Inhibition of Protein Phosphatase 2A Enhances Cytotoxicity and Accessibility of Chemotherapeutic Drugs to Hepatocellular Carcinomas

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common and therapeutically challenging malignancies worldwide. For patients ineligible for “curative resection” or liver transplantation, chemotherapy is an important minimally effective option. Strategies for chemosensitization are urgently needed. Here, we report that LB-100, a serine/threonine protein phosphatase 2A (PP2A) inhibitor, enhances the cytotoxicity of chemotherapy for HCC in vitro and in vivo. We found that LB-100 significantly enhanced inhibition of HCC by doxorubicin and cisplatin in vitro and in vivo in a PP2A-dependent way, while having little inhibitory activity when used alone. LB-100 promoted vascular endothelial growth factor secretion and vasculogenic mimicry, associated with increased microvessel density and blood perfusion of tumor cell xenografts. LB-100 also enhanced paracellular endothelial permeability to Evans Blue dye and doxorubicin in vivo and in vitro, presumably by altering vascular endothelial–cadherin contact between cells. Changes in permeability and perfusion were accompanied by increased accumulation of doxorubicin in HCC xenografts but not in normal liver tissue. In conclusion, LB-100 enhances chemotherapy by interfering with DNA damage–induced defense mechanisms and by increasing angiogenesis and drug penetration into tumor cells. The induction of angiogenesis and vascular permeability of tumor xenografts by inhibition of PP2A may be a novel approach for enhancing the cytotoxic treatment of HCC and potentially other cancers. Mol Cancer Ther; 13(8); 2062–72.

Introduction

Protein phosphatase 2A (PP2A), a family of the major serine/threonine phosphatases in cells, is widely considered a tumor suppressor (1, 2). Inhibition of PP2A is thought to be a precursor of malignant transformation of human cells and some PP2A inhibitors, such as okadaic acid, are associated with tumorigenesis and tumor progression (3, 4). Structurally, PP2A has three subunits and each subunit has alternative isoforms (5), resulting in over 60 heterotrimeric holoenzymes (6). Because of the complicated constitutive and various signaling pathways involving PP2A, this ubiquitous phosphatase may play distinct roles in different tissue and disease states. For instance, the B55α regulatory subunit of PP2A was shown to enhance the survival of human fibrosarcoma cells during glutamine deprivation (7), whereas inhibition of the B56γ subunit induces tumorigenic transformation of human embryonic kidney cells (8), thereby acting like B56α as a tumor suppressor (9). This diversity of PP2A function in tumorigenesis suggests that in certain circumstances targeting PP2A may be an effective cancer strategy.

Cantharidin, a natural product isolated from Mylabris sidae, and several cantharidin derivatives have PP2A inhibitory activity, and have been used as anticancer agents for decades (10–13). The mechanism by which PP2A exerts anticancer activity is believed to be abrogation of cell-cycle checkpoints and induction of mitotic catastrophe (14). LB-102, a synthetic cantharidin derivative and potent PP2A inhibitor, has been shown to significantly enhance the effects of classic chemotherapy on glioblastoma, neuroblastoma, and stem cell–derived aggressive sarcoma (15, 16).

Primary hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death. At diagnosis, HCCs are frequently unresectable and often seem to be chemotherapy resistant from the outset or readily developed chemoresistance (17). Many strategies of chemosensitization have been explored in HCC treatment (18–20), but...
more effective therapies are urgently needed (21). Moreover, PP2A overexpression was found in tumors of patients with hepatitis C virus infection (22). Because cantharidin has been reported to have significant activity against HCC (23), we tested whether a novel low molecular weight PP2A inhibitor LB-100, which has been proved to radiosensitize pancreatic cancers (24), enhances the effectiveness of chemotherapy for HCC.

This study showed that LB-100 enhances the cytotoxicity of doxorubicin and cisplatin in vitro and in vivo on HCC. Besides the mechanisms previously suggested for LB-102 potentiation of cytotoxic chemotherapy, namely blocking DNA damage-induced defense mechanisms of tumor cells, we found that LB-100 increases accessibility of chemotherapeutic drugs to tumor cells in vivo by promoting angiogenesis and paracellular permeability of vascular endothelial cells.

Materials and Methods

Cell culture and materials

The human umbilical vein endothelial cell (HUVEC), HL-7702 and HCC cell lines Huh-7, SNU-449, and Hep3B were obtained from the Shanghai Institute for Biological Science (Shanghai, China); HepG2 was obtained from American Type Culture Collection (ATCC). HepG2 and Huh-7 cells were authenticated by using short tandem repeat analysis and amelogenin analysis on October 28, 2012. Huh-7, HepG2, and Hep3B cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Sigma). 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°C ± 0.2°C in a humidified incubator (Thermal Tech) with 5.0% CO2.

LB-100, a water soluble homolog of 4-(3-carboxy-7-oxa-bicyclo[2.2.1]heptane-2-carbonyl) piperazine-1-carboxylic acid tertbutyl ester (15), was provided by Lixte Biotechnology Holdings, Inc. LB-100 is racemic, and the two enantiomeric structures are showed in Fig. 1A. Detailed information of LB-100, doxorubicin, and cisplatin is also described in the Supplementary Materials and Methods.

Cytotoxicity assays

Cytotoxicity was evaluated with the Cell Counting Kit-8 (CCK-8; Dojindo). The detailed information is showed in the Supplementary Materials and Methods. Relative cytotoxicity was expressed as a percentage of specific controls.

PP2A activity assays

Cultured Huh-7, HepG2, and HL-7702 cells were treated with 5 μmol/L 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 (Ningbo Scientz Biotechnology). Mice were treated intraperitoneally (i.p.) with 2 mg/kg of LB-100, sacrificed at the indicated time, 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 manufacturer's instructions. Assays for each cell line and mouse tissue were performed in triplicate.

Vasculogenic mimicry assays

Vasculogenic mimicry assays were conducted as described previously (25). Brief description is given in the Supplementary Materials and Methods.

Transient transfection

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

Immunoblotting, immunofluorescence, and immunohistochemistry

Immunoblotting, immunofluorescence staining, and immunohistochemistry staining were performed as described previously (26). The following primary antibodies were from Cell Signaling Technology: anti-Akt, anti-p-Akt, anti-VEGF, anti-cyclin D1, anti-p53, anti-PP2A, anti-CD31, anti-Ki-67, and anti-VE-cadherin. Anti-GAPDH antibody was purchased from Kangchen Biotechnology Holdings. Detailed information is described in the Supplementary Materials and Methods.

Concentration tests of VEGF and doxorubicin

The concentration of VEGF was determined by enzyme-linked immunosorbant assay (ELISA). The relative concentration of doxorubicin was measured by detecting its fluorescence, using a NightOWL II LB 983 NC320 in vivo Bioluminescence Imaging System (Berthold) at excitation wavelength of 480 nm. The concentration of doxorubicin in xenografts was obtained by using quantitative fluorescence. See detailed information in the Supplementary Materials and Methods.

Xenografts and drug treatment

BALB/c nude mice were injected subcutaneously in the right flank with 1 × 106 Huh-7 cells suspended in 200 μL PBS per mouse. After a tumor volume of 100 to 200 mm3 was reached, tumor-bearing mice were randomly allocated to four groups: control group, doxorubicin/cisplatin group, LB-100 group, and doxorubicin/cisplatin plus LB-100 group. For the doxorubicin plus LB-100 study (n = 6 to 8), doxorubicin and LB-100 were injected i.p. at...
1.5 and 2 mg/kg, respectively, on alternate days for a total of 16 days. For the cisplatin plus LB-100 study (n = 8 to 10), cisplatin and LB-100 were injected at 3 and 2.5 mg/kg, i.p., respectively; cisplatin was injected every 4 days and LB-100 was used every other day for 16 days. Control mice were injected with DMSO (in the doxorubicin plus LB-100 group) or PBS (in the cisplatin plus LB-100 group) on the same schedule as the drug-treated animals. Tumor size was monitored every 3 or 4 days, and was calculated by the formula: tumor volume = length/width/height/2.

All mice were sacrificed at day 16, and xenografts were obtained, weighed, and fixed with 10% formaldehyde.

**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.).

**In vitro HUVEC permeability assays**

*In vitro* HUVEC permeability assays using Evans Blue dye is similar with concentration test of doxorubicin but without using of flow control system, and is described in the Supplementary Materials and Methods.

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

*In vivo* permeability assays were conducted as described previously (27), and are described in the Supplementary Materials and Methods. For xenograft permeability assays, 6 mice were randomly allocated to two groups, one of which was treated with LB-100 (2 mg/kg, i.p., every other day) 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). Statistical analyses were performed using one-way ANOVA or F test following two-tailed unpaired Student 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 mean ± standard deviation (SD) of three independent experiments. For all tests, \( P < 0.05 \) was considered statistically significant.

Results

LB-100 inhibited PP2A activity and showed moderate cytotoxicity to cells

We first tested the PP2A inhibitory effect of LB-100 in vitro and in vivo. Four HCC cell lines, Huh-7, HepG2, Hep3B, and SNU-449, and an immortalized human fetal hepatic cell line (HL-7702) were studied. Dose-dependent cytotoxicity was shown in all four HCC cell lines, with a half maximal inhibitory concentration (IC\(_{50}\)) exceeding 10 \( \mu \text{mol/L} \) (Supplementary Fig. S1). Of note, doses up to 5 \( \mu \text{mol/L} \) did not inhibit viability of any of the cell lines. Thus, we used 5 \( \mu \text{mol/L} \) of LB-100 in subsequent experiments. Exposure to 5 \( \mu \text{mol/L} \) LB-100 for 2 hours reduced the activity of PP2A to about 70% in Huh-7, HepG2, and HL-7702 cells (Fig. 1B). In vivo, the injection of LB-100 (2 mg/kg, i.p.) decreased in a time-dependent manner the activity of PP2A in xenografts and livers in nude mice to 50% to 60% after 12 hours (Fig. 1C). LB-100 did not alter the expression of the three PP2A subunits (PP2A_A, PP2A_B, and PP2A_C) in cell lines, xenografts, or livers, as confirmed by immunoblotting (Fig. 1D). These results demonstrate that partial inhibition of PP2A activity by low concentrations of LB-100 does not significantly affect cell viability.

LB-100 synergized with chemotherapeutic drugs and altered cell cycle and cell signaling pathways

PP2A inhibition has been reported to enhance the efficacy of cancer treatment in model systems (15, 16). Thus, we explored a strategy to increase the effectiveness of drugs currently used for the treatment of HCC with low doses of LB-100. Using CCK-8 assays, we found that both doxorubicin (0.2 \( \mu \text{g/mL} \)) and cisplatin (2 \( \mu \text{g/mL} \)) alone and in combination with LB-100 were as expected cytotoxic to each of the four HCC cell lines. The addition of LB-100 (5 \( \mu \text{mol/L} \)) sensitized HCC cells to doxorubicin or and LB-100 treatments.
and cisplatin, whereas LB-100 alone did not show any cytotoxicity (Fig. 2A; Supplementary Fig. S2; Supplementary Tables S1 and S2). To further confirm that the chemosensitization of LB-100 was PP2A activity-dependent, we overexpressed PP2A catalytic subunit in Huh-7 cells. The chemosensitization of LB-100 was greatly attenuated in vitro (Supplementary Fig. S3A). In addition, in xenograft mouse model that was generated by PP2A_C overexpressed Huh-7 cells, LB-100 did not further reduced tumor volume (Supplementary Fig. S3B).

Interestingly, LB-100 did not enhance the cytotoxicity of doxorubicin to HL-7702 cells (Fig. 2B), which is considered an in vitro model of normal liver cells. Exposure of Huh-7 and HepG2 cells to LB-100 (5 μmol/L) for 24 hours was associated with an increase in abnormal nuclei (Fig. 2C and Supplementary Fig. S4). After doxorubicin (0.2 μg/mL) treatment (48 hours), both Huh-7 and HepG2 cells showed decreased ratios of p-Akt/Akt (1.00, 0.52, 0.70, and 1.10 in Huh-7, and 1.00, 0.78, 0.87, and 1.59 in HepG2, for control, doxorubicin, doxorubicin plus LB-100, and LB-100-alone exposures, respectively), whereas the p-Akt/Akt ratio was increased by inclusion of LB-100 (Fig. 2D), which could be explained by the fact that p-Akt is a direct substrate of PP2A. Doxorubicin stimulated the expression of p-p53 (ser15) and LB-100 attenuated this effect in HepG2 but not Huh-7 cells, suggesting an influence of p53 integrity upon cell-cycle control by PP2A. Notably, the expression of cyclin D1 also was upregulated by LB-100, consistent with the impaired p53-mediated cell-cycle arrest (15). In agreement with previous reports (15, 16, 28), our results suggest that LB-100 potentiates chemotherapy in HCC cells at least in part from induction of changes in cell cycle and aberrant mitosis.

**LB-100 enhanced effects of chemotherapy in HCC xenograft**

In vivo, LB-100 alone (2 and 2.5 mg/kg, i.p., every other day) did not significantly reduce tumor burden in Huh-7

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**Figure 3.** LB-100 enhances chemotherapeutic effect of doxorubicin in vivo. A, tumor volumes were significantly reduced in mice with doxorubicin (DOX; 1.5 mg/kg, every other day, i.p.) and LB-100 (2 mg/kg, every other day, i.p.) cotreatment, whereas doxorubicin alone did not show significant antitumor effect. 

n = 6, 6, 8, and 8 for control, LB-100, doxorubicin, and doxorubicin plus LB-100 groups, respectively. 

*, P < 0.05 as compared with the doxorubicin group; 

**, P < 0.05 as compared with the control group. B, representative tumors from each group. C, immunohistochemistry showed increased CD31 staining in mice with LB-100 treatment. 

*, P < 0.01; arrows, microvessels; scale bar, 50 μm. D, immunohistochemistry showed less Ki-67 staining in mice with doxorubicin and LB-100 cotreatment. 

*, P < 0.05; scale bar, 50 μm.
xenografts (Figs. 3 and 4), consistent with the results in vitro. However, the combination of doxorubicin (1.5 kg/mL, every other day) and LB-100 (2 mg/kg, every other day) significantly slowed the growth of tumors (Fig. 3A and B) with reduction of tumor volume in two animals with no effects on tumor growth in animals treated with single agents. Immunochemistry of the xenografts revealed enhanced expression of CD31 and downregulation of Ki-67 in the doxorubicin plus LB-100 group compared with the doxorubicin-alone group (Fig. 3C and D), suggesting that LB-100 enhanced microvesSEL density within tumors and reduced proliferation of tumor cells. At higher doses (2.5 mg/kg, every other day) of LB-100, there was an apparent increase in tumor volume; however, when combined with cisplatin (3 mg/kg, i.p., every 4 days), LB-100 significantly augmented cytotoxicity compared with cisplatin alone (Fig. 4A–C). We also monitored blood flow in tumor xenografts by using a laser Doppler apparatus. At day 16, the xenografts of animals treated with LB-100 had higher blood flow than xenografts in untreated animals (Fig. 4D), indicating better perfusion of xenografts in the LB-100 and cisplatin/LB-100 groups. The combination of an increased blood supply and increased inhibition of tumor growth raised the possibility that LB-100 enhances accessibility of chemotherapeutic drugs to tumor cells.

**LB-100 increased angiogenesis and the amount of chemotherapeutic drugs within xenografts**

To test the hypothesis that LB-100 has the capability of increasing the amount of chemotherapeutic drugs into tumor cells, we first evaluated its influence on angiogenesis. By ELISA assays, we observed that LB-100 increased the concentration of VEGF in medium in both DMSO control and doxorubicin-exposed cells (P < 0.001 for control vs. LB-100, P < 0.001 for doxorubicin vs. doxorubicin/LB-100; Fig. 5A). However, the expression of VEGF was not significantly altered after LB-100 treatment, suggesting that LB-100 might promote the secretion of VEGF. When PP2A activity was further altered by transfection of a siRNA targeting PP2A_C or a PP2A_C overexpression plasmid, VEGF secretion showed similar changes (Supplementary Fig. S5), which implied that PP2A inactivation was capable of promoting VEGF secretion. In Huh-7 cells, we also found that LB-100 could improve vasculogenic mimicry (Fig. 5B), which is considered an important source of blood supply for early tumors and might be the prelude to angiogenesis (29). To investigate the angiogenic effect of LB-100 in vivo, nude mice with or without LB-100 pretreatment (four times, 2 mg/kg, i.p., every other day), were injected with doxorubicin (0.12 mg in 0.4 mL PBS). Xenografts from mice with LB-100 pretreatment showed higher fluorescence intensity of doxorubicin (Fig. 5C), suggesting an increase...
in the amount of doxorubicin within xenografts. We also used Evans Blue dye, which combines with albumin, as an indicator of blood perfusion. Consistently, mice with LB-100 pretreatment had more Evans Blue per unit weight of xenografts (Fig. 5D). We calculated that treatment with LB-100 doubled the amount of doxorubicin per unit weight of tumor \( (P = 0.004) \) but LB-100 did not enhance the amount of doxorubicin in liver \( (n = 4; P = 0.89; \text{Fig. 5E}) \). These findings were compatible with increased angiogenesis and blood perfusion in tumors induced by LB-100.

**Enhanced paracellular permeability of vascular endothelial cells by LB-100**

An increase of blood supply could lead to tumor proliferation and be detrimental to cancer treatment. On the basis of higher blood perfusion with chemotherapeutic drugs and inhibited tumor growth, we speculated that...
LB-100 enhanced the vascular permeability, increasing the chemotherapeutic drugs accessible to tumor cells. To test this hypothesis, we developed a flow control system to mimic blood flow in vitro and used HUVECs as the barrier of vessel wall. LB-100–pretreated HUVECs showed higher permeability of doxorubicin (DOX) in the fluid flowing through the upper compartment, leading to higher concentration of doxorubicin in the lower compartment, as measured by fluorescence of doxorubicin (Fig. 6A). Exposure to doxorubicin with or without LB-100 did not significantly damage the HUVECs monolayer as judged by microscopic observation (Supplementary Fig. S6). We assessed the integrity of the HUVECs by measuring the immunofluorescence of vascular endothelial (VE)-cadherin, which is an endothelial specific adhesion molecule required for maintenance of the vascular endothelial barrier (30, 31). LB-100 altered VE-cadherin integrity between endothelial cells (Fig. 6B), potentially accounting for the increased permeability of HUVECs induced by LB-100 but not by doxorubicin. Evans Blue dye was again used to assess enhanced permeability of HUVECs induced by LB-100. Pretreatment of LB-100 resulted in a nearly 40% increase in dye passing through the HUVECs monolayer (Fig. 6C). To determine the enhanced permeability of vascular endothelial cells in vivo, Evans Blue dye was injected into blood circulation from retro-orbital venous sinus. Compared with PBS control, local injection of LB-100 significantly increased Evans Blue extravasation by more than 50% (Fig. 6D). Mice (n = 3) were injected with Evans Blue dye from retro-orbital venous sinus, followed by intradermal injection of LB-100 or PBS. Skins were cut after 1 hour and the dye was extracted by formamide and was quantified as fluorescence intensity. Relative fluorescence intensity was calculated by calibration to that of PBS control. Representative skins are shown (bottom). Data are shown as mean ± SEM (*, P < 0.05). E, scheme of mechanisms by which LB-100 enhances chemotherapy by inhibition of PP2A.

Figure 6. LB-100 elevated vascular permeability in vitro and in vivo. A, LB-100 enhanced HUVEC permeability for doxorubicin (DOX), reflected by stronger fluorescence of the medium in the lower compartment (top). A flow control system was used for evaluation of HUVEC permeability in vitro (bottom). Medium containing doxorubicin (0.2 μg/mL) flowed in the upper compartment for 6 hours with or without LB-100 (5 μmol/L) pretreatment on HUVECs monolayer for 6 hours. B, immunofluorescence showed loose contact of VE-cadherin in HUVECs monolayer of A. C, Evans Blue dye was used to reassess the permeability of HUVECs as treated in A, and more dye was indicated in lower compartment after pretreatment of LB-100 (n = 4). ***, P < 0.001. D, mice (n = 3) were injected with Evans Blue dye from retro-orbital venous sinus, followed by intradermal injection of LB-100 or PBS. Skins were cut after 1 hour and the dye was extracted by formamide and was quantified as fluorescence intensity. Relative fluorescence intensity was calculated by calibration to that of PBS control. Representative skins are shown (bottom). Data are shown as mean ± SEM. *, P < 0.05. E, scheme of mechanisms by which LB-100 enhances chemotherapy by inhibition of PP2A.
Discussion

The balance between phosphorylation and dephosphorylation is vital for all aspects of cell physiology, including growth, differentiation, survival, and function (32). Protein kinases and phosphatases are global regulators of protein activity, and their dysfunction or aberrant activation results in a wide spectrum of diseases including cancers (33, 34). Cancers are widely characterized by abnormal activation of protein kinases such as Akt, tyrosine kinase, casein kinase, etc. (35–37). Kinase inhibition was therefore believed to be a feasible strategy for cancer treatment, and to date, more than 10 kinase inhibitors have been approved for clinical use by U.S. Food and Drug Administration (38). As one of the few and most critical serine/threonine-specific phosphatases in cells, PP2A has been considered as cancer suppressor and PP2A activity is reported to positively correlate with effects of cancer treatment (39). However, PP2A inhibitors have also shown benefit for cancer treatment in certain conditions. For instance, PP2A inhibition has been shown to induce apoptosis in T-leukemia cells, pancreatic cancer, and prostate cancer (11, 40–42), and gene therapy strategy targeting PP2A has shown efficacy on HCC in vitro and in vivo (43). In addition, endothall, another PP2A inhibitor, even showed that growth inhibition preferentially inhibited HCC cell lines compared with normal fetal hepatocytes (44). Our results also suggest a possible selective inhibition of HCC cell lines compared with an immortalized hepatocyte cell line in vitro (Fig. 2A and B).

The discrepancy between unaltered PP2A expression and changed substrates (Figs. 1D and 2D) implied an activity-based rather than expression-based inhibition of PP2A by LB-100. LB-100 is in the family of modified cantharidins that are demonstrated to inhibit PP2A by direct interaction with the enzyme as described previously (13). These cantharidin-related structures are assumed to bind to a pocket needed for activity on the enzyme as has been demonstrated for naturally occurring but structurally unrelated molecules okadaic acid, microcstin, and fostrieacin (45).

LB-100 inhibition of PP2A in HCC cell lines may be due to cancer type heterogeneity as evidenced by a lower IC50 of LB-100 for pancreatic cancer cell lines compared with HCC cell lines (Zhang and Zhi; unpublished data). Also, the relatively higher activity of PP2A in the liver among other organs (Zhang; unpublished data), possibly related to its role in regulation of glycogen synthesis, may play a role in the relative resistance to LB-100 in HCC cell lines. Our results suggest that LB-100 alone is not cytotoxic to HCC cells in vitro nor in vivo at low doses, which, however, sensitize tumor cells to doxorubicin and cisplatin in vitro (Fig. 2A) and in vivo (Figs. 3A and 4A).

Enhancement of the effectiveness of LB-100 was shown for both doxorubicin and cisplatin (Figs. 2A–4). The potentiation of chemotherapy by PP2A inhibitors has been mechanistically explained as due to impairment of DNA damage–induced defense mechanisms, sustained activation of the NF-xB pathway, and activation of extrinsic and intrinsic apoptosis pathways involving JNK and p38 MAPK signaling pathways (11, 15, 16, 40, 41). We showed similar effects of LB-100 on cell cycle in vitro (Fig. 2D), as reported for its analog LB-102 (15, 16). The differences among cell lines may be related to p53 status (Y220C, wild-type, deleted and A161T in Huh-7, HepG2, Hep3B, and SNU-449, respectively). In vivo, our studies bring to light other possible mechanisms. We found abundant angiogenesis (Fig. 3C), higher blood perfusion (Fig. 4D), and greater amounts of doxorubicin (Fig. 5C and F) in HCC xenografts after LB-100 treatments and that LB-100 enhances VEGF release and increases vasculogenic mimicry in vitro. We propose that LB-100 enhances chemotherapy by facilitating drug entry into tumors. The rate of doxorubicin entry into Huh-7 and HepG2 cells was enhanced in the cotreatment group with LB-100 in vitro (Supplementary Fig. S7) culminating in increased concentrations of doxorubicin in tumor cells in vivo. Elevated paracellular permeability of vascular endothelial cells was also induced by LB-100, an effect that should increase drug delivery to tumor cells (Fig. 6). Others have noted increased endothelial permeability induced by PP2A inactivation (27). To the best of our knowledge, however, this is the first report of a cancer treatment strategy that induces both angiogenesis and increases vascular permeability. Although angiogenesis inhibitors have been considered a new generation of cancer drugs and indeed showed some clinical benefits (46), other studies note unexpected adverse events with this therapy including increased epithelial-mesenchymal transition and a higher incidence of metastasis (47). However, an increase in microvessel density could enhance drug delivery to tumor cells (48). Therefore, stimulating angiogenesis and increasing vascular permeability may provide another strategy for cancer control, particularly for solid cancers with low blood supply that respond poorly to angiogenesis inhibitors.

The relative selectivity of LB-100 on tumor cells in vitro (Fig. 2B) and on xenografts in vivo (Fig. 5E) is encouraging. Two possible mechanisms may account for this phenomenon. On one hand, angiogenesis is indispensable within tumors but is seldom in adult liver, changing blood distribution in body and leading more drugs toward tumors. On the other hand, liver-specific sinusoidal endothelial cells are the only type of endothelial cells that have fenestrations and lack basement membrane (49), so that the drug is easy to get into hepatocytes, and elevated paracellular permeability may hardly further increase the accessibility of drug to hepatocytes. Although it has a long way to go, the above-mentioned strategy may have tolerable enhanced side effects with chemoresensitization.

In conclusion, our work demonstrates that LB-100, a PP2A inhibitor, synergizes with doxorubicin and cisplatin to increase anticancer activity in HCC cell lines and xenografts. The effects of enhanced angiogenesis...
and elevation of vascular permeability may be responsible for LB-100–induced chemosensitization (Fig. 6E) offering a novel strategy for the treatment of solid tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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