Drug Development and Industrial Pharmacy, 32:719–726, 2006

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1080/03639040500529077

Taylor & Francis

Effect of Phospholipid Composition on Characterization of Liposomes Containing 9-Nitrocamptothecin

J. Chen, Q. N. Ping, J. X. Guo, X. Z. Chu and M. M. Song
Department of Pharmaceutics, China Pharmaceutical University, Nanjing, P. R. China

ABSTRACT 9-Nitrocamptothecin (9-NC), a newly developed camptothecin derivative, had poor solubility in any pharmaceutically acceptable solvents. One way of improving the solubility is to formulate the drug into liposomes. However, 9-NC has low affinity to lipid membranes resulting in a very low drug-to-liposome entrapment. We developed a novel liposome-based 9-NC formulation which was composed of soybean phosphatidylcholine (SPC), hydrogenated soybean phosphatidylcholine (HSPC), and cholesterol. Compared with conventional liposomes composed of only SPC and cholesterol, 9-NC/lipid molar ratio increased from 1:72 to 1:18 while incorporation efficiency was still maintained about 80%. In addition, after 9-NC was encapsulated into novel liposomes, pharmacokinetic results revealed an increase in area under the plasma concentration-time curve (AUC) and a decrease in distribution volume of 9-NC following intravenous administration to rats. Increased stability in plasma may account for the improved pharmacokinetic behavior of the novel liposomes. Effect of HSPC/SPC molar ratio on characterization of the novel liposomes was also investigated. Except for drug/lipid molar ratio and encapsulation efficiency, HSPC/SPC molar ratio had only a little effect on other properties of novel liposomes. In conclusion, the study suggests that the novel liposomes can act as promising carriers for hydrophobic substances such as 9-NC.

KEYWORDS Liposomes, 9-Nitrocamptothecin, Hydrogenated soybean phosphatidylcholine, Pharmacokinetics

Address correspondence to Professor Qineng Ping, Department of Pharmaceutics, China Pharmaceutical University, No. 24 Tong Jia Xiang, Nanjing 210009, P. R. China; E-mail: pingqn@mailbox.cpu.edu.cn or you might communicate with Jun Chen directly with email address: chenjun75@163.com, junchen_75@hotmail.com

INTRODUCTION

Camptothecin, a plant alkaloid isolated from *Camptotheca acuminata*, is the prototype of a novel class of antitumor angents which exert their activities exclusively by inhibition of topoisomerase I. Camptothecin analogs have shown significant activity against a broad range of tumors. Among them, irinotecan (CPT-11) and topotecan have been approved for the treatment of metastatic colorectal cancer and refractory ovarian cancer, respectively. The intact lactone ring has been proved to be the necessary function group for in vivo and in vitro activity of camptothecins

(Ulukan et al., 2002). For example, the lactone ring-opened carboxylate form of camptothecin has only one-tenth the potency of camptothecin lactone form (Hertzberg et al., 1989). However, the lactone ring is highly susceptible to facile ring opening under alkaline conditions, paticularly in the presence of serum albumin (Burke et al., 1994).

9-nitrocamptothecin (9-NC), a new analog of camptothecin, has been identified to be a very promising anticancer drug with high potency against a wide spectrum of human cancers in preclinical evaluation (Giovanella et al., 2002). Moreover, 9-NC was found to inhibit HIV-1 replication and had potential clinical utility for HIV-infection/AIDS (Hung et al., 2001). However, poor response rates have been obtained from a series of recently conclused clinical trials of oral 9-NC. The results showed that oral 9-NC was clinically inactive in the treatment of advanced soft-tissue sarcomas (Patel et al., 2003), glioblastoma multiforme (Raymond et al., 2002), colorectal cancer (Schoffski et al., 2002) and advanced small cell lung cancer (Punt et al., 2004). Only in the treatment of advanced pancreatic cancer, oral 9-NC was efficacious as first-line therapy (Konstadoulakis et al., 2001). The crucial obstacles to 9-NC clinical effectiveness are the poor oral absorption (Sha et al., 2004; Zhong et al., 2003) and the opening of the lactone ring in vivo (Cao et al., 1998). The chemical structure of 9-NC is shown in Fig. 1.

9-NC is poorly soluble in aqueous solutions, and is practically insoluble in most physiologically compatible and pharmaceutically acceptable solvents. Formulation of 9-NC in concentrated pharmaceutical delivery system for intravenous administration is thus difficult. One way of improving the solubility of 9-NC is to formulate the drug into liposomes.

Liposomes are known to (a) enhance drug cellular internalization, (b) generally decrease unwanted systemic toxic effects, and (c) increase drug solubility in biological fluids, modulating at the same time the drug release profile. Liposomal delivery of 9-NC has shown favorable pharmacokinetics and biodistribution in rats (Chow et al., 2000).

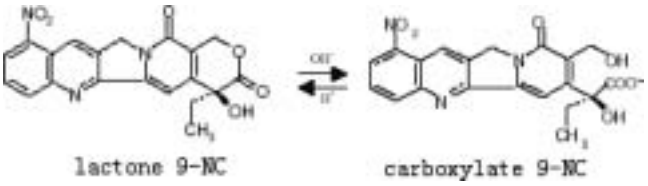

FIGURE 1 **Chemical Structures of Lactone and Carboxylate 9-NC.**

In our laboratory, 9-NC-containing conventional liposomes composed of soybean phospholipid (SPC) and cholesterol were prepared. However, it was found that 9-NC had a low affinity to lipid membranes and tended to precipitate into aqueous phase resulting in a very low drug-to-liposome entrapment. This low lipid affinity behavior of 9-NC has made the development of a liposome-based 9-NC formulation more challenging. To be useful as a pharmaceutical product, the liposomal formulation should have a high drug-to-lipid ratio.

Recently, a novel liposome-based drug delivery system capable of incorporating large amounts of hydrophobic substances has been invented (Kan et al., 2004). The novel liposomes were composed of cholesterol and two phospholipids such as an unsaturated phospholipid and a saturated phospholipid with different phase transition temperatures. Conventional paclitaxel-liposomes were prepared at drug/lipid molar ratio of approximately 3%. After encapsulated by the novel liposomes, the paclitaxel/lipid molar ratio increased up to 20% and incorporation efficiency was maintained above 80% (Kan et al., 2004). It is obvious that the novel liposomes for incorporating large amounts of hydrophobic substances may have promising application potential. However, the characterization of the novel liposomes is still unclear.

In this work, we assessed the feasibility of increase 9-NC/lipid molar ratio using the novel liposomes composed of two phospholipids, namely SPC and hydrogenated soybean phosphatidylcholine (HSPC). And characteristics of conventional liposomes and novel liposomes were compared. Moreover, in vitro and in vivo properties of the novel liposomes composed of different proportion of SPC/HSPC were also compared in order to investigate the effect of phospholipid composition on the characterization of the novel liposomes capable of incorporating large amounts of hydrophobic substances.

MATERIALS AND METHODS

Materials

Soybean phosphatidylcholine (SPC) and hydrogenated soybean phosphatidylcholine (HSPC) were from Lipoid Corp (Germany). 9-nitrocamptothecin (9-NC)

was supplied by the Department of Medicinal Chemistry, China Pharmaceutical University (purity>99%). Water was deionized and then distilled. Methanol was of HPLC-grade. Other reagents were of analytical grade.

Methods

Preparation of 9-NC-containing Liposomes

9-NC-containing conventional liposomes were prepared by the method of thin film hydration. Briefly, the hydrophobic excipients, such as SPC, cholesterol and 9-NC in a molar ratio of 72:24:1 were dissolved in ethanol and were transferred into a round bottom flask. The solution was evaporated under vacuum to remove the solvent and form a lipid film on the wall of the round bottom flask. The dry lipid film was maintained overnight to remove traces of ethanol. The lipid film was then hydrated with 5 mL of 66 mM phosphate buffer solution (pH6.0) for 90 min. Large multiamellar liposomes were then suspended, followed by probe sonicating using JY92-II ultrasonicator (Xinzhi Corp., Ningbo, China) for 10 min (200w) in order to obtain small unilamellar vesicles. The resulted liposomes were then filtered through a 0.45 μm membrane (Shanghai Institute of Pharmaceutical Industry, China). For the preparation of novel liposomes composed of SPC, HSPC, and cholesterol, HSPC was added in various quantities in ethanol to obtain different HSPC/SPC molar ratios and the quantity of SPC decreased correspondingly. In addition, the temperature for vacuum evaporation and hydration increased from 30°C to 60°C (above the phase transition temperature of HSPC). Other compositions and procedures were the same as those of conventional liposomes.

Vesicle Size Measurement

The particle size of the liposome was measured by dynamic light scattering (DLS), using a Zetasizer3000 (Malvern Instrument, U.K.). A sample of the formulation (0.25 mL) was diluted with the hydrating buffer and the intensity-weighted size was averaged from 10 runs.

HPLC Analysis of 9-NC

The HPLC system used for drug analysis consisted of Agilent 1100 modules (Hewlett Packard, USA). For 9-NC drug content assay, a UV variable detector at a wavelength of 370 nm and a reverse-phase C_{18} column (Diamonsil, 5 μm, 250 mm × 4.6 mm, Dikma, China) were utilized. The mobile phase was filtered using a vacuum filter system equipped with 0.45 μm filter and was delivered at a flow rate of 1.0 mL/min.

For the quantitation of 9-NC, the mobile phase consisted of a 7:3(v/v) mixture of methanol and water containing 0.1% acetic acid (pH3.5). The pH of the mobile phase was maintained at 3.5 to ensure that the analyte, 9-NC was in the closed lactone ring form during the assay. The retention time of lactone 9-NC was 5.1 min.

For the analysis of 9-NC in plasma samples, an inline guard column (5 μm, 100 mm × 4.6 mm, Hanbang, China) was coupled with C_{18} column. The mobile phase was a mixture of methanol and 1% triethylamine (adjusted to pH6.5 with glacial acetic acid) (65:35, v/v). The retention time of 9-NC lactone form was 7.0 min.

Entrapment Efficiency

The entrapment efficiency was determined by ultrafiltration method as described previously (Yang et al., 2002; Zhang et al., 2004). The centrifugal filter devices (Millipore, U.S.) were used. The 9-NC liposome suspension was ultrafiltrated with ultrafiltration membrane (MW 100,000, Millipore, U.S.). The concentration of 9-NC in liposome suspension and the ultrafiltrate were diluted with methanol-0.1% acetic acid (9:1, v/v) and assayed by HPLC.

In Vitro Drug Release

In vitro 9-NC release from liposomes was evaluated using dialysis bag diffusion technique (Yang et al., 1999). Dialysis bags (Wanqing Corp., Nanjing, China) with a molecular weight cut-off of 10, 000 were filled with 1 mL of 9-NC liposome suspension, and then placed into 100 mL isotonic phosphate buffer solution (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH7.4) containing 0.05% Tween 80, which was constantly stirred at 37 ± 1°C on a TP-3 magnetic stirrer with a thermostat (Xinlian Electronic Instrumental Factory, Nanjing, China). At various time intrevals, aliquot samples were withdrawn and assayed for 9-NC content by HPLC after acidification with acetic acid. To evaluate the effect of plasma albumin on drug release from liposomes, 1 ml of 9-NC liposome suspension

Effect of Phospholipid Composition

combined with 1 ml fresh rat plasma was placed in the dialysis bag.

In Vivo Pharmacokinetic Studies

Male Spargue-Dawley rats weighing 230–280 g were used in the study. Twenty-five rats were divided into five groups at random. One group of rats was given a single 1.5 mg/kg dose of 9-NC solution by the tail vein injection. 9-NC solution was composed of DMSO: PEG400: ethanol: 5% glucose (pH3.0) (3:3:2:2 by volume) (Chow et al., 2000; Scott et al., 1993). The solution was prepared by first dissolving 9-NC in DMSO followed by the addition of the other solvents and immediately administered to the rats after preparation. Another group of rats was given a single intravenous 1.5 mg/kg dose of 9-NC conventional liposomes. Other three groups of rats were given a single intravenous 1.5 mg/kg dose of 9-NC novel liposomes composed of different HSPC/SPC molar ratios (1:1, 1:3, and 1:9), respectively. Animals had free access to food and water throughout the experimentation period. At various time intervals, the rats were anaesthetized using ether and blood samples (about 0.25 mL) were collected from the retro-orbital plexus into heparinized microfuge tubes. The samples were immediately centrifuged at 4000 r/min for 3 min, and plasma was separated. 200 μl ice-cold (–20°C) methanol-acetonitrile (1:1, v/v) was added to 100 μl plasma sample. The mixture was vortexed for 1 min and centrifuged at 12000 r/min for 3 min. The supernatant was stored at –20°C until bioanalysis. Plasma concentrations of lactone and total 9-NC were determined using validated reverse-phase high-performance liquid chromatography (HPLC) with UV detection method (Warner et al., 1997). Briefly, a volume of 50 μl supernatant was injected into the HPLC system for the analysis of intact lactone 9-NC. For the determination of total 9-NC, glacial acetic acid was added to the supernatant (1:9, v/v) to cause the complete lactonization of 9-NC and then a volume of 50 μl was injected.

RESULTS

Phospholipid Composition and 9-NC Encapsulation Efficiency

9-NC encapsulation efficiency of conventional liposomes composed of 9-NC:SPC (1:72, molar ratio) was 89.55 ± 2.97% (n = 3). When drug/lipid molar ratio increased to 1:64, the efficiency decreased dramatically and was below 70%.

9-NC encapsulation efficiency into liposomes of the different phospholipid compositions at drug/lipid molar ratio of 1:24 or 1:18 is shown in Figs. 2 and 3. It can be easily seen that the use of HSPC resulted in a significantly higher 9-NC/lipid molar ratio by 3 ~ 4-fold. Furthermore, encapsulation efficiency was significantly influenced by HSPC/SPC molar ratio while 9-NC/lipid molar ratio was 1:18. The liposomes composed of HSPC/SPC at molar ratio of 1:3 exhibited the highest entrapment efficiency. When 9-NC/total lipid molar ratio decreased to 1:24, satisfactory encapsulation efficiency (above 80%) was achieved with HSPC/SPC molar ratio ranged between 1:1 and 1:9. Therefore, to compare the effect of HSPC/SPC molar ratio on characterization of the novel liposomes, the 9-NC/lipid molar ratio was set at 1:24 and the HSPC/SPC molar ratios were chosen to be 1:1, 1:3 and 1:9.

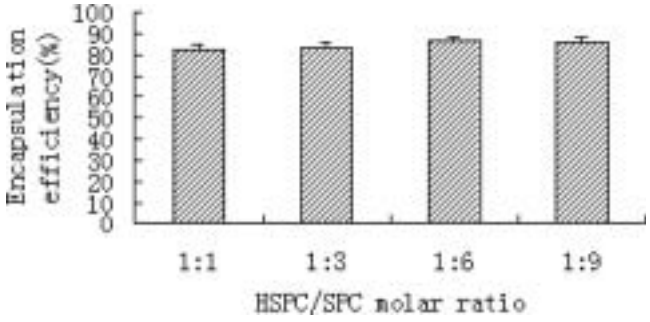

FIGURE 2 **Effect of HSPC/SPC Molar Ratios on Encapsulation Efficiency of 9-NC-Loaded Novel Liposomes (9-NC/lipid Molar Ratio is 1:24). The Encapsulation Efficiency is the Mean of Three Runs ±SD for Each HSPC/SPC Molar Ratio.**

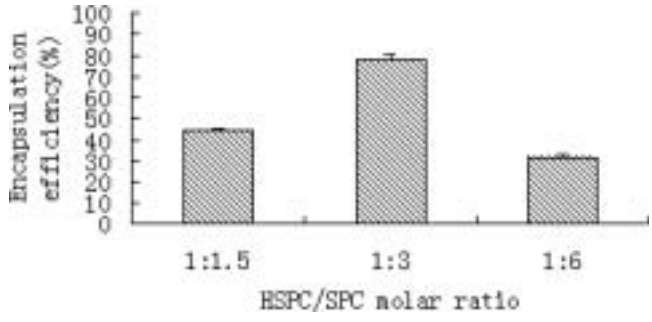

FIGURE 3 **Effect of HSPC/SPC Molar Ratios on Encapsulation Efficiency of 9-NC-Loaded Novel Liposomes (9-NC/lipid Molar Ratio is 1:18). The Encapsulation Efficiency is the Mean of Three Runs ±SD for Each HSPC/SPC Molar Ratio.**

The 9-NC-loaded conventional liposome formulation exhibited significant increase in solubilization of 9-NC, from the aqueous solubility of 0.0025 mg/mL to 0.5 mg/mL. In order to compare the characteristics of conventional and novel liposomes, the 9-NC concentration of novel liposomes was also set to 0.5 mg/mL. It was obvious that the 9-NC solubility might further increase by novel liposomes as the novel liposomes can incorporate larger amounts of 9-NC.

Effect of HSPC/SPC Molar Ratios on the Sizes of 9-NC-loaded Liposomes

The intensity-weighted particle sizes of 9-NC-loaded novel liposomes with different HSPC/SPC molar ratios are shown in Fig. 4. It could be seen that the sizes of liposomes slightly increased with the decrease of HSPC/SPC molar ratios. Such a difference, however, was not significant and the particle sizes of novel liposomes were in the range of 119 ~ 143 nm. In addition, the size of the conventional liposomes with no HSPC had an intermediate size compared with the novel liposomes.

Effect of HSPC/SPC Molar Ratios on the Drug Release from 9-NC-loaded Liposomes

The in vitro drug release profiles are shown in Figs. 5 and 6. Without the existence of rat plasma, the release of 9-NC from novel liposomes was accelerated after the adding of HSPC. And the release rate decreased with the increase of molar ratios of HSPC/SPC. However, with the existence of rat plasma, the release of drug from novel liposomes was sustained in comparsion with conventional liposomes. And the effect of HSPC/SPC molar ratios on the release rate was no longer obvious.

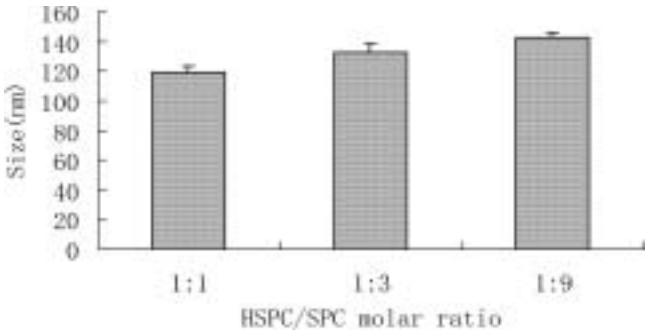

FIGURE 4 **Effect of HSPC/SPC Molar Ratios on Sizes of 9-NC-Loaded Novel Liposomes. The Sizes Are the Mean of Three Runs ± SD for Each HSPC/SPC Molar Ratio. Particle Size of 9-NC Conventional Liposomes Which Contained No HSPC Was 134.5 ± 10.9 nm (*n* = 3).**

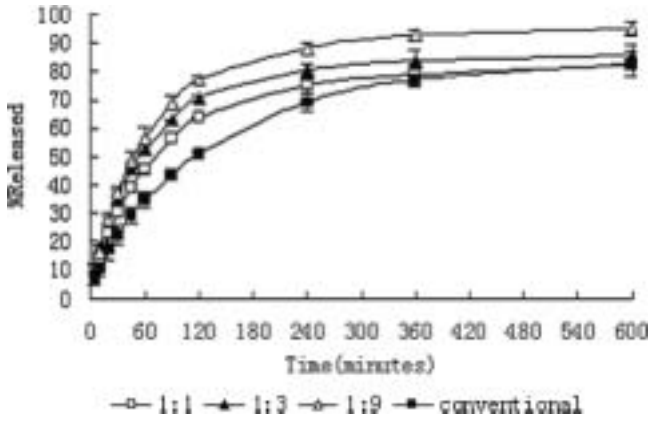

FIGURE 5 **In Vitro Release Profiles of 9-NC From Novel Liposomes Composed of Different HSPC/SPC Molar Ratios or Conventional Liposomes Composed of Only SPC.**

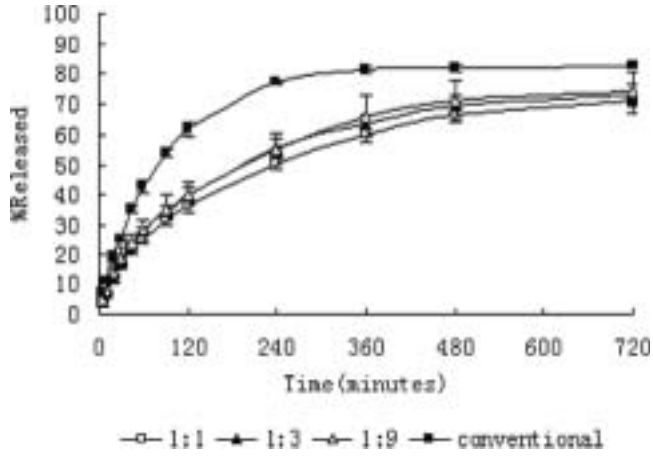

FIGURE 6 **In Vitro Release Profiles (with Rat Plasma) of 9-NC From Novel Liposomes Composed of Different HSPC/SPC Molar Ratios or Conventional Liposomes Composed of Only SPC.**

In Vivo Pharmacokinetic Studies

The lactone and total 9-NC plasma concentrations at various time intervals are depicted in Fig. 7. Plasma drug level profiles of liposomal drug were markedly higher compared to free 9-NC solution. Furthermore, it was also obvious that plasma concentration profiles of 9-NC-loaded novel liposomes composed of HSPC/SPC were higher and sustained compared with conventional 9-NC-loaded liposomes composed of SPC. Non-compartmental analysis of data was performed

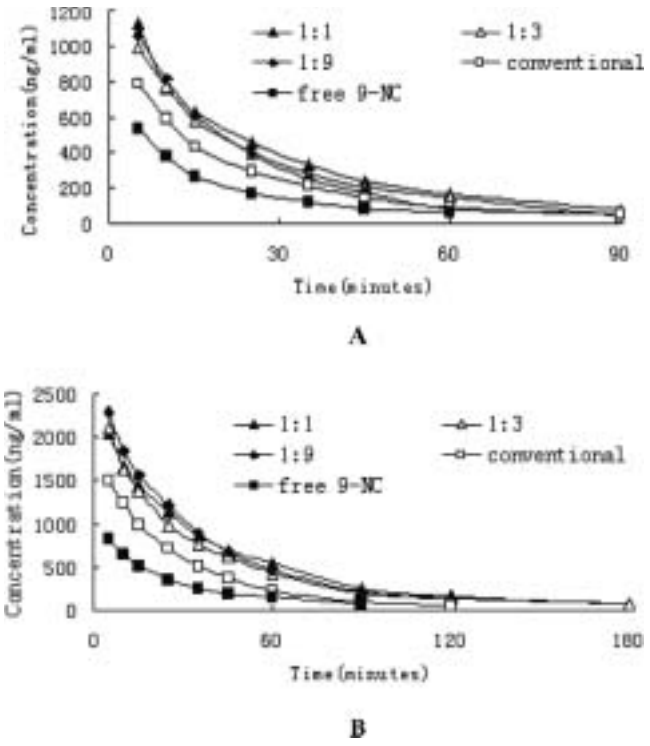

FIGURE 7 **Plasma Level Profiles of Lactone (A) or Total (B) 9-NC Following a Single I.V. Dose (1.5 mg/kg) of 9-NC Solution, 9-NC-Loaded Liposomes Composed of Different HSPC/SPC Molar Ratios or Conventional Liposomes Composed of Only SPC in Rats.**

using statistical moment theory. And calculated pharmacokinetic parameters were summarized in Tables 1 and 2.

Compared with 9-NC solution, the AUC of lactone or total 9-NC were increased significantly after conventional liposomal encapsulation. Moreover, after encapsulated into novel liposomes composed of HSPC and SPC, the AUC of lactone or total 9-NC was increased markedly and the mean retention time of total 9-NC was also prolonged obivously. However, the volume of distribution and total body clearance were decreased. Reduction in volume of distribution may prevent unexpected exposure of healthy cells to the cytotoxic drug, thereby reducing toxic side effects. Moreover, HSPC/SPC molar ratios had little effect on pharmaockinetics of 9-NC encapsulated into novel liposomes.

DISCUSSION

Hydrophobic compounds have a tendency to undergo concentration-dependent aggregation in hydrophobic environment by forming intermolecular hydrogen bonds (Balasubramanian et al., 1994), destablizing the conventional liposomes containing hydrophobic drugs. However, as to novel liposomes, the stability is different. At special ranges of two phospholipid combination and temperature, novel liposomes composed of two phospholipids such as an unsaturated phospholipid and a saturated phospholipid with different phase transition temperature are able to form two separated phases, gel phase and liquid-crystal phase, in the phospholipid bilayer. The two immiscible phases coexist in the novel liposomes and create discontinuous regions. Therefore, the hydrophobic compounds such as 9-NC can be held within the lipid bilayer. The phase boundary barrier between the regions of two immiscible phases is able to reduce lateral movement and aggregation of the hydrophobic compounds, thereby stabilizing the novel liposomes (Kan et al., 2004). The theory provides a rationale for the significantly increased drug/lipid molar ratio after HSPC is included in the phospholipid composition of liposomes.

In this study, characterizations of conventional and novel liposomes were compared. Besides the significant increase of drug/lipid ratio, other characteristics were also influenced. With the existence of rat plasma,

TABLE 1 **Pharmacokinetic Parameters of Lactone 9-NC After a Single I.V. Dose of 1.5 mg/kg in Rats**

Parameters	Free 9-NC	9-NC-loaded conventional liposomes	9-NC-loaded novel liposomes at different HSPC/SPC molar ratios		
			1:1	1:3	1:9
$t_{1/2}$ (h)	0.37 ± 0.07	0.45 ± 0.07	0.43 ± 0.08	0.32 ± 0.03	0.370.04
CL(ml/min/kg)	103 ± 11	62 ± 9	43 ± 8	49 ± 2	51 ± 8
Vss(L/kg)	2.8 ± 0.6	2.0 ± 0.4	1.40 ± 0.28	1.33 ± 0.24	1.33 ± 0.28
MRT(h)	0.46 ± 0.09	0.53 ± 0.07	0.55 ± 0.09	0.45 ± 0.07	0.44 ± 0.02
AUC_{0-t}(ng·h/ml)	214 ± 27	378 ± 68	549 ± 100	489 ± 22	477 ± 81
$AUC_{0-\infty}$(ng·h/ml)	246 ± 25	411 ± 65	596 ± 115	508 ± 22	504 ± 89

TABLE 2 **Pharmacokinetic Parameters of Total 9-NC After a Single I.V. Dose of 1.5 mg/kg in Rats**

Parameters	9-NC solution	9-NC-loaded conventional liposomes	9-NC-loaded novel liposomes at different HSPC/SPC molar ratios		
			1:1	1:3	1:9
$t_{1/2}$ (h)	0.42 ± 0.05	0.49 ± 0.05	0.63 ± 0.14	0.7 ± 0.4	0.49 ± 0.03
CL(ml/min/kg)	52 ± 4	30 ± 3	17 ± 4	18 ± 5	16.5 ± 2.1
Vss(L/kg)	1.97 ± 0.27	1.9 ± 0.4	0.75 ± 0.12	0.77 ± 0.22	0.63 ± 0.08
MRT(h)	0.53 ± 0.07	0.58 ± 0.13	0.76 ± 0.25	0.8 ± 0.4	0.64 ± 0.11
AUC_{0-t}(ng·h/ml)	436 ± 34	816 ± 85	1520 ± 529	1383 ± 326	1451 ± 180
$AUC_{0-\infty}$(ng·h/ml)	482 ± 34	843 ± 85	1587 ± 550	1484 ± 455	1535 ± 194

the drug release rate from conventional liposomes was significantly increased. The destruction effect of plasma albumin on liposomes may account for the acceleration of drug release rate. The drug release rate from the novel liposomes, however, decreased with the existence of plasma and was much lower than that of conventional liposomes. The results suggested that novel liposomes composed of SPC and HSPC resist the destruction effect of plasma albumin. Therefore, compared with conventional liposomes, the stability of novel liposomes in blood circulation in vivo may be improved correspondingly. The fact that plasma albumin can combine with camptothecin drugs, especially the carboxylate form (Burke et al., 1994), provided an explanation for the decrease of release rate with the existence of plasma in dialysis bags. Consequently, pharmacokinetics of encapsulated 9-NC were obviously improved in vivo. Plasma drug level profiles of novel liposomal 9-NC were higher and showed the sustained property compared to conventional liposomes.

Until recently, little research about the characterization of novel liposomes composed of an unsaturated phospholipid and a saturated phospholipid has been reported. In the present paper, effect of HSPC/SPC molar ratios on the characterization of novel liposomes was also investigated. HSPC/SPC molar ratios had significant effect on drug/lipid molar ratio and entrapment efficiency. When HSPC/SPC molar ratio was 1:3, 9-NC/lipid molar ratio was about four fold higher than that of conventional liposomes while entrapment efficiency was still maintained at about 80%. On the other hand, our results demonstrated that HSPC/SPC molar ratio had only a little effect on other properties of novel liposomes, such as particle size, drug release with the existence of plasma, and pharmacokinetics of liposomal encapsulated 9-NC.

CONCLUSIONS

In summary, we successfully obtained novel liposomes containing 9-NC, by adding HPSC into a formulation composed of SPC and cholesterol. Compared to conventional liposomes, the novel liposomes composed of HSPC and SPC were proved to be better carriers for the research of 9-NC intravenous delivery system. This means that the novel liposomes may have much developing potential.

REFERENCES

Balasubramanian, S. V., Alderfer, J. L., & Straubinger, R. M. (1994). Solvent- and concentration-dependent molecular interactions of Taxol (Paclitaxel). *Journal of Pharmaceutical Science, 83*, 1470–1476.

Burke, T. G., & Mi, Z. (1994). The structural basis of camptothecin interactions with human serum albumin: impact on drug stability. *Journal of Medicinal Chemistry, 37*, 40–46.

Cao, Z., Harris, N., Kozielski, A., Vardeman, D., Stelin, J. S., & Giovanella, B. (1998). Alkyl esters of camptothecin and 9-nitrocamptothecin: synthesis, in vitro pharmacokinetics, toxicity and antitumor activity. *Journal of Medicinal Chemistry, 41*, 31–37.

Chow, D. S., Gong, L., Wolfe, M. D., & Giovanella, B. C. (2000). Modified lactone/carboxylate salt equilibria in vivo by liposomal delivery of 9-nitrocamptothecin. *Annals NewYork Academy of Sciences, 922*, 164–174.

Giovanella, B. C., Stehlin, J. S., Hinz, H. R., Kozielski, A. J., Harris, N. J., & Vardeman, D. M. (2002). Preclinical evaluation of the anticancer activity and toxicity of 9-nitro-20(s)-camptothecin (Rubitecan). *International Journal of Oncology, 20*, 81–88.

Hertzberg, R. P., Caranfa, M. J., Holden, K. G., Jakas, D. R., Gallagher, G., Mattern, M. R., Mong S. M., Bartus, J. O., Johnson, R. K., & Kingsbury, W. D. (1989). Modification of the hydroxy lactone ring of camptothecin: inhibition of mammalian topoisomerase I and biological activity. *Journal of Medicinal Chemistry, 32*, 715–720.

Hung, C. L., Doniger, J., Palini, A., Snyder, S. W., Radonovich, M. F., Brady, J. N., Pantazis, P., & Sadaie, M. R. (2001) 9-Nitrocamptothecin inhibits HIV-1 replication in human peripheral blood lymphocytes: a potential alternative for HIV-infection/AIDS therapy. *Journal of Medical Virology, 64*, 238–244.

Kan, P., Wang, A. J., Chen, W. K., & Tsa, C. W. (2004). Liposome for incorporating large amounts of hydrophobic substances. US2004126886, July 1, 2004.

Konstadoulakis, M. M., Antonakis, P. T., Tsibloulis, B. G., Stathopoulos, G. P., Manouras, A. P., Mylonaki, D. B., & Golematis, B. X.

(2001). A phase II study of 9-nitrocamptothecin in patients with advanced pancreatic adenocarcinoma. *Cancer Chemotherapy and Pharmacology, 48*, 417–420.

Patel, S. R., Beach, J., Papadopoulos, N., Burgess, M. A., Trent, J., & Benjamin, R. S. (2003). Results of a 2-arm phase II study of 9-nitrocamptothecin in patients with advanced soft-tissue sarcomas. *Cancer, 97*, 2848–2852.

Punt, C. J., Jonge, M. J., Monfardini, S., Daugaard, G., Fiedler, W., Baron, B., Lacombe, D., & Fumoleau, P. (2004). RFS2000 (9-nitrocamptothecin) in advanced small cell lung cancer, a phase II study of the EORTC New Drug Development Group. *European Journal of Cancer, 40*, 1332–1334.

Raymond, E., Campone, M., Stupp, R., Meten, J., Chollet, P., Lesimple, T., Fety-Deporte, R., Lacombe, D., Paoletti, X., & Fumoleau, P. (2002). Multicentre phase II and pharmacokinetic study of RFS2000 (9-nitrocamptothecin) administered orally 5 days a week in patients with glioblastoma multiforme. *European Journal of Cancer, 38*, 1348–1350.

Schoffski, P., Herr, A., Vermorken, J. B., Van den Brande, J., Beijnen, J. H., Rosing, H., Volk, J., Ganser, A., Adank, S., Botma, H. J., & Wanders, J. (2002). Clinical phase II study and pharmacological evaluation of rubitecan in non-pretreated patients with metastatic colorectal cancer-significant effect of food intake on the bioavailiability of the oral camptothecin analogue. *European Journal of Cancer, 38*, 807–813.

Scott, D. O., Bindra, D. S., & Stella, V. J. (1993). Plasma pharmacokinetics of the lactone and carboxylate forms of 20(s)-camptothecin in anesthetized rats. *Pharmaceutical Research, 10*, 1451–1457.

Sha, X., & Fang, X. (2004). Transport characteristics of 9-nitrocamptothecin in the human intestinal cell line Caco-2 and everted gut sacs. *International Journal of Pharmaceutics 272*, 161–171.

Ulukan, H., & Swaan P. W. (2002). Camptothecins: A review of their chemotherapeutic potential. *Drugs, 62*, 2039–2057.

Warner, D. L., & Burke, T. G. (1997). Simple and versatile high-performance liquid chromatographic method for the simultaneous quantitation of the lactone and carboxylate forms of camptothecin anticancer drugs. *Journal of Chromatography B, 691*, 161–171.

Yang, S. C., Zhu, J. B., Lu, Y., Liang, B. W., & Yang, C. Z. (1999). Body distribution of camptothecin solid liquid nanoparticles after oral administration. *Pharmaceutical Research, 16*, 751–757.

Yang S. C., & Zhu, J. B. (2002). Preparation and characterization of camptothecin solid lipid nanoparticles. *Drug Development and Industrial Pharmacy, 28*, 265–274.

Zhang, J. A., Xuan, T., Parmar, M., Ma, L., Ugwu, S., Ali, S., & Ahmad, I. (2004). Development and characterization of a novel liposome-based formulation of SN-38. *International Journal of Pharmaceutics, 270*, 93–107.

Zhong, D. F., Li, K., Xu, J. H., Du, Y., & Zhang, Y. F. (2003). Pharmacokinetics of 9-nitro-20(s)-camptothecin in rats. *Acta Pharmacologica Sinica, 24*, 256–262