Supporting Materials and Methods

Cell culture and material

Human Umbilical Vein Endothelial Cell (HUVEC) cell line, HL-7702 and HCC cell lines Huh-7, SNU-449, and Hep3B were obtained from Shanghai Institute for Biological Science (Shanghai, China); HepG2 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 and Huh-7 cells were authenticated by using short tandem repeat analysis and amelogenin analysis in October 28th 2012. Huh-7, HepG2 and Hep3B cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA). SNU-449, HL-7702 and HUVEC cells were cultured in Roswell Park Memorial Institute (RPMI) Media 1640 (Gibco) with 10% heat-inactivated FBS and 1% penicillin/streptomycin. All cells were maintained at 37.0 ± 0.2 °C in a humidified incubator (Thermal Tech, Orlando, FL, USA) with 5.0% CO_2.

LB-100, a water soluble homolog of 4-(3-carboxy-7-oxa-bicyclo [2.2.1] heptane-2-carbonyl) piperazine -1- carboxylicacid tertbutyl ester,[1] was provided by Lixte Biotechnology Holdings, Inc (NY, USA). LB-100 is racemic, and the two enantiomeric structures are showed in Fig. 1A. LB-100 was stocked in 0.1M monosodium glutamate, pH 10.5, -20°C, and was diluted as needed into cold phosphate buffered saline (PBS; KeYi, Hangzhou, China). Doxorubicin and cisplatin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin and cisplatin were dissolved into dimethyl sulfoxide (DMSO; Sigma) and PBS at 37°C, respectively, as indicated concentrations.

Cytotoxicity assays

Cytotoxicity was evaluated with a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) and clonogenic survival assay. For CCK-8 assays, cells were seeded in 96-well plates a density of 3000 cells per well and were assessed after treatments following the CCK-8 protocol. Cells were incubated with CCK-8 solution for 3 hours, and absorbance was measured at 450 nm using a Varioskan Flash Multimode Reader (Thermo Scientific, CA, USA). Relative cytotoxicity was expressed as a percentage of specific controls.

For clonogenic survival assays, cells were treated with indicated chemicals for 48 hours, and were seeded in 6-well plates with a density of 200 cells per well (500 for HepG2 cells). The 6-well plate was embedded with 1.2% agarose (weight/volume), and cells were mixed with 0.7% agarose and 2 × media (containing 20% FBS). After 2 weeks, clones were stained with Giemsa and the number of clones was counted. The half-maximal inhibitory concentration (IC_{50}) was determined by fitting data to the equation: $Y = 100/(1+[X/IC_{50}]\text{^}p)$, where Y is the percentage of colonies, X is the concentration (μg/ml) of doxorubicin or cisplatin, and p is a parameter associated with the slope of the curve.

PP2A activity assays

Cultured Huh-7, HepG2 and HL-7702 cells were treated with 5 μM of LB-100 or equal volume of

vehicle for 2 hours. Cells were washed three times with normal saline, and were lysed with an ultrasonic cell disruptor (Scientz Biotechnology, Ningbo, China). Mice were treated intraperitoneally (i.p.) with 2 mg/kg of LB-100, sacrificed at the indicated times, and xenograft and liver tissue removed and homogenized in double distilled water. Lysates containing 30 μg of tumor cell protein or 150 μg of tissue protein were tested for PP2A activity using a Ser/Thr phosphatase assay kit (Millipore) according to the instructions. Assays for each cell line and mouse tissue were performed in triplicate.

Vasculogenic mimicry assays

The assays were conducted as described.[2] Briefly, a 24-well plate was coated with 40 μl of a gel matrix solution (Matrigel; BD, Franklin Lakes, NJ, USA; 3:2 diluted with DMEM) and placed to dry at 37 °C for at least 30 minutes. Huh-7 cells were pre-treated with doxorubicin or/and LB-100 for 48 hours and were seeded at a density of 2×10^5 per well. After culture at 37°C for 24 hours, cells were observed and photographed (Nikon, Tokyo, Japan).

Transient transfection

PP2A_C siRNA was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and PP2A_C overexpression plasmid was a gift from William Hahn from Dana-Farber Cancer Institute (Addgene #10689). Transfections of siRNA and plasmid were performed using X-tremeGENE siRNA Transfection Reagent and X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland), respectively, as previously reported. Medium was replaced after 6 hours of cell exposure to siRNA or plasmid. All siRNAs were transfected at 50 nM final concentration. Cells were lysed or used for further assays after another 48 hours.

Immunoblotting, immunofluorescence and immunohistochemistry

Immunoblotting was performed as described previously.[3] Briefly, Huh-7 and HepG2 cells were treated with LB-100 (5 μM) or/and doxorubicin (0.2 μg/ml) for 24 hours, collected, and lysed. Proteins were extracted and quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). After blocking with milk, the membranes were probed with specific primary antibodies against serine/threonine-specific protein kinase (Akt), p-Akt, cyclin D1, PP2A, p53, VEGF as well as GAPDH. Secondary antibodies were incubated overnight at 4 °C. Membranes were visualized using enhanced chemiluminescence (ECL, GE Healthcare, Piscataway, NJ, USA) and ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA). The following primary antibodies from Cell Signaling Technology (Danvers, MA, USA) were used at a 1:1000 dilution: anti-Akt, anti-p-Akt, anti-VEGF, anti-cyclin D1, anti-p53, anti-PP2A. Anti-GAPDH antibody was purchased from Kangchen Biotechnology (Shanghai, China), and was diluted at 1:5000. The secondary antibodies were goat anti-mouse and goat anti-rabbit antibodies conjugated with horseradish peroxidase (1:2000, GE Healthcare). Band intensity was detected via densitometry analysis using Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA).

For immunofluorescence staining, Huh-7 and HepG2 cells were treated with LB-100 (5 μM) or PBS for 24 hours. Cells were then fixed with cold 4% paraformaldehyde for 20 minutes, followed by 0.1% Triton X-100 for 30 minutes. After washing with PBS, cells were incubated with anti-VE-cadherin (1:100, Cell Signaling) at 4 °C overnight, followed by incubation with goat anti-rabbit FITC-conjugated secondary antibodies (1:1000, Invitrogen) for 30 minutes at room temperature. Counterstaining with DAPI (1:10,000, Sigma) was carried out for 3 minutes before inspection under a fluorescence microscope (Olympus, Japan). Staining without primary antibody was used as negative controls.

For immunohistochemistry staining, formalin-fixed, paraffin-embedded tumor tissue samples from nude mice were cut into 5 μm-thick serial sections as described earlier.[4] Briefly, the slides were incubated with CD31 (1:50) and Ki-67 (1:100) antibodies. The slides were then incubated with horseradish peroxidase (HRP)-conjugated antibodies against rabbit immunoglobulin using Histostain-Plus Kit (ZSGB-BIO, Beijing, China) and counterstained with hematoxylin. Negative controls were incubated with PBS instead of the specific primary antibody. The mean microvessel density was quantified in five random fields at ×100 magnification.

Concentration tests of cytokine and drugs

The concentration of VEGF was determined by enzyme-linked immunosorbant assay (ELISA). A total of 2×10^5 Huh-7 cells were treated with LB-100 (5 μM) or/and doxorubicin (0.2 μg/ml). After 48 hours, the supernatants were collected, and the concentration of VEGF was measured using an ELISA kit (Assay Biotechnology, Sunnyvale, CA, USA).

The relative concentration of doxorubicin was measured by detecting its fluorescence, using a NightOWL II LB 983 NC320 *in vivo* Bioluminescence imaging system (Berthold, Oak Ridge, TN) at excitation wavelength of 480 nm. Briefly, 24-mm diameter polycarbonate filters (0.4-μm pore) of Transwell chambers (Corning, NY, USA) for use in 6-well plates were seeded with HUVEC and kept at 37 °C until HUVEC completely covered the surface of the filters. Then 1 ml of RPMI Media 1640 was added to the upper and lower chambers. RPMI Media 1640 with 0.2 μg/ml of doxorubicin was flowed through the confluent monolayer surface of HUVECs at 80 ml/h for 6 hours. If the cell monolayer remained intact, the fluid in the lower compartment of the chamber was collected and its fluorescence measured. Three independent experiments were performed.

The concentration of doxorubicin in xenografts was obtained by using quantitative fluorescence. Xenografts were resected *en bloc*, weighed immediately, and soaked in 2 ml of formalin. After homogenization, the supernatant was collected by filtration and fluorescence measured with excitation wavelength 480 nm and emission wavelength 580 nm. A standard curve was plotted using doxorubicin solutions with concentrations ranging from 0.01 to 5 μg/ml. Triplicates with four mice in each group

were analyzed.

Determination of tumor blood flow

Mice were anesthetized with 4% chloral hydrate (Sigma) and tumor blood flow was measured at three sites on the surface of each tumor using a Laser Doppler Perfusion and Temperature Monitor (moorVMS-LDF1; Moor Instruments Ltd., Devon, UK).

***In vitro* HUVEC permeability assays**

HUVECs were seeded in Transwell inserts with 0.4-μm pores in a six-well plate. After a confluent monolayer was formed, HUVECs were treated with LB-100 (5 μM) or PBS for six hours. 0.04% of Evans Blue dye was then added to the upper compartments and maintained for overnight. Medium in upper and lower compartments was collected and optic density (OD) value was measured using a Multimode Reader at wavelength of 595 nm. Data were normalized to PBS controls and presented as mean ± SEM of three independent assays.

***In vivo* assays of blood vessel permeability in normal skin and HCC xenografts**

In vivo permeability assays were conducted as described.[5] Briefly, Evans Blue dye (1% in PBS; 4 ml/kg; Aladdin, Shanghai, China) was injected through the retro-orbital venous sinus, followed by intradermal injection of 20 μl of vehicle or LB-100 (100 μM) in each flank of the same mouse. After one hour, mice were sacrificed and skin samples (6 mm in diameter) were excised and photographed. Skin samples were further incubated with formamide in 56 °C for 3 days, and dye fluorescence assessed by excitation at 253 nm and measurement at 387 nm with a Multimode Reader. Extraction of tissues from non-injected sites was used as background control. For xenograft permeability assays, six mice were randomly allocated to two groups, one of which was treated with LB-100 (2 mg/kg, i.p., qod) for four doses. Mice were given Evans Blue dye as described previously. One hour later, xenografts were removed *en bloc* and weighed. Each xenograft was homogenized in equal volume of formamide, incubated and the dye concentration measured as described previously. The OD values were adjusted according to weight of xenografts.

Statistical analysis

Statistical calculations were performed using Prism 5 software (GraphPad, San Diego, CA, USA). Statistical analyses were performed using one-way ANOVA or *F* test following two-tailed unpaired Student's t-tests, as appropriate, unless otherwise specified. Data of *in vivo* tumor blood flow and vascular permeability assays are presented as mean ± standard error of the mean (SEM) of three mice. Other data are presented as means ± standard deviation (SD) or SEM as indicated. For IC_{50} comparisons, extra sum-of squares *F* test was used. For all tests, $P < 0.05$ was considered statistically significant.

References

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