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ORIGINAL ARTICLE

Liposomal borrelidin for treatment of metastatic breast cancer

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Abstract

Borrelidin is an inhibitor of threonyl-tRNA synthetase with both anticancer and antiangiogenic activities. Although borrelidin could be a potent drug that can treat metastatic cancer through synergistic therapeutic effects, its severe liver toxicity has limited the use for cancer therapeutics. In this study, we developed a liposomal formulation of borrelidin to treat metastatic breast cancer effectively through its combined anticancer and antiangiogenic effects while reducing the potential liver toxicity. The liposomal formulation was optimized to maximize loading stability and efficiency of lipophilic borrelidin in the liposomal membrane and its delivery efficiency to primary tumor in a mouse model of metastatic breast cancer. Liposomal borrelidin showed significant in vitro therapeutic effects on proliferation and migration of tumor cells and angiogenesis of endothelial cells. Furthermore, liposomal borrelidin exhibited superior inhibitory effects on primary tumor growth and lung metastasis in vivo compared to free borrelidin. More importantly, liposomal borrelidin did not induce any significant systemic toxicity in the mouse model after multiple injections.

Keywords Angiogenesis · Borrelidin · Chemotherapy · Liposome · Metastatic cancer

Introduction

Cancer metastasis is the spread of cancer cells from a primary tumor to other parts of the body. It has been estimated that metastasis accounts for approximately 90% of all cancer-related deaths [1]. Despite multidisciplinary efforts, metastasis has remained the biggest hurdle to be overcome for effective cancer therapy. At the early stage of metastasis cascade, angiogenesis is required to promote direct disposal of cancer cells into the blood stream [2]. Thus, in order to suppress metastasis into distant organs, antiangiogenic therapy could be an effective therapeutic option along with primary tumor removal and chemotherapy [3, 4].

Borrelidin, which is isolated from *streptomyces* and other bacteria [5], is a natural lipophilic small molecule drug with a molecular weight of 489.6 g/mol and a partition coefficient value of 5.6 [6]. Since borrelidin is an inhibitor of threonyl-tRNA synthetase, it induces disruption of threonine-containing protein synthesis, resulting in antibacterial, antimalarial, antibacterial, antiangiogenic, and anticancer effects [7, 8]. Its potent anticancer activity has been reported in the lung, mammary, lymphocytic leukemia, colon, melanoma, and pancreatic cancer [9–12]. In addition, borrelidin induces apoptosis in endothelial cells and inhibits tube formation during angiogenesis progress via activation of caspase 3/8 pathway [13, 14]. Thus, borrelidin could be a potent drug that can achieve synergistic therapeutic benefits in treating metastatic cancer by attacking both cancer and endothelial cells simultaneously. However, its severe in vivo toxicity such as dilatation of central and hepatic veins and swelling of hepatocytes has limited its use for cancer therapeutics [6].

In this study, we develop a liposomal formulation of borrelidin to leverage the potential of its combined anticancer and antiangiogenic activities for effective prevention and treatment of metastatic breast cancer. We take advantage of long-circulating liposomes to achieve efficient delivery of toxic borrelidin to hypervascularized regions of primary tumor

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via enhanced permeability and retention effect while minimizing its accumulation in normal tissues [15–20]. We engineer a liposomal formulation to maximize loading stability and efficiency of lipophilic borrelidin in the liposomal membrane and its delivery efficiency to primary tumor in a mouse model of metastatic breast cancer. We then evaluate in vitro therapeutic effects of liposomal borrelidin on proliferation and migration of tumor cells and angiogenesis of endothelial cells. Lastly, we assess in vivo therapeutic effects of liposomal borrelidin on primary tumor growth and lung metastasis.

Materials and methods

Cell culture

4T1 mouse breast cancer cells were cultured in RPMI supplemented with 10% FBS (Hyclone, UT, USA) and 1% penicillin/streptomycin (Hyclone, UT, USA). Human umbilical vein endothelial cells (HUVEC) were cultured in EGM-2 Bulletkit media (Youngscience, South Korea). HUVECs in passage 3~4 were used in experiments. All cells were incubated at 37 °C in 5% CO_2.

Preparation and characterization of liposomal borrelidin

Liposomal formulations of borrelidin were prepared with phosphatidylcholine-based backbone lipids and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG (2000), Avanti Polar Lipids) in the molar ratio of 95:5. The backbone lipids included L-α-phosphatidylcholine, hydrogenated (soy) (HSPC, Avanti Polar Lipids), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids), and L-α-phosphatidylcholine (egg) (EPC, Avanti Polar Lipids). Lipid solution containing 7 μmol of backbone lipids and DSPE-mPEG(2000) prepared in chloroform was mixed with borrelidin (LKT laboratories, USA) in the molar ratio of 100(lipid):2.91(borrelidin). The chloroform was evaporated for 24 h in a chemical hood. Completely dried lipid cake was hydrated with 1 mL of phosphate buffered saline (PBS) preheated above the phase transition temperature of backbone lipid, and then stirred with a magnetic bar for 30 min at 700 rpm at the transition temperature to form large multilamellar vesicles. The liposome size was controlled using an extrusion method with a 100 nm polycarbonate membrane at the transition temperature. After extrusion, free borrelidin was removed by Sephadex G-50 column (GE healthcare, USA). Doxil® mimicking liposomal formulation without doxorubicin was prepared with L-α-phosphatidylcholine, hydrogenated (soy) (HSPC, Avanti Polar Lipids), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG (2000), Avanti Polar Lipids), and cholesterol (ovine) (Avanti Polar Lipids), in the molar ratio of 56:5:39 [21, 22]. For fluorescently labeled liposomes, 10 μg of DiR (Invitrogen, USA) in 100% ethanol stock solution was added to the lipid solution before the lipid cake was prepared by solvent evaporation. All liposomes were stored in PBS at 4 °C until ready for use. Hydrodynamic size and polydispersity index (PDI) of liposomal formulations were measured by Dynamic Light Scattering (Zetasizer Nano ZS90, Malvern Instrument, MA, USA). Loading efficiency of borrelidin in the liposomal formulation was measured with UV-Vis spectrophotometer (Molecular Devices, CA, USA) after dissolving the liposomal borrelidin in 100% ethanol (absorption peak: 260 nm). The loading efficiency was calculated by dividing the final amount of borrelidin loaded in the liposome by the initial amount of borrelidin added to the lipid cake. These experiments were performed at least twice with different batches of liposomes.

Colloidal and borrelidin loading stability

To evaluate colloidal stability, liposomal borrelidin was incubated in PBS at 37 °C for 0, 6, 12, and 24 h ($n = 3$). The hydrodynamic size at each time point was measured by Dynamic light scattering. To evaluate borrelidin loading stability, liposomal borrelidin was incubated in PBS containing 10% serum at 37 °C for 0, 6, 12, and 24 h and the amount of borrelidin remaining in the liposomes was measured by UV-Vis spectrophotometer ($n = 3$). The solution containing borrelidin released from the liposomes was separated from the liposomes by using a centrifugal filter (100 kDa MWCO, Merck Millipore). The amount of borrelidin released from the liposomes at each time point was determined by measuring the absorbance of the solution and subtracting that of a control solution (PBS containing 10% serum). The amount of borrelidin remaining in the liposome was calculated by subtracting the amount of borrelidin released from the liposome from the amount of borrelidin loaded initially in the liposome.

In vitro cytotoxicity

1×10^4 4T1 cells were seeded in 48 well plates. After 24 h, the cells were treated with free or liposomal borrelidin at various concentrations (0, 6.25, 12.5, 25, 50, 100, 200, and 400 nM) for 48 h ($n = 3$). For MTT assay, the cells were treated with thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich, MO, USA) for 4 h and then the supernatants were removed. The violet crystals were dissolved in DMSO and the absorption was measured using UV-Vis spectrophotometer.

In vitro wound healing assay

1.5×10^5 4T1 cells or human umbilical vein endothelial cells (HUVEC) were seeded in 24-well plates. After 24 h, the cells were treated with empty liposome (the same amount of lipids with liposomal borrelidin), free or liposomal borrelidin at a borrelidin concentration of 30 nM (for 4T1) or 60 nM (for HUVEC) for 8 h ($n = 3$~4). After treatment, a scratch was made into a monolayer of cells using a yellow pipette tip and imaged by optical microscopy immediately and 12 h (for 4T1) or 6 h (for HUVEC) after wound formation [23]. The borrelidin concentration was chosen based on the IC_{50} value for 4T1 cell and the previous report for HUVEC [13]. Recovery level of the cells was analyzed by measuring the percentage wound closure of cells in randomly chosen spots.

In vitro capillary tube forming assay

Matrigel Basement Membrane Matrix (phenol red free) at concentrations of 18–22 mg/mL (BD bioscience, South Korea) was thawed in the ice overnight. Then, thawed matrigel was mixed with pre-cooled PBS in the volume ratio of 1:1.50 μL of matrigel solution was coated in each 48-well plate with pre-cooled pipette tips. Matrigel-coated 48-well plates were placed in 37 °C incubator for 30 min. 1×10^4 HUVEC were seeded on the matrigel-coated plates and treated with empty liposome (the same amount of lipid with liposomal borrelidin), free or liposomal borrelidin at a borrelidin concentration of 30 nM for 4 h ($n = 3$). The plates were imaged by optical microscopy immediately and 18 h after treatment [24, 25]. The borrelidin concentration was chosen based on the previous report [13]. Capillary tube forming level of the HUVEC was analyzed by measuring the total length of the tube in randomly chosen spots.

In vivo mouse tumor model

Seven-week-old female Balb/c wild type mice were purchased from Koatech (South Korea). 4T1 mouse breast cancer cells were cultured in RPMI with 10% FBS and 1% penicillin/streptomycin. 1×10^6 4 T1 cells were inoculated into the one of the fourth mammary fat pads of Balb/c mice. In vivo experiments were performed 7–10 days after inoculation when the tumors reached an average size of 50~80 mm^3. A caliper was used to measure the width (short) and length (long) of the primary tumor, and the tumor volume was calculated with the following equation: $V = (\text{width}) \times (\text{width}) \times (\text{length}) / 2$. All animal procedures were in agreement with the guidelines and protocols for rodent research provided by the Institutional Animal Care and Use Committee of the Korea Advanced Institute of Science and Technology (KAIST).

Biodistribution

DiR-labeled liposomal borrelidin or Doxil®-mimicking liposomes was intravenously injected into mice bearing 4T1 tumors at a lipid amount of 1 μmol (DiR of 1.43 μg, $n = 5$~7). At 48 h post-injection, the tumor, liver, spleen, kidney, lung, heart, and brain were harvested and imaged using a LI-COR Odyssey fluorescence imaging system (Biosciences, NE). To determine the liposome accumulation in the tumor, liver, and spleen, DiR fluorescence in each organ was quantified and divided by the sum of the fluorescence of all three organs.

In vivo tumor angiogenesis inhibition

PBS, free or liposomal borrelidin was intravenously injected into mice bearing 4T1 tumors once at a dose of 0.5 mg/kg (free and liposomal borrelidin) or 5 mg/kg (free borrelidin) ($n = 3$). Single dose was used because we wanted to see anti-angiogenesis effects of free or liposomal borrelidin without affecting tumor size. At 96 h post-injection, the tumors were harvested and rapidly frozen in OCT compound (Leica, Germany). The tumors were cryo-sectioned, blocked with a blocking solution (10% goat serum, 1% BSA and 0.02% Tween in PBS) for 30 min, incubated with rat anti-mouse CD31 antibody (1:50, Invitrogen) overnight at 4 °C, and then incubated with Alexa Fluor®488 goat anti-rat IgG antibody (1:200, Invitrogen) for 1 h at room temperature [26]. The blood vessels were observed in the GFP channel of fluorescence microscopy. To determine inhibitory effects of liposomal borrelidin on tumor angiogenesis, the average fluorescence intensity of blood vessels in the peripheral region of tumors (within 400 μm from edge [27]) was analyzed by Image J program.

In vivo tumor and lung metastasis therapy

PBS, free or liposomal borrelidin was intravenously injected into mice bearing 4T1 tumors at a dose of 0.4 mg/kg ($n =$ 4~6). The injections were done every 4 days for 20 days (total 6 injections). Changes in tumor volume and mouse body weight were observed before every injection. At 4 days after the last injection when the average tumor volume of control group was over 1000 mm^3, the mice were sacrificed. Lungs were excised and lung metastatic nodules with widths larger than 1 mm were counted.

In vivo organ histology

PBS, free or liposomal borrelidin was intravenously injected into mice bearing 4T1 tumors at a dose of 0.5 mg/kg ($n =$ 5~6). Higher injection frequency was used to study organ histology because it better mimics the clinical setting. The injections were done every 3 days for 27 days (total 10

injections). At 3 days after the last injection, the liver, kidney, spleen, and heart were harvested and rapidly frozen in OCT compound (Leica, Germany). The organs were cryo-sectioned, and stained with hematoxylin and eosin (H&E). To identify liver toxicity, dilation of hepatic veins was observed.

Results

Preparation and characterization of liposomal borrelidin

Borrelidin is a lipophilic molecule with partition coefficient value 5.65 [6]. In order to take advantage of nanoparticles to solubilize borrelidin in the physiological solution and enhance its tumor accumulation, we selected a liposomal formulation because it can load lipophilic drug in its lipid bilayer with high stability and circulate relatively long in the blood stream [20, 28, 29] (Fig. 1a). Furthermore, the PEGylated liposomal formulation of doxorubicin has been approved as Doxil® in FDA for cancer treatment [19, 30]. Various liposomal formulations were prepared with phosphatidylcholine(PC)-based backbone lipids (DMPC, DPPC, HSPC, EPC, or DOPC), DSPE-mPEG(2000), and borrelidin in the molar ratio of 95:5:2.91 (Table 1). Briefly, lipids and borrelidin were mixed in chloroform and evaporated to form a borrelidin-incorporated lipid film. The lipid film was then hydrated with PBS, extruded with 100-nm membrane filter, and washed on the desalting column. Hydrodynamic sizes of all liposomal formulations appeared in the range of 100–130 nm whereas the borrelidin loading efficiencies were higher in the DPPC- and HSPC-based liposomal formulations prepared with higher number of saturated carbon chains (Table 1). We speculate that lipophilic borrelidin could be embedded more readily between longer saturated lipid tails. DPPC- and HSPC-based liposomal formulations retained their hydrodynamic sizes over the 24-h incubation period (Fig. 1b). Additionally, they retained more than 80% of borrelidin after 24-h incubation in the physiological condition (Fig. 1c). Thus, these results demonstrate that DPPC- and HSPC-based liposomal formulations packaged considerable amount of borrelidin with high stability.

Next, we evaluated the biodistribution of DPPC- and HSPC-based liposomal borrelidin in the metastatic mouse model. A 4T1 orthotopic breast cancer spontaneous metastasis mouse model was chosen in this study because it is one of

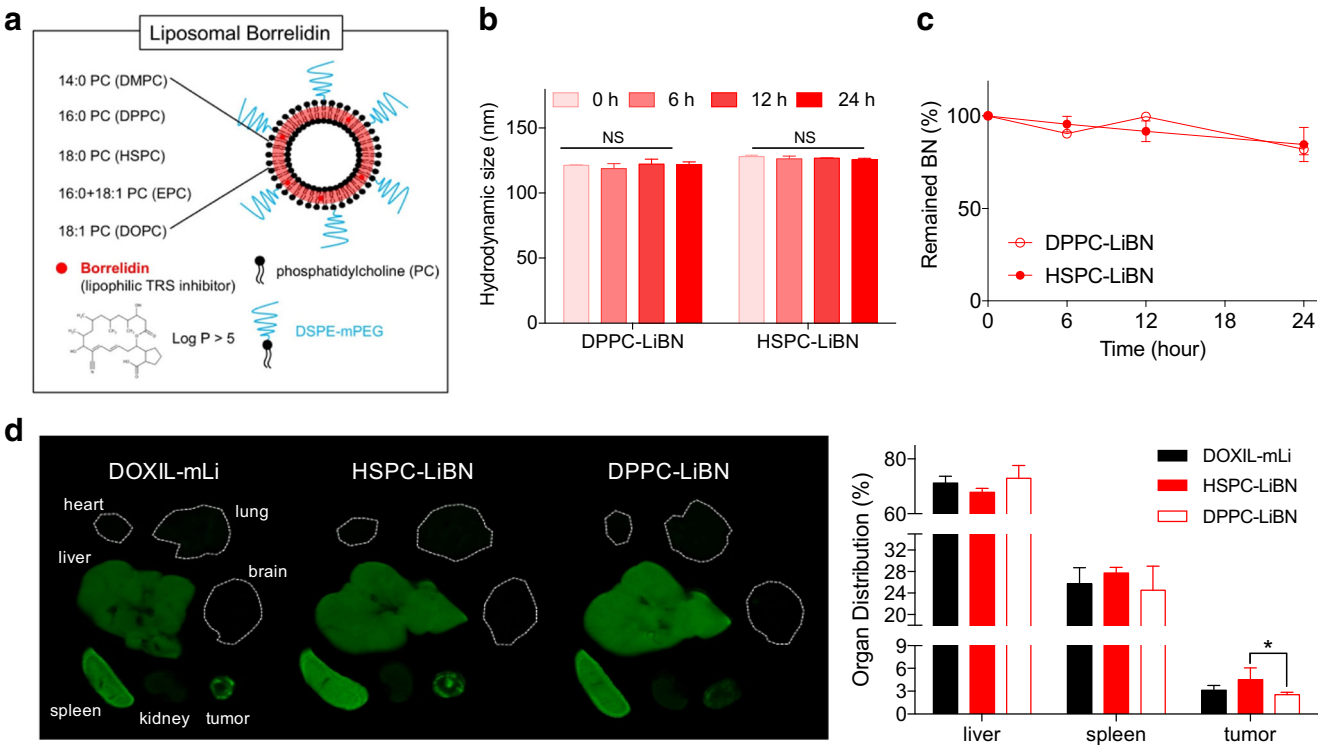

Fig. 1 Preparation and optimization of liposomal borrelidin. **a** Scheme of liposomal borrelidin. **b** Colloidal stability of liposomal borrelidin in PBS at 37 °C. **c** Borrelidin loading stability of DPPC-LiBN and HSPC-LiBN in PBS containing 10% serum at 37 °C. **d** Organ distribution of DiR-labeled DPPC-LiBN and HSPC-LiBN, and DOXIL-mLi. At 48 h post-injection, the tumor, kidney, spleen, liver, brain, lung, and heart were harvested and imaged using a near-infrared fluorescence imaging system (left). The DiR fluorescence was quantified in the liver, spleen, and tumor (right). DPPC-LiBN, HSPC-LiBN, and DOXIL-mLi indicate DPPC-based and HSPC-based liposomal borrelidin, and DOXIL®-mimicking liposome, respectively. Data represent mean ± S.D. ($n = 3$, NS, not significant, one-way ANOVA with Bonferroni's multiple comparison test for **b**; $n = 3$ for **c**; $n = 5$~7, $^*p < 0.05$, one-way ANOVA followed by the Bonferroni's multiple comparison test for **d**)

Table 1 Lipid compositions and physicochemical properties of liposomal borrelidin used in this study

Liposome name	Lipid composition (molar ratio)		Hydrodynamic size[b] (nm)	PDI[a]	Borrelidin loading efficiency (%)[c]
	Backbone lipid[a]	DSPE-PEG (2000)			
DMPC-LiBN	95 [DMPC (14:0)]	5	110.43 ± 7.08	0.083 ± 0.023	0.75 ± 0.73
DPPC-LiBN	95 [DPPC (16:0)]	5	106.23 ± 1.96	0.085 ± 0.020	15.35 ± 5.51
HSPC-LiBN	95 [HSPC (~18:0)]	5	110.83 ± 1.36	0.102 ± 0.011	31.0 ± 3.20
EPC-LiBN	95 [EPC (~16:0/18:1)]	5	118.47 ± 12.62	0.062 ± 0.012	3.27 ± 1.52
DOPC-LiBN	95 [DOPC (18:1)]	5	127.50 ± 4.04	0.057 ± 0.012	1.00 ± 0.78

[a] *DMPC*, *DPPC*, *HSPC*, *EPC*, and *DOPC* indicate 1,2-dimyristoyl-sn-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, L-α-phosphatidylcholine, hydrogenated (soy), L-α-phosphatidylcholine (egg), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, respectively

[b] Mean hydrodynamic sizes and polydispersity indexes (PDI) of the liposomal formulation were determined based on dynamic light scattering measurements ($n = 3$)

[c] Loading efficiency of borrelidin in the liposomal formulation was calculated by dividing the final amount of borrelidin loaded in the liposome by the initial amount of borrelidin added to the lipid cake. The amount of borrelidin loaded in the liposome was determined based on UV-Vis absorption measurements after dissolving the liposomal borrelidin in 100% ethanol (260 nm, $n = 2$)

triple negative breast cancer models needed for new therapeutic strategies and represents the most aggressive metastasis in lung [31, 32]. Doxil®-mimicking formulation was also prepared for comparison. All liposomal formulations were labeled with lipophilic DiR fluorophore to analyze organ accumulation based on the fluorescence. Mice bearing 4T1 tumors were intravenously injected with liposomal formulations. At 48 h post-injection, the heart, lung, brain, liver, spleen, kidney, and tumor were imaged by using a NIR fluorescence imaging system and the DiR fluorescence in each organ was quantified. Tumor accumulation of HSPC-based liposomal borrelidin was significantly greater than that of DPPC-based liposomal borrelidin and comparable to that of Doxil®-mimicking formulation (Fig. 1d). However, substantial quantities of liposomal formulations were accumulated in the liver and spleen regardless of liposomal type due to macrophage-mediated clearance of circulating nanoparticles in the mononuclear phagocyte system [33]. Thus, HSPC-based liposomal borrelidin was chosen as an optimal liposomal formulation for borrelidin and used for following experiments because it maximized primary tumor accumulation as well as borrelidin loading stability and efficiency.

Inhibition of tumor cell proliferation and migration in vitro

We first tested cytotoxic effects of liposomal borrelidin on tumor cells to determine its therapeutic potential against primary tumor growth. 4T1 murine breast cancer cells were treated with free or liposomal borrelidin for 48 h, and the cell viability was analyzed by MTT assay. IC_{50} values were similar in both free and liposomal borrelidin (22.07 nM and 25.57 nM, respectively) (Fig. 2a). These IC_{50} values were significantly lower than that of doxorubicin which is one of the conventional drugs for breast cancer treatment (170 nM against 4T1 cells) [34], indicating that borrelidin-based therapy could be more effective against triple-negative breast cancer than doxorubicin-based therapy. This superior therapeutic effect of borrelidin was also found in MDA-MB-231 human triple negative breast cancer cells based on their IC_{50} values (17.06 nM for free borrelidin and 36.81 nM for liposomal borrelidin vs. ~200 nM for doxorubicin [35]) (Supplementary Fig. 1A). We next performed a wound healing assay to evaluate antimetastatic potential of liposomal borrelidin. The wound healing assay allows us to study inhibitory effects of liposomal borrelidin on tumor cell proliferation and migration in vitro [36, 37]. A scratch was made into a monolayer of 4T1 cells after the cells were treated with empty liposome, free or liposomal borrelidin for 8 h. After 12 h, the width of wounded cell monolayer was measured to determine the recovery of tumor cells. Both free and liposomal borrelidin showed significant inhibitory effects on tumor cell migration (Fig. 2b). The recovery percentages of tumor cells were similar in both free and liposomal borrelidin. Empty liposomes alone did not inhibit the tumor cell migration significantly, indicating that borrelidin-mediated therapeutic effects mainly determined the inhibition of tumor cell migration. This inhibitory effect was also found in MDA-MB-231 human breast cancer cells (Supplementary Fig. 1B). Thus, these results suggest that cytotoxic effects of liposomal borrelidin can be used to inhibit both primary tumor growth and metastasis.

Inhibition of endothelial cell migration and tube formation in vitro and angiogenesis in vivo

In order to evaluate the antiangiogenic potential of liposomal borrelidin, we first studied its inhibitory effects on endothelial

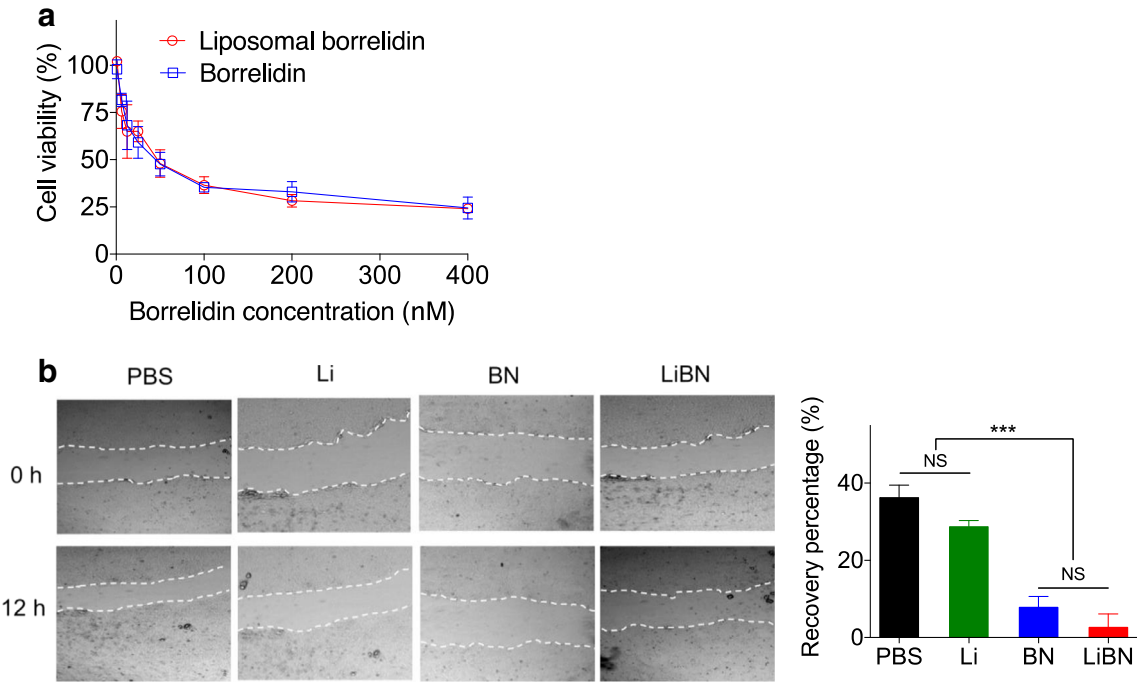

Fig. 2 Inhibition of tumor cell proliferation and migration in vitro. **a** Cytotoxicity of free and liposomal borrelidin against 4T1 mouse breast tumor cells. The cells were treated with free or liposomal borrelidin at various concentrations for 48 h and their viability was determined by MTT assay. **b** Wound recovery of 4T1 tumor cells treated with empty liposome, free or liposomal borrelidin. The cells were treated with empty liposome, free or liposomal borrelidin for 8 h and then a scratch was made into a monolayer of 4T1 cells. After 12 h, the width of wounded cell monolayer was imaged (left) and quantified (right) to determine the recovery of tumor cells. Li, BN, and LiBN indicate empty liposomes, free and liposomal borrelidin, respectively. Scale bar indicates 200 μm. Data represent mean ± S.D. ($n = 3$~4, NS not significant, ***$p < 0.001$, one-way ANOVA with Bonferroni's multiple comparison test)

cell proliferation and migration in vitro using a wound-healing assay. Human umbilical vein endothelial cells (HUVEC) were treated with empty liposome, free or liposomal borrelidin for 8 h. A scratch was then made into a monolayer of HUVEC. After 6 h, the width of wounded cell monolayer was measured to determine the recovery of endothelial cells. As observed in the tumor cell result, liposomal borrelidin possessed significant inhibitory effects on endothelial cell proliferation and migration, which was comparable of that of free borrelidin (Fig. 3a).

We next evaluated the antiangiogenic potential of liposomal borrelidin in vitro and in vivo. We first employed a tube formation assay to determine the ability of liposomal borrelidin to inhibit capillary-like structure formation of HUVEC in vitro. HUVECs were seeded on the matrigel-coated plates and treated with empty liposomes, free or liposomal borrelidin. At 18 h post-treatment, the capillary tube formation of HUVEC was imaged by optical microscopy to measure the total tube length. Optical microscopy and quantification data revealed that liposomal borrelidin significantly inhibited the capillary tube formation of HUVEC (Fig. 3b), indicating that the borrelidin was delivered efficiently to endothelial cells using HSPC-based liposomal formulation to induce the antiangiogenic effect. We next studied whether liposomal borrelidin can disrupt angiogenic capillary vessels effectively in the primary tumor by taking advantage of nanoparticle-mediated tumor accumulation. Mice bearing 4T1 tumors were intravenously injected with free or liposomal borrelidin. At 96 h post-injection, the tumors were stained and imaged by fluorescence microscopy, and the vessel confluence was quantified in the angiogenic tumor periphery. Fluorescence microscopy and quantification data revealed that liposomal borrelidin significantly reduced the vessel density in the tumor periphery (Fig. 3c). Importantly, vascular disrupting effect of liposomal borrelidin was significantly higher than that of free borrelidin injected at a 10 times higher dose, indicating the enhanced tumor delivery efficiency of liposomal borrelidin. These results suggest that liposomal borrelidin accumulated in the perivascular regions of primary tumor can effectively induce the antiangiogenic effect to prevent intravasation of tumor cells into the circulation.

Inhibition of primary tumor growth and lung metastasis in vivo

We then assessed in vivo therapeutic effects of liposomal borrelidin on primary tumor growth and lung metastasis. Mice bearing 4 T1 tumors were intravenously injected with free or liposomal borrelidin at a borrelidin dose of 0.4 mg/kg every 4 days (total six injections) while the tumor volume and

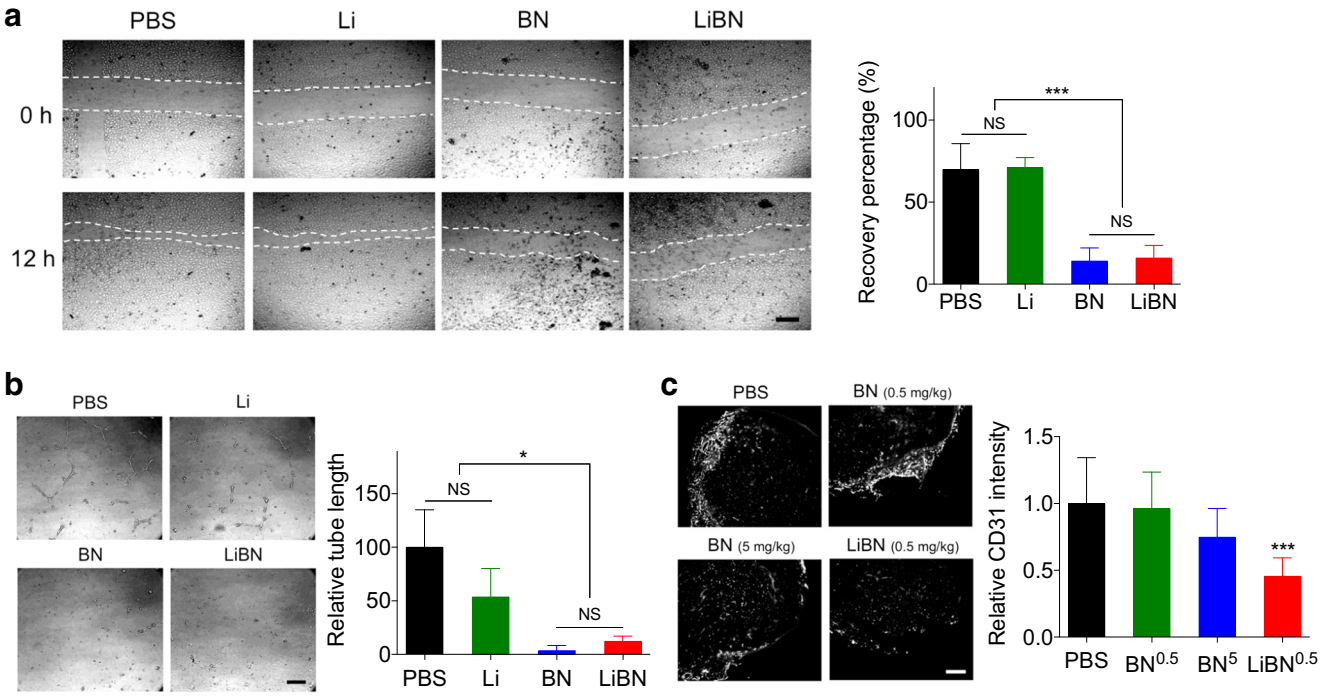

Fig. 3 Inhibition of endothelial cell migration and tube formation in vitro and angiogenesis in vivo. **a** Wound recovery of HUVEC treated with empty liposome, free or liposomal borrelidin. The width of wounded cell monolayer was imaged by optical microscopy (left) and quantified to determine the recovery percentage of endothelial cells (right). **b** Capillary tube formation of HUVEC treated with empty liposome, free or liposomal borrelidin. The capillary tube formation of HUVEC was imaged by optical microscopy (left) and quantified to measure the total tube length (right). **c** Fluorescence images and quantification of tumor vessels after intravenous injection of PBS, free or liposomal borrelidin. The vessel confluence was quantified in the angiogenic tumor periphery. Li, BN, and LiBN indicate empty liposomes, free and liposomal borrelidin, respectively. The superscript in the sample name indicates the injection dose (mg/kg). Scale bars indicate 200 μm in **a** and 500 μm in **b** and **c**. Data represent mean ± S.D. ($n = 3$, NS, not significant, $*p < 0.05$, $***p < 0.001$, one-way ANOVA with Bonferroni's multiple comparison test)

mouse body weight were recorded. When the average tumor volume of control group was over 1000 mm^3, the mice were sacrificed, and lung metastatic nodules with widths larger than 1 mm were counted. Liposomal borrelidin showed significantly greater therapeutic effects on the primary tumor growth and lung metastasis than free borrelidin (Fig. 4a, b). Mouse body weight was not changed significantly with both treatments (Fig. 4c). We further examined the potential organ toxicity of liposomal borrelidin at a higher injection frequency. Mice bearing 4T1 tumors were intravenously injected with PBS, free or liposomal borrelidin at a borrelidin dose of 0.5 mg/kg every 3 days (total ten injections) and the main organs were stained with hematoxylin and eosin. Histopathological analysis revealed that the organs treated with liposomal borrelidin

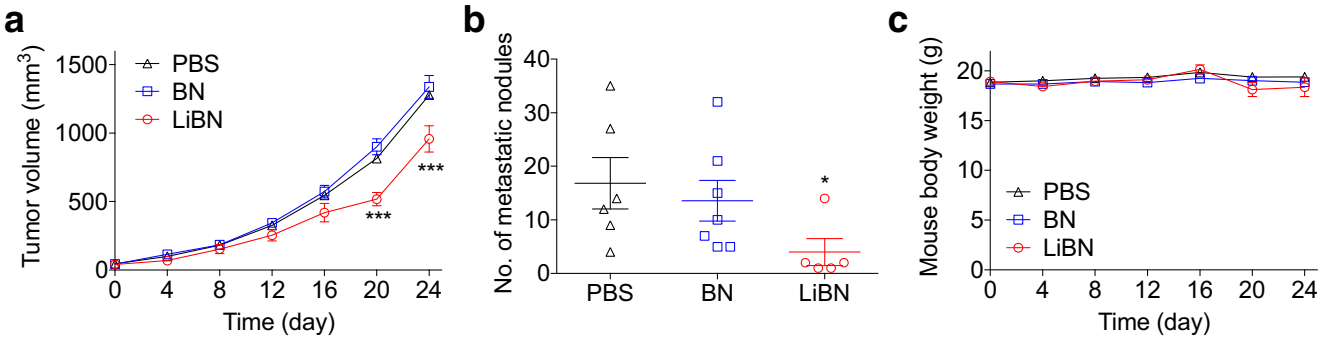

Fig. 4 Inhibition of tumor growth and metastasis in vivo. **a** Primary tumor growth inhibition by liposomal borrelidin in mice bearing 4T1 orthotopic mouse breast tumors. The mice were intravenously injected with free or liposomal borrelidin every 4 days (total six injections). **b** Lung metastasis inhibition by liposomal borrelidin in mice bearing 4T1 tumors. When the average tumor volume of control group was over 1000 mm^3, the mice were sacrificed and lung metastatic nodules with widths larger than 1 mm were counted. **c** Body weight change of the mice injected with free or liposomal borrelidin. BN and LiBN indicate free and liposomal borrelidin, respectively. Data represent mean ± S.E.M. ($n = 4$~6, $***p < 0.001$, two-way ANOVA with Bonferroni's posttest for **a**, $*p < 0.05$, Student's t test for **b**

showed no significant change compared to control organs while the hepatic vein in the liver treated with free borrelidin showed dilation (Fig. 5). Thus, these results suggest that liposomal borrelidin can treat metastatic breast cancer effectively through its combined anticancer and antiangiogenic effects while reducing the in vivo organ toxicity of borrelidin.

Discussion

Borrelidin is a natural small molecule drug with a nanomolar IC_{50} value against many types of cancer cells [6, 7]. It inhibits protein synthesis during active metabolism of cancer cells [5, 8]. In addition, borrelidin is known to effectively kill angiogenic endothelial cells through activating caspase 8/3 pathway [13, 14]. Since the tumor cell migration and angiogenesis are essential steps of the metastatic cascade [3, 4], borrelidin can be leveraged to inhibit metastasis through its combined therapeutic effects. However, it has been challenging to maximize synergistic therapeutic benefits of borrelidin by delivering it efficiently to the proper regions of primary tumor. In this study, we harnessed a PEGylated liposomal formulation to achieve efficient tumor accumulation by packaging lipophilic borrelidin in the liposomal membrane and prolonging its circulation time in blood. We found the optimal liposomal borrelidin composed of HSPC and DSPE-mPEG (2000) in the molar ratio of 95:5 based on three criteria: borrelidin loading efficiency and stability, and tumor accumulation. This liposomal formation can accumulate in the perivascular regions of tumor and interact easily with both tumor and endothelial cells. In in vitro experiments, liposomal borrelidin showed significant inhibitory effects on tumor cell proliferation and migration at the nanomolar concentration. In addition, liposomal borrelidin displayed significant inhibitory effects on endothelial cell migration and tube formation. Such in vitro therapeutic effects were comparable of those of free borrelidin at the same concentration. Furthermore, liposomal borrelidin exhibited superior inhibitory effects on primary tumor growth and lung metastasis in vivo compared to free borrelidin. Particularly, liposomal borrelidin prevented lung metastasis at total injection dose of 2.4 mg/kg (six injections of 0.4 mg/kg) which was much lower than lethal dose 50% (LD50) of borrelidin (39 mg/kg when intravenously injected in mouse) [38]. More importantly, liposomal borrelidin did not show any significant change in mouse body weight and organ histopathology. Taken together, these results suggest that liposomal borrelidin can be used as a therapeutic option to treat metastatic breast cancer without significant side effects. We believe that this effective antimetastatic activities result from enhanced accumulation of liposomal borrelidin in the primary tumor, particularly in the perivascular regions. In addition, empty core of liposomal borrelidin can be used to load hydrophilic drugs such as doxorubicin and cisplatin or RNA-based drugs such as siRNA and miRNA to further

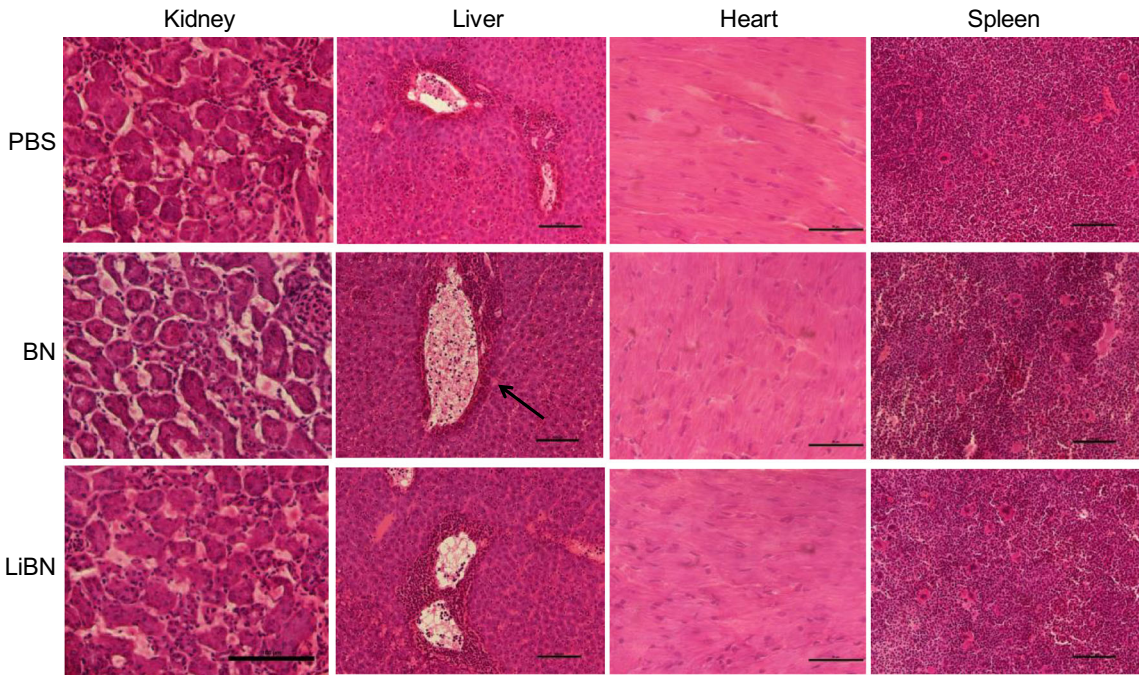

Fig. 5 Microscopic images of the kidney, liver, heart and spleen from the mice injected with liposomal borrelidin or borrelidin. Organs were excised, sectioned, and stained with hematoxylin and eosin (H&E) to examine the organ toxicity. BN and LiBN indicate free and liposomal borrelidin, respectively. Arrow indicates the dilated hepatic vein. Scale bars indicate 100 μm in the kidney and spleen, 500 μm in liver, and 50 μm in heart

enhance both antitumor and antimetastatic effects. Therefore, the liposomal borrelidin has great potential to prevent and treat various types of metastatic cancer.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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