Electronic Supplementary Material (ESI) for ChemComm.

Engineering of Lipid-Polymer Nanoarchitectural Platform for Highly Effective Combination Therapy of Doxorubicin and Irinotecan

Thiruganesh Ramasamy [a], Himabindu Ruttala [b], Ju Yeon Choi [a], Tuan Hiep Tran [a], Jeong Hwan Kim [a], Sae Kwang Ku [c], Han-Gon Choi [d], Chul Soon Yong [a,§], Jong Oh Kim [a,§]

[a] College of Pharmacy, Yeungnam University, 214-1, Dae-dong, Gyeongsan, 712-749, South Korea

[b] College of Pharmacy, Gachon University, 191 Hambakmoe-ro, Incheon, 406-799, South Korea

[b] College of Korean Medicine, Daegu Haany University, Gyeongsan, 712-715, South Korea

[d] College of Pharmacy**,** Institute of Pharmaceutical Science and Technology, Hanyang University, 55, Hanyangdaehak-ro, Sangnok-gu, Ansan 426-791, South Korea

[§]Corresponding author: College of Pharmacy, Yeungnam University, 214-1 Dae-dong, Gyeongsan 712-749, South Korea

E-mail: jongohkim@yu.ac.kr (Jong Oh Kim)
Tel: +82-53-810-2813, Fax: +82-53-810-4654
E-mail: csyong@ynu.ac.kr (Chul Soon Yong)
Tel: +82-53-810-2812, Fax: +82-53-810-4654

1. Materials and Methods

1.1. Materials

PEG-*b*-PAsp (MW 12000) was purchased from Alamanda Polymers, Inc. (Huntsville, AL, USA). The block lengths were 113 and 50 repeating units for PEG and PAsp, respectively. DOX (doxorubicin hydrochloride) was a gift from the Dong-A Pharmaceutical Company (Yongin, South Korea). IRI (irinotecan hydrochloride) was a kind gift from Hanmi Pharmaceuticals, Co. Ltd. (Hwaseong, South Korea). The SCC-7, MCF-7, HeLa, A-549, and MDA-MB-231 cell lines were originally obtained from the Korean Cell Bank (Seoul, South Korea). All other chemicals were of reagent grade and were used without further purification.

1.2. Preparation of Dual Drug-Loaded L-PCN

PCN/DI were formulated by a simple self-assembly process. Briefly, aqueous solutions of drugs and PEG-*b*-PAsp copolymer were prepared and mixed at various molar ratios of drug to copolymer, followed by incubation for 24 h at room temperature. Lecithin was then employed to form vesicles around the PCN/DI. Lecithin was dissolved in chloroform and a micellar solution of PCN/DI was added to this organic solution in a round-bottomed flask. The mixture was shaken for 1 min to produce a milky dispersion, followed by immediate evaporation of organic solvent in a rotary evaporator (40°C for 1 h). The final dispersion was probe-sonicated for 3 min to form uniform L-PCN/DI. The L-PCN/DI was stored at 4°C until further analysis. Unbound drugs were removed by ultrafiltration using Amicon YM-10 centrifugal filter devices (MWCO 10,000 Da, Millipore). The concentrations of DOX and IRI in the filtrates and in the nanoparticles were determined by high-performance liquid chromatography (HPLC). The mobile

phase (water:acetonitrile:methanol, 50:25:25, pH 3.5) was run at 1 mL/min with a detection absorbance of 254 nm.

1.3. Dynamic Light Scattering Analysis and Drug Loading

The Z-averaged hydrodynamic size (nm), polydispersity index (PDI), and ζ-potential (mV) of the micelles were determined using dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

1.4. Physical Characterization

Fourier transform-infra red (FT-IR) spectra were recorded using a Thermo Scientific Nicolet Nexus 670 FT-IR Spectrometer and Smart iTR, with a diamond window (Thermo Fisher Scientific Inc., Waltham, MA). The sample crystallinity index was obtained by X-ray diffraction (XRD) using an X'Pert PRO MPD diffractometer (Almelo, the Netherlands) with a copper anode (Cu Kα radiation) as the source of radiation.

1.5. Morphological Analysis

The morphology of L-PCN/DI was examined by transmission electron microscope (TEM) imaging (CM 200 UT, Philips, MA, USA) at an accelerating voltage of 100 kV. Briefly, the nanoparticle dispersion was applied to a carbon-coated copper grid (300 mesh) and allowed to

adhere to the carbon substrate. A drop of 2% phosphotungstic acid solution was applied as a negative stain, followed by air-drying or infrared radiation.

1.6. *In Vitro* Release Study

The release of DOX and IRI from L-PCN/DI was evaluated in phosphate-buffered saline (PBS, pH 7.4, 0.14 M NaCl) and acetate-buffered saline (pH 5.0, 0.14 M NaCl) using a dialysis method (Spectra/Por; 3,500 Da cutoff). At specific time intervals, the medium was completely withdrawn and replaced with fresh medium to maintain sink conditions. The concentration of the released drug was determined by HPLC, as described above.

1.7. *In Vitro* Cytotoxicity Studies

The *in vitro* cytotoxicity of the free drugs, PCN/DI, and L-PCN/DI was evaluated using an MTT assay, as reported previously [13]. The cell lines (SCC-7, HeLa, MCF-7, A-549, and MDA-MB-231) were seeded into 96-well microtiter plates at a density of 1×10^4 cells/well and allowed to attach for 24 h. The cells were exposed to various concentrations of free drug or L-PCN/DI and incubated for 24 h at 37°C. MTT (100 μL, 1.25 mg/mL in medium) was added to each well and incubated for 3 h at 37°C in the dark before measuring the absorbance at 570 nm in a microplate reader (Multiskan EX, Thermo Scientific, MA, USA). The concentrations causing half-maximal (50%) toxicity (IC_{50}) were calculated using GraphPad Prism software.

The combination index (CI) value based on the Chou and Talalay method was obtained using CalcuSyn software (Biosoft, Cambridge, UK). The CI values were determined at the IC_{50}

for all cell lines. The interaction between treatment modalities was calculated using the median-effect equation and CI analysis. This analysis determined the synergistic, additive, and antagonistic effects of the drug combination against SCC-7, HeLa, MCF-7, A-549, and MDA-MB-231 cell lines. Values of CI > 1 represented antagonism, whereas CI < 1 represented synergistic effects.

1.8. Cellular Uptake Study

The *in vitro* cellular uptake of L-PCN/DI into SCC-7 cell lines was studied using confocal laser scanning microscopy (CLSM) (Nikon, Japan). The cells were seeded onto 12-well culture plates at a density of 1×10^5 cells/well and incubated for 24 h. The cells were incubated with Lysotracker Green (100 nM) for 10 min, followed by L-PCN/DI (with a total drug concentration of 0.5 μg/mL) treatment for 10 min, followed by fixing with 4% paraformaldehyde for 10 min. The cells were then observed by CLSM.

Cellular uptake efficiency was also evaluated in SCC-7, MCF-7, and MDA-MB-231 cell lines by flow cytometry. Cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated for 24 h at 37°C. The cells were treated with a free drug cocktail or L-PCN/DI (equivalent concentration of 20 μg/mL) for 30 or 60 min, respectively.

1.9. Apoptosis Assay and Cell-Cycle Analysis

SCC-7 cells (1×10^6 cells/well) were incubated overnight and treated with individual free drugs, drug cocktail, or L-PCN/DI (0.5 and 1.0 μg/mL) for 24 h. The cells were trypsinized and

resuspended in PBS. The cells were stained using the annexinV/propodium iodide Apoptosis Kit, according to the manufacturer's instructions. The cells were then analyzed using the FACS Calibur system with CellQuest software.

For cell cycle analysis, 1×10^6 cells/well were incubated overnight and treated with individual free drugs, drug cocktail, or L-PCN/DI (0.5 and 1.0 μg/mL) for 6 or 24 h, respectively. Cells were washed, collected, centrifuged, and fixed with 70% ice-cold ethanol for 30 min. Cells were washed again and treated with Rnase A and propidium iodide (1 mg/mL) for 1 h in the dark. The percentage of cells in each cycle phase was measured by the FACS Calibur system.

1.10. Pharmacokinetic Study

All animal care and experimental protocols were performed in accordance with the guidelines of the Institutional Animal Ethical Committee, Yeungnam University, South Korea. The rats (~ 250 g) were cannulated to administer drug formulations and to withdraw blood samples. Approximately 250 μL of blood was collected and immediately centrifuged (Eppendorf, Hauppauge, NY, USA) at 13,000 rpm for 10 min. The plasma (150 μL) was mixed with acetonitrile (150 μL) and vortexed for 30 min. This sample was centrifuged and the supernatant layer was separated and vacuum evaporated (Modul 3180C, Buchon, South Korea) at 40°C. The residue was reconstituted with mobile phase and injected into the HPLC column. Plasma DOX and IRI were analyzed using a Hitachi HPLC system (Tokyo, Japan). Different mobile phases were used for each drug; the mobile phase for DOX consisted of methanol:water:acetic acid (50:49:1), pH 3; and the mobile phase for IRI consisted of water:acetonitrile:methanol (65:17.5:17.5), pH 3.5. The UV absorption wavelength was 254 nm. The area under the plasma

concentration-time curve (AUC), K_{el}, and $t_{1/2}$ were calculated using non-compartmental analysis (Win-Nonlin; professional edition, version 2.1; Pharsight Co., MountainView, CA, USA). Levels of statistical significance ($p < 0.05$) were assessed by analysis of variance (ANOVA).

1.11. *In Vivo* Antitumor Study

An SCC-7 tumor xenograft mouse model was prepared using 7-week-old female BALB/c nude mice. The mice were divided into 6 groups, with 6 animals in each group. An SCC-7 cell suspension (1×10^6 cells in 0.1 mL PBS) was subcutaneously injected into the right flank of each mouse. The mice were then treated with the following formulations (i) untreated control, (ii) free DOX, (iii) free IRI, (iv) free DOX/IRI cocktail, (v) PCN/DI, or (vi) L-PCN/DI at a dose of 5 mg/kg. The mice were injected (via the tail vein) with these formulations once every 3 days (day 1, 4, 7, 10) until the 4th injection. The tumor volume (V) was measured using the equation; V = 0.5 × longest diameter × shortest diameter^2. The study was continued for up to 21 days, after which mice were sacrificed according to the ethical guidelines.

1.12. Histopathological Analysis

Xenografted tumor masses were cut and fixed in 10% neutral buffered formalin, embedded in paraffin, serially sectioned (3-4 μm), and stained with hematoxylin and eosin (H & E) prior to examination of their histopathological profiles under a light microscope (Nikkon, Tokyo, Japan). The tumor cell volumes and intact tumor cell-occupied regions (%/mm^2 of tumor mass) were calculated using a computer-based automated image analyzer (iSolution FL ver 9.1, IMT i-solution Inc., Quebec, Canada).

1.13. Immunohistochemical Analysis

The apoptotic markers, caspase-3 and poly(ADP-ribose) polymerase (PARP), were investigated immunohistochemically using purified primary antibodies and biotinylated secondary antibodies, with avidin-biotin-peroxidase complex (ABC) and a peroxidase substrate kit (Vector Labs, Burlingame, CA, USA). Briefly, endogenous peroxidase activity was blocked with methanol and 0.3% H_2O_2 for 30 min, and non-specific immunoglobulin binding was blocked with normal horse serum blocking solution in 10 mM citrate buffer (pH 6.0). Tissue sections were incubated with primary antisera overnight at 4ºC in a humidity chamber, and then incubated with biotinylated universal secondary antibody and ABC reagents for 1 h at room temperature. Finally, sections were reacted with the peroxidase substrate kit for 3 min at room temperature. Cells were counted as immunopositive if they had > 20% immunoreactivity to the apoptotic marker (caspase-3 or PARP). The percentage region occupied by caspase-3- and PARP-positive cells within the tumor mass (%/mm^2 of tumor mass) was measured by automated image analyzer.

1.14. Statistical Analysis

Multiple comparison tests were conducted to compare the dose groups. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtained data were analyzed by one way ANOVA followed by a least-significant differences (LSD) multi-comparison test to determine which pairs of groups were significantly different. When the Levene test identified significant deviation from variance homogeneity, a Kruskal-Wallis H non-parametric test was conducted. When a significant

difference was observed in the Kruskal-Wallis H test, the Mann-Whitney U (MW) test was conducted to compare specific pairs of groups. Statistical analyses were conducted using SPSS for Windows (Release 14.0K, SPSS Inc., USA). Differences were considered significant at $p < 0.05$.

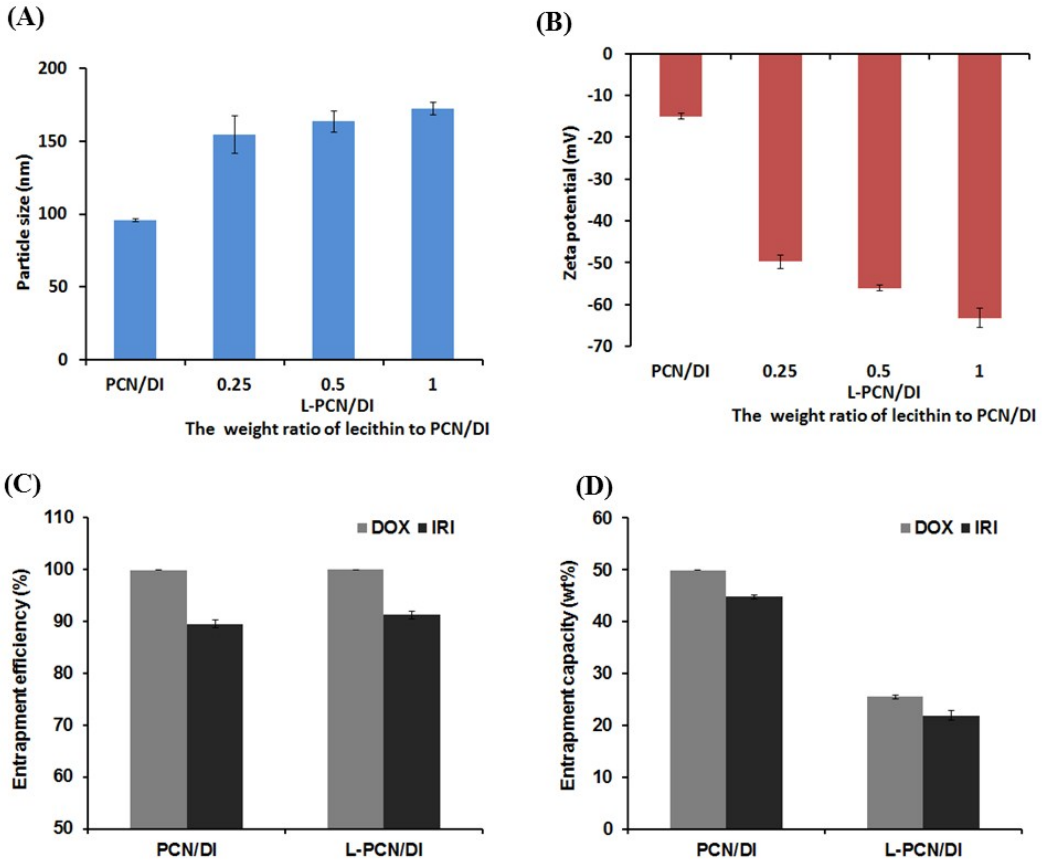

Fig. S1. (A) Particle size and (B) ζ-potential after lecithin assembly on PCN/DI. (C) Entrapment efficiency and (D) loading efficiency of L-PCN/DI.

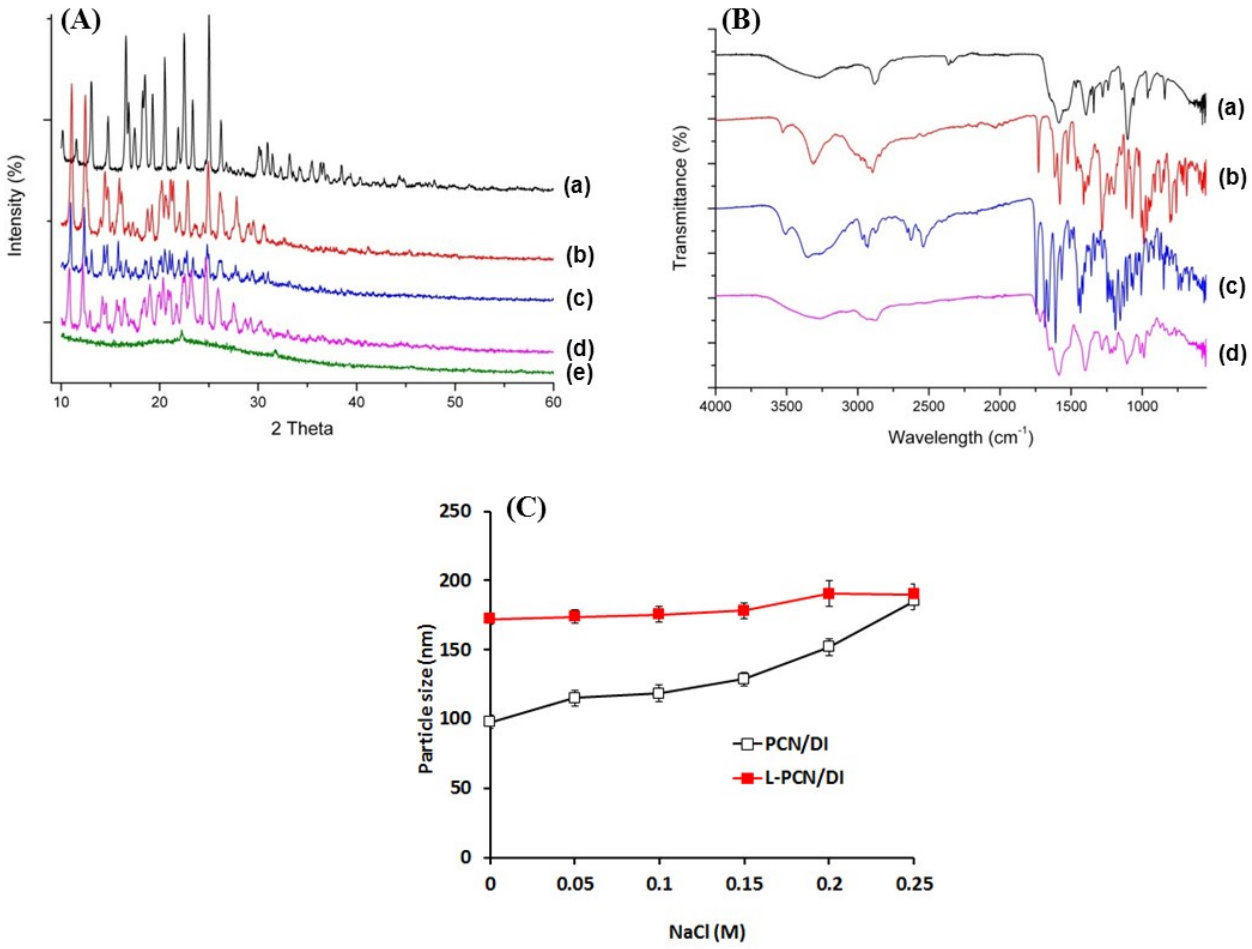

Fig. S2. (A) XRD patterns of (a) DOX, (b) IRI, (c) DOX/IRI cocktail, (D) physical mixture of drug/polymer, and (e) L-PCN/DI. (B) FT-IR spectrum of (a) PEG-*b*-PAsp, (b) DOX, (c) IRI, and (d) L-PCN/DI. (C) Effect of salt on L-PCN/DI and PCN/DI particle sizes.

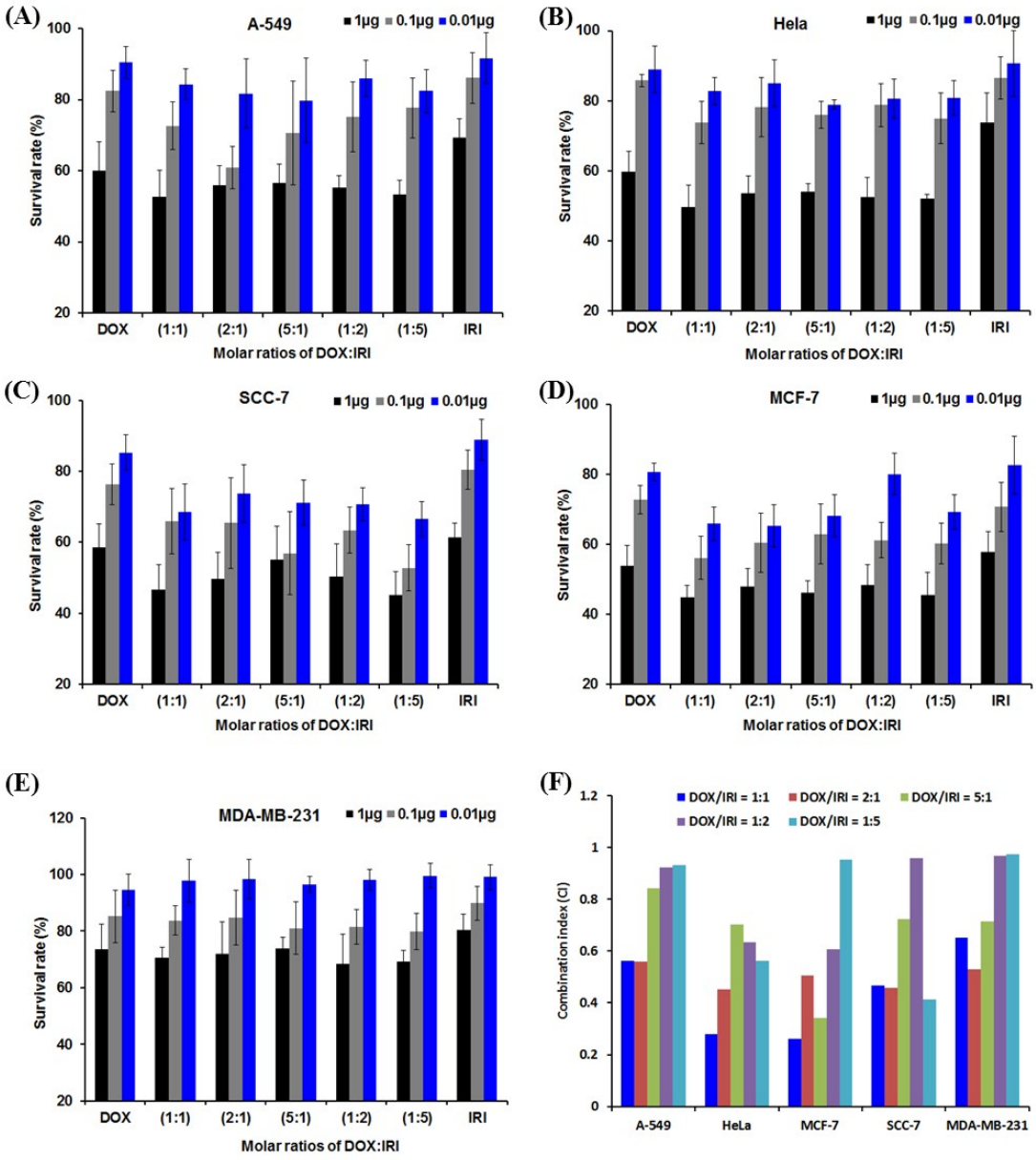

Fig. S3. (A) *In vitro* cell viability of (A) A-549, (B) HeLa, (C) MCF-7, (D) SCC-7, (E) MDA-MB-231 cells, following treatment with single and combined free DOX and IRI, as indicated. (F) Combination index (CI) values in the indicated cell lines at DOX:IRI molar ratios of 1:1, 2:1, 5:1, 1:2, and 1:5. Data are presented as mean ± SD.

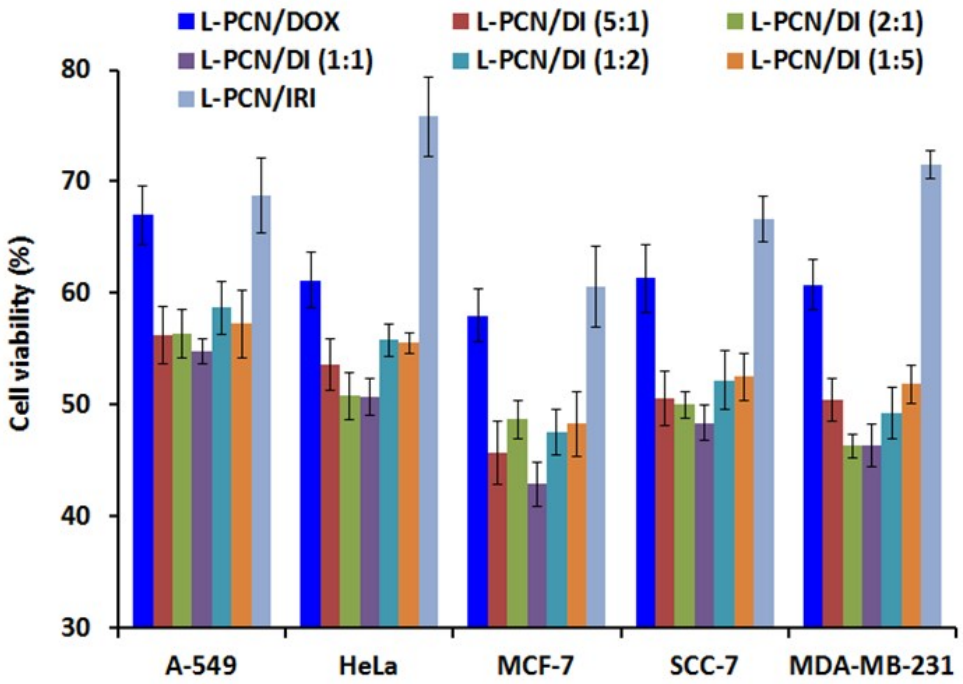

Fig. S4. *In vitro* cell viability of L-PCN/DI with various DOX:IRI molar ratios at a concentration of 1 μg/mL in A-549, HeLa, MCF-7, SCC-7, and MDA-MB-231 cells. Data are presented as mean ± SD.

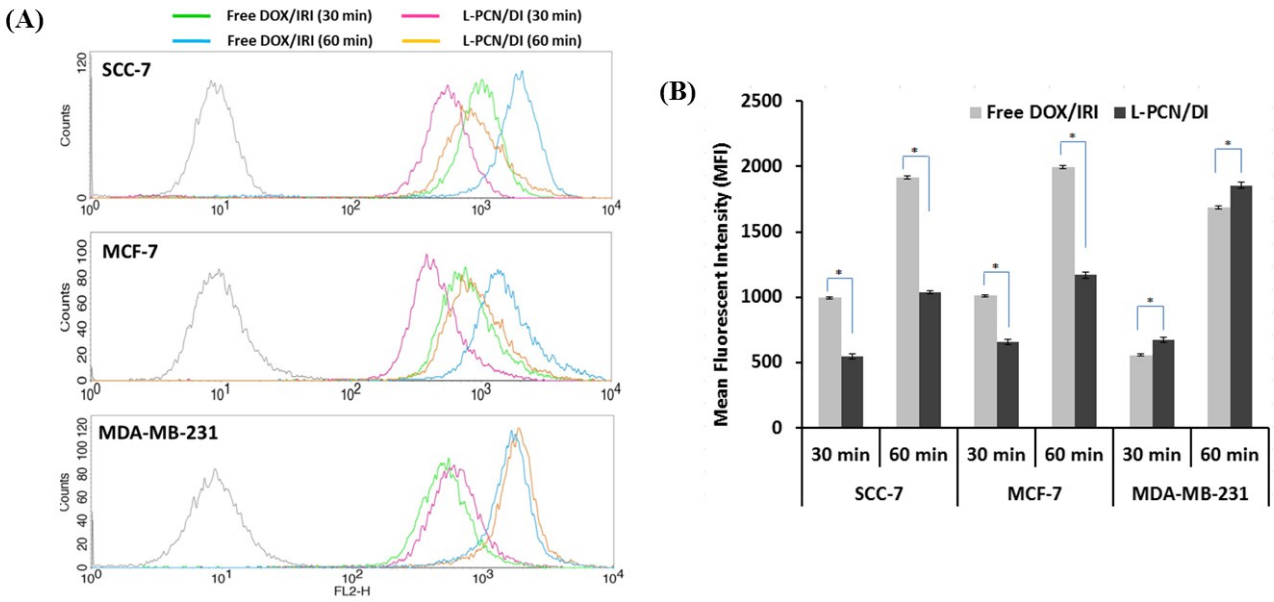

Fig. S5. (A) Flow cytometry analysis of the cellular uptake efficiency of L-PCN/DI in SCC-7, MCF-7, and MDA-MB-231 cells. (B) Mean fluorescence intensity of DOX in the indicated cancer cell lines.

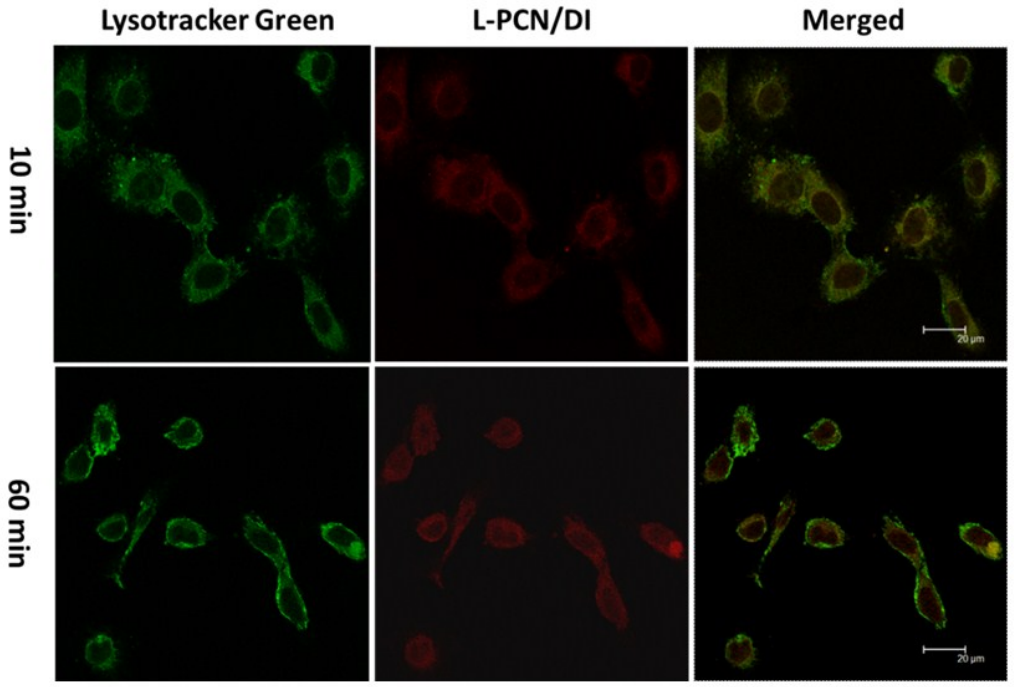

Fig. S6. *In vitro* cellular uptake in SCC-7 cells incubated with L-PCN/DI for 10 min or 60 min.

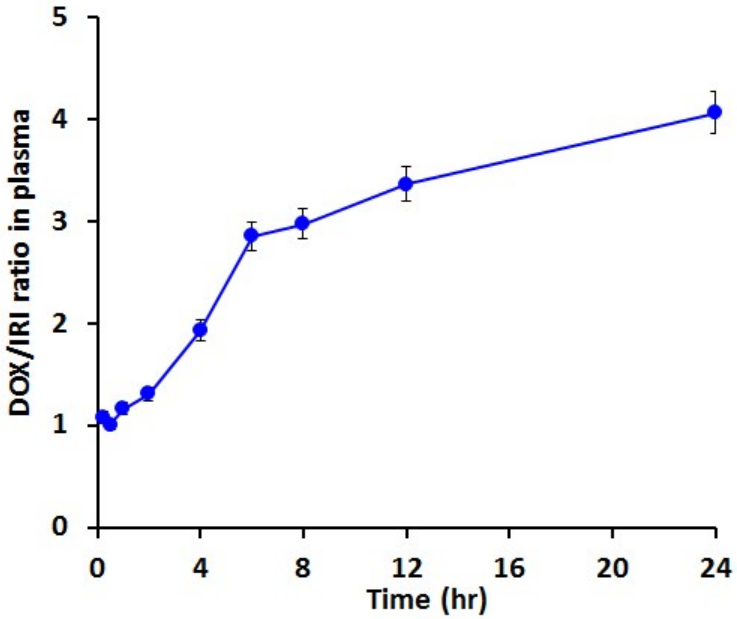

Fig. S7. DOX:IRI ratio in plasma after IV administration of a free DOX/IRI cocktail, PCN/DI, or L-PCN/DI to rats at a dose of 5 mg/kg. Each value represents the mean ± SD (n = 6). L-PCN/DI and PCN/DI had a DOX:IRI loading ratio of 1:1 and were prepared at pH 7.0.