

A Simple and Improved Active Loading Method to Efficiently Encapsulate Staurosporine into Lipid-Based Nanoparticles for Enhanced Therapy of Multidrug Resistant Cancer

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Received: 9 September 2015 / Accepted: 29 December 2015 / Published online: 12 January 2016
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ABSTRACT

Purpose This study was aimed at developing a new active loading method to stably encapsulate staurosporine (STS), a water insoluble drug, into lipid-based nanoparticles (LNPs) for drug targeting to tumors.

Methods A limited amount of DMSO was included during the active loading process to prevent precipitation and facilitate the loading of insoluble STS into the aqueous core of a LNP. The drug loading kinetics under various conditions was studied and the STS-LNPs were characterized by size, drug-to-lipid ratio, drug release kinetics and in vitro potency. The antitumor efficacy of the STS-LNPs was compared with free STS in a mouse model.

Results The drug loading efficiency reached 100% within 15 min of incubation at a drug-to-lipid ratio of 0.31 (mol) via an ammonium gradient. STS formed nano-aggregates inside the aqueous core of the LNPs and was stably retained upon storage and in the presence of serum. A 3-fold higher dose of the STS-LNPs could be tolerated by BALB/c mice compared with free STS, leading to nearly complete growth inhibition of a multidrug resistant breast tumor, while free STS only exhibited moderate activity.

Conclusion This simple and efficient drug loading method produced a stable LNP formulation for STS that was effective for cancer treatment.

KEY WORDS Multidrug resistant cancer · Liposome · Active loading · Staurosporine · Water insoluble drug

ABBREVIATIONS

AUC	Area under the concentration curve
CHOL	Cholesterol
CI	Clearance
DMEM	Dulbecco's Modified Eagle's medium
DSPC	1,2-distearoyl- <i>sn</i> -glycero-3-phosphatidylcholine
DSPE-PEG	1,2-distearoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-[methoxy(poly ethyleneglycol)-2000
EPR-effect	Enhanced permeability and retention effect
FBS	Fetal bovine serum
LNPs	Lipid-based nanoparticles
MDR	Multi-drug resistance
MWCO	Molecular weight cut-off
PDI	Polydispersity index
STS	Staurosporine
$t_{1/2}$	Half life
UPLC	Ultra-performance liquid chromatography
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometer

Electronic supplementary material The online version of this article (doi:10.1007/s11095-015-1854-4) contains supplementary material, which is available to authorized users.

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INTRODUCTION

Staurosporine (STS), a universal kinase inhibitor, exhibits potent activity against many tumor cells *in vitro* (1). However, the clinical application of STS for cancer therapy has been hindered due to poor solubility and the lack of selectivity, which result in significant side effects (2). To improve the solubility and specificity, STS derivatives have been synthesized such as UCN-01. Unfortunately, UCN-01 has failed clinical trials due to its narrow therapeutic window (3). An alternative approach is to employ a nanoparticle (NP) system to carry STS in an aqueous medium as this enhances its tissue selectivity toward tumors via the enhanced permeability and retention (EPR) effect.

Lipid-based nanoparticles (LNPs), such as liposomes, are attractive systems for targeted delivery of drugs with a number of products approved clinically, including Doxil, Ambisome, DaunoXome and Marqibo (4). There are two major methods to encapsulate a drug into LNPs. First, a hydrophilic agent and a hydrophobic drug can be *passively* loaded into the aqueous core and the lipid bilayer of a LNP, respectively. Second, an amphiphilic weak base or acid can be *actively* loaded into the aqueous core of a LNP through a chemical gradient such as pH, ammonium sulfate or calcium acetate (5–7). The active loading method has been demonstrated superior to the passive loading method for providing improved drug loading efficiency (which refers to increased drug-to-lipid ratio) and enhanced stability *in vitro* and *in vivo* (which refers to reduced drug leakage during storage and prolonged blood circulation) (8). This is because the actively loaded drug molecules form crystals or complexes with specific ions (sulfate, calcium, copper, and EDTA) or oligomer/polymer (sucrose sulfate, dextran sulfate) in the aqueous core of LNPs (6,8–20). This mechanism leads to efficient and stable drug loading, which eventually prolongs the drug half-life (11,21–23). However, not all compounds are suitable for active loading. For instance, the drug has to be amphiphathic, as it should not only be soluble in the outer aqueous phase of LNPs, but also permeable through the lipid bilayer and interact with the ions or oligomer/polymer in the inner aqueous phase, in order to form stable crystals or complex. This particular formation limits the leakage of drug back to the outer phase (Fig. 1a). Furthermore, the drug needs to contain a functional group to interact with specific ions or molecules in the aqueous core, in order to take advantage of this active loading mechanism (5,6,8). Active loading of STS into LNPs is challenging as STS exhibits little water solubility. Active loading of this drug was first pursued by Mukthavaram et al. (24) STS was dissolved in a liposome suspension exhibiting a reverse pH gradient (inner pH 7.4, outer pH 3), and incubated the mixture at 50°C for 20 min. Approximately 70% drug encapsulation was achieved at a drug-to-lipid ratio (D/L) of 0.09 (mol/mol). In this system, STS containing a secondary amine group was ionized and solubilized at pH 3

in the outer phase, but precipitated inside the liposomes once penetrated into the inner phase (pH 7.4). However, the STS-liposomes (DSPC:DOPE:Chol:DSPE-PEG2000 = 6:6:6:1) produced with this method only achieved a moderate drug loading efficiency, and the drug leakage was fast when incubated with PBS and serum (>70% in 24 h).

This study was focused on the development of a simple and improved active loading method for STS into LNPs. One of the critical challenges is that the active loading process requires the drug to be dissolved with the preformed LNPs containing a chemical gradient. However, STS is insoluble, and reducing the pH cannot significantly improve the solubility. We hypothesized that introducing a limited amount of dimethyl sulfoxide (DMSO) to the LNPs could prevent precipitation of STS in the active loading process, and would also facilitate the permeation of the drug into the inner core of LNPs. Additionally, STS containing a secondary amine could be actively loaded into LNPs via an ammonium sulfate gradient (inner aqueous phase) and complex with sulfate to form stable nano-aggregates (Fig. 1b). The final product would then be produced after removal of DMSO by gel filtration or dialysis.

Herein, we report the development and optimization of this improved active loading method for STS. The drug retention in serum, cytotoxic potency, pharmacokinetics and the *in vivo* antitumor efficacy of the STS-LNPs were determined and compared with free STS.

MATERIALS AND METHODS

Reagents

1,2-Distearoyl-sn-glycero-3-phosphatidylcholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphatylethanol-amine-N-[methoxy (polyethyleneglycol)-2000] (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Alabaster, AL). Staurosporine (STS) was purchased from LC laboratories. Free cholesterol E assay kit was purchased from Wako Chemicals USA, Inc. Cholesterol (Chol), dimethyl sulfoxide (DMSO), XTT reagent, formalin solution (10%), anhydrous ethanol and sodium acetate were purchased from Sigma-Aldrich (Oakville, ON). All other reagents were analytical grade.

Resistant Cancer Cell Lines

PC-3 (human prostate cancer) cells were obtained from the American Type Culture Collection (ATCC), and the docetaxel (DTX)-resistant variant was generated by incubating the cells with gradually increasing concentrations of DTX until the cells were completely resistant to 100 nM DTX as described previously (25). EMT6 (murine breast tumor) and

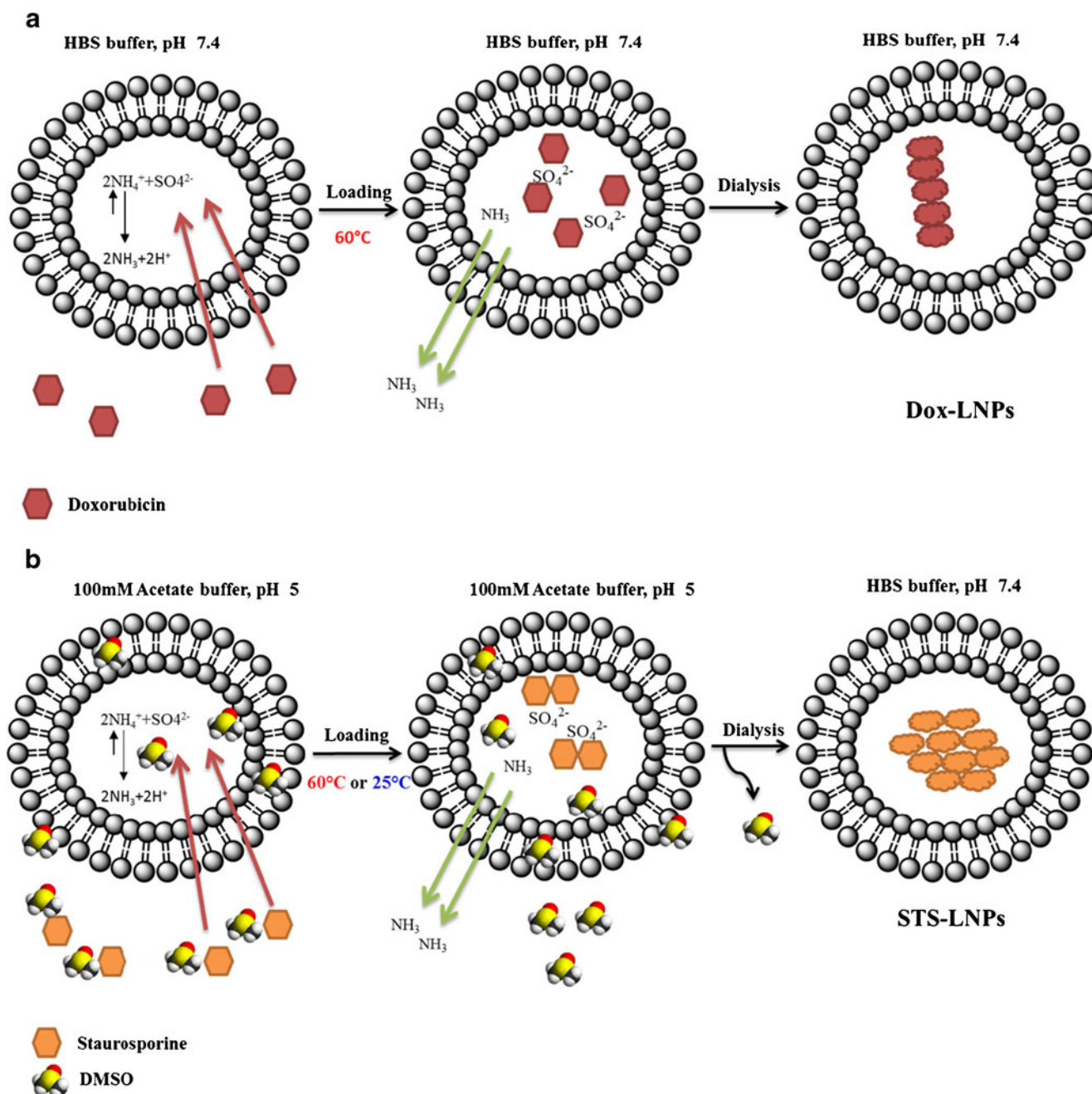


Fig. 1 Illustration of active loading of weak base drugs into LNPs. **(a)** Loading of doxorubicin (DOX) via an ammonium sulfate gradient. **(b)** Loading of staurosporine (STS) via an ammonium sulfate gradient in the presence of DMSO.

the resistant variant, EMT6/AR1 cells overexpressing P-glycoprotein were a gift from Ian Tannock (Princess Margaret Hospital, Toronto) (26). All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 nM DTX.

Mice

Female BALB/c mice (6–8 weeks old) purchased from the Jackson Laboratory (Bar Harbor, ME) were used for the

in vivo study. All animal studies were conducted at the Animal Resources Centre at the University Health Network (Toronto, ON, Canada) with approved protocols in compliance with the guidelines developed by the Canadian Council on Animal Care.

Ultra-performance Liquid Chromatography Analysis

The ultra-performance liquid chromatography (UPLC) analysis was performed with an ACQUITY UPLC System

equipped with a Photodiode Array (PDA) detector, a binary solvent system and an auto-sampler. Diluted samples were injected into the UPLC system and separated on a Waters ACQUITY CSH™ C18 column (2.1 × 50 mm, 1.7 μm) with mobile phase (A: MilliQ water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid). The eluent was monitored at 291 nm at a flow rate of 0.4 mL/min with the following designed gradient (0 min: A/B (90/10), 1.8 min: A/B (90/10), 2.3 min: A/B (90/10), 2.5 min: A/B (90/10), 3 min: A/B (90/10)). The concentration of STS was determined by integrating the peak area and compared with a standard curve.

Solubility Test

Three mg STS was dissolved in 1 mL of acetate buffer (100 mM, pH 5), water or HEPES buffered saline (HBS, 25 mM HEPES, 0.9% NaCl, pH 7.4) in the absence or presence of 5% DMSO. The drug suspension was sonicated for 30 min and incubated for 30 min at room temperature. Drug precipitates were removed by 0.2 μm amicon centrifugal filter units (12,000 rpm, 15 min). The filtrate was diluted with methanol and analyzed by UPLC using the method described in 2.4.

Preparation of LNPs with an Ammonium Sulfate Gradient

One hundred mg of DSPC/Chol/DSPE-mPEG₂₀₀₀ (55/40/5, mole ratio) was dissolved in 1 mL ethanol in a flask. Ethanol was removed by rotary evaporation at 60°C to form a thin film. The thin film was dried under heavy vacuum overnight and then hydrated with 1 ml of 350 mM ammonium sulfate at 60°C. The extruder was equilibrated at 70°C and the lipid suspension was extruded through 200, 100, 80 nm stacked Nucleopore polycarbonate membranes 20 times each to control the size. The external medium of the LNPs was replaced with acetate buffer (100 mM, pH 5) by dialysis (Slide-A-Lyzer Dialysis Cassettes 10 kDa MWCO, Pierce Biotechnology, Rockford, IL). The size and zeta potential of the empty LNPs were measured by a particle analyzer (Zetasizer Nano-ZS, Malvern Instruments Ltd, Malvern, UK) and stored at 4°C.

Optimization of STS Loading into the LNPs

Various amounts of the LNPs were mixed with 1 mg of STS in the presence of 5% DMSO in a total volume of 1 ml (diluted in 100 mM acetate buffer, pH 5). The mixture was incubated at 60°C for 1 h and then quenched on ice. The STS-LNPs were purified by Sepharose CL-4B gel filtration, and eluted with HBS. To

determine the drug loading efficiency, the following equation reported previously was applied (27).

$$\text{Loading efficiency}(\%) = \frac{[D]_{\text{purified}}/[Chol]_{\text{purified}}}{[D]_{\text{initial}}/[Chol]_{\text{initial}}} \times 100\%$$

To determine STS concentration ([D]) in the formulations, the LNPs were diluted with methanol to an adequate extent and the drug was quantified by UPLC using the method described in 2.4. Cholesterol concentration ([Chol]) was measured using a cholesterol E assay kit.

Active Loading Kinetics

STS (1 mg) was incubated with the LNPs containing 5 mg total lipid (D/L = 0.31/1, mol, L is referred as total lipid throughout the article) in the presence or absence of 5% DMSO at room temperature or 60°C for 5–60 min. The formulations were then purified with gel filtration (Sepharose CL 4B), and the drug loading efficiency was measured as described above.

Active Loading in the Presence of 5–60% DMSO

One mg STS was incubated with the LNPs containing 5 mg of total lipids (D/L = 0.31/1, mol) in the presence of 5–60% DMSO at room temperature for 1 h. The formulations were then purified with gel filtration (Sepharose CL 4B), and the drug loading efficiency was measured as described above.

Cryo-Transmission Electron Microscopy (Cryo-TEM) Imaging

The morphology of the LNPs was imaged by a FEI Tecnai G20 Lab6 200 kV TEM (FEI, Hillsboro, OR) following a previously described method (11). The instrument was operated at 200 kV in bright-field mode. Digital images were recorded under low dose conditions with a high-resolution FEI Eagle 4 k CCD camera (FEI, Hillsboro, OR) and analysis software FEI TIA. A nominal underfocus of 2–4 μm was used to enhance image contrast. Sample preparation was performed using the FEI Mark IV Vitrobot. Approximately 2–4 μL of LNP at 10–15 mg/mL total lipid was applied to a copper grid and plunge-frozen in liquid ethane to generate vitreous ice. The frozen samples were then stored in liquid nitrogen until imaged. All samples were frozen and imaged at the UBC Bioimaging Facility (Vancouver, BC).

Drug Retention Study

STS released from the LNPs was measured using the fluorescence de-quenching method as previously described with minor modifications (28,29). The STS-LNPs (500 μg STS/ml in

200 μl of 50% FBS/50% HBS) were incubated at 37°C at various time points, and were immediately cooled in an ice bath, diluted by 100-fold and transferred into a 96-well plate. The release of STS was determined using a BioTek Synergy H1 Multi-Mode Reader by measuring the fluorescence (Ex 296 nm/Em 396 nm). The percentage of the released STS was calculated as $(I_T - I_0)/(I_{100} - I_0) \times 100\%$, in which I_T is the fluorescence at time point T, I_0 is the fluorescence at the start of the incubation time, I_{100} is the fluorescence after the addition of 10 μl of 5% Triton X-100.

In Vitro Cytotoxic Potency of STS and STS-LNPs

EMT6/AR1 and PC-3-RES cells were plated in a 96-well plate (1000 ~ 5000 cells/well). After 24 h of incubation, the cells were treated with different concentrations of DTX (final DMSO <0.1%), free STS (final DMSO <0.1%) and STS-LNPs. After 72 h of treatment, cell viability was assayed by the XTT assay as described previously (25). The IC_{50} was determined by nonlinear regression analysis using GraphPad Prism Software (San Diego, CA).

Dose Escalating Study

A dose escalating study was performed in order to determine the maximum tolerated doses (MTDs) for STS-LNPs and free STS. Free STS was dissolved in acetate buffer (pH 5) containing 1% DMSO (*v/v* %). Free STS was found to damage the tail vein of mice after i.v. injections. Alternatively, i.p. injections were employed for administering free STS. The i.p. route for STS administration has been demonstrated to be well-tolerated by mice even the injection vehicle contains DMSO (30). On the other hand, STS-LNPs can be administered intravenously to BALB/c mice. The mice received various doses of free STS and STS-LNPs and were monitored in accordance with the established clinical health score chart (31). This health score chart includes the change of body weight, and the mice status of activity, appearance, breathing, posture, hydration, and elimination. The health status of the mice was scored until they recovered to the baseline score, and mice that reached humane endpoints were euthanized. The MTD was defined as the maximum dose that did not induce any humane endpoints in the treated animals.

In Vivo Efficacy Study

The MTDs for i.v. DTX (dissolved in Tween80/ethanol/0.9% saline, and then diluted in normal saline), i.v. STS-LNPs and i.p. free STS (dissolved in DMSO and then diluted in 100 mM acetate buffer, pH 5) were 12 mg/kg (twice weekly for 3 doses), 3 mg/kg (twice weekly for 3 doses) and 0.6 mg/kg (5 consecutive days), respectively. The MTDs were obtained from the dose escalating study described above or reported

previously (2,32). Female BALB/c mice were s.c. inoculated with EMT6/AR1 cells (2×10^5 cells/injection, $n = 10$ mice per group). One week later, when tumors reached the size of about 50 mm^3 , the mice were treated with HBS (i.v., twice weekly for 3 doses), DTX (12 mg/kg twice weekly for 3 doses, i.v.), free STS (0.6 mg/kg, i.p., daily for 5 consecutive days) and the STS-LNPs (3 mg/kg twice weekly for 3 doses, i.v.). Tumor volume and body weight of the treated animals were monitored every two days. Mice were euthanized when tumor volume reached the end point ($>1000 \text{ mm}^3$) or open tumors were discovered.

Storage Stability

The sterile filtered STS-LNPs with a D/L of 0.31 (mol/mol) at 1 mg STS/mL were stored in a glass vial at 4°C. At selected time points, an aliquot of the STS-LNPs was collected, and the size and loading efficiency were measured following the methods described above.

Statistical Analysis

All data are expressed as mean \pm SD. Statistical analysis was conducted with the two-tailed unpaired *t* test for two-group comparison or one-way ANOVA, followed by the Tukey's multiple comparison test by using GraphPad Prism (for three or more groups). A difference with *P* value < 0.05 was considered to be statistically significant.

RESULTS

Solubility of STS

STS was insoluble in water and HBS (pH 7.4) (solubility <0.78 $\mu\text{g}/\text{mL}$), and reducing the pH to 5 using acetate buffer increased the solubility to 120 $\mu\text{g}/\text{mL}$ (Fig. 2). Solubility of STS in acetate buffer was significantly improved to 1 mg/mL in the presence of 5% DMSO (Fig. 2). Including 5% DMSO in water and HBS failed to increase the solubility of STS to a significant extent (<50 $\mu\text{g}/\text{mL}$).

STS was Efficiently Loaded into the LNPs Via an Ammonium Gradient in the Presence of 5% DMSO

We hypothesized that DMSO could be used as a solubilizing agent to prevent STS precipitation during the active loading process and increase the permeability of lipid membrane, allowing STS to pass through the lipid bilayer and get encapsulated effectively. As shown in Fig. 2 and Supplementary Fig. 1, 1 mg/mL of STS in 100 mM acetate buffer (pH 5) required at least 5% DMSO to avoid drug precipitation. Next, we incubated STS with a LNP formulation containing an ammonium

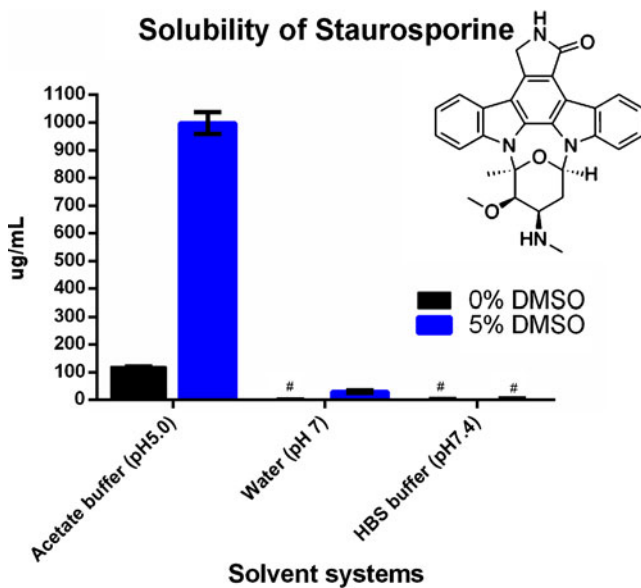


Fig. 2 Structure and solubility of STS in aqueous solvent systems. Data = mean \pm S.D., $n = 3$. # [STS] is lower than the detection limit: $0.78 \mu\text{g/mL}$.

sulfate gradient (inner: 350 mM ammonium sulfate; outer: 100 mM acetate buffer, pH 5) in the presence of 5% DMSO at 60°C for active loading of STS. The highest drug-to-lipid ratio (D/L, mol/mol) that produced 100% encapsulation efficiency was 0.31. When the D/L increased to 0.39, the encapsulation efficiency was reduced to $\sim 50\%$ (Fig. 3).

Drug Loading Kinetics

We then studied the STS loading kinetics into the LNPs in the presence and absence of 5% DMSO at a D/L of 0.31 at either room temperature or 60°C . As shown in Fig. 4, complete drug loading was achieved within 5 min of incubation in the

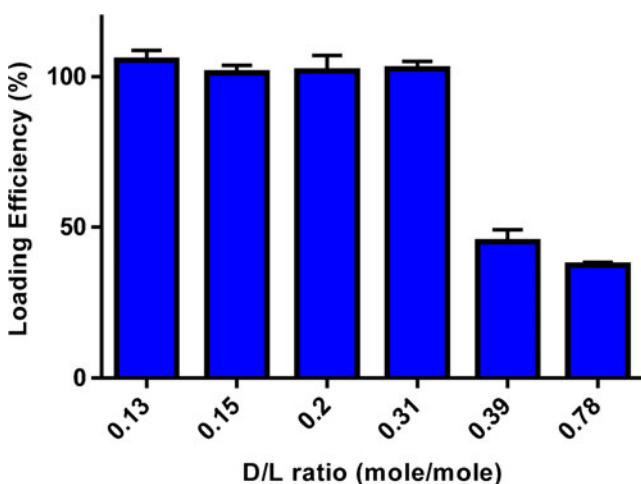


Fig. 3 Loading efficiency with different drug-to-lipid (D/L) ratios. Data = mean \pm S.D., $n = 3$.

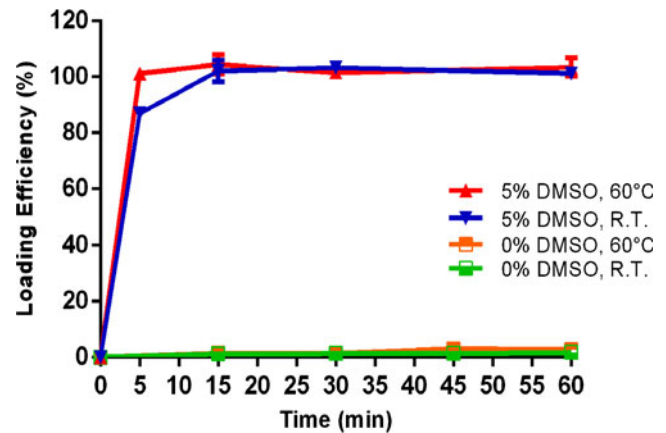


Fig. 4 Comparison of STS loading kinetics in the presence and the absence of 5% DMSO at room temperature and 60°C . Data = mean \pm S.D., $n = 3$.

presence of 5% DMSO at 60°C , a temperature that is higher than DSPC lipid transition temperature (T_m , 55°C). At room temperature (lower than the T_m), although the loading rate was decreased, the loading efficiency reached 100% by 15 min. Only a trace amount of STS ($<3\%$) was loaded into the LNPs without the assistance of DMSO (1.7% at room temperature and 2.8% at 60°C).

Drug Loading in the Presence of Different Amounts of DMSO

We also examined whether an excess amount of DMSO in the active loading system would adversely affect the drug loading. As shown in Fig. 5, although only 5% DMSO was required to completely dissolve STS in this system (1 mg/ml), the presence of an excess amount of DMSO (up to 60%) did not reduce the drug loading. Complete STS loading was produced via an ammonium sulfate gradient in the presence of 5–60% DMSO at room temperature. Moreover, the size (~ 100 nm) and the polydispersity index ($\text{PDI} < 0.06$ for all samples) of the final STS-LNPs produced with various amounts of DMSO were comparable and remained unchanged compared to the LNPs

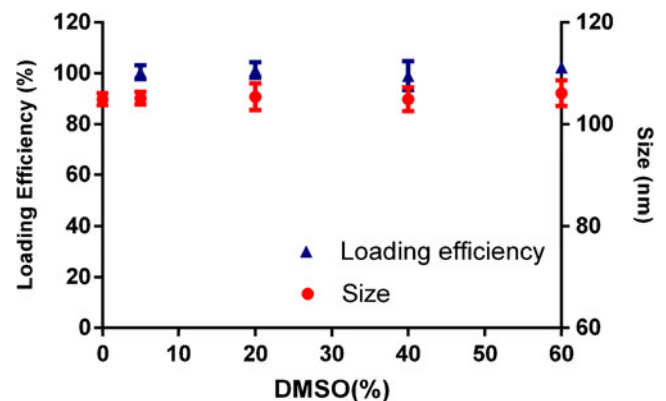


Fig. 5 STS loading in to the LNPs in the presence of various amounts of DMSO (5–60%) at room temperature. Data = mean \pm S.D., $n = 3$.

before drug loading (0% DMSO represents the empty LNPs). However, when less than the required amount of DMSO (2%) was employed in the active loading process, the loading was incomplete, yielding a significant amount of drug precipitates in the final product (data not shown).

Characterization of the STS-LNPs

The STS-LNPs prepared with a D/L of 0.31 (mol/mol) were further characterized and the key parameters of the formulation are reported in Table 1.

STS-LNPs Displayed Nano-aggregates in the Aqueous Core of the LNPs

Cryo-TEM was used to image the drug free LNPs and the STS-LNPs prepared with a D/L of 0.31 in the presence of 5 or 60% DMSO (Fig. 6). The STS-LNPs were dialyzed against HBS to produce the final products for cryo-TEM imaging. Nano-aggregates were observed in the aqueous core of the LNPs after STS loading.

Drug Retention of the STS-LNPs in HBS and FBS

The release of STS from the LNPs was determined by using the fluorescence de-quenching assay. The fluorescence intensity increased linearly while the drugs released from the LNPs. As shown in Fig. 7, there was limited drug leakage (<5% in 7 days) from the LNPs when incubated in HBS or 50% FBS at 37°C.

In Vitro Cytotoxic Potency

As shown in Table 2, free STS and the STS-LNPs exhibited comparable cytotoxic potency against EMT6/AR1 and PC-3-RES cells with IC_{50} values between 3 and 70 nM. STS and the STS-LNPs were 2- to 300-fold more potent compared to DTX. In addition, EMT6/AR-1 cells were founded to be more sensitive to STS compared to PC3-RES (IC_{50} are ~3

and ~60 nM, respectively). Therefore, EMT6/AR1 was selected for the following animal studies.

The Dose Escalating Study

As shown in Table 3, the MTDs for free STS and STS-LNPs were 0.6 mg/kg (5 consecutive i.p. injections) and 3 mg/kg (twice weekly for 3 doses), respectively. The accumulated MTDs for free STS and STS-LNPs were 3 mg/kg and 9 mg/kg, respectively. The LNP formulation increased the accumulated MTD by 3-fold relative to free STS.

In Vivo Antitumor Efficacy Study

The MTDs for i.v. DTX (dissolved in Tween80/ethanol/0.9% saline, and then diluted in normal saline) and the STS-LNPs were 12mg/kg and 3mg/kg (twice weekly for 3 doses), respectively. The dosing regimen for free STS (dissolved in DMSO and then diluted in acetate buffer) was adopted from the previous report (2), and the MTD for which was 0.6 mg/kg consecutively for 5 days by i.p. injection. The antitumor activity of DTX, free STS and the STS-LNPs was compared at their MTDs in a rapidly growing EMT6/AR1 mouse breast cancer model in BALB/c mice (Fig. 8a). This tumor model was highly resistant to DTX, which showed no antitumor activity. The STS-LNPs exhibited significantly improved antitumor activity in this highly resistant breast cancer model compared with free STS and DTX. After 3 doses of the STS-LNPs, the tumor growth was effectively impeded and the average tumor volume was controlled by 150 mm³ in comparison to ~800 mm³ in the free STS group by day 18, while all tumors in the DTX and buffer treated mice all exceeded the endpoint size (1000 mm³) or displayed open ulceration before day 14. The STS-LNPs treatment did not cause significant body weight loss (Fig. 8b). On the other hand, 3 mice in the free STS group reached humane endpoints on day 9 possibly due to the drug toxicity, and there was ~5% body weight loss in the mice treated with DTX.

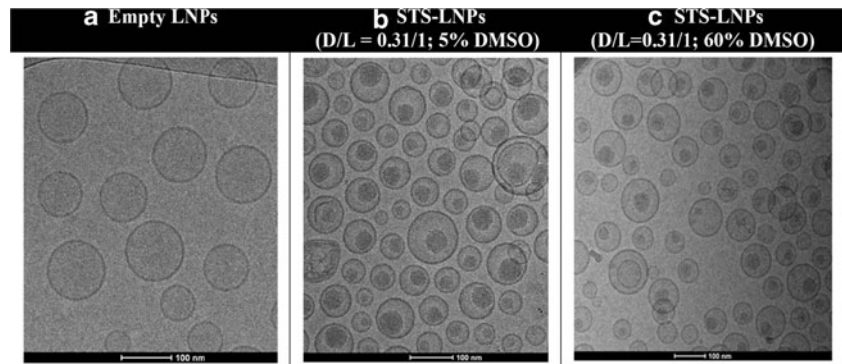
Table 1 Characterization of the STS-LNPs With/Without DMSO Involved During Drug Loading

Composition		DMSO (%)	Temperature (°C)	Particle size	PDI	Zeta potential (mV)	Loading efficiency (%)	Final drug/total lipid (mol/mol) ^a
DSPC/Chol/DSPE-PEG ₂₀₀₀ (55/40/5)	Before loading	–	–	101.5 ± 1.7	0.035 ± 0.016	–15.5 (±0.3)	–	–
	After loading	5%	r.t.	102.5 ± 1.5	0.068 ± 0.027	–24.7 (±1.5)	100.4 ± 2.7	0.31:1
			60	102.4 ± 1.2	0.052 ± 0.022	–26.5 (±1.3)	101.1 ± 1.3	
	After loading	0%	r.t.	102.2 ± 2.2	0.060 ± 0.011	–18.1 (±0.5)	1.7 ± 0.1	0.005:1
60			100.8 ± 1.2	0.045 ± 0.012	–15.2 (±0.4)	2.8 ± 0.2	0.008:1	

Data = mean ± S.D., *n* = 3

^a Drug/total lipid (w/w) = 0.2/1

Fig. 6 Cryo-TEM images of the drug free LNPs (a) and the STS-LNPs in the presence of 5% (b) and 60% (c) of DMSO during the STS loading. Scale bar represents 100 nm.



Storage Stability of the STS-LNPs

The STS-LNPs were stored at 4°C and the size and loading efficiency were measured after 1, 4, and 6 months (Table 4). The samples did not show visible precipitates, and during the 6-month period, no apparent change in size or drug encapsulation was determined.

DISCUSSION

Most of the clinically approved liposomal products employ the active loading approach to stably encapsulate the drug, including Doxil, DaunoXome and Marqibo. Taking Doxil as an example, doxorubicin (DOX) is actively loaded into the LNPs via an ammonium sulfate gradient, forming crystals with the sulfate ions inside the liposomal core. As a result, a high loading efficiency ($D/L = 2/15$, w/w) can be achieved, and the formation of drug aggregates inside the LNPs reduces the leakage of the drug, leading to improved PK and storage stability. This active loading method involves dissolving and incubating the drug with preformed LNPs containing a chemical gradient (i.e. the compositions of the inner and outer aqueous phases are different) at the temperature that is slightly above the transition temperature (T_m) of the lipid bilayer. The

drug needs to be soluble in the outer aqueous phase first and permeable through the lipid bilayer into the inner phase to efficiently interact with the ions inside to form stable crystalline or complex. Therefore, the drug needs to possess specific properties to be a candidate for the active loading approach. First, the drug needs to exhibit good water solubility and membrane permeability. Second, the drug has to contain certain functional groups that can interact with specific ions for forming stable complex, for example, amino group and sulfate; carboxylate and calcium. Like DOX, STS contains a weak base secondary amine group that may interact with sulfate to form stable complex, and its high lipophilicity ($\log P$ 4.17) suggests good membrane permeability. However, STS is not a good candidate for the active loading method as it is not water soluble, and reducing the pH does not increase the solubility to a desirable extent. We hypothesized that adding a limited amount of a water miscible solvent in the active loading system could solubilize STS in the outer phase as well as improve its penetration through the lipid bilayer into the inner core. STS is dissolved freely in DMSO, which is miscible with water. We determined that 5% DMSO is minimally required to dissolve STS in the outer phase of the LNPs (100 mM acetate buffer, pH 5) at 1 mg/ml. When STS was incubated with the LNPs containing an ammonium sulfate gradient in the presence of 5% DMSO at 60°C, complete drug loading was reached within 5 min at a D/L of 0.31/1 (mol/mol). STS loading could also be achieved at room temperature, but required a longer time (15 min), suggesting the presence of DMSO might increase the membrane permeability of STS. On the other hand, in the absence of DMSO, <3% of the

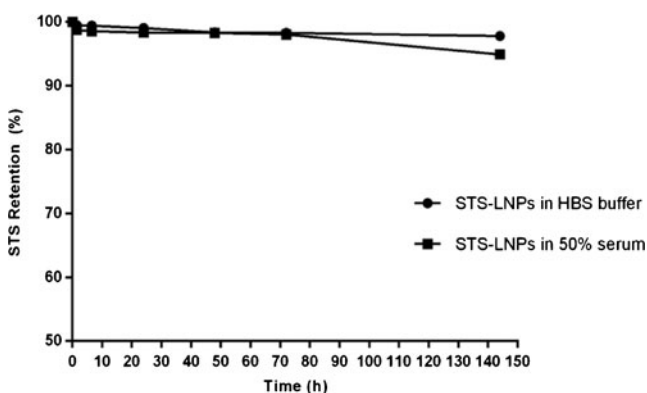


Fig. 7 Drug retention in the STS-LNPs when incubated in 50% serum and HBS at 37°C. Data = mean \pm SD ($n = 3$).

Table 2 Cytotoxicity of Different Agents Against Drug Resistant Tumor Cells

	IC ₅₀ (nM) DTX	IC ₅₀ (nM) STS	IC ₅₀ (nM) STS-LNPs
EMT6/ARI	1091 (\pm 45)	3.3 (\pm 0.5)	3.5 (\pm 0.8)
PC-3-RES	903 (\pm 50)	56 (\pm 5)	65 (\pm 2)

Data = mean (\pm SD), $n = 3$

Table 3 Dose escalating Results of STS-LNPs and Free STS

	Dose (mg/kg)	Survival rate	Maximum change of body weight (%)
Free STS	0.6	3/3	-2.1 ± 2.0
	0.8	0/3	-16.0 ± 3.0
STS-LNPs	0.6	3/3	0.1 ± 1.1
	3	3/3	0.8 ± 1.4
	5	1/3	-8.0 ± 2.1

N = 3 in each group

drug was loaded into the LNPs, indicating that DMSO was required for efficiently loading of STS into the LNPs. We also compared the drug loading in the presence of 5–60% DMSO at room temperature and discovered that even with 60% DMSO in the system, complete STS loading was reached at a D/L of 0.31/1, suggesting the ammonium sulfate gradient could be maintained even in the presence of 60% DMSO at room temperature. However, if the system contained only 2% DMSO which was not sufficient to dissolve STS, complete drug loading could not be achieved even after 1 h incubation at 60°C, with significant precipitates in the final product. These results confirm the importance of completely dissolving STS by DMSO in the system for efficient drug loading. The Bally lab previously reported that ethanol could facilitate

DOX loading into liposomes containing a pH gradient (33). However, the ethanol method could only be applied to cholesterol free liposomes, and the loading was needed to be performed at 37°C in the presence of a narrow range of ethanol content (10–15%).

In the final step, the STS-LNPs were purified by gel filtration or extended dialysis against HBS. As revealed in cryo-TEM, STS formed spherical nano-aggregates inside the LNPs, resulting in high drug retention during 37°C incubation in buffer or 50% serum. This stable drug encapsulation also resulted in the prolonged storage stability at 4°C, with no significant size change or drug leakage detected during 6 months of storage (study ongoing). It is worth mentioning that cryo-TEM examines the LNP morphology on a fixed plane of a frozen liquid sample, which reveals the detailed structure of the LNPs. However, this fixed plane could be at the edge or center of a LNP. Therefore, the variation in size in cryo-TEM images does not result from the real size variation, but the images at the edge or center of the LNPs. The size variation of the LNPs was measured by dynamic light scattering and the PDIs were all below 0.1, suggesting uniform size distribution.

Prior studies using liposomes to encapsulate STS and its derivative (UCN-01) encompass two drug loading methods: passive loading using the reverse evaporation (RE) method (34), and the active loading via a normal or reversed pH gradient (24,35). These methods provided low to moderate drug loading efficiency, with a D/L of 0.008 (mol/mol) for the RE method, 0.035 (mol/mol) for the normal pH gradient, and 0.09 (mol/mol) for the reversed pH gradient method. Moreover, the stability of the liposomes (DSPC/DOPE/cholesterol/DSPE-PEG₂₀₀₀) prepared with the reverse pH gradient method was poor, leaking ~70% of STS when incubated with PBS or serum at 37°C for 1 day, possibly due to the unstable drug loading and/or lipid formulation with decreased transition temperature. This result may also suggest a compromised storage stability, which may diminish the potential of developing this formulation into a pharmaceutical product. The method and the LNP formulation disclosed in this manuscript provided improved STS loading and stability. In addition to these attempts, developing an active loading method for water insoluble drugs has been an actively pursued topic of research. (24,27,35–40). Zhigaltsev et al. (27) chemically conjugated a

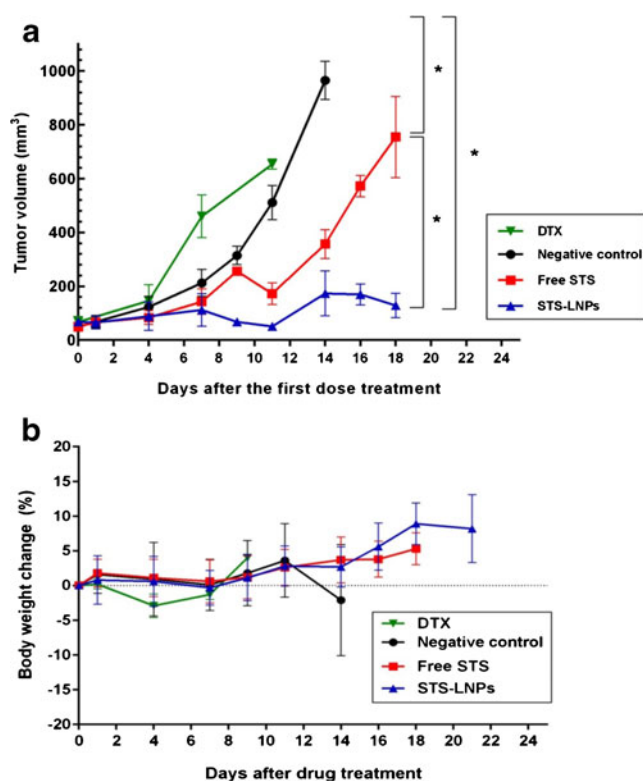


Fig. 8 In vivo antitumor efficacy against murine EMT6/AR1 tumor: (a) tumor size; (b) body weight. Data = mean \pm SD (n = 10). * indicates p value < 0.05.

Table 4 Drug retention, Size and Polydispersity Index (PDI) of the STS-LNPs upon Storage at 4°C

Day	Size	PDI	Loading efficiency
0	96.7 ± 1.1	0.023 ± 0.032	100.4 ± 2.5
1 month	98.2 ± 1.2	0.033 ± 0.012	97.2 ± 1.8
4 month	100.5 ± 0.3	0.030 ± 0.023	98.1 ± 3.1
6 month	102.3 ± 0.2	0.039 ± 0.014	96.7 ± 2.4

piperazine group to docetaxel to introduce a weak base functional group (tertiary amine) to improve the drug solubility at pH 5 (1.7 mg/mL) as well as enable the prodrug to form complex with the sulfate ions inside the LNPs. A D/L of 0.4 (w/w) was achieved with this method. Sur et al. (36) chemically modified the hydrophilic surface of cyclodextran with an alkyl amino group and employed this ionizable cyclodextran as a solubilizing agent in the active loading process. The ionizable cyclodextran solubilized a water insoluble compound by incorporating it inside the hydrophobic chamber and carried the drug through the lipid bilayer into the aqueous core, where the cyclodextran was ionized by a low pH and trapped. Our method also involves inclusion of a solubilizing agent (i.e. DMSO) in the active loading system. As many water insoluble compounds are freely soluble in DMSO, it is anticipated that our method could be employed to actively load many other water insoluble compounds into LNPs for targeted drug delivery. This new loading technology, although derived from the existing technique, offers more than incremental improvements, as it enables stable encapsulation and delivery of STS and possibly many other water insoluble drugs that otherwise cannot be formulated with the standard active loading method. Compared to the Zhigaltsev and Sur methods, our approach does not need chemical modification of a solubilizing agent or the drug, and allows drug loading at room temperature.

In order to evaluate the potential of the STS-LNPs for delivering STS for cancer therapy, we compared the activity of DTX, free STS and the STS-LNPs against resistant tumor cells *in vitro* and *in vivo*. Free STS and STS-LNPs exhibited comparable potency against EMT6/AR1 and PC-3-RES cells, and were 2- to 360-fold more potent than DTX. The overall dose of the STS-LNPs that could be safely administered to mice was 3 times higher than free STS. As a result, the STS-LNPs exhibited significantly improved antitumor activity against the DTX-resistant EMT6/AR1 tumor in mice compared to free STS at their MTDs.

CONCLUSION

The standard active loading technique cannot formulate water insoluble drugs into LNPs. Insoluble drugs account for a large percentage of therapeutic agents and require advanced technologies for effective delivery. We have developed a new active loading method to encapsulate a water insoluble drug STS into LNPs at a D/L of 0.31/1 (mol/mol). STS was loaded into the LNPs via an ammonium sulfate gradient in the presence of a water miscible solubilizing solvent, DMSO, forming a spherical drug aggregate inside the core of LNPs with improved drug retention upon 4°C storage or 37°C incubation with 50% serum. The complete drug loading could be achieved within 15 min, and could be performed at room

temperature. Compared to free STS, the STS-LNPs exhibited comparable *in vitro* cytotoxic potency, but the STS-LNPs could be safely dosed 3 times higher. As a result, the *in vivo* efficacy of the STS-LNPs against a resistant tumor model was significantly improved compared to free STS and DTX. This simple and efficient drug loading method may potentially transform STS into an effective therapeutic agent against resistant cancer.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was funded by grants from the Canadian Institutes of Health Research (MOP-119471 and PPP-130153). S.D. Li is a recipient of a Coalition to Cure Prostate Cancer Young Investigator Award from the Prostate Cancer Foundation and a New Investigator Award from Canadian Institutes of Health Research (MSH-130195).

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