Supporting Information

Overcoming Ovarian Cancer Drug Resistance with a Cold Responsive Nanomaterial

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**Materials and Methods**

**Materials:** The drug resistant A2780ADR cell line, Pluronic F127 (PF127), and poly(N-isopropylacrylamide-co-butylacrylate) (PNIPAM-B) were purchased from Sigma (St. Louis, MO, USA). Hyaluronic acid (HA, Mw: 66-90 kDa) was purchased from Lifecore Biomedical (Chaska, MN, USA). Polyvinyl Alcohol (PVA, Mw: 100 kDa) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Chitosan oligosaccharide of pharmaceutical grade (Mw: 1.2 kDa, 95% deacetylation) was purchased from Zhejiang Golden Shell Biochemical Co. Ltd (Yuhuan, Zhejiang, China). Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). The CCK-8 cell proliferation reagent was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, Eagle's minimum essential medium (EMEM) and Dulbecco's modified eagle's medium/ham's nutrient mixture F-12 (DME/F12) were purchased from ATCC (Manassas, VA, USA). HUVECs and the EGM-Plus Growth Medium for culturing the HUVECs were purchased from Lonza (Allendale, NJ, USA). Doxorubicin hydrochloride (DOX) was purchased from LC laboratories (Woburn, MA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless specifically mentioned otherwise.

**Preparation of chitosan-modified PF127 (chitosan-PF127)**

The chitosan-PF127 was prepared using a previously reported procedure\(^1\). Briefly, PF127 was dissolved in 30 ml of benzene (26 mM) and the solution was added dropwise into 30 ml of 4-nitrophenyl chloroformate (4-NPC) solution (160 mM in benzene). The PF127 and 4-NPC were allowed to react for 3 h in N\(_2\) atmosphere at room temperature. The activated PF127 was precipitated in excess ice-cold diethyl ether first and then washed with ice-cold diethyl ether for
three times before dried under vacuum overnight. Chitosan-PF127 was synthesized by adding 10 ml of chitosan solution (200 mg/ml) in deionized (DI) water into 10 ml of the activated PF127 solution (400 mg/ml, in DI water). After reacting for 12 h, this mixture was dialyzed (molecular weight cut off or MWCO: 7 kDa) against DI water for at least 24 h. Lastly, the chitosan-PF127 polymer was freeze-dried for 48 h to remove water for preparing nanoparticles.

**Preparation of nanoparticles**

To prepare nanoparticles using PF127+DPPC and PNIPAM-B+DPPC, the double emulsion (water in oil in water or W-in-O-in-W) method was used as previously reported with slight modification\(^2\). Briefly, 5 mg of DPPC and 10 mg of PF127 (or PNIPAM-B) were dissolved in 2 ml of dichloromethane and emulsified with 0.4 ml of DI water for 1 min using a Branson 450 sonifier. This initial emulsion was then emulsified with 4 ml of 2% polyvinyl alcohol (PVA) solution (in DI water) for 2 min using the Branson 450 sonifier. The resultant mixture was slowly dropped into 6 ml of 0.6% PVA (in DI water) and stirred for 10 min at room temperature. Rotary evaporation was used to remove the organic solvent in the emulsion mixture. The samples prepared with PNIPAM-B could be collected by centrifugation at 13,800 g for 10 min at room temperature and washed twice with DI water to remove non-encapsulated drug. The samples prepared with PF127 had to be collected by using freeze-dry.

To prepare LPN and HCLPN (with or without drug) nanoparticles, the procedure is the same as that mentioned above except that 5 mg of DPPC, 10 mg of PNIPAM-B, and 20 mg of PF127 were dissolved in 2 ml of dichloromethane and 4 ml of the aqueous solution of either PVA (for LPN nanoparticles) or the mixture of chitosan-PF127 and HA (for HCLPN nanoparticles) were used for the second emulsion. The nanoparticles were also collected by centrifugation (13,800 g for 10 min) at room temperature and washed twice with DI water to remove non-encapsulated
drug. To prepare nanoparticles without DPPC, the same procedure as that for preparing HCLPN nanoparticles was used except that no DPPC was used during the first W-in-O emulsion.

**Encapsulation of DOX in the nanoparticles and in vitro drug release.**

The encapsulation efficiency (EE) of DOX in HCLPN nanoparticles was calculated with the following equation:

\[ EE = \frac{W_{\text{encapsulated}}}{W_{\text{fed}}} \times 100\% \]  

where \(W_{\text{encapsulated}}\) represents the amount (in weight) of agents encapsulated into nanoparticles and \(W_{\text{fed}}\) is the initial total amount of agents fed for encapsulation. The amount of DOX was determined spectrophotometrically using a Beckman Coulter (Indianapolis, IN, USA) DU 800 UV-Vis spectrophotometer based on their absorbance at 486 nm.

To study the in vitro drug release, drug-laden nanoparticles (20-30 mg) dissolved in PBS (pH 7.4) were loaded into dialysis bags (MWCO: 20 kDa) and placed in 50 ml centrifuge tube with 30 ml of PBS solution. The tube was kept at 37 °C and stirred at 110 rpm. The dialysate was collected at various times and the concentration of the released DOX in the dialysate was determined based on its UV-Vis absorbance at 486 nm. For ice-triggered or pH-induced drug release, the nanoparticle solutions in tube were incubated in ice for 5 min or the nanoparticles reconstituted in a pH 5.0 phosphate buffer for 5 min at 5 h. The samples were centrifuged at 13,800 g and the released DOX in the supernatant was determined based on its absorbance at 486 nm. After the ice or low-pH treatment, the samples were centrifuged and reconstituted in PBS (pH 7.4) at 37 °C to monitor drug release for 2 more hours.

**Characterization of nanoparticles**

The size distribution of nanoparticles in aqueous environment was assessed using a Brookhaven 90 Plus/BI-MAS dynamic light scattering (DLS) instrument with nanoparticle concentration of 1
mg/ml in DI water or phosphate-buffered saline (PBS) at pH 7.4, 6.5 or 5.0. The morphology of nanoparticles was imaged using transmission electron microscopy (TEM). Briefly, the nanoparticles were examined using an FEI (Moorestown, NJ, USA) Tecnai G2 Spirit transmission electron microscope after negatively stained with uranyl acetate solution (2%, w/w).

**Cell culture and in vitro cell viability**

Multidrug resistant NCI/RES-ADR (ATCC) and A2780ADR ovarian cancer cells were cultured in EMEM and RPMI 1640, respectively, and supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO₂ incubator. To obtain CSC-enriched spheres, single cancer cells of 20,000 cells/ml were obtained and then cultured in 24-well ultralow attachment plates (Corning, Lowell, MA, USA) in 1 ml of CSC medium in each well. The CSC medium was made of DMEM/F12-K medium supplemented with 5 μg/ml insulin, 20 ng/ml epidermal growth factor (EGF), 20 ng/ml basic fibroblast growth factor (bFGF), 1x B27 (Invitrogen), and 0.4% (w/v) bovine serum albumin (BSA)⁴. After 10 days, the CSC-enriched spheres were collected for further experimental use. In order to maintain the multidrug resistant capability, DOX (1.5 μg/ml) was added in the medium for both the 3D sphere and 2D culture.

To determine the viability of 2D cultured cells, 10,000 cells were cultured in 96-well plates for 12 h, and then treated with various drug formulations for either 24 or 48 h. For quantifying the cell viability in 3D spheres, the spheres cultured in 24-well plates as aforementioned were treated with various drug formulations directly. For the treatments with ice cooling, cells after 12 h of incubation in the plates were cooled on ice for 5 or 10 min and then put back in incubator for further culture. Right before ice cooling, the medium with nanoparticles was collected and replaced with pure medium. Immediately after cooling, the pure medium was removed and the
collected medium with nanoparticles was put back to culture with the cells for additional 12 or 36 h (24 or 48 h of culture in total). Cells cultured in pure medium without any treatment are prepared as control. The cell viability after each treatment as compared to control cells was quantified using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instruction by measuring absorbance at 450 nm with a PerkinElmer VICTOR X4Multilabel plate reader.

**In vitro imaging**

Cells were seeded onto collagen-coated cover glasses (Nunc, Thermo Fisher Scientific Inc., Waltham, MA, USA) at a density of $2 \times 10^5$ cells/well in 6-well plate and cultured at 37 °C for 12 h. The medium was then replaced with fresh medium without (for control group) or with different drug formulations. After incubation at 37 °C for 3 h, the HCLPN-D nanoparticles treated cells were replaced with fresh medium and incubated on ice for 5 or 10 min. For free DOX treatment, cells were directly incubated on ice for 5 or 10 min. For experiments with loperamide, cells were cultured with loperamide (5 µM) for 1 h and then cultured with free DOX or HCLPN-D nanoparticles together with loperamide (5 µM) for another 3 h. The cells treated with the various drug formulations were then mounted onto a glass slide with anti-fade mounting medium (Vector Laboratories Burlingame, CA, USA) and imaged using an Olympus FluoView™ FV1000 confocal microscope. For imaging CD44 and CD133, cells cultured on collagen-coated cover glasses were fixed with 4% paraformaldehyde for 20 min at room temperature. After washing, fixed cells were incubated in 3% BSA and 0.1% TritonX-100 in 1 × PBS at room temperature for 1 h to block potential nonspecific binding and permeabilize the cell plasma membrane, respectively. Following that, the fixed and permeabilized cells were incubated overnight at 4 °C with the CD44 and CD133 antibodies at the dilution ratio of 1:200.
Unbounded antibody was removed by washing with $1 \times PBS$ for three times. Cells were then incubated with secondary antibody at the dilution ratio of 1:200 in PBS with 1% BSA at room temperature for 1 h, and washed three times with $1 \times PBS$. The cover glass attached with cells was mounted onto a glass slide with anti-fade mounting medium (Vector Laboratories Burlingame, CA, USA) for examination.

**Immunohistochemical staining**

For immunohistochemical staining of CD44 and CD133, collected tumors were put in frozen with the Tissue-Tek (Sakura Finetek, Torrance, CA, USA) O.C.T. Compound and Cryomold for 24 h at -80 °C. The tumors were then cut into slices of 7 µm thick using a cryo-microtome and transferred onto microscope slides. Immunohistochemical staining of CD44 and CD133 were conducted according to the instruction of Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC Kit (Abcam, Cambridge, MA, USA). Briefly, Tumor slides were first incubated with Hydrogen Peroxide Block solution for 10 min. After washed with buffer for 2 times, the tumor tissues were blocked with the Protein Block solution for 10 min, followed by overnight incubation at 4 °C with primary antibodies of CD44 or CD133. The slides were then washed with buffer for four times and incubated with Streptavidin Peroxidase for 10 min at room temperature. The slides were then washed four times and incubated with DAB Chromogen for 10 min at room temperature. Afterward, the slides were washed with buffer for four times and covered with anti-fade mounting medium for examination.

**TEM imaging of cells**

Cells were cultured in the Nunc™ Lab-Tek™ II Chamber Slide™ System (Thermo Fisher Scientific Inc., Waltham, USA) at a density of $1 \times 10^6$ cells/ml, and incubated with HCLPN-D nanoparticles for 3 h. Ice treatment was applied in the same way as that for cell viability studies.
Samples were prepared for TEM according to standard procedures and examined using a FEI
(Mooresstown, NJ, USA) Tecnai G2 Spirit transmission electron microscope.

**Flow cytometry**

To characterize the expression of CD44, 2D cultured MDA-MB-231 cells, NCI/RES-ADR cells
and 3D CSC-enriched spheres were dissociated enzymatically with trypsin/EDTA, and further
dissociated by gentle pipetting. Single cells were washed with PBS and incubated with 3% BSA
for 3 h to block nonspecific binding, and then stained with CD44-FITC (Invitrogen, Carlsbad,
CA) antibodies according to the manufacturer's instructions. Lastly, the stained samples were
studied using a BD LSR II Flow Cytometer and the data were analyzed using the BD FACSDiva
software.

**Animals and xenograft of drug resistant tumors**

Athymic female NU/NU nude mice of 6-week old were purchased from Charles River
(Wilmington, MA, USA) and maintained on a 16:8 h light-dark cycle. All procedures for animal
use were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio
State University and all efforts were made to minimize animal suffering. To obtain xenograft of
multidrug resistant tumor in the nude mice, cells detached (with trypsin) from CSC-enriched
spheres were suspended at 2 x 10^5 cells/ml in a mixture (1:1) of 1x PBS and matrigel (BD
Biosciences, MD, USA). A total of 20,000 cells in 100 µl of the mixture were subcutaneously at
the dorsal side of the upper hindlimb of nude mice of each 7-week-old mouse.

**In vivo imaging and biodistribution**

For *in vivo* imaging, after the tumor reached ~5 mm in long diameter, mice were injected with
100 µl of saline, free ICG, and HCLPN-G nanoparticles (ICG: 50 µg) in 100 µl of saline. After
intravenous injection *via* the tail vein, the mice were taken at 1, 3, 6, and 9 h using a
PerkinElmer (Waltham, MA, USA) IVIS instrument. The excitation at 780 nm was applied and the fluorescence emission of ICG was collected with an 830 nm filter. After imaging, the mice were sacrificed and tumor, liver, kidney, lung, spleen, and heart were collected for further *ex vivo* fluorescence imaging of ICG using the same IVIS instrument.

**In vivo antitumor efficacy and safety**

After tumors reached ~5 mm in long diameter, mice were treated with 100 µl of saline or HCLPN nanoparticles, DOX without or with ice treatment for 10 min (DOX: 3 mg/kg body weight), and HCLPN-D nanoparticles without and with ice treatment for 10 min (DOX: 3 mg/kg body weight). A total of 100 µl of saline was used as the carrier for all the drug formulations and mice were injected with various drug formulations for 5 times in total on days 1, 8, 15, 22 and 29. The ice treatment was conducted at 12 h after the intravenous drug injection. Immediately before and after ice treatment, mice were checked for the whole body temperature using infrared thermography. Tumor size was measured every 7 days using a caliper. The tumor volume (V) was calculated as: $V = (L \times W^2)/2$, where L is long diameter and W is short diameter. The mice were euthanized on day 59 after drug injection. Tumors, livers, lungs, hearts, spleens, and kidneys were collected, formalin fixed, paraffin embedded, and haematoxylin&eosin (H&E) stained for further standard histological analysis.

For establishing the orthotopic metastasis model of ovarian cancer, NCI/RES-ADR cells were suspended at 1 x $10^7$ cells/ml in a mixture (1:1) of 1x PBS and matrigel (BD Biosciences, MD, USA). A total of 2 x $10^6$ cells in 200 µl of the mixture were intraperitoneally injected into peritoneal cavity of each nude mouse of 7-week-old on day 1. Mice were treated with 100 µl of saline, free DOX (DOX: 3 mg/kg body weight), and HCLPN-D nanoparticles (DOX: 3 mg/kg body weight) at days 7, 14 and 21. All the mice received ice treatment at 12 h after intraperitoneal injection of the saline and drug formulations. Immediately before and after ice
treatment, mice were checked for their body temperature using infrared thermography. All mice were euthanized on day 32. Tumors, livers, lungs, hearts, spleens, and kidneys were collected, formalin fixed, paraffin embedded, and H&E stained for further standard histological analysis.

**Statistical analysis**

All data are reported as mean ± standard deviation (SD) from at least three independent runs. The Kruskal-Wallis $H$ test and the Mann-Whitney $U$ test were used to assess the overall among-group and two-group differences, respectively. All statistical analyses were carried out with the IBM (Chicago, IL, USA) SPSS 22 software.

**References:**

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4. Rao, W.; Zhao, S.; Yu, J.; Lu, X.; Zynger, D. L.; He, X., Enhanced enrichment of prostate cancer stem-like cells with miniaturized 3D culture in liquid core-hydrogel shell microcapsules. *Biomaterials 2014, 35* (27), 7762-7773.
Figure S1. TEM image of nanoparticles prepared by using PF127 and PNIPAM-B (without the lipid DPPC). The multicore-shell structure of the HCLPN nanoparticles is not evident in these nanoparticles, suggesting the lipid used during the first time emulsion may interact with PF127 and PNIPAM-B to form the multicore structure in the HCLPN nanoparticles.
Figure S2. Characterization of nanoparticle synthesis and drug encapsulation. (a) A typical photograph of the centrifuged samples obtained after preparing nanoparticles for encapsulation DOX with Pluronic F127 and DPPC (PF127+DPPC-D), PNIPAM-B and DPPC (PNIPAM-B+DPPC-D), and all the five materials for preparing the HCLPN-D nanoparticles. The larger pellet of HCLPN-D sample indicates more HCLPN-D nanoparticles can be made than the PF127+DPPC-D and PNIPAM+DPPC-D nanoparticles. The stronger red color in the pellet of HCLPN-D sample indicates more DOX can be encapsulated in the HCLPN-D than PF127+DPPC-D and PNIPAM+DPPC-D nanoparticles. (b) Encapsulation efficiency (EE) of DOX in the centrifuged pellet of PF127+DPPC-D, PNIPAM-B+DPPC-D, and HCLPN-D nanoparticles. It is worth noting that the small amount of PF127+DPPC-D and PNIPAM-B+DPPC-D nanoparticles also contributes to their low EE of DOX compared with HCLPN-D nanoparticles. Error bars represent S.D. (n = 3).
Figure S3. Visual and microscopic characterization of nanoparticles. (a) A typical picture of the PF127+DPPC-D, PNIPAM-B+DPPC-D, and HCLPN-D nanoparticles responded in deionized water after removing free DOX. Stronger red color can be seen in the sample with HCLPN-D nanoparticles than the samples with the other two nanoparticles. (b) A typical photograph of the aqueous samples of PF127+DPPC-D, PNIPAM-B+DPPC-D, and HCLPN-D nanoparticles after shining a red laser beam through them in the dark. As a result of the Tyndall effect (i.e., scattering of laser beam by nanoparticles in solution), a bright light track (indicated by arrow) is visible in the dark in the sample with PNIPAM-B+DPPC-D and HCLPN-D nanoparticles while the light track is weak in the samples with PF127+DPPC-D nanoparticles. (c) TEM images showing the PF127+DPPC-D nanoparticles is not uniform and many of them have liposome-like structure (indicated by arrows), suggesting many of the nanoparticles are made of DPPC alone. (d) TEM image showing inhomogeneous size distribution of the PNIPAM-B+DPPC nanoparticles with some being as large as ~400 nm.
Figure S4. Size distribution of HCLPN-D nanoparticles measured by dynamic light scattering (DLS) at different pH values. The results show that the size distribution of the HCLPN-D nanoparticles in pH 6.5 solution is similar to that in pH 7.0 solution (Figure 1g). However, the nanoparticle size increases in pH 5.0 solution with a new secondary peak at ~2.5 \( \mu \text{m} \). The latter suggests aggregate formation at pH 5.0.
Figure S5. Stability of HCLPN-D nanoparticles. The nanoparticles are stable in aqueous solution at room temperature for at least 49 days. The size of the nanoparticles (2 mg/ml) was determined using DLS. Error bars represent S.D. (n = 3). The nanoparticles could also be evaporatively dried at room temperature for further long-term storage.
Figure S6. Uptake of free DOX by multidrug resistant cancer cells. Confocal micrographs of 2D cultured NCI/RES-ADR multidrug resistant cancer cells after incubating them with either free DOX (10 µg/ml) for 3 h at 37 °C, followed by either continuing to culture in incubator (37 °C) or cooling with ice (+I) for 5 or 10 min. The images show minimal uptake of free DOX by the NCI/RES-ADR cells under all the different conditions at the given DOX concentration (10 µg/ml).
Figure S7. Uptake of HCLPN-D nanoparticles by multidrug resistant cancer cells and the effect of ice cooling. Confocal micrographs of 2D cultured NCI/RES-ADR multidrug resistant cancer cells after incubating them with either HCLPN-D nanoparticles (DOX concentration: 10 μg/ml) for 3 h at 37 °C, followed by either continuing to culture in incubator (37 °C) or cooling with ice (+I) for 5 or 10 min. The images show significant uptake of the HCLPN-D nanoparticles by the NCI/RES-ADR cells under all the different conditions at the given DOX concentration (10 μg/ml). Moreover, without ice cooling, DOX stays in the cytoplasm while DOX can enter the cell nuclei after ice cooling (particularly for 10 min).
**Figure S8.** Thermal characterization with infrared thermography. Infrared thermographic maps in 35 mm dish showing temperature distribution in the dish during incubating in incubator and after cooling with ice for 5 or 10 min. The data show the temperature of samples treated with free DOX or HCLPN-D nanoparticles is ~37 °C in incubator, drops to ~40 °C after ice cooling for 5 or 10 min, and returns to ~37 °C when incubated in incubator for 5 min.
Figure S9. Thermal characterization with thermocouple. The measured thermal history of samples with ice cooling for 5 (a) or 10 (b) min, followed by putting them back in incubator (37 °C) for 5 min. Error bars represent S.D. (n = 3).
Figure S10. Fluorescence emission spectra of free DOX and HCLPN-D nanoparticles at various pH values. The data show the fluorescence intensity of DOX decreases by ~20% after encapsulated inside the nanoparticles and it also reduces slightly with the decrease of pH (by ~5% from pH 7.0 to 5.0). DOX concentration was 50 µg/ml. Free DOX or HCLPN-D nanoparticles was dissolved in PBS at the different pH values.
Figure S11. Confocal images of CSC-enriched spheres derived from the multidrug resistant cancer cells after incubating them with free DOX for 3 h at 37 °C, followed by either continued culturing in incubator (37 °C) or ice cooling (+I) for 5 or 10 min. The images show that the CSC-enriched spheres are resistant to DOX.
Figure S12. Quantitative analysis of DOX in cell nuclei as shown in Figures 3 and S11. The data reveals that the concentration of DOX in cell nuclei significantly increases after treated with HCLPN-D nanoparticles and ice-cooling for 5 or 10 min (*: p < 0.05).
Figure S13. Confocal micrographs showing uptake of free DOX by 2D cultured NCI/RES-ADR multidrug resistant cancer cells. The cells were incubated with free DOX at different concentrations for 3 h at 37 °C either with or without ice cooling for 10 min. The images show minimal uptake of free DOX by the NCI/RES-ADR cells under all the different concentrations of DOX.
**Figure S14.** Confocal micrographs showing uptake of free DOX by 3D cultured NCI/RES-ADR spheres. The cells were incubated with free DOX at different concentrations for 3 h at 37 °C either with or without ice cooling for 10 min. The images show minimal uptake of free DOX by the NCI/RES-ADR spheres under all the different concentrations of DOX.
Figure S15. Uptake of HCLPN-D nanoparticles by multidrug resistant cancer cells. Confocal micrographs of 2D cultured NCI/RES-ADR multidrug resistant cancer cells after incubating them with HCLPN-D nanoparticles at different concentrations for 3 h at 37 °C either with or without ice cooling for 10 min. The images show most of the DOX enter the nuclei after ice cooling and the fluorescence intensity increases with the increase of DOX concentration.
**Figure S16.** Uptake of HCLPN-D nanoparticles by CSC-enriched NCI/RES-ADR spheres. Confocal micrographs of 3D cultured NCI/RES-ADR spheres after incubating them with HCLPN-D nanoparticles at different concentrations for 3 h at 37 °C either with or without ice cooling for 10 min. The images show most of the DOX enter the nuclei after ice cooling and the fluorescence intensity increases with the increase of DOX concentration.
Figure S17. Quantitative analysis of DOX in the nuclei of 2D cultured NCI/RES-ADR cells or 3D cultured NCI/RES-ADR spheres as shown in Figures S13-16. The data show that the concentration of DOX in the cell nuclei significantly increases after treating with HCLPN-D nanoparticles and ice cooling for 10 min, and the concentration of DOX in cell nuclei is dependent on the drug concentration.
Overcoming cancer drug resistance with cold-triggered burst drug release from HCLPN-D nanoparticles at the pH (6.5) of tumor microenvironment. Confocal micrographs of (a) 2D cultured NCI/RES-ADR multidrug resistant cancer cells and (b) CSC-enriched spheres derived from the multidrug resistant cancer cells after incubating them with HCLPN-D nanoparticles in pH 6.5 PBS solution for 3 h at 37 °C, followed by either continued culturing in incubator (37 °C) or ice cooling (+I) for 10 min. DOX could enter the cell nuclei only when treated with both HCLPN-D nanoparticles and ice cooling, indicating the cold-triggered burst drug release from the HCLPN-D nanoparticles could be used to overcome the drug resistance of the 2D cultured cancer cells and their CSCs in the slightly acidic (pH 6.5) tumor microenvironment.
Figure S19. Characterization of CD44 expression on 2D cultured NCI/RES-ADR cancer cells and cells in 3D CSC-enriched spheres. Typical flow cytometry peaks (a) and mean intensity (b) showing that the NCI/RES-ADR cancer cells are CD44 positive and the expression of CD44 is significantly increased on cells in the 3D CSC-enriched spheres. The MDA-MB-231 cancer cells are used as positive control.
Figure S20. Confocal micrographs of CD44 on saline (control) and HCLPN-D treated NCI/RES-ADR cancer cells. The confocal data indicate that CD44 is dominantly located on the surface of the cells while they may be internalized into the cytoplasm after incubated with HCLPN-D nanoparticles. This suggests the HCLPN-D nanoparticles can bind with CD44 and be taken up by the cells. After ice cooling, some line structures with CD44 are observable. This is probably because polymers bound with CD44 in the HCLPN-D nanoparticles could form fibers after cooling-induced disassembly of the nanoparticles (Figure 1e).
Figure S21. HA on the HCLPN-D nanoparticles enhances delivery of DOX into multidrug resistant cancer cells. Confocal images of the NCI/RES-ADR multidrug resistant cancer cells (a) after incubated with LPN-D and HCLPN-D nanoparticles for 3 and 6 h at 37 °C and then (b) further cooled with ice for 5 min. The data show that HA decorated on the HCLPN-D nanoparticles could be used to improve the delivery of DOX into the cells compared with LPN-D nanoparticles without HA on their surface. With ice cooling, more DOX delivered with the HCLPN-D nanoparticles could enter the cell nuclei than DOX delivered with the LPN-D nanoparticles.
Figure S22. Thermal characterization of 96-well plates with ice cooling for cell viability studies. (a) Near infrared thermographs and (b) thermocouple measurement of the temperature in the medium in 96-well plate before and after ice cooling for 5 or 10 min. Both 5 and 10 min of ice cooling could decrease the sample temperature to below 10 °C (~4-0 °C for the 5 and 10 min of ice cooling).
Figure S23. Viability of NCI/RES-ADR cells without or with ice treatment for 5 or 10 min. The results indicate that the ice cooling has no effect on the cell viability at both 24 and 48 h after the treatment. Error bars represent S.D. (n = 3).
Figure S24. Ice cooling enhances uptake of free DOX by multidrug resistant cancer cells. NCI/RES-ADR multidrug resistant cancer cells were incubated with free DOX at high concentrations (50, 80 and 160 µg/ml) in incubator (37 °C) for 3 h either without or with ice cooling for 5 or 10 min. More DOX could enter into the cells after cooling them with ice for 10 min, suggesting the ice cooling (for 10 min) could reduce the pumping activity of membrane transporters in the multidrug resistant cancer cells. However, little DOX could enter the cell nuclei, suggesting the diffusion of free DOX into the multidrug resistant cancer cells via their plasma membrane might activate some other mechanisms (in addition to efflux pump) to immobilize the DOX in the cytoplasm of the cells.
**Figure S25.** Ice cooling of multidrug resistant cancer cells for 1 h enhances uptake of free DOX. NCI/RES-ADR multidrug resistant cancer cells were incubated with free DOX (10 µg/ml) on ice for 1 h. More DOX could enter the cells after cooling them with ice for 1 h than 5-10 min (Figure 3a), suggesting ice cooling could reduce the pumping activity of membrane transporters in the multidrug resistant cancer cells. However, the free DOX mainly stays the cytoplasm, suggesting the multidrug resistant cancer cells may possess some mechanism to immobilize free DOX diffused into the cytoplasm of the cells via their plasma membrane.
Figure S26. Viability of NCI/RES-ADR cells after treated with free DOX at high concentrations. NCI/RES-ADR multidrug resistant cancer cells were incubated with free DOX at high concentrations (80 or 160 µg/ml) in incubator (37 °C) for 3 h either without or with ice cooling for 5 or 10 min. The cytotoxicity of free DOX is significantly higher when the cells are cooled with ice for 10 min, which is consistent with the free drug uptake data shown in Figure S9. Error bars represent S.D. (n = 3). *: p < 0.05 and **: p < 0.01 (Kruskal-Wallis H test).
Figure S27. Viability of NCI/RES-ADR cells after treated with free DOX combined with 5 µM loperamide. As a substrate of P-gp, loperamide could enhance the cytotoxicity of free DOX to both 2D cultured NCI/RES-ADR cells and 3D CSC-enriched spheres. However, the cell viability data suggest that the combination of loperamide and free DOX is not as efficient as HCLPN-D nanoparticles with ice cooling for killing the multidrug resistant cells (Figure 4a-b). Error bars represent S.D. (n = 3).
Figure S28. Confocal images of the 2D cultured NCI/RES-ADR cancer cells and 3D CSC-enriched spheres after incubated with free DOX and HCLPN-D nanoparticles with or without loperamide for 3 h at 37 °C. For the treatment with loperamide, it was incubated with the cells for 1 h before drug treatment and further incubated with free DOX and HCLPN-D nanoparticles in the presence of 5 μM loperamide for 3 h. The results indicate that free DOX can enter cells when incubating the cells with the free DOX and loperamide. However, most of the free DOX stays in the cytoplasm. Similarly, incubation of HCLPN-D nanoparticles with loperamide does not facilitate the entry of DOX into the cell nuclei.
Figure S29. Photograph of tumors collected on day 59 after different treatments. The tumors from the HCLPN-D+I treatment group are smaller than that from all the other treatment groups. I: ice cooling for 10 min.
Figure S30. Histology images of the tumors collected on day 59 after different treatments. The tissues were stained by hematoxylin and eosin (H&E). Extensive necrosis can be seen in the tumors from the HCLPN-D+L treatment group while tumors from all the other groups appear more viable. I: ice cooling for 10 min.
**Figure S31.** Confocal micrographs showing CD44 and CD133 expression on control (without any treatment) and HCLPN-D nanoparticles treated NCI/RES-ADR cancer cells under 2D culture. The cells were treated either with HCLPN-D nanoparticles for 12 h at 37 °C, or cooled with ice for 10 min and further incubated at 37 °C for 12 h. The data indicate that the expression of both CD44 and CD133 on NCI/RES-ADR cancer cells is decreased after the treatment with HCLPN-D nanoparticles and ice cooling, although their expression is only slightly reduced after the treatment with the HCLPN-D nanoparticles alone.
**Figure S32.** Confocal micrographs showing CD44 and CD133 expression on control (without any treatment) and HCLPN-D nanoparticles treated NCI/RES-ADR spheres. The cells were treated either with HCLPN-D nanoparticles for 12 h at 37 °C, or cooled with ice for 10 min and further incubated at 37 °C for 12 h. The data indicate that the expression of both CD44 and CD133 on cells in the NCI/RES-ADR spheres is decreased after the treatment with HCLPN-D nanoparticles and ice cooling, although their expression is only slightly reduced after the treatment with the HCLPN-D nanoparticles alone.
Figure S33. Immunohistochemical staining of CD44 and CD133 in tumor with different treatments showing diminished expression of both CD44 and CD133 after the treatment with HCLPN-D nanoparticles and ice cooling (HCLPN-D+I).
Figure S34. Histology images of the critical organs collected on day 59 after different treatments. The tissues were stained by hematoxylin and eosin (H&E). The data show that although the treatment with free DOX via intravenous injection could induce significant damage to heart, liver, kidney, and lung, no appreciable damage to these critical organs could been observed for the HCLPN-D+L treatment. These observations are in accordance with the body weight data shown in Figure 6h.
Figure S35. Histology images of critical organs collected on day 32 after different treatments. The tissues were stained by hematoxylin and eosin (H&E). The data show that encapsulation of DOX in the HCLPN-D nanoparticles could greatly reduce its systemic toxicity after intraperitoneal injection, which is in accordance with the body weight data shown in Figure 7f.
**Figure S36.** Confocal images of non-cancerous HUVECs and NCI/RES-ADR cancer cells after incubated with free DOX and HCLPN-D nanoparticles. Free DOX could enter the HUVECs but not the NCI/RES-ADR drug resistant cancer cells. In contrast, uptake of HCLPN-D nanoparticles by the HUVECs is minimal compared to the NCI/RES-ADR cells either without (middle panels) or with (bottom panels) ice cooling.
Figure S37. Overcoming cancer drug resistance with cold-triggered burst drug release from HCLPN-D nanoparticles. Confocal micrographs of drug resistant A2780ADR cancer cells after incubating them with (a) free DOX or (b) HCLPN-D nanoparticles for 3 h at 37 °C, followed by either further culturing at 37 °C or ice cooling for 10 min. The DOX concentration for all the studies is 10 µg/ml. The data show treating the A2780ADR cells with HCLPN-D nanoparticles and ice cooling could overcome their drug resistance for delivering DOX into their nuclei.
Figure S38. Quantitative analysis of DOX in the nuclei of A2780ADR cells as shown in Figure S37. The data show that the concentration of DOX in the cell nuclei significantly increases after treating with HCLPN-D nanoparticles and ice cooling for 10 min.
Figure S39. Enhanced *in vitro* anticancer capacity by HCLPN-D nanoparticles with ice cooling for overcoming drug resistance. Viability of drug resistant A2780ADR cancer cells after treating them with blank nanoparticles (HCLPN), free DOX, simple mixture of HCLPN nanoparticles and DOX, and HCLPN-D nanoparticles without or with ice cooling for 10 min. The viability of control cells cultured in medium without any drug or nanoparticles is 100%. Error bars represent S.D. (n = 3). *: $p < 0.05$ (Kruskal-Wallis H test), which indicates the viability of cells treated with HCLPN-D nanoparticles and ice cooling for 10 min is significantly lower than that of cells treated with other treatments at the same drug concentration.
Figure S40. Ice cooling of drug resistant A2780ADR cancer cells for 1 h enhances their uptake of free DOX. The drug resistant cancer cells were incubated with free DOX (10 μg/ml) on ice (+I) for 1 h. More DOX could enter the cells after cooling them with ice for 1 h than 10 min (Figure 37), suggesting ice cooling could reduce the pumping activity of the membrane transporters in the drug resistant cancer cells.