Supporting Information

Spatiotemporally Targeted Nanomedicine Overcomes Hypoxia-Induced Drug Resistance of Tumor Cells after Disrupting Neovasculature

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MATERIALS AND METHODS

Materials. Dimethylmaleic anhydride-modified methoxy poly(ethylene glycol)-*block*-poly(L-lysine) (mPEG-*b*-PLL/DMMA) and poly(L-lysine)−poly(L-phenylalanine-*co*-L-cystine) (PLL−P(LP-*co*-LC)) (NP) were synthesized according to the protocols reported in our previous work.[1] Combretastatin-A4 phosphate (CA4P) and bortezomib (BTZ) were purchased from Beijing Huafeng United Technology Co., Ltd. (Beijing, P. R. China). Methanol, ethanol, acetone, *N*,*N*-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were all purchased from Beijing Chemical Works (Beijing, P. R. China). 4-Methylumbelliferyl phosphate (4-MUP) and DiO were bought from Aladdin Reagent Co., Ltd. (Shanghai, P. R. China). Cyanine5.5 (Cy5.5) was purchased from Lumiprobe Corporation (Hunt Valley, MD, USA). Methyl thiazolyl tetrazolium (MTT) and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich (Shanghai, P. R. China).

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were provided by Gibco BRL Life Technology (Grand Island, NY, USA). Penicillin and streptomycin were obtained from Huabei Pharmaceutical Co. Ltd. (Shijiazhuang, P. R. China). LysoTracker Green was purchased from Molecular Probes (Eugene, OR, USA). The 4%−12% polyacrylamide gels were prepared using the SDS-PAGE Gel preparation kit (Beyotime, Shanghai, P. R. China). The 0.2 μm poly(vinylidene difluoride) (PVDF) membrane was purchased from Thermo Fisher Scientific (Indianapolis, IN, USA). Anti-CD31, anti-HIF-1α, anti-ABCG2, anti-GAPDH, anti-caspase-3, and anti-active caspase-3 primary antibodies were all purchased from Abcam (Cambridge, MA, USA). Hypoxyprobe^{TM}-1 kit for the detection of tissue hypoxia was purchased from Hypoxyprobe, Inc. (Burlington, MA, USA). Proteasome-Glo™ kit for the detection of chymotrypsin-like proteasomes was bought from Promega Corporation (Madison, WI, USA).

Preparation and Characterizations of $_{CA4P}NP_{BTZ}$. 5.0 mg of BTZ and 100.0 mg of NP were dissolved in 10.0 mL of DMF and stirred at room temperature overnight for efficient diffusion. 10.0 mL of water was then added to the above solution, and the mixture was stirred for another 12 h at room temperature. The solution was then dialyzed with water (molecule weight cut-off = 3500 Da). The loading content of BTZ in NP_{BTZ} was then analyzed through the amount of element boron (B) detected by inductively coupled plasma optical emission spectroscopy (ICP-OES). And then, NP_{BTZ} was mixed with CA4P at a different mass ratio in phosphate-buffered saline (PBS) for further characterizations of transmittance, size, and zeta potential. Subsequently, $_{CA4P}NP_{BTZ}$ was mixed with the shell at the mass ratio of 1:2 to form $S_{CA4P}NP_{BTZ}$.

Transmission electron microscopy (TEM) microimages of different formulations were captured by JEOL-1011 TEM (JEOL Ltd., Tokyo, Japan). *In vitro* release profiles of BTZ and CA4P under different conditions were carried out by detecting the amounts of drugs released from the dialysis bag filled with 10.0 mL of $S_{CA4P}NP_{BTZ}$ at the concentration of 0.05 mg mL^{-1} for BTZ and 0.5 mg mL^{-1} for CA4P to the outside media at different conditions by high-performance liquid chromatography (HPLC). HPLC was performed on a Waters e2695 HPLC system equipped with a Waters 2489 UV detector and the Symmetry C18 column (Waters, Milford, MA, USA). Methanol−water (70:30, *V*/*V*) was used as a mobile phase with a flow rate of 1.0 mL min^{-1}.

Cells, Animals, and Tumor Models. A549 tumor cells were cultured in DMEM supplemented with 10% (*V*/*V*) of FBS and 100.0 μg mL^{-1} penicillin-streptomycin.

BALB/c nude mice (male, 18.0−20.0 g, 5−6 weeks) and Sprague-Dawley rats (male, 200.0−220.0 g, 5−6 weeks) were purchased from the Beijing HFK Bioscience Co., Ltd. (Beijing, P. R. China), and all mice used in this study were handled under the protocol approved by the

Institutional Animal Care and Use Committee of Jilin University. All efforts were made to minimize suffering.

The human A549 pulmonary adenocarcinoma xenograft mouse model was constructed by injecting 2.0×10^7 cells in 100.0 μL of PBS into the right flank of the BALB/c nude mice. PDX colon tumor model was built by a subcutaneous implant of small fragments (~ 4 mm^3) of the 9th generation of established PDX colon tumor.

Intratumoral Distribution of 4-MP and Cy5.5. 4-MUP and Cy5.5 co-loaded SNP ($S_{4\text{-}MUP}NP_{Cy5.5}$) was prepared in a similar approach as described above and stored at the concentration of 2.0 mg mL^{-1}. Mice bearing A549 tumors were intravenously injected with 100.0 μL of above $S_{4\text{-}MUP}NP_{Cy5.5}$. The mice were then anesthetized with pentobarbital by intraperitoneal (*i.p.*) injection. An arc-shaped incision was made around the subcutaneous tumor, and the skin flap was elevated without injuring the feeding vessels. The mouse was then directly placed on the coverslip. The coverslip was attached with just enough pressure to flatten the tumor surface. All microimage acquisitions were performed using a Zeiss LSM780 CLSM instrument (Zeiss Co., Oberkochen, Germany). At the end of the experiment, the mice were sacrificed. The tumors were collected and cut into 10.0 μm slices. The collected tumor sections were further stained by the anti-CD31 antibody following the standard protocol of immunofluorescence staining for further evaluation of the distribution of 4-MP and Cy5.5.

Induced Overexpression of ABCG2 in A549 Cells under Hypoxia. A549 cells were cultured in 6-well plate at a density of 100,000 cells per well for 24 h and then transferred to the modular incubator chamber (Billups-Rothenberg, Inc., USA) filled with a mixture of 1% (*V*/*V*) oxygen (O_2), 5% (*V*/*V*) carbon dioxide (CO_2), and 94% (*V*/*V*) nitrogen (N_2) for different periods. The membrane and cytosol proteins were isolated by the membrane and cytosol protein

extraction kit (Beyotime, Shanghai, P. R. China). The protein concentrations were quantitated and normalized by a bicinchoninic acid (BCA) method. The extracted protein was applied in the electrophoresis gel on 4%−12% polyacrylamide gels under the voltage of 120 V for 1 h. Then the protein was transferred to a PVDF membrane under 4 °C for 2 h. The membranes were blocked by 5% skimmed milk, incubated with primary antibody overnight, and then incubated with horseradish peroxidase (HRP)-conjugated second antibody. The membranes were visualized using the BeyoECL Plus enhanced chemiluminescence Western blotting (ECL-WB) detection system (Beyotime, Shanghai, P. R. China).

The location of ABCG2 was also evaluated by immunofluorescence by co-staining of the membrane by DiO. The A549 cells under hypoxia or normoxia for 24 h were first incubated with DiO at a concentration of 10.0 μM for 10 min. The cells were then fixed by cold acetone and stained with anti-ABCG2 antibody and DAPI. CLSM also captured the microimages.

Overall Hypoxia and Overexpression of ABCG2 in A549 Xenograft Tumor Induced by VDAs. The A549 tumor-bearing mice were treated with PBS or $S_{CA4P}NP_{BTZ}$ at the doses of 1.0 mg (kg BW)$^{-1}$ for BTZ and 10.0 mg (kg BW)$^{-1}$ for CA4P by intravenous (*i.v.*) injection. After 24 h of *i.v.* injection, the mice were treated with 60.0 mg (kg BW)$^{-1}$ of pimonidazole hydrochloride. After 1.5 h, the mice were sacrificed, and the tumors were collected. Then, the tumors were cut into 5-μm slices, and the sections were fixed in cooled acetone for 20 min at 4 °C, washed thrice with PBS, blocked with 10% goat serum, and incubated with mouse IgG1 monoclonal antibody conjugated to Dylight™549 fluorophore in Hypoxyprobe™-1 kit overnight at 4 °C. Then, the sections were further stained with DAPI, anti-CD31 antibody, and anti-ABCG2 antibody, and CLSM captured the microimages.

Endocytosis and Intracellular Drug Release of NP. A549 cells cultured in 6-well plate at a density of 50,000 per well were first incubated under normoxia and hypoxia for 24 h. Free Cy5.5 or Cy5.5-loaded NP ($NP_{Cy5.5}$) at the same Cy5.5 concentration of 1.0 μg mL^{-1} was administrated to the above medium for 3 or 12 h. The cells were then incubated with LysoTracker green for another 1 h at 37 °C. Finally, the cells were fixed with cold acetone, stained with DAPI, and captured by CLSM.

Similarly, A549 cells cultured in 6-well plate at a density of 200,000 per well were treated with free BTZ or NP_{BTZ} at the BTZ concentration of 1.0 μg mL^{-1} under normoxia or hypoxia for 3 or 12 h. Then the cells were lysed by 0.1% (*W*/*V*) Triton X-100, and the total protein concentration per well was quantitated by BCA method. The proteins were then extracted by methanol. LC-MS analyzed the concentration of BTZ.

Cell Cytotoxicity under Normoxia and Hypoxia. A549 cells were cultured in a 96-well plate at a density of 5,000 per well. After incubation at normoxia or hypoxia for 24 h, the cells were treated with free BTZ or NP_{BTZ} at different BTZ concentrations. After another 24 h of incubation, the cell viability was analyzed *via* a MTT assay. The cell viability was calculated by the following Equation S1.

$$\text{Cell viability}\ (\%) = \frac{\mathrm{OD_{sample}} - \mathrm{OD_{blank}}}{\mathrm{OD_{control}} - \mathrm{OD_{blank}}} \times 100\% \qquad \text{(S1)}$$

In Equation S1, OD_{blank}, OD_{sample}, and $OD_{control}$ represent the optical density (OD) of the blank plate well, drug-treated well, and PBS-treated well measured at a wavelength of 570 nm, respectively.

Distribution of Tumor Hypoxia and Apoptosis after Treatment. Mice bearing A549 tumors were treated with PBS, free BTZ+CA4P, or $S_{CA4P}NP_{BTZ}$ at the doses of 1.0 mg (kg BW)$^{-1}$ for BTZ and 10.0 mg (kg BW)$^{-1}$ for CA4P by *i.v.* injection. For Western blot, the mice

were sacrificed, and the tumors were collected. The total proteins were isolated by radioimmunoprecipitation assay (RIPA) lysis buffer and quantitated by BCA. The expression of caspase-3 and active caspase-3 were then evaluated by Western blot according to a common protocol. For immunofluorescence staining, the mice were treated with 60.0 mg (kg BW) $^{-1}$ of pimonidazole hydrochloride after 48 h of *i.v.* injection. After 1.5 h, the mice were sacrificed, and the tumors were collected and stained by Dylight™549 fluorophore in Hypoxyprobe^{TM}-1 kit, as described above. Then the sections were further stained with an anti-active caspase-3 antibody according to a standard protocol.

Pharmacokinetics and Biodistribution. *Pharmacokinetics*: Sprague-Dawley rats were administrated with free BTZ+CA4P or $S_{CA4P}NP_{BTZ}$ at the doses of 2.0 mg (kg BW)$^{-1}$ for BTZ and 20.0 mg (kg BW)$^{-1}$ for CA4P by *i.v.* injection. At predetermined time intervals, 500.0 μL of blood was collected and centrifuged to obtain the plasma. Subsequently, 1.0 mL of acetonitrile was added into the plasma for protein settlement. After centrifugation, the supernatant was collected, dried, and redissolved in methanol for HPLC analysis as described above.

Biodistribution Assays: Free BTZ+CA4P or $S_{CA4P}NP_{BTZ}$ at the doses of 2.0 mg (kg BW)$^{-1}$ for BTZ and 20.0 mg (kg BW)$^{-1}$ for CA4P were administrated by *i.v.* injection. The mice were sacrificed after 12 h. Then the major organs, including the heart, liver, spleen, lung, and kidney, as well as the tumor, were excised and washed with cold normal saline. The tissues were homogenized and then extracted by methanol. LC-MS then analyzed the concentrations of BTZ and CA4P.

Therapeutic Effects on Multiple Tumor Models. A549 tumors that grew to around 60 to 100 mm^3 were treated with PBS, BTZ, CA4P, CA4P+BTZ, SNP_{BTZ}, $S_{CA4P}NP$, or $S_{CA4P}NP_{BTZ}$ at the doses of 1.0 mg (kg BW)$^{-1}$ for BTZ and 10.0 mg (kg BW)$^{-1}$ for CA4P *via i.v.* injection every

four days. Tumor growth was monitored by measuring the perpendicular diameter of the tumor using calipers. The following Equation S2 estimated tumor volume (mm^3).

$$V = \frac{a \times b^2}{2} \quad \text{(S2)}$$

In Equation S1, *a* and *b* were the major and minor axes of the tumor measured by a caliper.

The synergy index of BTZ and CA4P was calculated by Equation S3.[2]

$$q = \frac{\mathrm{E(A+B)}}{\mathrm{EA+EB-EA \times EB}} \quad \text{(S3)}$$

In Equation S2, E(A+B) represents the inhibition rate of combined A and B. EA and EB represent the inhibition rate of drug A and B alone. When $q = 1 \pm 0.15$, the combination of A and B is regarded as an addition effect, and the value of $q > 1.15$ indicates a synergistic effect between factors A and B.

The treatment of the PDX tumor model was carried out after about two months of the inoculation. The PDX tumors were treated with PBS, CA4P+BTZ, or $S_{CA4P}NP_{BTZ}$ at the doses of 1.0 mg (kg BW)$^{-1}$ for BTZ and 10.0 mg (kg BW)$^{-1}$ for CA4P *via i.v.* injection every four days.

Histopathological Analysis. At the end of all treatments, the tumors were collected and fixed in 4% (*W*/*W*) PBS-buffered paraformaldehyde overnight and then embedded in paraffin. The paraffin-embedded tissues were then cut into about 5.0 μm slices for hematoxylin and eosin (H&E) staining and immunohistochemical analysis. The H&E staining was detected by a Nikon Eclipse *Ti* microscope (Optical Apparatus Co., Ardmore, PA, USA).

Statistical Analysis. All experiments were performed at least three times, and the data were presented as mean ± standard deviation (SD). Differences between experimental groups were assessed by one-way analysis of variance with statistical software SPSS 17.0 (SPSS Inc., Chicago, USA). $*P < 0.05$ was considered statistically significant, and $**P < 0.01$ and $***P < 0.001$ were considered highly significant.

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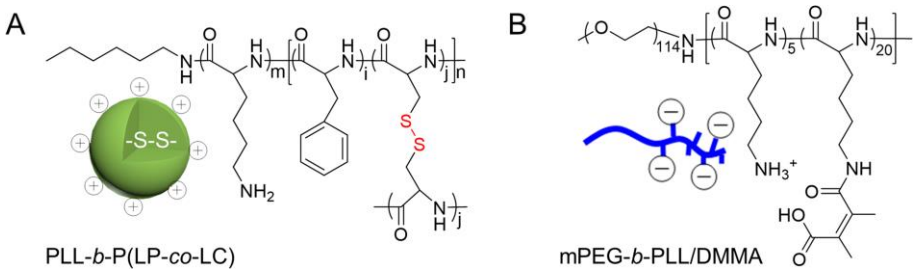

Scheme S1. Chemical structures of PLL-*b*-P(LP-*co*-LC) and mPEG-*b*-PLL/DMMA.

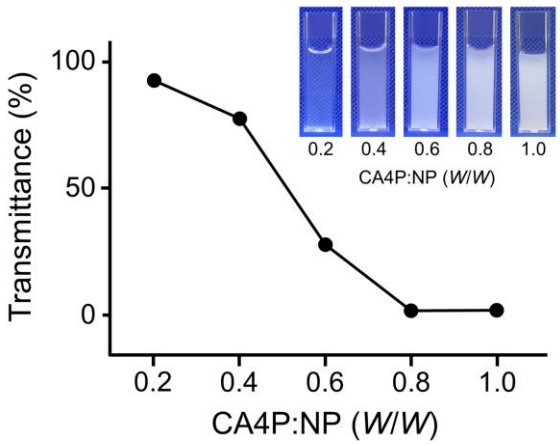

Figure S1. Transmittance of $_{CA4P}$NP under different mass ratios of CA4P and NP.

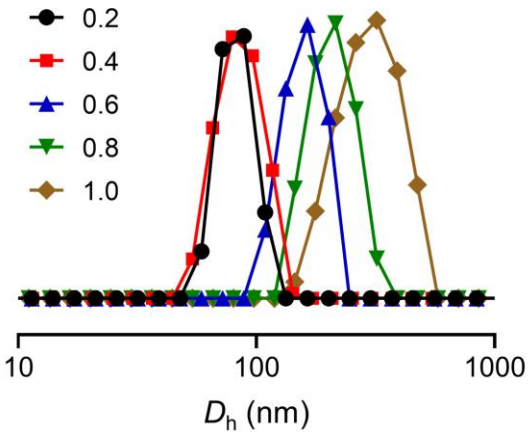

Figure S2. Size of $_{CA4P}$NP under different mass ratios of CA4P and NP.

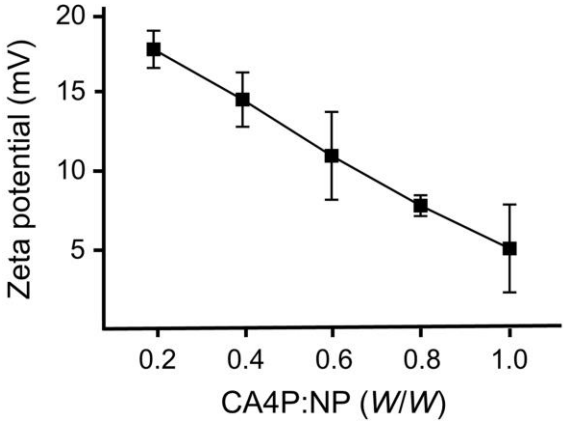

Figure S3. Zeta potential of $_{CA4P}$NP under different mass ratios of CA4P and NP.

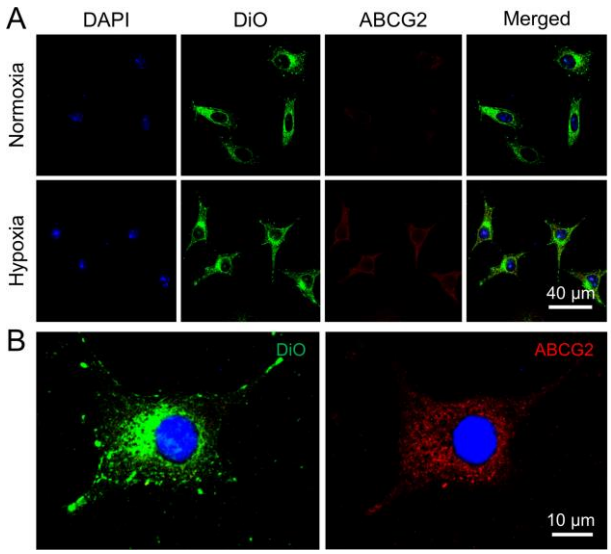

Figure S4. Relative distribution of DiO and ABCG2 in A549 cells.

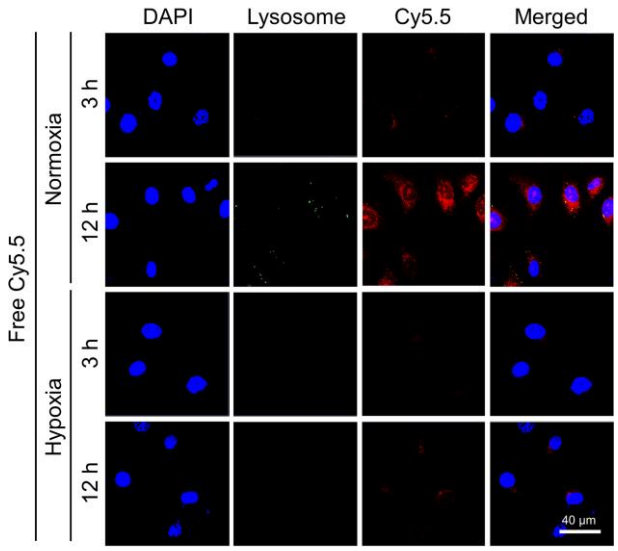

Figure S5. CLSM microimages of A549 cells treated with free Cy5.5 under normoxia and hypoxia for 3 and 12 h.

Table S1. Major parameters in pharmacokinetics.

Entry	BTZ		CA4P	
	Free BTZ+CA4P[a]	$S_{CA4P}NP_{BTZ}$[b]	Free BTZ+CA4P[a]	$S_{CA4P}NP_{BTZ}$[b]
$t_{1/2}$ (h)[a]	0.2	N/A	0.2	N/A
$t_{1/2\alpha}$ (h)[b]	N/A[c]	0.4	N/A	0.3
$t_{1/2\beta}$ (h)[b]	N/A	8.5	N/A	6.3
AUC_{0-t} (μg mL^{-1} h)	1.5	21.0	9.1	224.5
AUC_{0-inf} (μg mL^{-1} h)	1.6	23.5	9.1	235.3

[a]Calculated by a one-compartment model.

[b]Calculated by a two-compartment model.

[c]N/A is represented as not available.