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ORIGINAL RESEARCH

Folic Acid-Targeted Liposome-Based Nanoparticle Loaded with Sorafenib for Liver Cancer Therapy

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Introduction: Sorafenib (SF) is a small molecule involved in tumor proliferation and angiogenesis. SF is inhibitor of several kinases, including RAF, VEGFR, and PDGFR. However the weak targeting ability of SF for liver tumor tissues is the major problem in clinical therapy. Therefore, a SF-loaded folic acid-targeted liposome drug delivery system was devised for targeting liver tumor therapy in this study.

Methods: Folic acid (FA), HSPC, DSPE-PEG_{2k}, CHO, and SF were composed to prepare a folic acid-targeted SF-loaded liposome (LSF) drug delivery system. LSF and drug loading content was established through thin-film-hydration technique and HPLC, respectively. The particle size and stability of LSF were examined by dynamic light scattering (DLS). The inhibition effect of LSF was elucidated in vitro on liver cancer cells through cell cytotoxicity and apoptosis experiments. The tumor-inhibiting efficacy was measured on liver xenograft model.

Results: The drug loading content (DLC) of LSF was 3.6%. The diameter of LSF was 197.1±16.6 nm, and LSF was stable during 24 h. Liver cancer cells could be effectively inhibited by LSF in vitro. LSF could substantially induce apoptosis. Also, LSF could inhibit tumor growth effectively in vivo. LSF could reduce side effects of SF demonstrated by bio-safety tests.

Conclusion: LSF is a FA-targeted drug delivery system that could effectively inhibit the progression of liver cancer.

Keywords: folic acid, liver cancer, liposome, sorafenib, targeting delivery

Introduction

Liver cancer is a prevalent primary malignancy, affecting the liver and ranking high in global cancer-related mortality. Underlying liver disease limits therapeutic efficacy.[1] Although topical therapies play an important role in the therapy of liver cancer, more and more patients are identified at advanced stage and require systemic therapy as the primary treatment.[1]

Folic acid (FA) was first purified in 1931 by Lucy Wills, and belongs to the vitamin B9 (VB9) family member.[2] As a one-carbon (1C) unit carrier, FA could enhance the transport of 1C units, which is closely related with important physiological processes, including the synthesis of purine and thymidine, the homeostasis of amino acids, epigenetic maintenance, and redox defense.[3] In living organisms, the main form of FA is tetrahydrofolate (THF) which is used as a carrier, with the 1C unit involved in biosynthesis and metabolism. The deficiency of FA could inhibit the delivery of 1C units, thus the synthesis of nucleic acid and the metabolism of amino acid could be affected.[4] FA is a potent ligand that targets the folate receptor α (FR α), which is overexpressed on the tumor cell surface. Overexpressed folate receptors promote cell proliferation by increasing folate uptake, leading to tumor growths.[5]

Sorafenib (SF) is an inhibitor of the vascular endothelial growth factor (VEGF) pathway which was first approved by FDA, and is a novel oral multikinase inhibitor which could inhibit Raf kinase through its anti-proliferative property.[6] SF could also inhibit tyrosine kinases receptor of multiple proangiogenic factors, such as VEGFR1, VEGFR2, VEGFR3, and platelet-derived growth factor receptor β (PDGFR-β).[7] SF is an attractive drug in cancer therapy through its anti-proliferative and anti-angiogenic properties.[8] SF inhibits pathways like VEGF and RAF kinases,[9] which leads to

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impaired angiogenesis and nitric oxide signaling dysregulation,[10] and results in increasing hypertension, vascular tone, and thrombotic events. It causes a range of serious cardiovascular events, including myocardial ischemia, left atrial hypofrequency, coronary artery spasm, and congestive heart failure.[11] Through inhibiting RAF, VEGF, and PDGFR-β, SF could inhibit tumor vascular increase and tumor growth.[12–18] These factors were caused by weak targeting of SF for disease tissues and limit the use of sorafenib in clinical therapy. Therefore, improving the targeting ability of SF for disease tissues or cells through the drug delivery system is necessary and important.

Lipid manifestations are considered for use as nanocarriers for delivery of hydrophobic and hydrophilic molecules because of high biocompatibility,[19] biodegradability, and low immunogenicity. Liposomes also enhance drug solubility and controllable distribution, as well as having the ability to surface modify targeted, elongated, and sustained release.[20] Liposomes have a lipid bilayer, are spherical particles, and at nanoscale.[10] As delivery vehicles, liposomes could load both hydrophobic and hydrophilic agents, where hydrophobic drugs could be loaded in the membrane and hydrophilic drugs could be loaded in the water core.[11] In previous studies, Morgan et al used indium-111 to label liposomes for imaging.[21] Abelcet and AmBisome, approved in the 1990s for the therapy of fungal infections, were liposomal amphotericin B.[22–24] Doxil/Caelyx, used for Kaposi's sarcoma, was PEGylated liposomal doxorubicin.[25] The specific functions of liposomes could be used in different synthesis strategies.[26] The capacity of liposomes for drug delivery is influenced by a variety of factors, such as surface modification, lipid composition, particle stability, particle size, and so on. Liposome could increase the stability and circulation time of small-molecule drugs. Based on above advantages of lipid delivery system, a lipid drug delivery system would improve the targeting ability of SF for disease tissues or cells.

In this study, a FC targeted SF loaded liposome (LSF) drug delivery system was prepared (Scheme 1). LSF could be targeted for delivery to liver tumor cells through FC targets. Sorafenib-equipped liposomes exhibit structural stability and drug release persistence, and the drug showed strong inhibitory activity against liver cancer cells in vitro and in vivo. Compared to sorafenib alone for the treatment of liver cancer, the drug has reduced hepatotoxicity and reduced side effects. These results suggested that this drug might be a drug delivery strategy for therapy of liver cancer for further clinical applications.

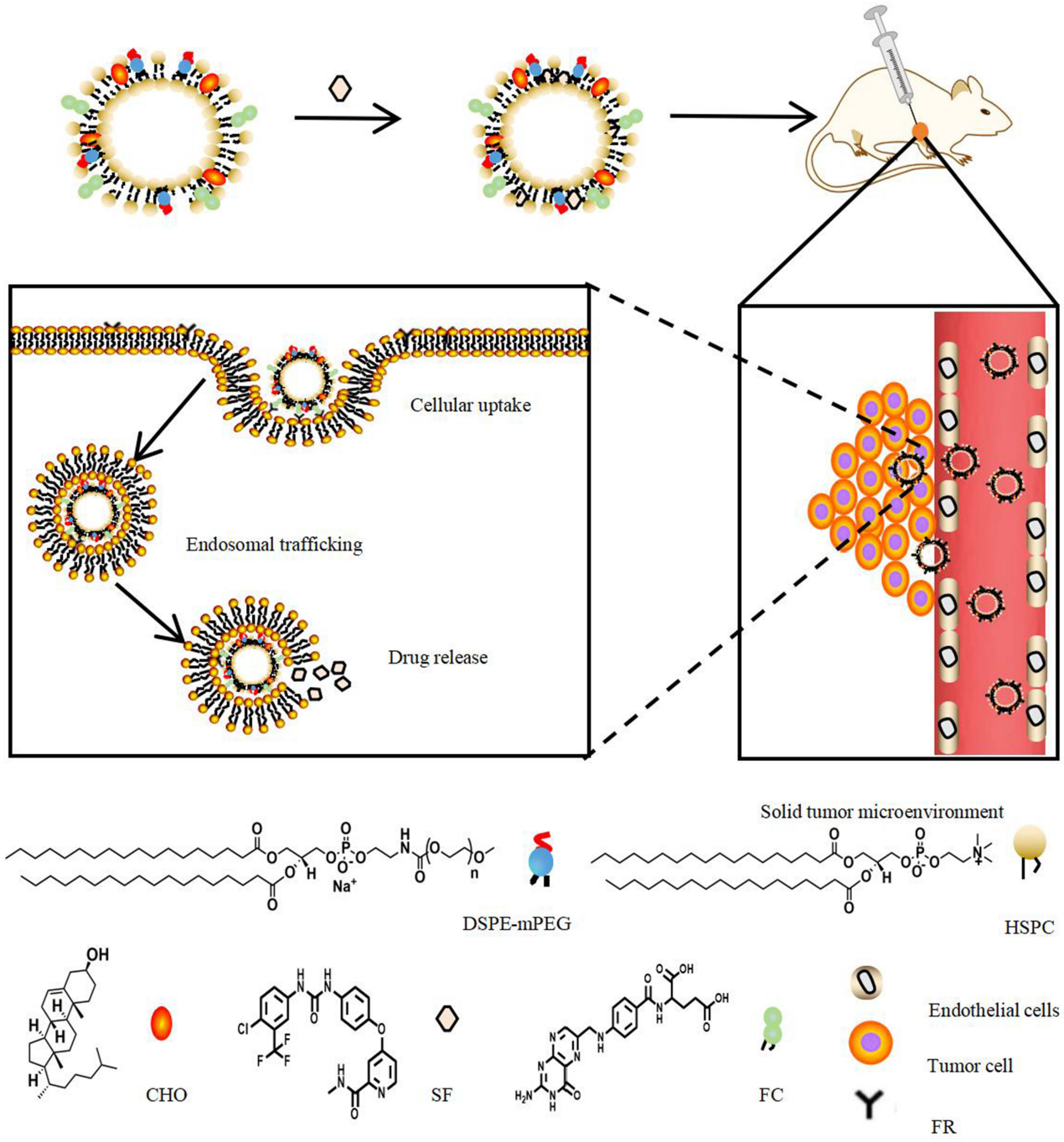
Materials and Methods

Chemicals and Reagents

The components were purchased from Yuanye Bio-Technology (Shanghai, China), such like folic acid (FA), sorafenib (SF), trehalose, rhodamine B (Rh), hydrogenated soybean phospholipids (HSPC), and cholesterol (CHO). The component purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) was 1.2-distearoyl-sn-glycero-3-phosphoethanolamine-N-Polyethyleneglycol-2000 (sodium salt) (DSPE-PEG2k). The kits purchased from TransGen Biotech (Beijing, China) were TUNEL cell apoptosis detection kit and Annexin V-FITC/PI cell apoptosis detection kit. The kits purchased from Solarbio Science and Technology Company (Beijing, China) were hematoxylin-eosin (H&E) staining and MTT reagent kit. The antibodies purchased from Affinity Bioscience (Jiangsu, China) were anti-Ki67 (Cat#AF0198), anti-cleaved-Caspase-3 antibody (Cat#AF7022), and HRP-conjugated Goat anti-Rabbit IgG secondary antibodies (Cat#S0001).

Preparation of LSF

After accurately weighing, the formula amounts of HSPC, DSPE-mPEG$_{2k}$, FA, SF, and CHO (molar ratio of 3.30:0.30:0.15:1.00:2.30) were mixed with methyl alcohol in a 250 mL round bottom bottle.[14] Then methyl alcohol was taken out by evaporation, forming a uniform film on the bottle wall. Certain amount of PBS solution was added, and the membrane was rotated for about 15 min to obtain the liposome suspension. Finally, the suspension was sonicated for a period of time to prepare a more uniform suspension of light yellow folate liposome. Sorafenib is then loaded into liposomes by physical interaction. LSF was obtained through dialyzing to remove free sorafenib, filtration, and lyophilization, sequentially. For protecting the LSF nanoparticle structure, trehalose was added during lyophilization. High-performance liquid chromatography (HPLC) was used to measure loading content of SF. The diameter of LSF was analyzed by DLS.



Scheme 1 Illustration of liver cancer treatment by FC targeted SF liposomes (LSF). LSF could target FC receptor through FC with FC receptor which overexpressed on liver tumor cells.

The Release of LSF

Freeze-dried LSF (5 mg) was dissolved and loaded in dialysis bags (MWCO 3500 Da) with different pH phosphate buffered saline (PBS) (pH 7.4, pH 6.8, pH 5.0) in 5 mL, respectively.[16] And then, the samples were incubated in beakers with 45 mL released media, which was the same as the media in dialysis bag correspondingly, and shaken at 100 rpm at 37 °C. At 0 h, 1 h, 2 h, 4 h, 6 h, 24 h, 48 h, and 72 h, 5 mL media in a beaker was taken out and replaced with fresh media. The released free small molecular SF from LSF was extracted after freeze-drying and analyzed by HPLC.

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HPLC analysis condition: the ratio of acetonitrile and H_2O (containing 0.05% acetic acid) was 65:35. The flow rate of solution was 1 mL/min, and the ultraviolet (UV) detector was 263 nm.

The Stability of LSF

The stability of LSF was evaluated in pH 7.4 PBS.[16] DLS was used for measuring the diameter of LSF at different time points, such as 0 h, 1 h, 2 h, 4 h, 6 h, and 24 h. LSF was stable during 24 h in PBS (pH 7.4) as results revealed.

Cell Culture

The mouse liver carcinoma cell H22 was purchased from Procell Life Science & Technology Company (Hubei, China). RPMI 1640 (HyClone, USA) was used for cell culturing, 100 μg/mL streptomycin and 100 units/mL penicillin were added during culturing. Fetal bovine serum (FBS, Gibco, USA) was added, 10%. Cell culture condition was in 5% CO_2 at 37 °C.

Cell Viability Assay

CCK8 assay was used for measuring the cytotoxicity of free drug SF and LSF in H22 mouse liver carcinoma cells. H22 cells were cultured in 96-well plates at 3×10^3 cells/well.[17] And then, different drugs with different concentrations were incubated with cells for 24 h, 48 h, and 72 h. Culture media was replaced with fresh media, and CCK8 was added. The absorbance was quantified at 450 nm (Cytation 5; Biotek, Winooski, VT, USA).

Apoptosis Assay

Cells were cultured in 6-well plates with 3×10^5 cells in each well.[17] Different content SF or LSF was added and co-incubated for 24 and 48 h. Then cells were incubated with Annexin V binding buffer, which contains Annexin V-FITC and PI. BD FACS Calibur (BD, San Jose, CA, USA) was used for analyzing the stained cells after culturing with 400 μL binding buffer, and Modfit LT 5 software was used to visualize the results.

Animal Study

The protocols and conditions used with in vivo animal experiments were approved by the experimental animal ethics committee of Jilin University (Changchun, China). Arrival guidelines and the national institutes of health guidelines were complied with during animal studies with the care and use of laboratory animals.[18] Female BALB/c mice were purchased (Beijing Vital river Laboratory Animal Technology Co., Ltd) and reared with five animals in each cage.

To establish H22 liver xenograft model, 2×10^6 H22 cells (in pH 7.4 PBS) were injected into the right back of mice subcutaneously. The mice were divided into three groups randomly: control (Ctr), SF, and LSF when the tumor volume reached 100–150 mm^3. And eight mice were in each group. The mice were injected with 200 μL saline, SF (3 mg/kg), or LSF (3 mg/kg on SF basis) intraperitoneally once every 3 d.

The tumor volume and mouse body weight were measured every 3 d. The formula ($V = ab^1 / 2$) was used to calculate mouse tumor volume. In this formula, "a" represents the longest radius, b represents the shortest radius of the mouse tumor.

Three treatments later, all mice were euthanized. All tumor samples were separated, and some tumor samples were stored at −80 °C, and others were fixed in 10% formalin. The major organs were also separated and fixed in 10% formalin, such as heart, liver, spleen, lungs, and kidneys.

Release of SF From LSF in Tumors

The mice with 100–150 mm^3 H22 tumor were divided into two groups randomly, LSF (4 mg/kg) and LSF (4 mg/kg on SF basis). After 48 h of injection with different drugs, the mice were sacrificed. Tumor samples were collected, and the content of SF was analyzed. A 0.1 g of tumor tissue was separated and added with 0.1 mL double-distilled water (dd-H_2O). After grinding, tumor tissue homogenates were mixed with methanol (1:3, v/v) and centrifuged. HPLC was used to measure the content of SF in tumor tissues.

H&E, TUNEL, and Immuno-Histochemical Staining

Tumor tissue samples were paraffin-embedded and cut into consecutive sections. Different stainings, like H&E, TUNEL, and immuno-histochemical staining, were performed as per the manufacturer's protocols.[12]

Tumor samples were prepared with dewaxing and hydration in water for H&E staining. Tumor tissue sections were stained with related solution. Having been dehydrated and covered, the tumor sections were observed and imaged by microscope (Olympus, Tokyo, Japan).

For TUNEL staining, tumor tissues sections with added 0.1% Triton X solution were labeled with TdT solution. Tumor samples were imaged with fluorescence microscope (Olympus).

At last for immuno-histochemical staining, tumor sections were deparaffinized and hydrated firstly. Tumor tissue samples were heated with EDTA (Solarbio, Cat#C1033), and then were performed with 2% BSA. Then, samples were immuno-stained with primary antibodies including anti-cleaved-Caspase-3 (1:100), anti-Bcl-2 (1:500), anti-BAX (1:500), and anti-Ki67 (1:100). HRP-conjugated secondary antibody was incubated with tumor sections. Tumor section images were taken by microscope.

Statistical Analysis

ANOVA was used in statistical analysis. $P < 0.05$ represents statistical significance. GraphPad Prism 8 was used for analysis procedures and graphs. Data are shown as the mean ± SD.

Results

Preparation and Characterization of LSF

After accurately weighing the formula amount of lecithin and cholesterol mixed with an appropriate amount of methyl alcohol, all these were put in a 250 mL flask to evaporate and remove methyl alcohol under reduced pressure, so that a uniform film was formed on the wall of the flask. Then a certain amount of PBS solution was added, and the membrane was rotated for about 15 min to obtain a liposome suspension. Finally, the suspension water bath was ultrasounded for a period of time to prepare a more uniform pale yellow folic acid liposome suspension.[1] Sorafenib (SF) was loaded through physical interactions, and LSF was obtained after lyophilization. Free small molecule SF could be removed through dialysis. To maintain the structure of LSF, trehalose was added during lyophilization. The DLC of SF in LSF was evaluated by high-performance liquid chromatography (HPLC), and the DLC was 3.6%. The diameter of LSF was 197.1 ± 16.6 nm measured by DLS (Figure 1A). The zeta potential of LSF was 0.21 ± 0.35 mV. The positive zeta potential of LSF could increase the circulation time in body. The stability of LSF was evaluated in different pH PBS such as pH 7.4, 6.8, and 5.0 (Figure 1B). DLS was used to measure the diameter of LSF at different time points, and the results showed that LSF was stable in PBS (pH 7.4) during 24 h (Figure 1C).

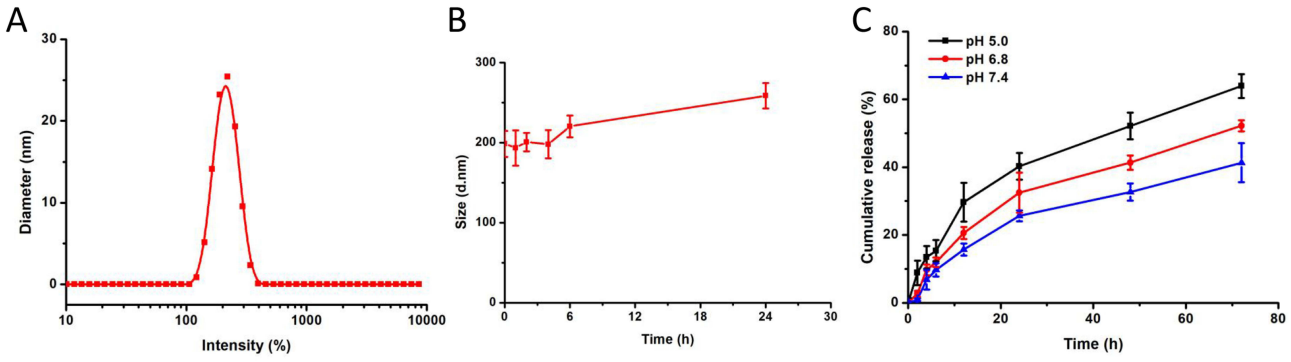

Figure 1 The characterization of SF liposome (LSF). (**A**) The diameter of LSF. (**B**) Stability of LSF. (**C**) The in vitro release of LSF. Data are shown as the mean ± SD, $n = 3$.

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Effects of LSF in vitro

The cytotoxic effects of SF and LSF on the liver cancer cell line were determined though CCK8 experiment. Cells were co-cultured under SF and LSF with different concentrations from 0 to 12 μM at 24 h, 48 h, and 72 h. During 24 h, the cell inhibition rates did not reach 50% in SF or LSF groups (Figure 2A). The cytotoxicity of SF and LSF was enhanced significantly dependent on time and dose. The results showed that 6.0 μM SF could induce about 50% inhibitory rate on H22 cells at 48 h (Figure 2B). At 72 h, 1.0 μM SF and 2.0 μM LSF could induce almost 50% inhibitory rate on H22 cells (Figure 2C). This inhibitory trend was significant in both SF and LSF treatment groups. The inhibition effect of LSF on H22 cells was not as effective as SF because of the slow release behavior of SF from LSF liposomes.

Cell apoptosis was measured to research the mechanism on SF and LSF affecting the viability of H22 cells. The cell apoptotic rate of SF and LSF was significantly higher than that in the control group (Figure 2D). We further counted the value of the apoptotic rate in SF and LSF groups (Figure 2E and F). Furthermore, we co-loaded rhodamine B in LSF to prepare LSF-RB nanoparticles for testing cell uptake of LSF. The results showed that cell uptake of LSF was greater at 48 h than that at 24 h (Figure S1). The results showed that LSF could induce cell apoptosis; however, the effect was not significant with SF because of slow release behavior as above CCK8 assay. The apoptosis proteins of c-caspase3 (c-cas3), c-caspase9 (c-cas9), BAX, and BCL2 were detected in tumor cells at 24 h and 48 h to research LSF mediated tumor suppression pathway (Figure S2). The results showed that LSF could increase the expression of c-cas3, c-cas9, BAX, and BCL2 at 24 h. And LSF could further increase the expression of c-cas3 and BAX at 48 h which was higher than that at 24 h. All these showed that LSF might mediate tumor suppression pathway through

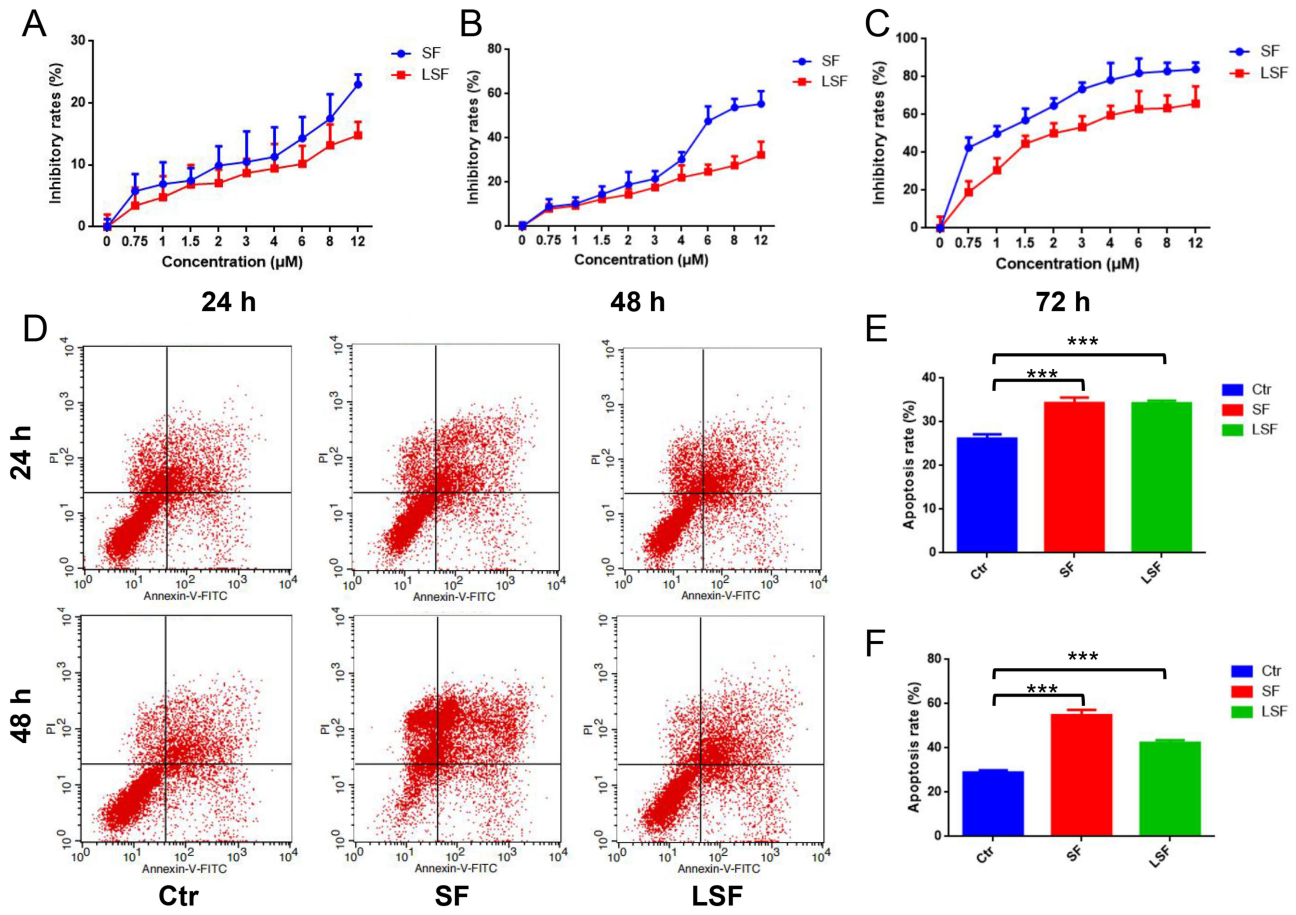

Figure 2 Effects of SF and LSF in vitro. (**A**–**C**) Inhibitory effects of SF and LSF with different doses on H22 cells with different cultured time. (**D**) Cell apoptosis of H22 measured by flow cytometric and statistical analysis (**E** and **F**). Data are shown as the mean ± SD, $n = 3$. ***$P<0.001$ compared with Ctr.

BAX/BCL2 axis. However, the expression of c-cas9 at 48 h was the same as that at 24 h. The expression of BCL2 at 48 h was the same as that at 48 h. We are researching the mechanism further.

Antitumor Effect of LSF

Distribution of small molecule SF in tumor tissues was measured by HPLC to analyze targeting release of LSF. After 48 h post injection, small molecule SF was measured in SF and LSF group. The results revealed that SF in tumor tissues in LSF group was significantly higher than that in the SF group (Figure 3A). The therapeutic ability prevalent primary malignancy of LSF on liver cancer was using liver cancer xenograft model. After SF or LSF treatment, tumor volume was inhibited significantly in SF and LSF groups compared with that in control group. Tumor growth was inhibited better in the LSF group than in the SF group (Figure 3B and C). Mice body weight in LSF group was similar to that in Ctr group, and there was no wave motion when compared with that in SF treatment group (Figure 3D). The results indicated that LSF could reduce the side effect compared with free small molecule SF. All these results showed that LSF could target delivery of SF to tumor tissues and reduce SF side effect.

To further verify whether SF and LSF could induce apoptosis in vivo, the staining of TUNEL was performed. Figure 4 shows the level of green fluorescence signal which represented apoptotic cells. The green fluorescence signal indicated that tumor apoptosis was markedly induced in the LSF group compared with SF and Ctr group.

Immuno-histochemical staining was researched to evaluate the level of Ki67 which was proliferation marker, cleaved-Caspase-3 (C-Cas 3) which was apoptosis marker, Bcl-2, and BAX (Figure 5). The levels of Ki67 and Bcl-2 were observed to be high in the Ctr group compared with the SF and LSF groups. The levels of C-Cas 3 and BAX were increased in LSF group compared with the SF or Ctr groups. All these results suggested that SF and LSF could inhibit liver cancer growth through inducing apoptosis in vivo.

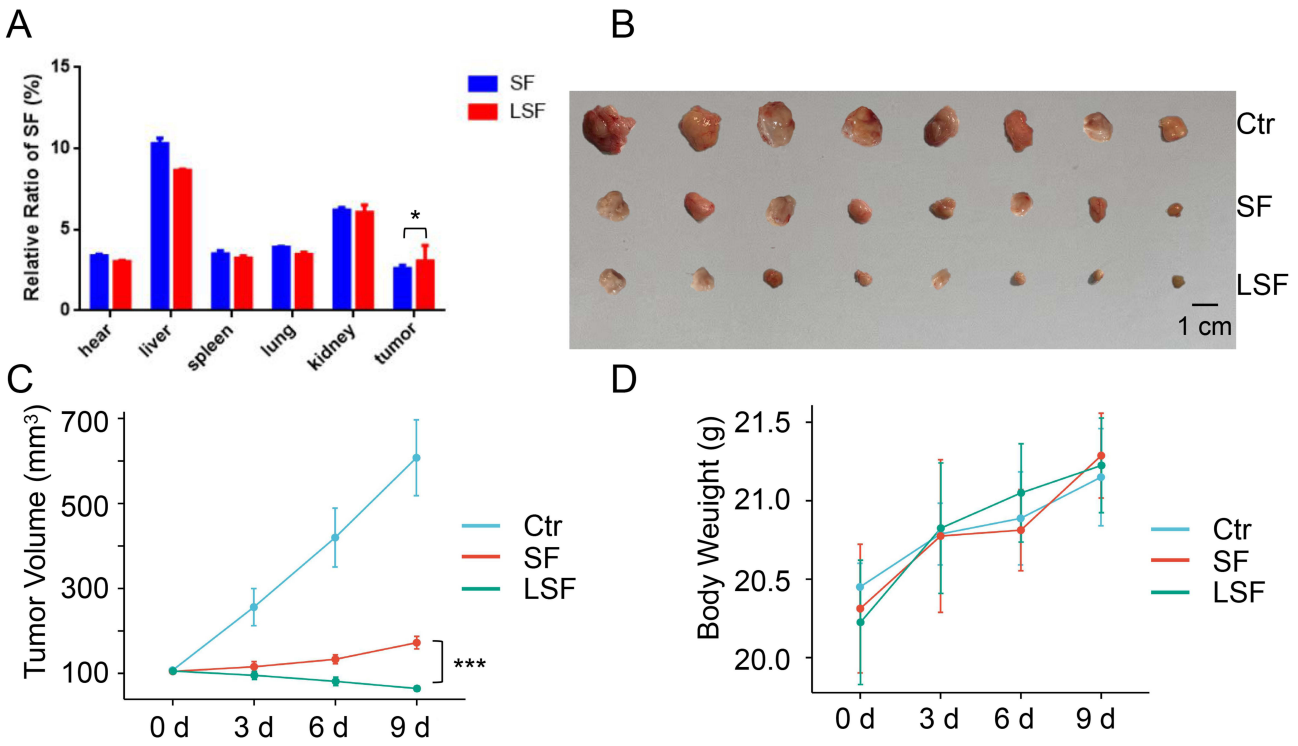

Figure 3 Distribution of free SF and in vivo tumor inhibition with LSF. (**A**) Free small molecule SF distribution in different organs. (**B**) Tumor tissues at the 9th day. (**C**) Tumor volume of mice during treatments. (**D**) Mice body weight in each group during treatments. Data are shown as the mean ± SD. $n = 3$, *$P<0.05$ compared with SF. $n = 8$, ***$P<0.001$ compared with SF.

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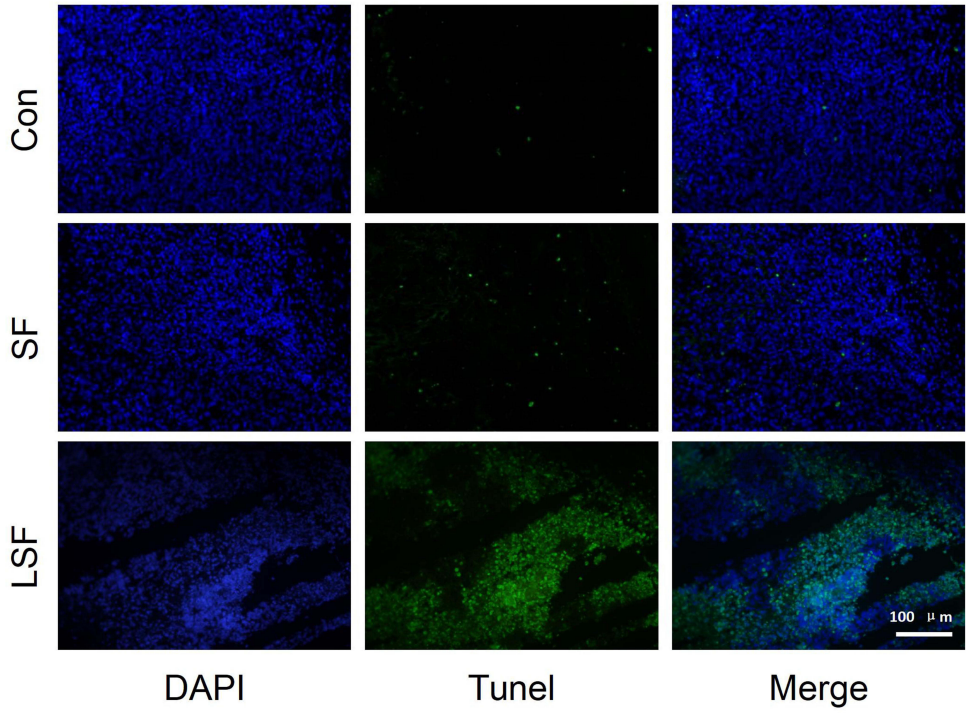

Figure 4 The TUNEL staining of tumors.

Safety Assessment of LSF

Small molecule SF could induce some serious side effects on tumor-bearing mice as seen in previous experiments. In this study, bio-safety assessments were necessary to evaluate the side effect induced by LSF compared with that induced by SF. In Figure 3D, SF could decrease mice body weight significantly. However LSF treatment did not exhibit this side effect. H&E staining showed that there was no significant changes on major organs like heart, liver, spleen, lung, and kidney in LSF group compared with that in Ctr group (Figure 6B). In tumor tissues, necrotic and vacuous areas were observed in LSF group compared with Ctr and SF groups (Figure 6A). Biochemical parameter analyses of liver function indexes ALT and AST, and kidney function indexes BUN and UA were carried out (Figure S3). These results demonstrated that LSF could effectively increase SF tumor inhibition effect and alleviate SF side effects.

Discussion

In this study, LSF, a new drug delivery system, could enhance tumor therapeutic effect, and reduce side effects and improve bio-availability compared with SF treatment in liver cancer.

The growth and metastasis of tumors are related to the tumor cells' proliferation and the process of blood vessels in tumor tissues. The RAS/ERK signaling pathway is an important pathway to regulate tumor cell proliferation and angiogenesis. RAS gene mutations, V599E gene mutations in BRAF genes, and overexpressed receptor tyrosine kinases (RTKs) could activate RAS, and the activated RAS further activates the cascade of RAF/ERK signaling pathway, bringing growth factor signals into the nucleus, thereby playing a role in regulating gene transcription and promoting cell proliferation. On the one hand, SF could inhibit tumor cell proliferation through inhibiting the activity of B-Raf and Raf-1 kinases, thereby blocking the RAS/ERK pathway. On the other hand, SF inhibits the growth of tumor cells by inhibiting the activity of several tyrosine kinase receptors (containing C-KIT, PDGFR-b, VEGFR-2, VEGFR-3, and

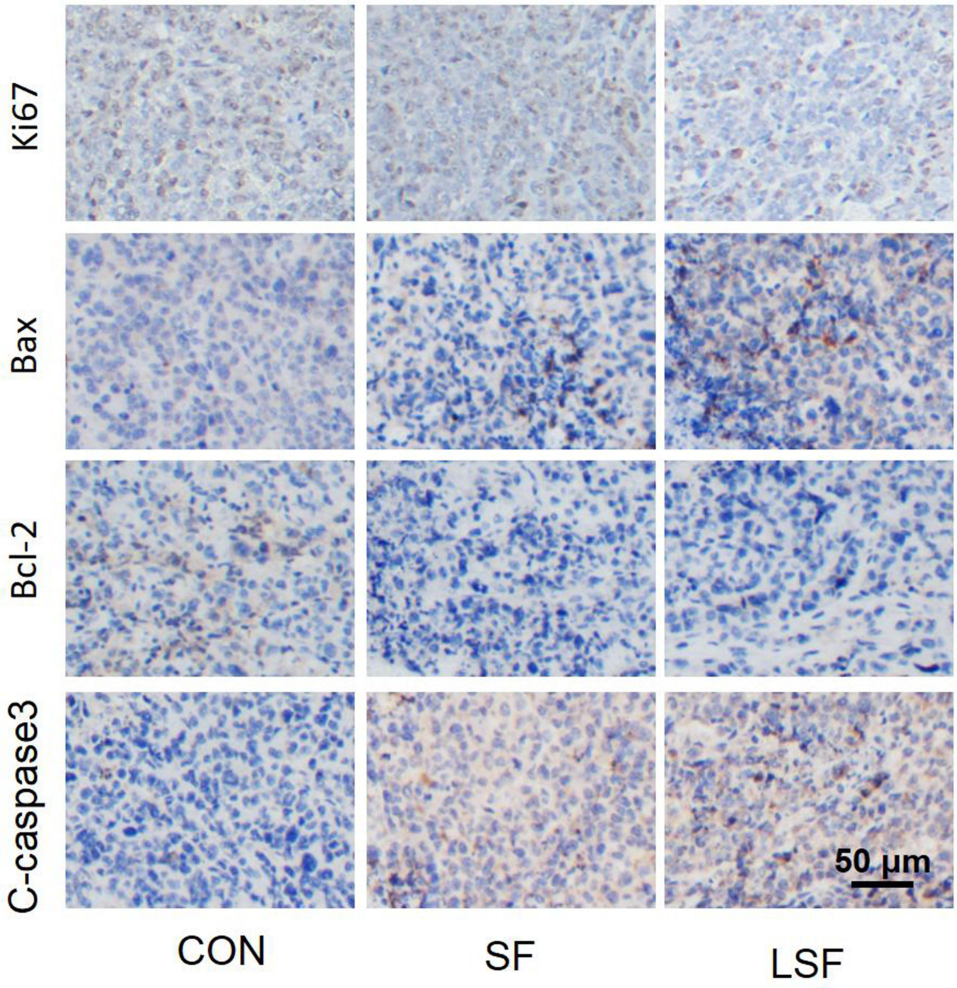

Figure 5 The immuno-histochemical staining of Ki-67, BAX, Bcl-2, and C-caspase-3 in tumors.

FLT3) related to neo-angiogenesis and tumorigenesis, blocking tumor neo-angiogenesis and indirectly inhibiting the growth of tumor cells, thereby playing an anti-tumor role.[1]

Studies have revealed that SF has shown a wide range of anti-tumor effect. As reported, the efficacy of SF in kidney cancer and liver cancer had been identified in phase II/III clinical trials, mainly reflected in good disease control rate and significantly prolonged PFS. SF has also shown certain efficacy in clinical studies of other solid tumors, but has not achieved the expected satisfactory results in non-small cell lung cancer phase III clinical trials, melanoma, and other tumors. On the one hand, the reason might be related to the interaction of tumor signaling pathways. Tumors were mediated by a variety of signals, and there were many direct or indirect cross-effects between each pathway. These cross-talkers could be distracting influencing factors of SF's action. On the other hand, it might be related to genetic mutations. Gene mutations are related to the occurrence and development of tumors.

Our results show that FC targeted liposomes could provide SF with favorable DLC, diameter, release and stability characteristics. The inspiring results of this LSF system were that LSF not only enhanced the anti-tumor effect but also reduced the side effects of SF compared to SF treatment. LSF could effectively inhibit liver cancer increase in vitro and in vivo through increasing apoptosis and inhibiting proliferation of liver tumor cells. Protection of body weight was the most intuitive manifestation of the safety of LSF. These results showed that this strategy was effective and better than previous similar work. And this revealed that this strategy could be transformed to clinical use relatively easily.

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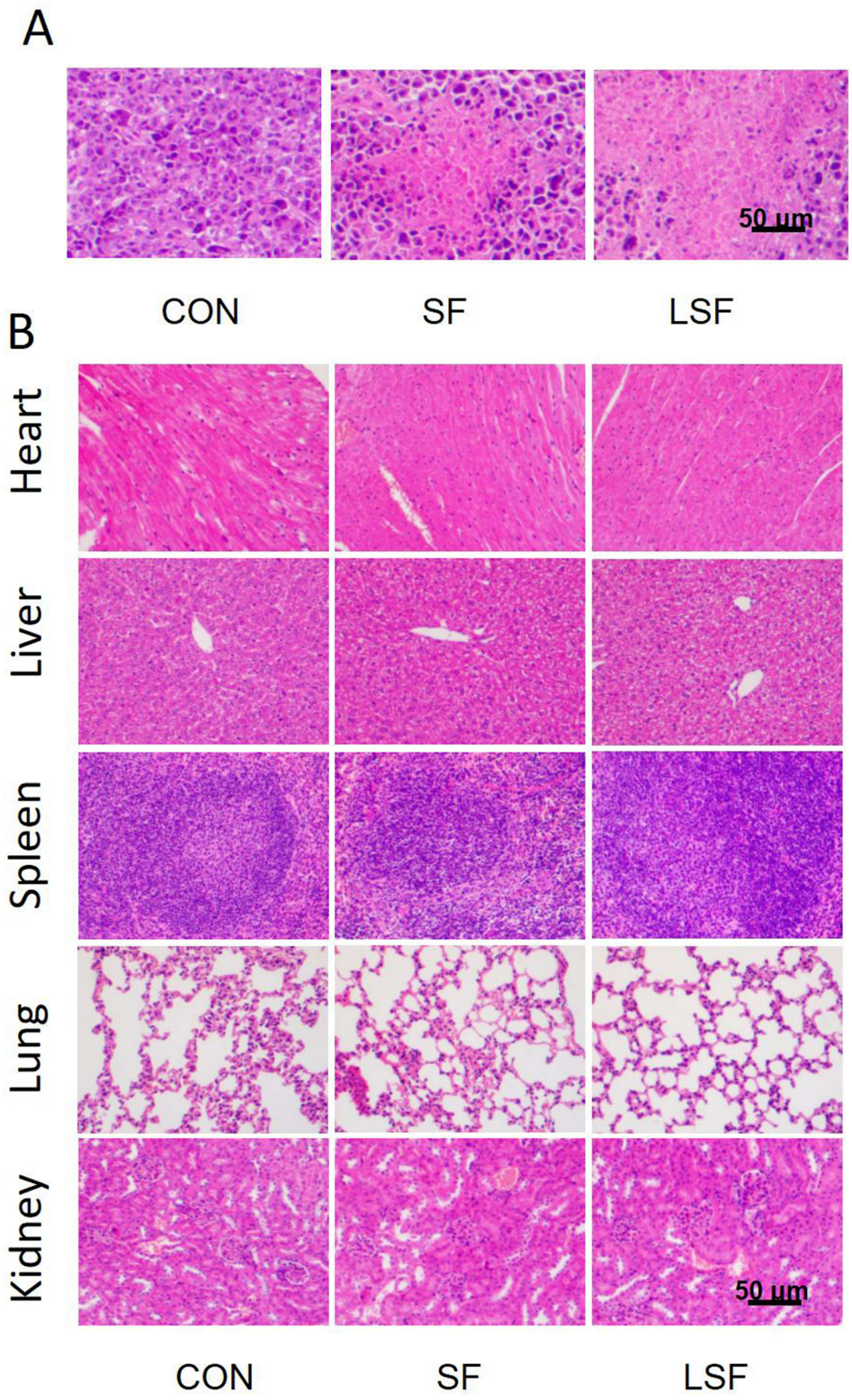

Figure 6 (**A**) The H&E staining of tumors. (**B**) The H&E staining of major organs like heart, liver, spleen, lung, and kidney.

Conclusion

Targeted by folic acid, SF-equipped liposome (LSF) exhibited structural stability and drug release persistence, and the drug showed strong inhibitory activity against liver cancer cells in vitro and in vivo. Compared to SF alone for the

treatment of liver cancer, LSF reduced reduced side effects of SF. These results suggested that LSF might be a drug delivery system for the treatment of liver cancer for further clinical applications.

Data Sharing Statement

The data used and analyzed in this study were available from the corresponding author.

Ethics Approval and Consent to Participate

Animal experiments were approved and researched under the animal ethics committee of Jilin University (Changchun, China).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas. All authors took part in drafting, revising and critically reviewing the article. All authors gave final approval of the version to be published and had agreed on the journal to which the article has been submitted, and agreed to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests during this work.

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