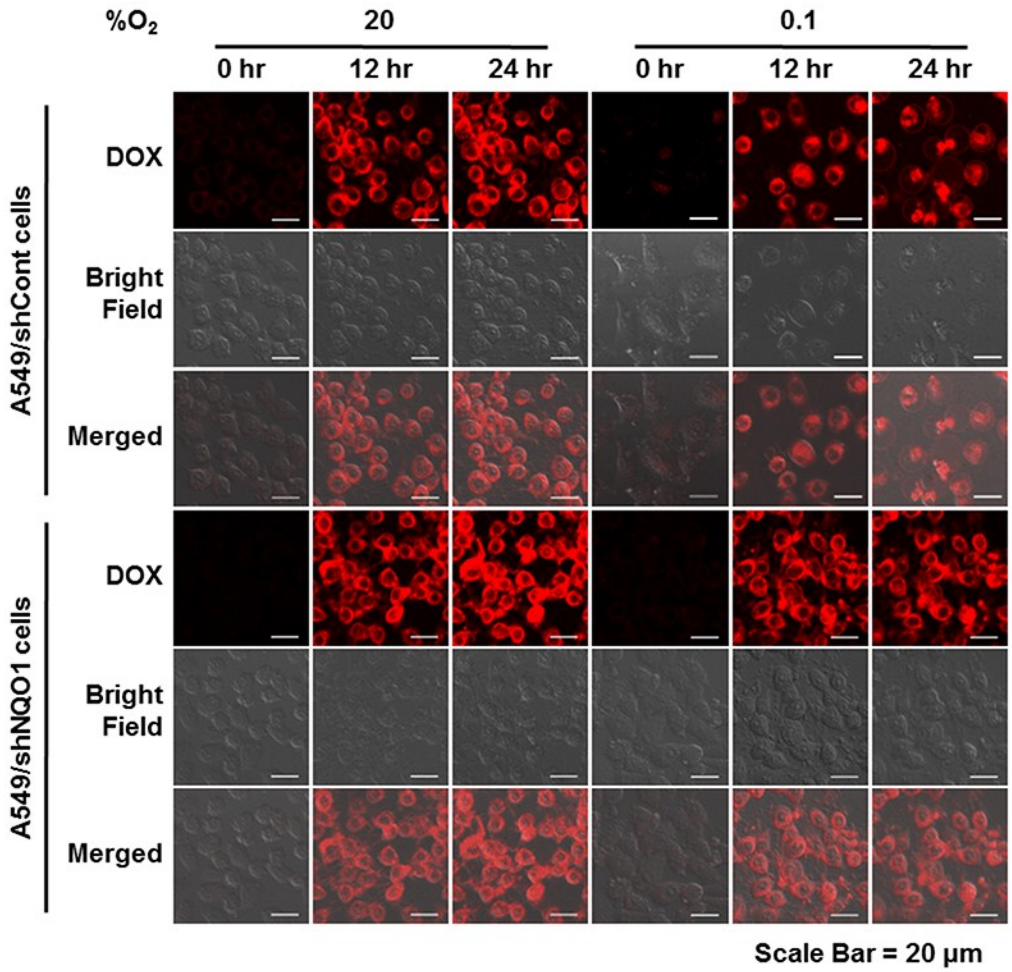

Fig. S8. Confocal laser-scanning microscopy (CLSM) images showing the time course of changes in DOX fluorescence intensity in A549/shCont and A549/shNQO1 cells incubated for 24 h with DOX-loaded (5 μM) Si-Azo-CD-PEG.

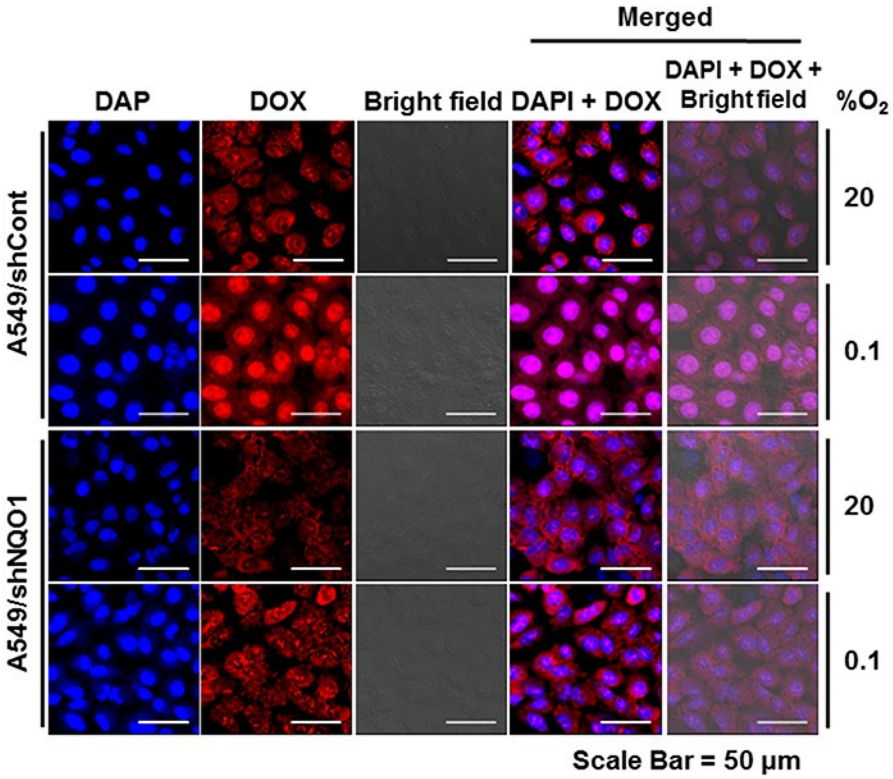

Fig. S9. Effect of hypoxia on the NQO1-induced release of DOX from Si-Azo-CD-PEG in A549 cells with NQO1 loss-of-function. A549/shCont and A549/shNQO1 cells were treated with DOX-loaded (5 μM) Si-Azo-CD-PEG for 12 h. The cells were fixed with 3.7% PFA, washed three times with PBS, and stained with DAPI. The fluorescence intensities of DOX and DAPI-stained nuclei were examined using a TE2000E laser-scanning confocal microscope (Nikon, Tokyo, Japan).

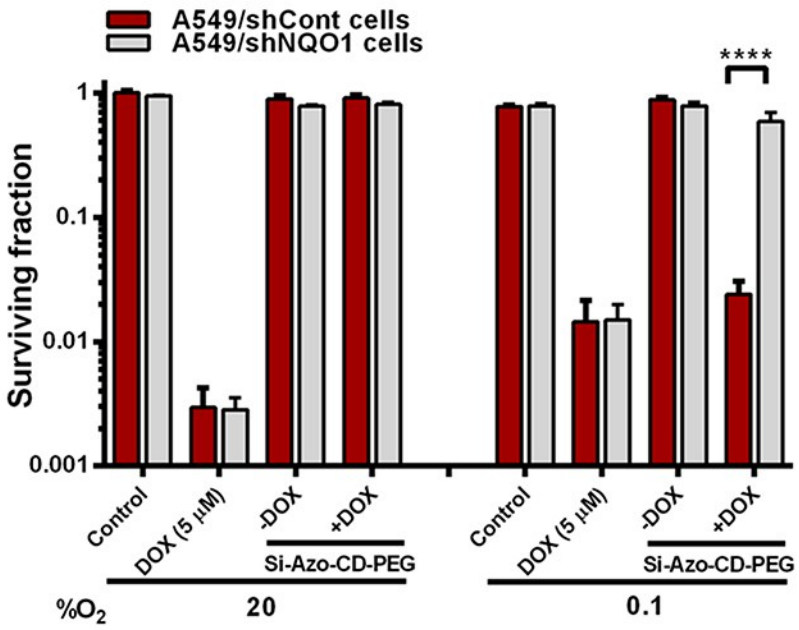

Fig. S10. Clonogenic survival of A549/shCont and A549/shNQO1 cells incubated for 6 h with 5 μM free DOX, Si-Azo-CD-PEG without DOX, or Si-Azo-CD-PEG loaded with 5 μM DOX under normoxic and hypoxic conditions. After incubation, the cells were washed three times with PBS and cultured for an additional 14 d; survival proportions were then calculated.

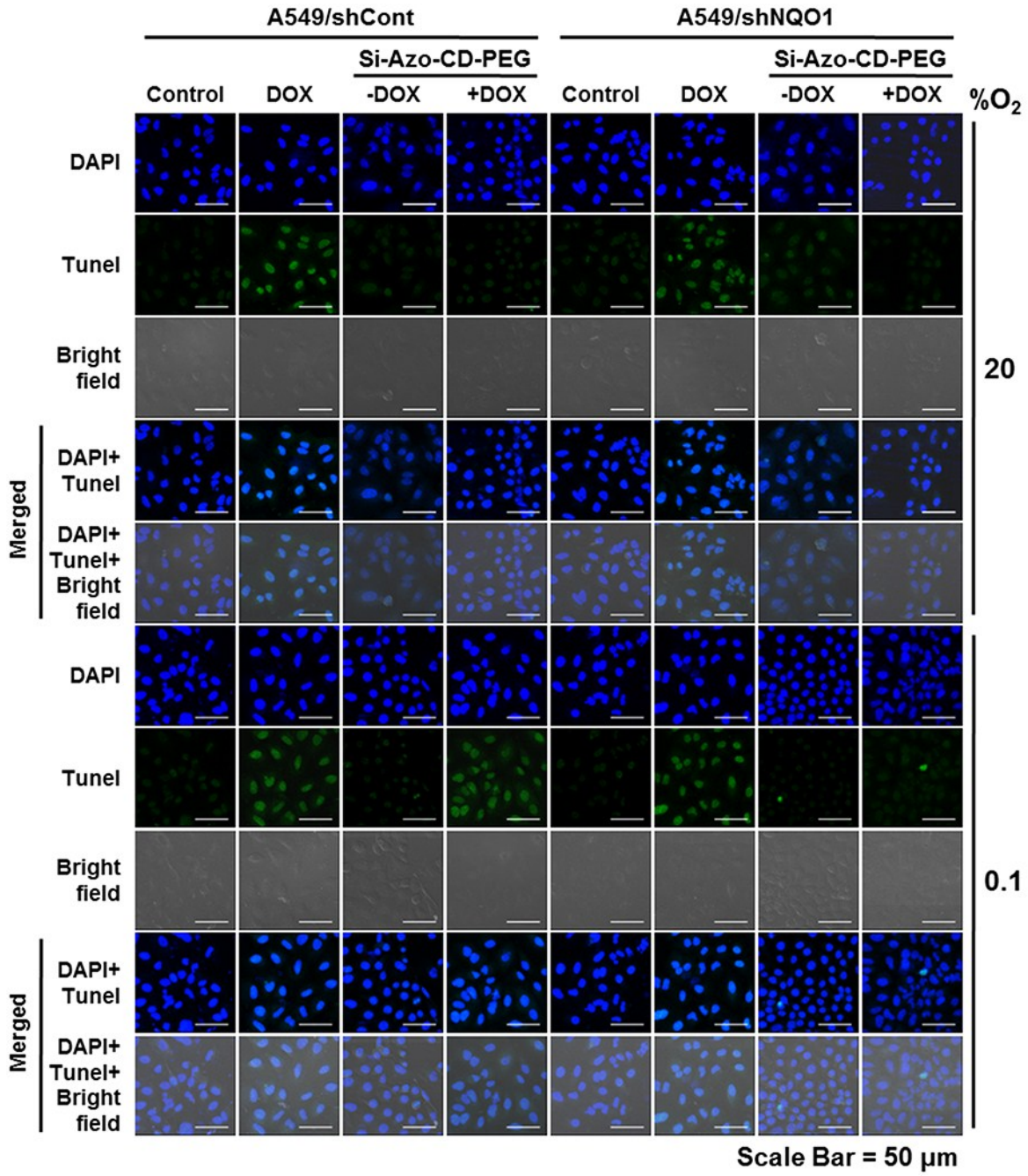

Fig. S11. Representative photomicrographs of TUNEL-positive (apoptotic) cells. A549/shCont and A549/shNQO1 cells were treated with 5 μM free DOX, PEG-WCGKC-SS-Si without DOX or PEG-WCGKC-SS-Si loaded with 5 μM DOX for 12 h under normoxic and hypoxic conditions. TUNEL-positive cells were examined by confocal microscopy.

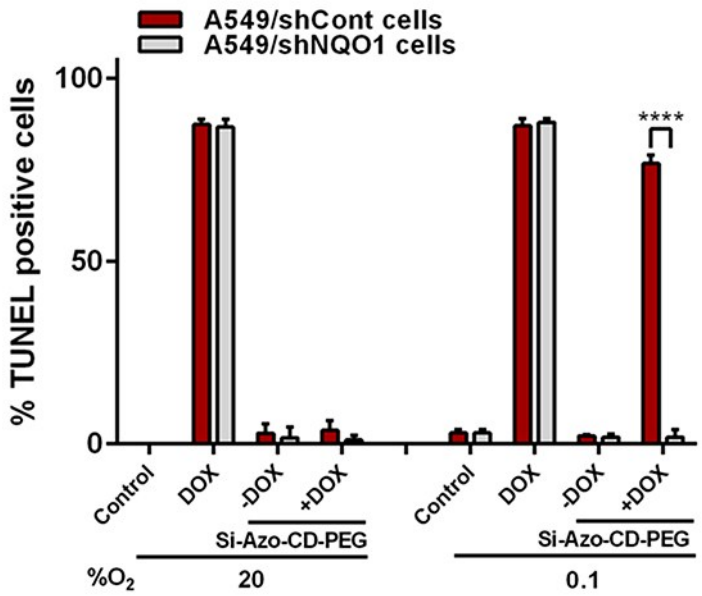

Fig. S12. The percentage of TUNEL-positive (apoptotic) cells was quantified and normalized to cells stained with DAPI. Data are expressed as the means of three independent experiments ± standard deviation. Columns represent compiled data derived from three independent experiments ± standard deviation. ****$P < 0.0001$.

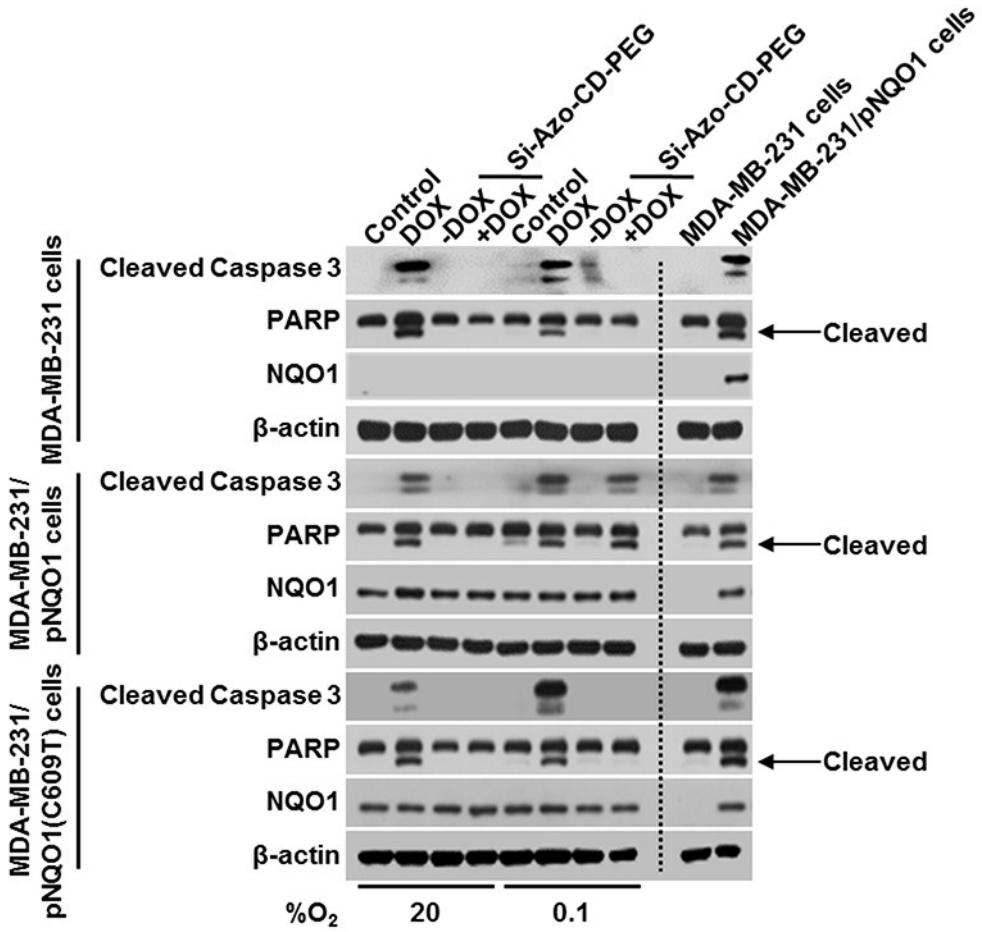

Fig. S13. Effects of hypoxia-induced activation of NQO1 on the intracellular release of DOX from Si-Azo-CD-PEG in MDA-MB-231 cells overexpressing NQO1, and on caspase activity in cells treated with 5 μM free DOX, Si-Azo-CD-PEG without DOX, or Si-Azo-CD-PEG loaded with 5 μM DOX for 24 h. Protein levels were determined by immunoblot analysis.

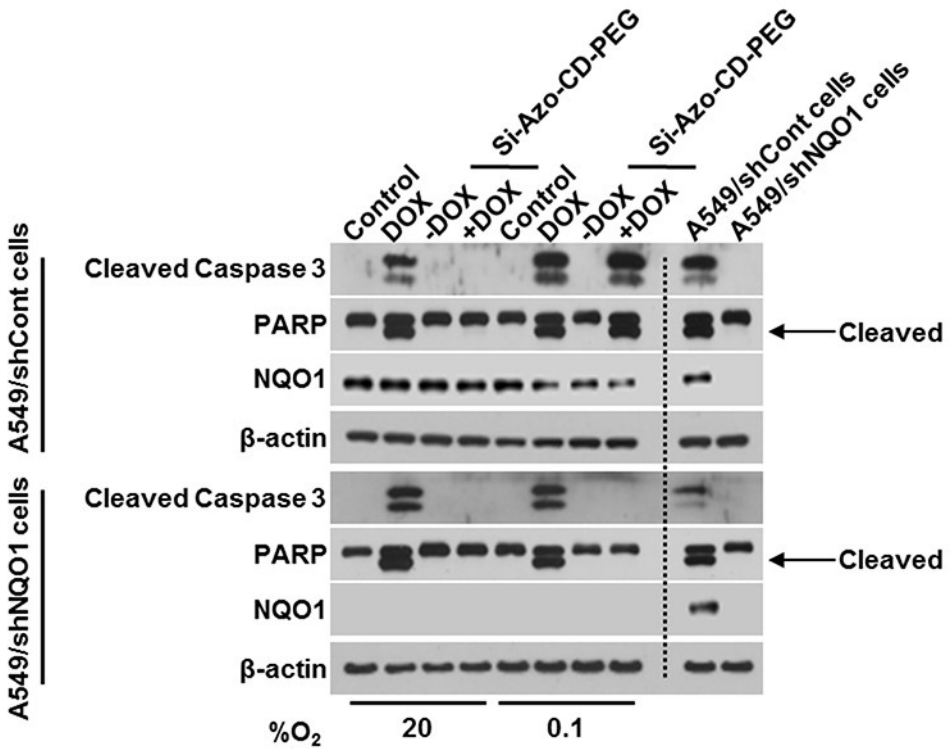

Fig. S14. Effect of hypoxia-induced activation of NQO1 enzyme activity on the intracellular release of DOX from Si-Azo-CD-PEG in A549 cells with NQO1 loss-of-function and the caspase activity in cells treated with 5 μM DOX, Si-Azo-CD-PEG w/o or Si-Azo-CD-PEG loaded with 5 μM DOX for 24 hr. Protein levels were determined by immunoblot analysis.

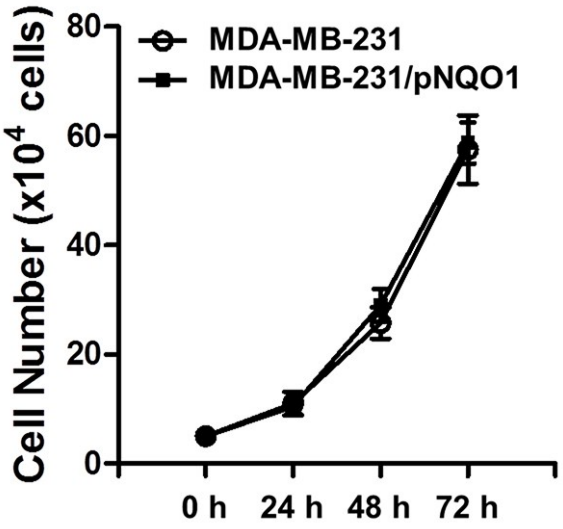

Fig. S15. Cell proliferation rates in MDA-MB-231 and MDA-MB-231/pNQO1 cells under normoxia. Cell proliferation rate was determined by cell counting after 24, 48, and 72 h culture. Each point represents the mean ± SD.

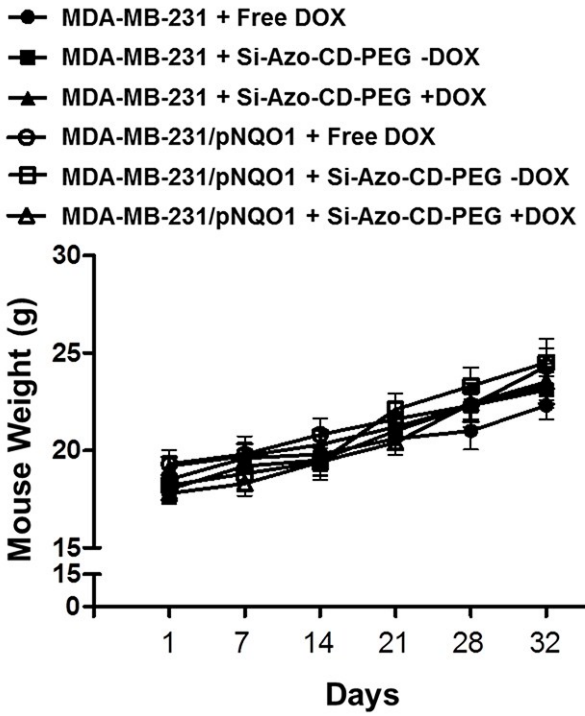

Fig. S16. Average changes of body weights of MDA-MB-231 and MDA-MB-231/pNQO1 xenograft mice. Body weights of the mice were measured every 4 days for 32 days.

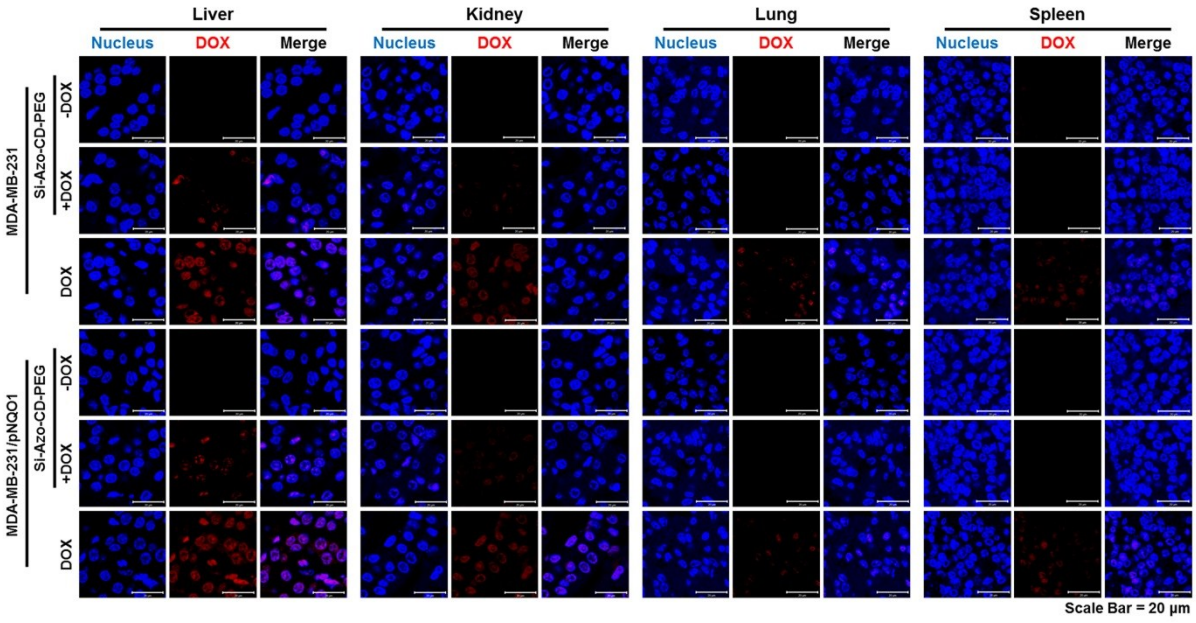

Fig. S17. Major organs (liver, kidney, lung and spleen) were excised from MDA-MB-231 and MDA-MB-231/pNQO1 tumor-bearing mice after administration of free DOX, Si-Azo-CD-PEG without DOX, or DOX-loaded Si-Azo-CD-PEG, and imaged to assess DOX fluorescence (red); nuclei were counterstained with DAPI (blue).

References

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