Supplementary Data

Supplementary Tables

Object description: Clonogenic survival assays were performed to assess the chemosensization of LB-100. Four hepatocellular carcinoma cell lines were used. LB-100 significantly reduced half-inhibitory concentration (IC_{50}) of doxorubicin (Table S1) and cisplatin (Table S2). Clonogenic survival assays and Cell Counting Kit-8 assays reflect different aspects of cell viabilty. Clonogenic survival assays are prone to show stem cell-like characteristic of proliferation of cells, while Cell Counting Kit-8 assays are inclined to show total metabolic activity of cells. These two tables suggest that chemotherapy-induced inhibition of proliferation can be faciliated by LB-100.

Table S1 IC_{50} values and statistical analyses of doxorubicin and doxorubicin plus LB-100 treatments in HCC cell lines.

Cell lines	IC_{50}*		Extra sum-of squares *F* test	
	Doxorubicin	LB-100 + doxorubicin**	$F_{(1,32)}$	*p* value
HepG2	0.24 (0.19-0.29)	0.12 (0.10-0.14)	30.05	< 0.001
Huh-7	0.22 (0.19-0.24)	0.14 (0.12-0.15)	48.44	< 0.001
Hep3B	0.36 (0.26-0.45)	0.19 (0.15-0.23)	18.31	< 0.001
SNU-449	1.63 (0.38-2.88)	0.83 (0.58-1.08)	4.003	0.054

* IC_{50} values show doxorubicin concentration [μg/ml, mean (95% CI)].

** The concentration of LB-100 here was 5 μM. The unit of doxorubicin/cisplatin was μg/ml as above.

Table S2 IC_{50} values and statistical analyses of cisplatin and cisplatin plus LB-100 treatments in HCC cell lines.

Cell lines	IC_{50}*		Extra sum-of squares *F* test	
	Cisplatin	LB-100 + cisplatin**	$F_{(1,32)}$	*p* value
HepG2	1.66 (1.43-1.88)	1.26 (1.12-1.40)	10.53	0.003
Huh-7	1.98 (1.71-2.24)	1.27 (1.09-1.45)	22.67	< 0.001
Hep3B	2.09 (1.74-2.44)	1.31 (1.15-1.47)	22.02	< 0.001
SNU-449	2.79 (2.43-3.14)	1.82 (1.62-2.02)	28.23	< 0.001

* IC_{50} values show doxorubicin concentration [μg/ml, mean (95% CI)].

** The concentration of LB-100 here was 5 μM. The unit of doxorubicin/cisplatin was μg/ml as above.

Supplementary Figures

Figure S1

Object description: Cell Counting Kit-8 (CCK-8) assays (A) and Clonogenic assyas (B) were performed to test the cytotoxicity of LB-100 on various hepatocellular carcinoma (HCC) cell lines. CCK-8 assays showed that the IC_{50} of LB-100 on these four HCC cell lines is more than 20 μM, while clonogenic showed IC_{50} not less than 10 μM. Particullarly, both assays showed that 5 μM of LB-100 did not do harm to HCC cell viability or capacity of clonogenesis.

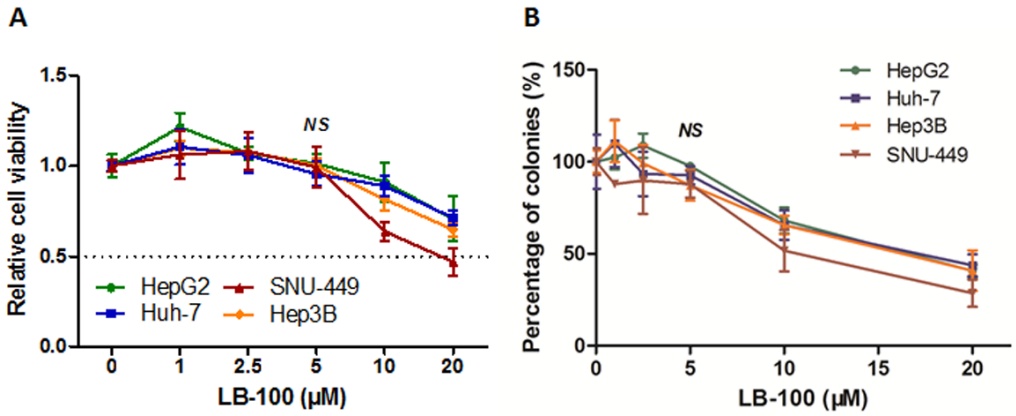
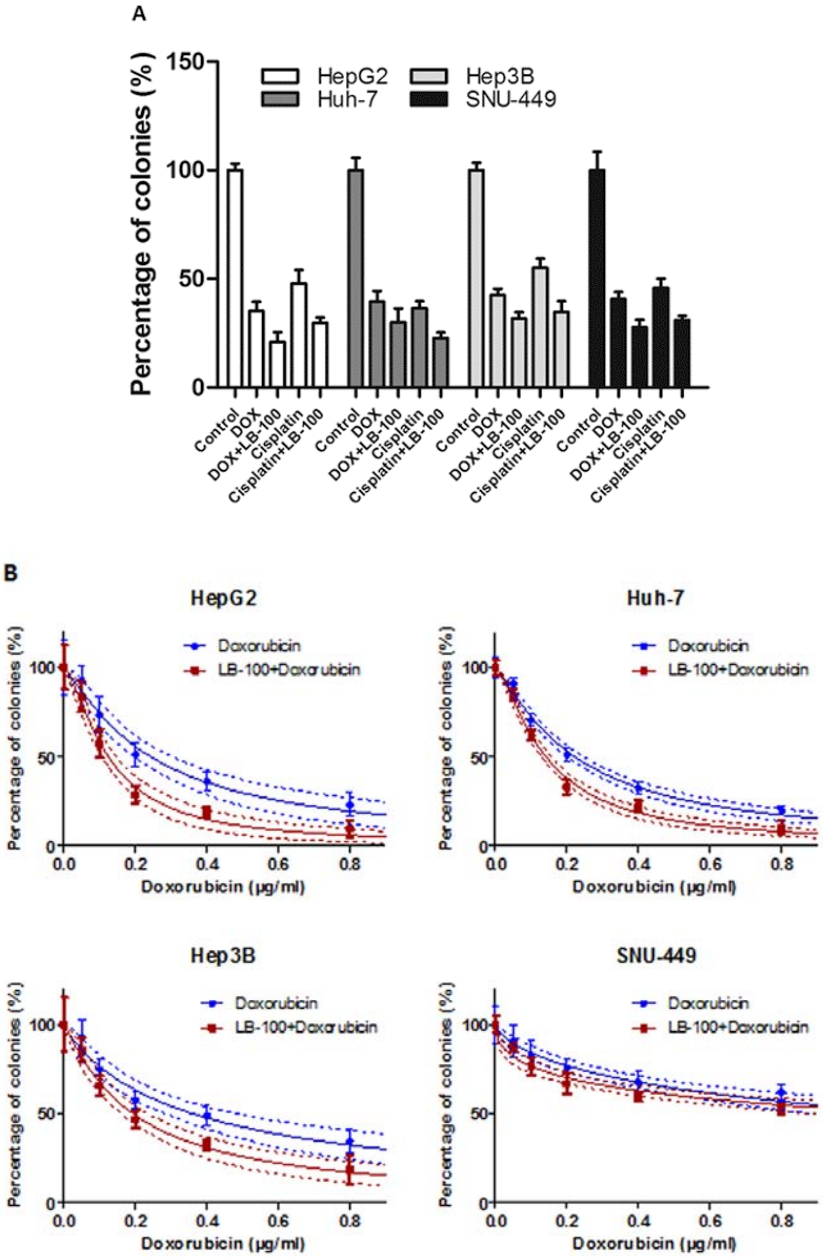


Fig. S1. Toxicity of LB-100 in different cell lines. Four liver cancer cell lines were treated with indicated concentration of LB-100 for 48 h. (A) Cell viability was assessed by CCK-8 assays. (B) Cells were seed in 6-well plate in a density of 200 cells per well (500 cells for HepG2). After 2 weeks, colonies were stained with Giemsa stain and the number of colonies was calculated. All data were normalize to cells without LB-100 treatment. Doses up to 5 μM of LB-100 did not show any toxicity to cells. N = 3. Data are presented as mean ± SD.

Figure S2

Object description: Clonogenic assays were additionally performed to verify the chemosensization effects of LB-100 on doxorubicin and cisplatin treatments. The role of LB-100 in combinative chemotherapy showed similar pattern with the results of CCK-8 assays (Fig. S2A). In the presence of LB-100, the half-maximal inhibitory concentration (IC_{50}) of doxorubicin and cisplatin was significantly reduced (Fig. S2B and S2C). Taken together, these results showed a convinced chemosensization of LB-100.



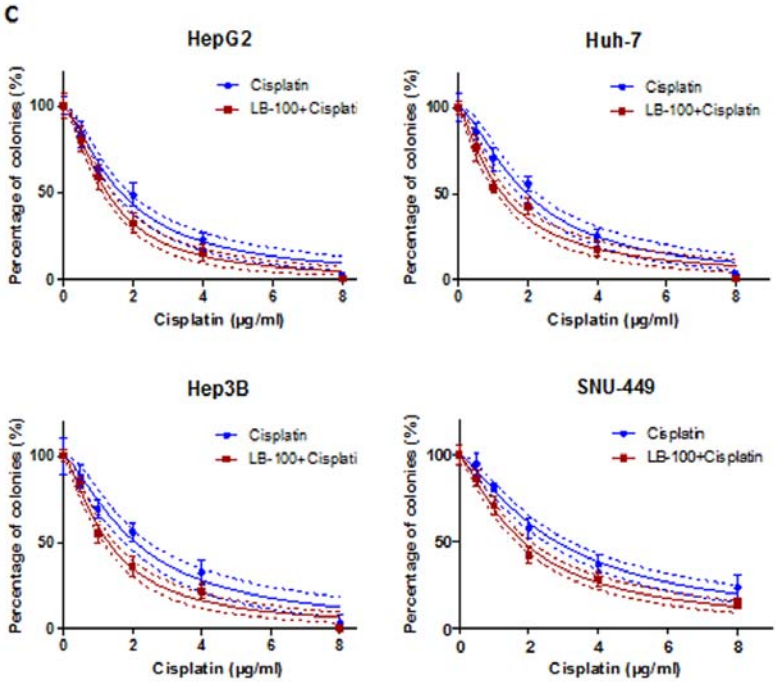


Fig. S2. Clonogenic assays of LB1 chemosensization. (**A**) Cells were treated with doxorubicin (0.2 μg/ml) or cisplatin (2 μg/ml), with or without LB-100 (5 μM) for 24 hours. Cells were seeded in 6-well plate, and after 2 weeks colonies were stained with Giemsa. The number of colonies were calcualted and normalized to control group. N = 3. Data are presented as mean ± SEM. Cells were treated with varied concentrations of doxorubicin (**B**) or cisplatin (**C**), with or without LB-100 (5μM) for 48 hours. Clonogenic survival assays were then performed. After 2 weeks, colonies were calculated. Relative cell survival rate was calculated as percentage of controls without doxorubicin treatment. N = 3. Data are presented as mean ± SD, 95% confidence interval (CI) was plotted.

Figure S3

Object description: To confirm whether LB-100-induced chemosensization was PP2A-dependent, we use a plasmid to upregulate the expression of PP2A catalytic subunit (PP2A_C). PP2A_C overexpression was confirmed by immunoblotting (Fig. S3A, upper panel). CCK-8 assays were perfomed to evaluated the effects of PP2A_C overexpression on LB-100-induced chemosensization *in vitro*. As expected, PP2A_C overexpression blocked LB-100-induced chemosensization in Huh-7 cells (Fig. S3A lower panel). *In vivo*, mice were inoculated with Huh-7 cells transfected with PP2A_C overexpression plasmids, and doxorubicin with or without LB-100 were used to treat mice. Doxorubicin showed mild therapeutic effect as compared to control group (also with PP2A_C transfection); however, LB-100 was not able to enhance the therapeutic effect of doxorubicin (Fig. S3B). Together with the previous data (Figs. 2A, 3A and 4A), these results suggested that LB-100 sensitized chemotherapy by a PP2A-dependent way.

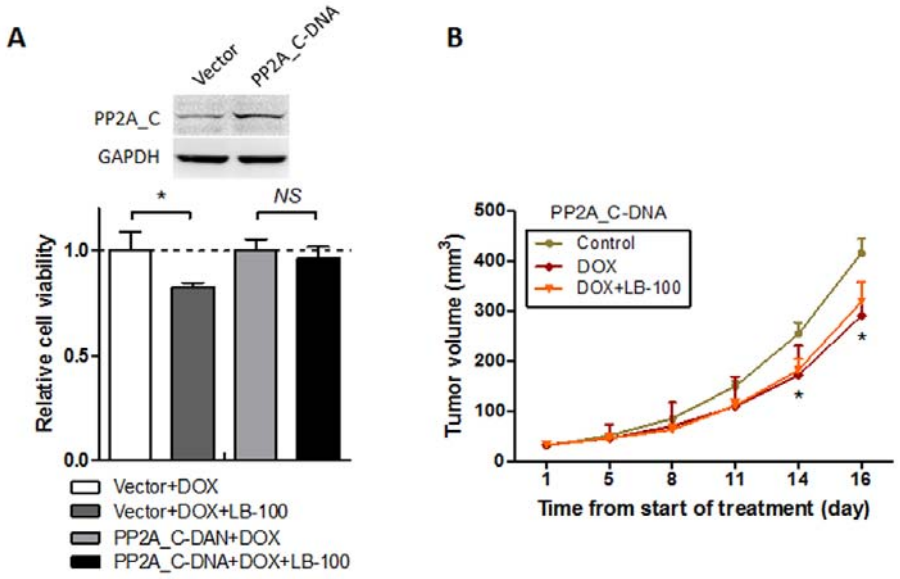


Fig. S3. PP2A_C overexpression attenuated LB-100 chemosensitization *in vitro* and *in vivo*. (A) PP2A_C overexpression plasmid (PP2A_C-DNA) or vector was transfected into Huh-7 cells. LB-100 enhanced cytotoxicity of doxorubicin (DOX) in cells with vector transfection, however, this chemosensization effect of LB-100 was blocked in cell with PP2A_C-DNA transfection. N = 3. Data are presented as mean±SEM. * $P < 0.05$. (B) Huh-7 cells were transfected with PP2A_C-DNA before subcutaneously injected to generate xenograft mouse model. All mice were allocated into three groups: Control, DOX, DOX plus LB-100. DOX (1.5 mg/kg, qod) and LB-100 (2 mg/kg, qod) were intraperitoneally injected, and tumor volume was detected. N = 6. Data are presented as mean±SEM. * $P < 0.05$, DOX or DOX+LB-100 *vs.* Control.

Figure S4

Object description: LB-100 significantly increased the percentage of cells with abnormal nuclei in HepG2 and Huh-7 cells.

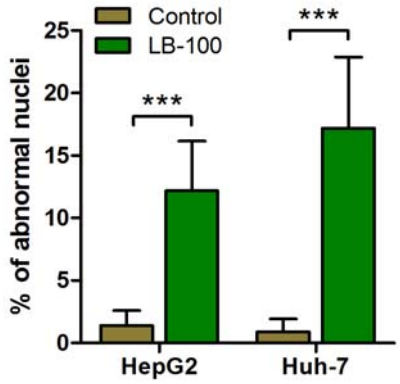


Fig. S4. Percentage of cells with abnormal nuclei. HepG2 and Huh-7 cells were treated with LB-100 (5 μM, 24 h) or vehicle, followed by DAPI staining for 5 min. Abnormal nuclei were quantified in five random vision (× 200) under microscope. Data are presented as mean ± SD, *** $P < 0.001$.

Figure S5

Object description: To explore whether VEGF secretion influenced by LB-100 was PP2A-dependent, we used a siRNA and an overexpression plasmid to change the level of PP2A catalytic subunit (PP2A_C) in Huh-7 cells. Expression of PP2A_C was detected by immunoblotting (Fig. S5A), and Expression of PP2A_C positively correlated with PP2A activity (Fig. S5B). Notably, VEGF secretion was enhanced when PP2A_C expression decreased, and vice versa ($P < 0.05$; Fig. S5C).

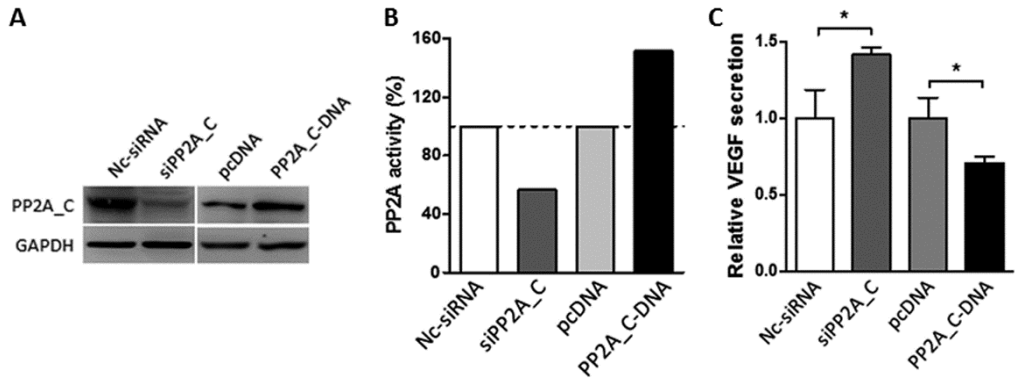


Fig. S5. PP2A activity is associated with VEGF secretion. Huh-7 cells were transfected with PP2A_C siRNA (50 nM; Santa Cruz Biotechnology) and PP2A_C overexpression plasmid as well as their controls (Nc-siRNA and plasmid vehicle, respectively). (A) After 48 hours, cells were lysed and immunoblotting was performed. (B) PP2A activity of the transfected cell was evaluated. (C) VEGF in medium was assessed by ELISA assays. N = 3, data are presented as mean±SD. PP2A activity and VEGF concentration were normalized to controls as indicated. * $P < 0.05$.

Figure S6

Object description: Before performing vascular permeability assays *in vitro*, the integrity of human umbilical vein endothelial cell (HUVEC) monolayer had to be verified. The intact monolayers were confirmed in all groups.

Fig. S6. Pictures of HUVECs monolayers before and after doxorubicin treatment as indicated in Fig. 6A. The intact monolayers could be seen in all groups (× 40).

Figure S7

Object description: LB-100 increased vascular permeability and led to higher concerntration of chemotherapeutic drugs within tumors, however, only higher concentration and/or longer stay of drugs within tumor cell made great sense. We quantified the amount of doxorubicin within cells in Huh-7 and HepG2 cells in different time after doxorubicin treatment. LB-100 significantly promoted early entry of doxorubicin into cells, resulting in more doxorubicin within cells in 12 hours after treatment (Fig. S7A). Fluoresence of doxorubicin by laser confocal microscope confirmed this effect of LB-100, showing higher fluoresence intensity in cells with LB-100 addition ($P < 0.05$; Fig. S7B).

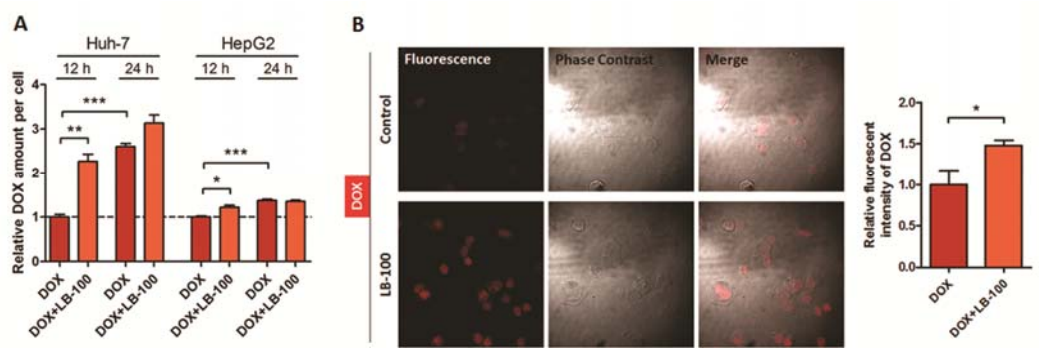


Fig. S7. LB-100 facilitated early entry of doxorubicin into tumor cells. (A) Huh-7 and HepG2 cells were treated with doxorubicin (DOX; 0.2 μg/ml) with or without LB-100 (5 μM). After indicated times, cells were washed with PBS sufficiently and lysed. The amount of doxorubicin in lysates was quantified as fluorescence measured with excitation wavelength 480 nm and emission wavelength 580 nm. All results were calibrated to cell number and normalized to doxorubicin group in 12 hours. LB-100 increased intracellular doxorubicin in 12 hours but not 24 hours. Data are presented as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) Huh-7 cells was treated with DOX (5 μM) with or without LB-100 (5 μM) for 8 hours. Cells was washed and observed by a confocal microscope, with excitation wavelength 480 nm and emission wavelength 580 nm. The fluorescent intensity of DOX was quantified all results were normalized to DOX alone group. N = 3 or 4. Data are presented as mean ± SEM. * $P <$ 0.05.