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Nigericin-mediated liposome loading of topotecan: Is nigericin a potential drug release regulator?

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ABSTRACT

Topotecan was loaded into large unilamellar vesicles using nigericin-generated pH gradient or with triethylammonium (TA) ion gradient. Despite that in both loading methods, the encapsulated counter ion was 5-sulfosalicylate (5ssa), the resultant formulations exhibited distinct properties. In NaCl-containing release buffer, vesicles prepared with nigericin/Na^+ system could release topotecan at a faster rate than 5ssa–TA vesicles, whereas in the absence of Na^+, there was no difference in the drug release kinetics. In plasma, both formulations could prolong the circulation halftime of topotecan, but 5ssa–TA vesicles were more able to stabilize the encapsulated topotecan, as evidenced by the increased $t_{1/2}$ and decreased conversion of lactone to carboxylate form. However, the improved drug retention did not mean the elevated safety and efficacy. In L1210 ascitic tumor model, the administration of 5ssa–TA vesicles at 10 mg/kg induced the early death of ~60% mice at 6–7 days. In contrast, the treatment with nigericin/Na^+ vesicles at the same dose level resulted in a mean survival time of ~18.0 days, ~1.38-fold of that of free topotecan. In addition, the formulation was safer than free topotecan. The results indicated that nigericin could be used as release regulator to optimize drug release kinetics, resulting in safe and efficacious liposome-based topotecan formulation. The results were promising since no liposome topotecan formulations with reduced toxicity were reported.

1. Introduction

Encapsulation of various antineoplastic drugs into liposomes has been shown to decrease toxic side effects while increasing or maintaining therapeutic activity (Drummond et al., 1999). However, not all the liposome formulations could improve therapeutic index of entrapped drugs. A desired liposome formulation must possess stable drug encapsulation and long circulation characteristics. Many means could mediate drug loading, but the methods that rely on pH gradient have distinct advantages over other methods and thus have been extensively employed. To create transmembrane pH gradient, at least three kinds of methods could be used, including (1) the formation of large lamellar vesicles (LUVs) in acidic buffer followed by exchanging vesicles into neutral buffers (Cullis et al., 1991; Maurer-Spurej et al., 1999; Mayer et al., 1986; Redelmeier et al., 1989); (2) the employment of "self-generating" systems such as vesicles with entrapped ammonium sulfate (Haran et al., 1993; Lasic et al., 1995) and (3) ionophore-mediated liposome loading techniques (Chakrabarti et al., 1992; Fenske et al., 1998; Wheeler et al., 1994).

The drug loading technique based on the ion-translocating properties of the ionophores (e.g., nigericin and A23187) was first introduced by Wheeler et al. (Fenske and Cullis, 2005; Fenske et al., 1998; Wheeler et al., 1994) and has been applied to the loading of various drugs such as vinca alkaloids, camptothecin derivatives and so on (Abraham et al., 2002, 2004; Chiu et al., 2005; Fenske and Cullis, 2005; Johnston et al., 2006; Mayer et al., 1986; Messerer et al., 2004; Semple et al., 2005; Taggar et al., 2006; Waterhouse et al., 2005; Zhigaltsev et al., 2005, 2006). The method involves the following steps: (1) the preparation of LUVs in metal ion salt solutions; (2) the formation of salt gradient by solvent exchange and (3) uptake of desirable drugs in the presence of ionophores (Fenske and Cullis, 2005; Fenske et al., 1998; Wheeler et al., 1994).

Topotecan is a semi-synthetic derivative of camptothecin, which has been approved for the treatment of solid tumors and leukemia (Dennis et al., 1997; Herben et al., 1996; Jones and Burris, 1996). As

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with all camptothecins, topotecan specifically inhibits the activity of topoisomerase I by stabilizing the topoisomerase I-DNA complex, resulting in lethal DNA strand breaks (Jones and Burris, 1996).

In aqueous solution, topotecan undergoes a pH-dependent hydrolysis of the lactone ring to form a relatively inactive carboxylate. This rapid conversion is also observed in patients after systemic injection of the drug (Burke and Gao, 1994). A potential solution to this problem is to encapsulate topotecan within a liposome. Previous studies have proved that liposomes with an acidic internal media could stabilize topotecan as its lactone form (Burke and Gao, 1994; Liu et al., 2002; Taggar et al., 2006). Moreover, liposomes could deliver more drugs to malignant zones due to "EPR" effects and prolong the exposure time of tumor cells to topotecan, resulting in enhanced efficacy (Drummond et al., 2010; Hao et al., 2005; Liu et al., 2002; Subramanian and Muller, 1995; Tardi et al., 2000).

To date, various liposomal topotecan formulations have been developed with different drug loading strategies. Nevertheless, all reported liposomal topotecan formulations had high toxicity relative to free topotecan despite that antineoplastic effects might be improved (Drummond et al., 2010; Hao et al., 2005; Liu et al., 2002; Subramanian and Muller, 1995; Tardi et al., 2000).

In the present work, we have encapsulated topotecan in the aqueous interior of the liposome using an nigericin-induced proton gradient. This approach provides efficient loading at high drug to lipid ratios, and an acidic environment stabilizing the lactone species. Since nigericin was hard to be removed from vesicles and might affect pharmaceutical and pharmacological properties of the resultant formulations, the drug release properties, pharmacokinetic behavior and anticancer efficacy of liposomal formulations were investigated in a systemic manner. Significantly compared to reported liposomal formulations, the achieved formulation was efficacious and safe.

2. Materials and methods

2.1. Materials

Topotecan hydrochloride was provided by Chengdu Tianyuan Natural Product Co., Ltd. (Chengdu, China). Hydrogenated soybean phosphatidylcholine (HSPC) was a kind gift from Degussa (Freising, Germany). N-(carbonyl-methoxypolyethyleneglycol$_{2000}$)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (DSPE-PEG) was obtained from Genzyme Pharmaceuticals (Liestal, Switzerland). Cholesterol (Chol), nigericin and Sephadex G-75 (medium) were obtained from the Sigma Chemical Company (St. Louis, MO). Nucleopore polycarbonate filters (47 mm, 0.10 μm pore size) were obtained from Northernlipids, Inc. (Canada). All other chemicals used in this study were analytical or high-performance liquid chromatography (HPLC) grade.

The L1210 cell line was originally purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). KM mice (8–10 weeks old) were obtained from Hebei Medical University. DBA/2 mice were purchased from Vitalriver (Beijing, China). All animal experiment protocols were approved by Institutional Animal Care and Use Committee and comply with Code of Ethics for animal experiments.

2.2. Preparation of liposomes

Liposomes were prepared according to the following procedure. Briefly, the mixtures of HSPC, Chol and DSPE-PEG (60.2:39.5:0.3, molar ratio) were solubilized in chloroform and dried to a thin lipid film under a stream of N_2 gas, followed by incubation overnight under vacuum to remove residual solvent. The dried lipid films were subsequently hydrated with 150 mM sodium 5-sulfosalicylate or triethylamine salt of 5-sulfosalicylic acid (pH 5.5). The hydration process was performed at 60 °C for 1 h. The dispersion was extruded 8 times through polycarbonate filters of 0.10 μm employing a LiposoFast-100 jacketed extruder obtained from Avestin (Ottawa, Canada). This procedure formed large unilamellar vesicles. The zeta average size of vesicles was typically about 100 nm based on quasi-elastic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK).

2.3. Remote loading of liposomes

A transmembrane metal ion gradient was generated across the LUVs by exchanging the extraliposomal buffer using Sephadex G-75 columns. The buffer employed in the experiments was sucrose (300 mM)–histidine (20 mM) buffer (pH 6.0). All the loading experiments were performed at 60 °C. For samples hydrated with sodium 5-sulfosalicylate, nigericin was incubated with empty liposomes at 60 °C for 10 min before the addition of topotecan. In contrast, 5ssa–TA sample was mixed directly with topotecan in the absence of nigericin. Following the addition of topotecan, the mixtures were incubated at 60 °C for desired time. Samples of the mixtures were taken at specific time points and unentrapped topotecan was removed by size exclusion chromatography for determining the loading efficiency.

2.4. In vitro release studies

Topotecan release from different vesicles was monitored using a fluorescence dequenching assay. Prior to analysis, an aliquot of liposomal topotecan was diluted 1000-fold by injection into a quartz cuvette containing a solution of isotonic NaCl/histidine (pH 6.5 or 7.5). A Hitachi F-4600 fluorescence spectrophotometer was employed. The fluorescence intensity data were collected continually with a time interval of ~8 s. The ex/em wavelengths were 381/525 nm, respectively. Temperature was controlled at 37 °C using a jacketed sample holder, which was connected to a circulating water bath. The achieved intensity data were converted to concentration data according to a fluorescence intensity (FI) versus concentration standard curve. % release was determined by $100 \times ([\text{topo}]_t - [\text{topo}]_0)/[\text{topo}]_{\text{total}}$, where $[\text{topo}]_t$ and $[\text{topo}]_0$ is free topotecan concentration at time points t and 0, $[\text{topo}]_{\text{total}}$ was the total topotecan concentration, including both free and liposomal topotecan. To quantitatively compare the difference in drug release kinetics, % release was plotted as a function of time.

2.5. Pharmacokinetic studies

Plasma pharmacokinetic analysis was performed in male KM mice. KM mice received injections of 10 mg/kg single i.v. bolus dose of liposomal topotecan formulations or free topotecan via tail vein. Blood samples were obtained via cardiac puncture under anesthesia and collected in Eppendorf tubes containing sodium heparin as an anticoagulant at various time points after administration. Blood samples were centrifuged at 2500 rpm for 10 min to separate the plasma. The plasma samples were stored at −20 °C until HPLC analysis.

Topotecan concentrations in plasma samples were determined using HPLC method. For 50 μL plasma, 450 μL ice-cooled methanol was added to precipitate proteins and solubilize liposomes. The resulting mixture was vortexed and permitted to precipitate at −20 °C for at least 1 h; and then centrifuged at 20,000 × g for 10 min. The supernatant was collected for analysis. The injection volume for plasma samples was 20 μL. These conditions were found to stabilize lactone form and thus permit to determine both lactone and carboxylate simultaneously.

A Shimadzu HPLC system controlled by LC solution software was used for chromatographic analysis, which was composed of DGU-20A5 degasser, LC-20AT liquid chromatograph, SIL-20A auto-sampler, RF-10AXL detector and CTO-20A column oven. The auto-sampler and the column compartment were maintained at 25 °C. The HPLC separations were achieved using a Diamonsil C18 column (150 mm × 4 mm i.d., 5 μm particle size) from Dikma with a run time of 15 min at a flow rate of 1 ml/min. A guard column (Diamonisl C18, 4 mm × 8 mm) was installed ahead of the analytical column. The ex/em wave lengths were 381/525 nm, respectively.

The mobile phase was a mixture of acetonitrile (A) and aqueous phase (B) (3% triethylamine solution adjusted to pH 5.5 with acetic acid). The elution gradient consisted of a mixture of A: B in the following ratios: 15%:85% for 0–6 min, 25%:75% for 6–10 min, followed by 15%:85% for 10–15 min. Under the HPLC conditions outlined above, the carboxylate species of topotecan elutes at 3 min and the lactone species elutes at 7 min.

Standard curves for the two species of the drug were generated by dissolving the drug in either 40% methanol:60% 10 mM citrate buffer (pH 3) for the lactone species or 40% methanol:60% 10 mM borate buffer (pH 9) for the carboxylate species. The recovery of both species was >95% and the standard curve with an r value of 0.999.

The difference in topotecan concentrations among different formulations was examined by a series of independent-samples T-test (SPSS 11.5 software). In all cases, $p < 0.05$ was considered to be statistically significant.

2.6. Anti-tumor efficacy

Male DBA/2 mice were inoculated intra-peritoneally with 5×10^5 L1210 murine tumor cells, derived from the ascitic fluid of a previously infected DBA/2 mouse. Free topotecan or liposome-encapsulated topotecans were administrated via a lateral tail vein 24 h after tumor cell inoculation. Animal weights were monitored daily and mortality determined up to 60 days. The data was analyzed with SPSS 11.5 version software (survival analysis).

3. Results

3.1. Drug loading experiments

Nigericin could exchange one Na^+ from the interior of vesicles with one H^+ from external buffer (Fenske and Cullis, 2005; Fenske et al., 1998). Thus, a transmembrane pH gradient could be generated followed incubation of nigericin with Na^+-containing empty vesicles, in response to which topotecan could be loaded into LUVs. As revealed by Fig. 1, when the nigericin to HSPC mass ratio was >1 ng/mg HSPC, >95% loading could be achieved within 5 min and the loading was stable overall the incubation time. When the nigericin/HSPC (ng/mg) ratio ranged from 0.01% to 0.2, % loading continuously increased with increasing incubation time. Moreover, drug loading rate and efficiency were positively correlated with nigericin content.

Since previous studies reported that drug loading profiles into LUVs in response to pH gradient were governed by $EE(t) = EE(eq)(1 - e^{-kt})$ (Boman et al., 1993; Cullis et al., 1997), $LN(1 - EE/EE(eq))$ was plotted versus time to determine the loading rate constant k. It is interesting to find that the data could be well fitted into the exponential equation with a r value of >0.99. Therefore, k could be easily calculated by means of regression analysis.

Moreover, the plot of k vs. nigericin content was linear with an r^2 value of ~0.9993; namely, k increased linearly with increasing nigericin/HSPC ratio. Although it is not clear why such a mathematic relationship existed, it is convenient to determine nigericin content and its removal efficiency from LUVs using the relationship.

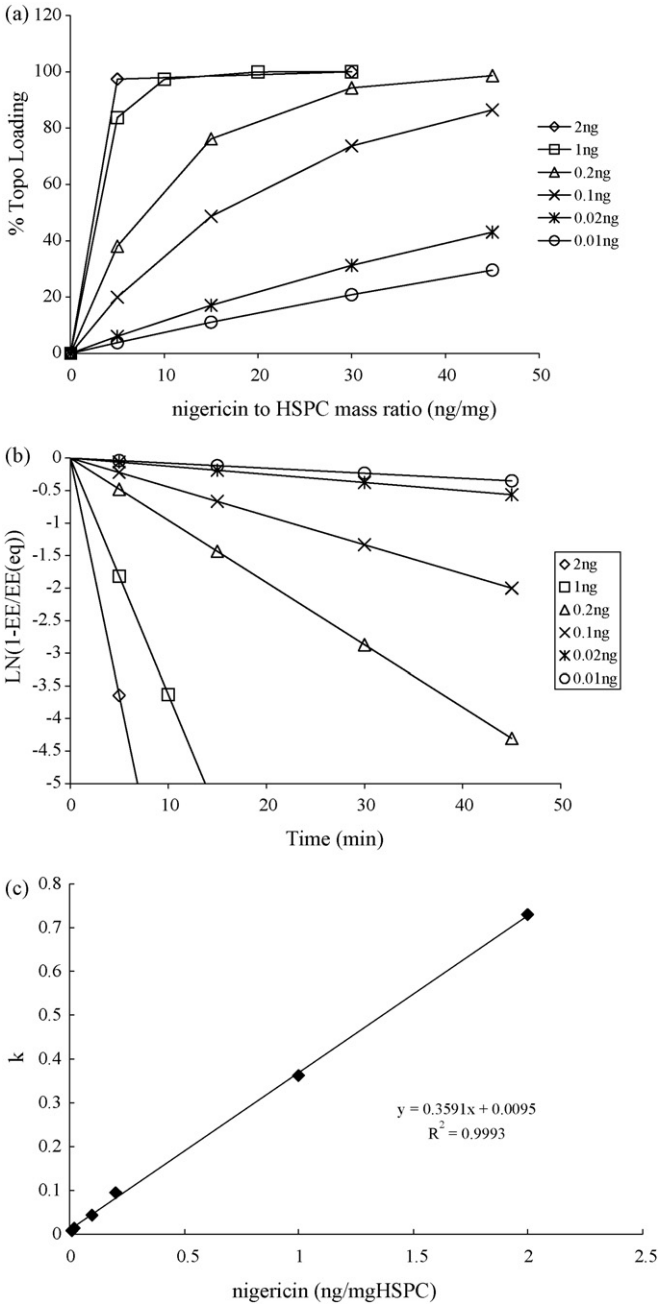

Fig. 1. (A) Time-course of topotecan uptake into 100 nm HSPC/chol vesicles in the presence of various amount of nigericin. Prior to drug loading, nigericin was mixed with empty vesicles with entrapped sodium salt of 5-sulfosalicylic acid (300 mM) and incubated at 60 °C for 10 min. The mass ratio of nigericin to HSPC ranged from 0.01 to 2 ng/mg. All the samples were loaded at 60 °C with a drug to HSPC ratio of ~0.2. At specific time points, an aliquot of sample was taken for the determination of % loading using size chromatography. (B) Plot of $LN(1 - EE/EE(eq))$ vs. time, where EE is encapsulation efficiency at time t. The slopes of these lines give the rate constant (k) for the transportation of topotecan across the lipid membrane. (C) Plot of k vs. nigericin content. The slope of this line is 0.3591.

1–2 ng nigericin was first incubated with LUVs for ~10 min, and then the resulting mixture was loaded onto Sephadex column. After elution with sucrose/histidine solution, the LUV fraction was concentrated and employed to load topotecan. It is interesting to find that drug loading profiles with or without column separation were almost superimposed, indicating that nigericin was hard to be removed from LUVs.

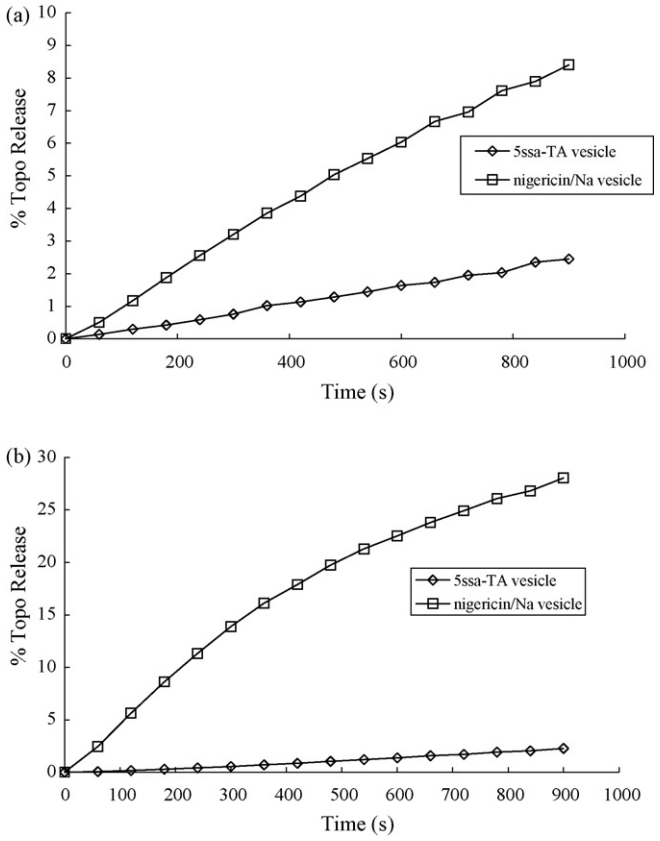

Fig. 2. In vitro release of different liposomal topotecan formulations. The release experiments were performed in (a) pH 6.5 or (b) 7.5 isotonic NaCl/histidine buffers at 37 °C. Prior to analysis, an aliquot of liposomal topotecan was diluted 1000-fold by injection into a quartz cuvette containing a solution of isotonic NaCl/histidine A Hitachi F-4600 fluorescence spectrophotometer was employed. The fluorescence intensity data were collected continually with a time interval of ~8 s. The ex/em wavelengths were 381/525 nm, respectively. Temperature was controlled at 37 °C using a jacketed sample holder, which was connected to a circulating water bath. Data points represent the mean values calculated from 3 samples.

3.2. Drug release rate

Since nigericin had high affinity for LUVs and was hard to be removed, it might affect drug release rate, especially in media with high concentration of electrolyte. To test this, drug release experiments were first performed in glucose/histidine (290/10 mM) buffer; both formulations exhibited no difference in topotecan release. However, in the release medium composed of NaCl/histidine (150/10 mM, pH 6.0 or 7.5), the drug release rate from vesicles loaded with nigericin/Na^+ combination was considerably faster than that from vesicles loaded with 5ssa–TA gradient. As shown in Fig. 2, during the experimental period, the drug released from 5ssa–TA vesicles slowly increased, with a final release ratio of ~2%. The pH-independent release might be ascribed to the "dilution effects". In contrast, topotecan was released at a fast rate from nigericin/Na^+ vesicles in a pH dependent manner. % release of topotecan from nigericin/Na^+ vesicles could reach ~8% and ~25% in pH 6.5 and 7.5 release media, respectively. This phenomenon might be ascribed to the pH dependent activity of nigericin.

3.3. PK studies

Analogous to previous studies, following i.v. injection, free topotecan was rapidly cleared from plasma. Liposomal encapsulation could markedly prolong the plasma halftime of topotecan, resulting in increased AUC values, regardless of the loading method

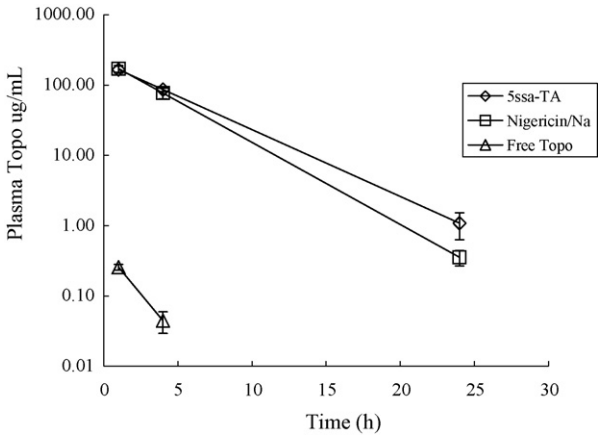

Fig. 3. Plasma concentrations of topotecan in KM mice injected i.v. with liposome-encapsulated topotecans and free topotecan. The topotecan dose was 10 mg/kg and the total topotecan levels were assayed using HPLC method. The data are shown as mean ± SD ($n = 3$).

(Fig. 3). Moreover, following the administration of liposomal formulations, topotecan mainly existed in the form of lactone (>90%), which was considerably compared to free topotecan group, where a substantial amount of topotecan was in carboxylate form (~30%). Therefore, liposomal encapsulation could prevent topotecan hydrolysis due to low intraliposomal pH and slow drug release rate. However, different liposomal formulations exhibited different plasma pharmacokinetics. After injection of nigericin/Na^+ vesicles, topotecan was cleared from plasma at a fast rate compared to that of the 5ssa–TA group. The $t_{1/2}$ values of these two groups were 2.58 h and 3.17 h, respectively. This phenomenon might be attributed to the rapid drug release kinetics of nigericin/Na^+ vesicles.

Since at plasma physiological pH value (7.35–7.45), the lactone ring is liable to hydrolysis, leaked topotecan will rapidly convert to its open ring form. Therefore, increased drug release rate might lead to decreased lactone/carboxylate ratio. Based on our results, at the same time points, % lactone values in nigericin/Na^+ group were significantly lower than those in 5ssa–TA group, indicating the rapid drug release rate of the former formulation (Table 1).

3.4. Antineoplastic effects

The antineoplastic effects were investigated using L1210 ascitic model, a fast growth tumor model. Liposomal topotecan and free topotecan were injected into DBA/2 mice at a dose of 10 mg/kg. Sucrose/histidine buffer was used as control. The inoculation of L1210 cells into DBA/2 induced early death of control mice, with a mean survival time of ~10 days (Table 2).

Similarly, treatment with empty vesicles also resulted in survival time of ~10 days. In contrast, free topotecan was thera-

Table 1
Analysis of plasma topotecan after injection of free or liposomal topotecan.

Time	Total (lactone plus carboxylate) plasma concentration (μg/ml)	Carboxylate (% of total)
Free topotecan		
1 h	0.257	21.11
4 h	0.045	38.14
Nigericin/Na^+		
1 h	179.86	3.64
4 h	80.99	5.84
24 h	0.37	10.19
5ssa–TA		
1 h	165.75	1.81
4 h	86.43	3.18
24 h	1.08	7.23

Table 2
Anti-tumor efficacies of topotecan formulations against L1210 leukemia cell line in DBA/2 mice. Mice were inoculated with 5×10^5 cells i.p. on day 0 and treated on day 1.

Treatment group	Dose (mg/kg)	No. of survivors (day 60)	Mean survival time[a]	Median survival time[a]	% ILS[b]	L/F[b]
Control	10	0/10	10.60 ± 0.40	10		
Empty vesicles	10	0/10	10.50 ± 0.31	10	0	
Free Topo	10	0/10	14.30 ± 0.78	13	30	
5ssa–TA	10	0/10	13.7 ± 3.23	7.00 ± 0.31	−30	0.54
Nigericin/Na^+	10	0/10	20.3 ± 1.29	18.00 ± 0.62	80	1.38

[a] To calculate mean and median survival time, survivors after 60 days were assigned survival times of 60 days.
[b] Values for ILS (increased life span) and liposomal/free (L/F) were calculated using median survival data.

peutically active. After treatment, the survival time increased ~30% relative to that of control group. Surprisingly, in 5ssa–TA vesicle group, 60% mice died at 6–7 days post administration, thus resulting in decreased mean and medium survival time. The early death in this group might be ascribed to the toxicity associated with topotecan since the empty vesicles exhibited no toxicity signs. Of all the formulations tested, vesicles loaded with nigericin/Na^+ method were the most promising because the formulation could significantly prolong the survival time of mice and was also less toxic. Based on body weight versus time curves, the toxicity could be arranged as follows:

$$\text{Empty vesicles} \approx \text{control} < \text{nigericin/Na}^+ \text{ vesicles} \leq \text{free topotecan} \ll \text{5ssa–TA vesicles.}$$

4. Discussion

Weakly basic antineoplastic drugs could be loaded into LUVs exhibiting transmembrane cationic ion gradient in the presence of ionophores (Fenske and Cullis, 2005; Fenske et al., 1998; Mayer et al., 1986; Wheeler et al., 1994). Typically, two well-characterized ionophores (nigericin and A23187) were employed. Since Na^+ is more physiologically acceptable than other ions, in this study nigericin/Na^+ system was selected to load topotecan.

Kinetic analysis of drug loading process revealed that the process was governed by the equation $EE(t) = EE(eq)(1 - e^{-kt})$. The phenomenon has been observed when weakly basic drugs were loaded into vesicles exhibiting pH gradient (Boman et al., 1993; Cullis et al., 1997). Since in our studies, pH gradient was set up prior to drug loading, it is not surprising to observe the same phenomenon. However, to our surprise, the calculated rate constant k was positively proportional to the amount of ionophore.

Although it is hard to expound why such a relationship existed, the linear relationship between k and nigericin content made it possible to quantitatively determine the amount of nigericin in the system even at very low concentration. Unlike A23187, nigericin has no UV–VIS absorption and cannot emit fluorescence, so it is hard to quantify its content. Based on our observation, it is easy to resolve the problem. Unknown amount of nigericin could be added to LUVs with entrapped Na^+ ions to perform drug loading experiments. After mathematic treatment of drug loading curves, k could be achieved, which can be used to calculate nigericin content. Using this method, it is found that nigericin had great affinity for lipid membrane and was hard to be removed from LUVs.

Since nigericin could stably associate with lipid bilayer, it might affect drug release kinetics especially when liposomes were injected into plasma. The high serum concentration of ions such as Na^+ and K^+ may cause reverse transport, leading to the collapse of pH gradient and the release of drugs. This has been proved by plasma kinetics studies. In circulation, vesicles loaded with nigericin/Na^+ system could release topotecan at a rapid rate relative to vesicles loaded with 5ssa–TA method. However, the accelerated release induced by nigericin might be advantageous.

In L1210 ascitic tumor model, liposomal topotecan loaded with 5ssa–TA method was more toxic than free topotecan. This result is not surprising in view of the fact that liposomal encapsulation could stabilize topotecan as the lactone form and deliver more lactone form to normal tissues and tumors. The lactone form, not its hydrolysis product, could effectively diffuse into cells and inhibit topoisomerase I, exerting cytotoxic effects (Jones and Burris, 1996). Accordingly, at the equivalent dose level, liposomal topotecan usually exhibited high toxicity and efficacy relative to free topotecan. It was also the case in our studies. In the group treated with 5ssa–TA vesicles, if the mouse did not die from drug toxicity, they could survive a relatively long time, indicating the enhanced therapeutic effects.

In contrast to this, liposomes loaded with nigericin/Na^+ system had enhanced antineoplastic effects and slightly improved safety. Perhaps, it was the first liposomal topotecan formulation with reduced toxicity compared to free topotecan. Why did the formulation exhibit distinct properties? Nigericin present in lipid membrane might play a role.

As revealed by previous studies, leaked drugs, not liposome-encapsulated drugs, were bioavailable, and the antineoplastic effects of liposomal drugs were the result of the interplay of both targeting efficacy and drug release rate (Cui et al., 2007). Both factors determine the tissue AUCs (normal or malignant zones) of bioavailable drugs. If relative to free drugs, liposomal formulations have elevated tumor and normal tissue AUCs of bioavailable drugs, they would exhibit increased toxicity and efficacy. As for whether therapeutic index (TI) is improved or not, the ratio of tumor AUC_b (AUC of bioavailable drugs) to tissue AUC_b is critical. If the value is larger than that of free drug, liposomal formulation will has elevated TI value. Of course, when liposomal drug has increased tumor AUC_b and reduced (or equivalent) tissue AUC_b, the formulation will possess improved efficacy and safety. It is possible that liposomal topotecan loaded with nigericin belonged to the latter cases.

Indeed, nigericin present in the bilayer should be regarded as a drug release regulator. In plasma, it induced additional release of topotecan, thus reducing the amount of lactone form that was delivered to normal tissues. Despite that the amount of lactone form that was delivered to tumor zone also reduced, it might still be sufficient to exert therapeutic effects. Accordingly, this formulation had not only improved safety but also enhanced efficacy. Moreover, the ion-translocation activity of nigericin was pH dependent. At slightly basic pH, nigericin had higher activity to translocate ions and reverse pH gradient, thus permitting more drug release in plasma. Interestingly, due to their rapid growth, tumor zones usually have relatively lower pH. Thus nigericin might selectively reduce drug release rate at malignant zone, which may be advantageous to realize the effects of liposomal topotecan. Therefore, nigericin/Na^+ system was not so disadvantageous as previously expected. Reversing ion gradient and increasing drug release in plasma might be helpful under certain conditions, which could provide novel option for optimization of drug release.

As for toxicity concerns about nigericin, it also appears to be unwarranted at present. Due to its high potency, the amount used (0.5 μg nigericin/kg mouse, corresponding to 10 ng nigericin/mg

HSPC) would correspond to dose approximately 4 orders of magnitude below established toxic levels (LD_{50} in mice, 10–15 mg/kg i.p.). Indeed, no additional toxicity signs had been observed when nigericin-containing empty vesicles were administrated into mice.

5. Conclusions

In this study, it is found that nigericin present in lipid bilayer could be used as drug release regulator, which could modify drug release rate in response to external pH and metal ions. Perhaps, it is just because of the presence of nigericin in bilayer that a formulation with increased efficacy and safety was achieved. It might be the first liposomal topotecan formulation with reduced toxicity. Thus, nigericin/Na^+ system is worthy of more investigation (esp. its functions as drug release regulator), despite that A23187/Mg^{2+} combination was previously thought to be more promising due to the easy removal of A23187 from vesicles.

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