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Lactone Stability and Tissue Distribution of Free and Liposomal Encapsulated 9-Nitrocamptothecin in Rats Following Intravenous Injection

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9-Nitrocamptothecin (9-NC) is a newly developed but poorly soluble derivative of camptothecin (CPT), which has a wide spectrum of anticancer activity in preclinical evaluation. Lactone moiety is a key structural feature for the antitumor activity of CPT analogs including 9-NC. Lactone stability versus time profiles of 9-NC in vivo following intravenous (i.v.) administration of free and liposomal encapsulated 9-NC has been investigated in this article. After i.v. injection of 9-NC solution, it was found that lactone stability of 9-NC in liver was the poorest in vivo and even worse than that in plasma. In other tissues, lactone stability of 9-NC was better than that in plasma. After liposomal encapsulation, both lactone and total 9-NC concentrations in reticuloendothelial system (RES) tissues, for example, spleen, liver, and lung, were significantly increased. In particular, liposomal encapsulation had a significant improving effect on the lactone stability of 9-NC in the liver. The lactone percentage was increased from 39.11 ± 16.93% to 65.57 ± 9.73% ($p < .05$) at 10 min and from 30.99 ± 6.54% to 51.22 ± 11.10% ($p < .01$) at 30 min. On the basis of these results, a theoretical explanation of lactone stability in vivo was discussed. In summary, liposomal encapsulation, which resulted in passive targeting and a significant improvement of lactone stability in the liver, might have clinical utility.

Keywords 9-nitrocamptothecin; liposomes; tissue distribution; lactone

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INTRODUCTION

Camptothecin (CPT) is a plant alkaloid extracted from the leaves and fruits of *Camptotheca acuminata* and exerts its antitumor activity by specifically inhibiting the human topoisomerase I enzyme. One structural requirement for successful interaction with the topoisomerase I target as well as the antitumor potency in vivo is a closed lactone moiety (Ulukan & Swaan, 2002). Unfortunately, the closed lactone ring is unstable and easily hydrolyzed under neutral or alkaline conditions, particularly in the presence of serum albumin, and the ring is opened to form an almost inactive carboxylate form. It has been proven that carboxylate of CPT has only one-tenth the antitumor potency of the lactone of CPT (Hertzberg et al., 1989).

9-NC, a new analog of CPT, 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). 9-NC has also been found to inhibit HIV-1 replication and has potential clinical utility for HIV infection/AIDS (Hung et al., 2001). However, one of the crucial obstacles to 9-NC clinical effectiveness is the opening of the lactone ring (Figure 1) (Cao et al., 1998), which means that the human tumor is exposed to a relative low concentration of active lactone 9-NC in vivo.

9-NC is barely soluble in aqueous solutions and is practically insoluble in most physiologically compatible and pharmaceutically acceptable solvents. Therefore, up to now, 9-NC

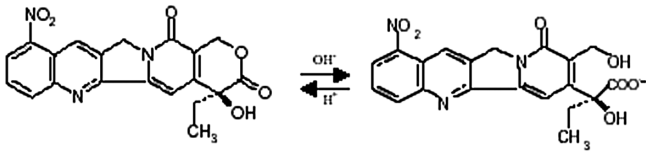

FIGURE 1. Chemical structures of lactone 9-nitrocamptothecin (9-NC) and carboxylate 9-NC.

is mainly administered by oral route. Unfortunately, the absolute bioavailability is very low (Sha & Fang, 2004; Zhong, Li, Xu, Yue, & Fan, 2003) and the variability in oral absorption leads to high interpatient and intrapatient variability (Jung et al., 2004). Consequently, formulation of 9-NC in concentrated pharmaceutical delivery systems for parenteral administration is necessary. One way of improving the solubility of 9-NC is to formulate the drug into liposomes. Chow and coworkers have demonstrated the merits of liposomal 9-NC formulation by its favorable pharmacokinetic and biodistribution characteristics in rats (Chow, Gong, Wolfe, & Giovnella, 2000).

In the present work, we investigate the lactone percentage, an index of lactone 9-NC stability (Cao et al., 1998), over time profiles in vivo both in plasma and in tissues. To improve lactone stability and modify biodistribution, we prepared 9-NC-loaded liposomes and compared their lactone stability and biodistribution with those of 9-NC solution in rats in vivo by intravenous (i.v.) injection. Finally, the theoretical speculation focused on the lactone stability in vivo was discussed based on the results of these studies.

MATERIALS AND METHODS

Drugs and Reagents

Soybean phosphatidylcholine (SPC) was purchased from Lipoid Corp (Ludwigshafen, Germany, Lot number 256172-1). 9-Nitrocamptothecin (9-NC) was supplied by the Department of Medicinal Chemistry, China Pharmaceutical University (purity > 99%). CPT was provided by Lishizhen Pharmaceutical Co, Ltd (Wuhan, China). Water was deionized and then distilled. Acetonitrile was of high-performance liquid chromatography (HPLC) grade. Other reagents were of analytical grade.

Liposome Preparation

9-NC-loaded liposomes were prepared by the method of thin film hydration (Chen, Ping, Guo, Chu, & Song, 2006a). 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 flask wall. The flask was kept in vacuum desiccator overnight. The lipid film was then hydrated with an aliquot of 66-mmol phosphate buffer solution (pH 6.0) for 90 min. The coarse suspension containing large multilamellar liposomes formed, followed by probe sonicating for 15 min to obtain small unilamellar vesicles. The resulted liposomes were then filtered through a 0.45-μm membrane. The entrapment efficiency was 89.55 ± 2.97% (n = 3), determined by ultrafiltration method (Yang & Zhu, 2002; Zhang et al., 2004). The particle size was 134.5 ± 10.9 nm (n = 3), measured by dynamic light scattering (Zetasizer 3000, Malvern Instrument, Worcestershire, UK).

Tissue Distribution Study

9-NC solution was composed of dimethyl sulfoxide (DMSO):polyethylene glycol (PEG) 400:ethanol:5% glucose (pH 3.0) (3:3:2:2 by volume) (Chow et al., 2000; Scott, Bindra, & Stella, 1993). The 1.5 mg/mL 9-NC solution was prepared by dissolving 9-NC in DMSO followed by the addition of the other solvents and immediately administered to the rats after preparation. The resulting 9-NC solution was sufficiently acidic to prevent the lactone ring from opening prior to administration.

Male Spargue–Dawley rats from the Laboratory Animal Centre of China Pharmaceutical University (Nanjing, China) were acclimatized for at least 1 week in a 12-h light/dark cycle with free access to standard chow and water. Forty rats (200–240 g) were randomly divided into eight groups. The rats were fasted overnight but had free access to water. Four groups were injected with 9-NC solution (6 mg/kg) through tail vein, and the other four groups were injected with 9-NC liposomes (6 mg/kg). At each sampling time point (10, 30, 60, or 120 min), one group treated with 9-NC solution and another with 9-NC liposomes were anesthetized by using ether, and blood samples (about 0.25 mL) were collected from the retro-orbital plexus into heparinized tubes. Then the rats were killed and the tissues (heart, liver, spleen, lung, kidney, stomach, and small intestine) were excised, washed, weighed, and stored at –20°C. Blood samples were immediately centrifuged at 2220 g for 3 min, and plasma was separated. Plasma samples were treated immediately according to the analysis procedures.

Sample Treatment

Because the 9-NC lactone↔carboxylate conversion rate is minimal at pH 6.5 (in our laboratory, we had proved $t_{1/2}$ > 1,700 min for both forward and reverse directions at 0°C), tissue samples were homogenized in isotonic PBS solution (pH 6.5) using a tissue blender with ice bath. 250 μL ice-cold (–20°C) methanol–acetonitrile (1:1, vol/vol) and 50 μL CPT (1.35 μg/mL) in methanol were added to 150 μL tissue or plasma sample. The mixture was vortexed for 1 min and centrifuged at 12,000 rpm for 3 min. The supernatant was stored at –20°C until bioanalysis.

HPLC Method for the Simultaneous Analysis of Lactone 9-NC and Total 9-NC in Biosamples

Plasma concentrations of lactone of 9-NC and total 9-NC were determined using validated reverse-phase HPLC with UV detection method. Analyses were carried out using a HP1100 series model (Hewlett Packard, Santa Clara, USA) on a reverse-phase column (Diamonsil C_{18}, 5 μm, 250 mm × 4.6 mm, Dikma, Beijing, China). The mobile phase was a mixture of acetonitrile and 1% triethylamine (adjusted to pH 6.5 with glacial acetic acid) (45:55, vol/vol). pH 6.5 was chosen as the pH of mobile phase for HPLC analysis.

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. The column temperature was 40°C and the UV detector was set at 370 nm.

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, vol/vol) to cause a complete lactonization of 9-NC, and then a volume of 50 μL was injected.

The ratio of the peak area of 9-NC to that of internal standard, CPT, was plotted versus the concentration of 9-NC. The data were fitted using a $1/x^2$ weighted least squares linear regression. Drug concentrations in biosamples were determined from calibration curve. The lowest limit of quantitation was 48 ng/mL.

Lactone 9-NC Percentage Calculations

Lactone stability of 9-NC in tissues and plasma was evaluated by the calculation of lactone percentage, namely the ratio of lactone concentration to total concentration. Both lactone 9-NC and total 9-NC concentrations above were at the same time point and in the same biosample.

Statistical Analysis

All data are expressed as mean ± *SD*. Statistical analysis of the data was performed using the *t* test. $p < .05$ was considered as statistically significant.

RESULTS

Tissue Distribution of Free and Liposomal Encapsulated 9-NC

It has been reported that pharmacokinetics of total 9-NC are independent of dose following i.v. administration in the range from 1.5 to 6 mg/kg (Zhong et al., 2003). Our other work also confirms that dose has little effect on lactone/carboxylate equilibrium in vivo as well as pharmacokinetic behaviors of lactone, carboxylate, and total 9-NC (Chen, Ping, Guo, Chu, & Song, 2006b). Therefore, the increase of dose in the study of tissue distribution, facilitating quantitation of lactone in tissues, will not influence the results.

The results of tissue distribution after i.v. injection of 9-NC solution or liposomes (6 mg/kg) are shown in Figure 2. In the reticuloendothelial system (RES) tissues such as spleen, liver, and lung, the concentrations of lactone 9-NC and total 9-NC after liposomal encapsulation increased significantly as compared with those of 9-NC solution. However, in other tissues such as heart, stomach, and kidney, liposomal encapsulation had little effect on the drug concentrations. In addition, the intestine drug concentrations were the highest among all tissues at 60 and 120 min after i.v. administration of 9-NC solution or 9-NC liposomes.

Lactone Stability of Free and Liposomal Encapsulated 9-NC Versus Time Profiles in Tissues and Plasma

The results demonstrated (Table 1) that lactone stability of 9-NC was different in different tissues following i.v. injection of 9-NC solution. 9-NC existed mainly as the lactone form in most tissues. Lactone stability in heart, lung, spleen, and stomach were comparatively good so that lactone percentages were all higher than 60%. On the whole, lactone stability in tissues was better than that in plasma. However, 9-NC in liver existed mainly as the carboxylate form and lactone percentage was the lowest in all tissues, even lower than that in plasma. In plasma, kidney, and small intestine, the lactone percentages decreased obviously with time. After liposomal encapsulation, the lactone percentage in liver was increased from 39.11 ± 16.93% to 65.57 ± 9.73% ($p < .05$) at 10 min and from 30.99 ± 6.54% to 51.22 ± 11.10% ($p < .01$) at 30 min. However, there was not much improvement of lactone percentage in other tissues. In addition, liposomal encapsulation had little effect on lactone stability in plasma. Only at 10 min, lactone percentage in plasma increased obviously ($p < .05$).

DISCUSSION

For the preparation of solution formulation, because of scarce solubility of 9-NC, DMSO and PEG 400 were included and the optimal pH for lactone stability should be adjusted to 3.0–3.5. With an apparent pH of 3.0–3.5, the resulting solution was sufficient acidic to prevent the lactone ring from opening prior to administration (Zhong et al., 2003).

The interconversion kinetics of lactone and carboxylate 9-NC in liposomes under different pH conditions were measured in our previous work (Chen, Ping, Guo, & Ding, 2005a). It was found that almost 100% 9-NC existed as lactone form under pH 6.0 condition.

For the simultaneous analysis of 10-hydroxylatecamptothecin (10-HCPT) lactone and carboxylate forms, a pH 6.5 mobile phase was chosen for HPLC analysis because the lactone ↔carboxylate conversion rate was minimal at this pH (e.g., $t_{1/2}$ = 89 min and 82 min for forward and reverse directions, respectively) (Shenderova, Burke, & Schwendeman, 1997). For 9-NC also, it had been found that the lactone↔carboxylate conversion rate was also minimal at pH 6.5 (e.g., $t_{1/2}$ = 247.5 and 216.6

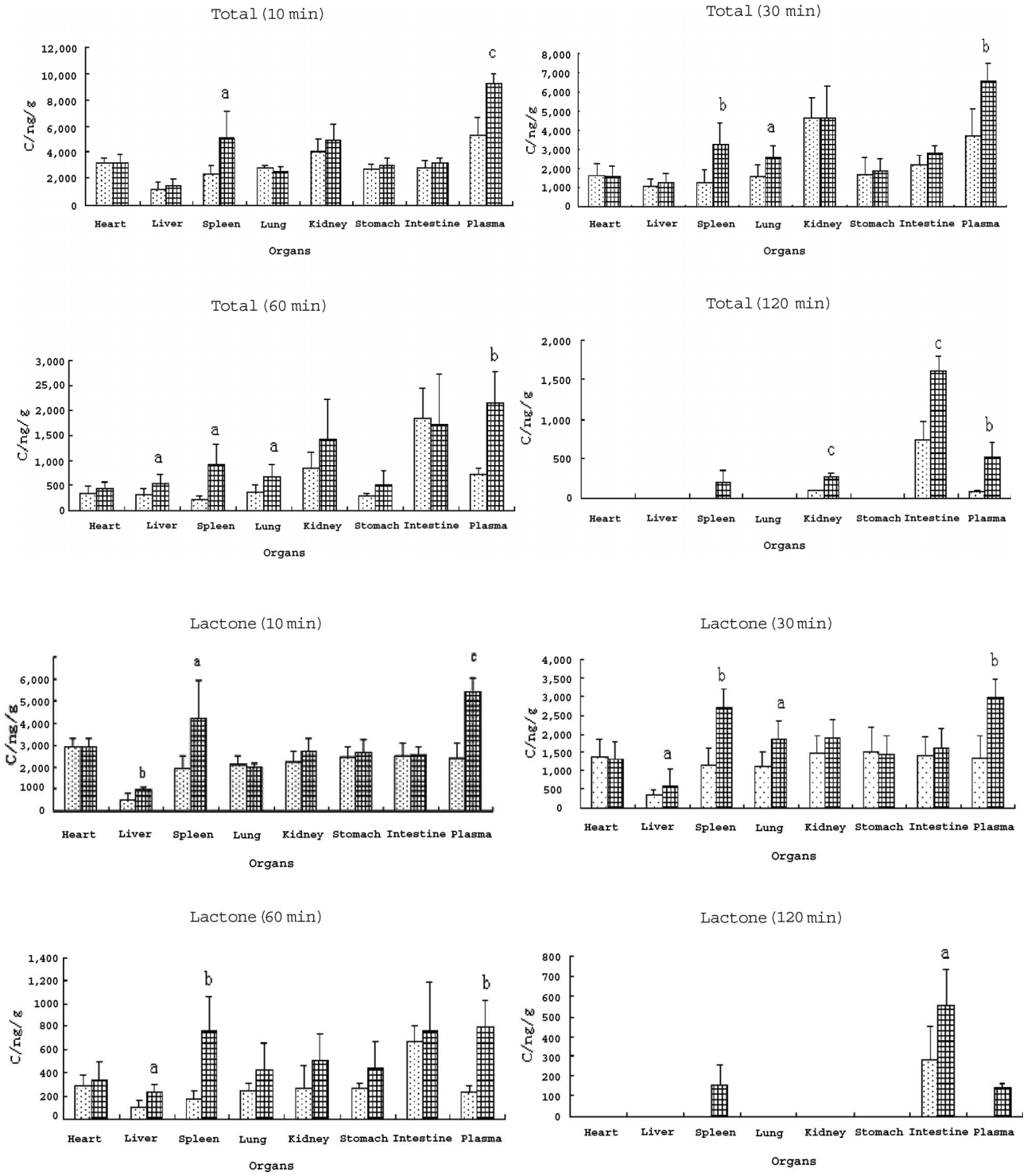

FIGURE 2. Concentrations of lactone 9-nitrocamptothecin (9-NC) and total 9-NC in tissues and plasma of rats at different times after a single intravenous dose (6 mg/kg) of 9-NC liposomes or 9-NC solution. $n = 5$, mean + *SD*. $^{a}p < .05$, $^{b}p < .01$, $^{c}p < .001$ versus free 9-NC (9-NC solution) in the same tissue.

TABLE 1
Lactone Percentages of 9-NC in Tissues and Plasma of Rats after a Single Dose (6 mg/kg) of 9-NC Liposomes or 9-NC Solution (n = 5, Mean ± *SD*)

	10 min		30 min		60 min		120 min	
Tissues	Free 9-NC	9-NC liposome	Free 9-NC	9-NC liposome	Free 9-NC	9-NC liposome	Free 9-NC	9-NC liposome
Heart	90.98 ± 2.86	91.13 ± 5.73	83.36 ± 7.13	81.24 ± 2.54	83.47 ± 7.68	81.34 ± 4.72	–	–
Liver	39.11 ± 16.93	65.57 ± 9.73[a]	30.99 ± 6.54	51.22 ± 11.10[b]	30.15 ± 6.49	37.48 ± 5.94	–	–
Spleen	84.40 ± 4.65	84.89 ± 3.58	89.40 ± 4.49	84.02 ± 3.92	79.74 ± 15.41	81.64 ± 9.71	–	76.70 ± 1.56
Lung	75.87 ± 10.72	80.52 ± 2.16	72.06 ± 1.93	71.25 ± 11.48	67.95 ± 14.40	60.79 ± 8.79	–	–
Kidney	55.98 ± 4.63	57.37 ± 3.48	30.14 ± 4.81	39.46 ± 4.00[a]	29.56 ± 12.31	36.99 ± 7.63	–	–
Stomach	90.87 ± 2.81	91.29 ± 2.89	89.06 ± 2.14	86.57 ± 6.80	91.17 ± 6.16	88.69 ± 4.26	–	–
Intestine	89.21 ± 7.16	81.78 ± 8.31	62.48 ± 13.50	58.73 ± 3.95	37.20 ± 6.93	41.67 ± 5.15	37.14 ± 9.50	34.42 ± 2.87
Plasma	50.05 ± 3.80	58.37 ± 5.19[a]	40.63 ± 3.57	44.62 ± 4.91	31.75 ± 9.01	36.85 ± 3.07	–	30.28 ± 7.62

9-NC, 9-nitrocamptothecin.
[a]$p < .05$, [b]$p < .01$ versus 9-NC solution in the same tissue.

min for forward and reverse directions at 25°C, respectively) (Chen et al., 2005a). The conversion rate of 9-NC was slower and almost one third of that of 10-HCPT. Therefore, pH 6.5 was also chosen as the pH value of the mobile phase and tissue homogenates in this article to assure that neither the lactone nor the carboxylate forms of 9-NC in samples were converted in the course of both tissue sample treatment and HPLC analysis.

In the previous report (Chow et al., 2000), based on the AUC measurements, the lactone 9-NC constituted 50% of circulating total 9-NC from the lipo-9-NC delivery, but only 27% with the treatment of free 9-NC. It was concluded that the liposomal 9-NC formulation was developed to circumvent the drawback of lactone instability in blood circulation. The author did not describe the composition of 9-NC liposome; it is hard to compare the results' differences among these works. However, in Chow's report, free 9-NC was solubilized in a co-solvent system of a DMSO:PEG 400:ethanol:normal saline (3:3:2:2 by volume), and in the neutral pH condition, it is obvious that lactone 9-NC could be easily converted into the carboxylate form before i.v. administration. Therefore, compared with free 9-NC, the improvement effect of liposomal 9-NC on lactone stability in vivo might be overestimated.

The comparison of pharmacokinetics of free 9-NC and liposomal encapsulated 9-NC was reported by us (in our laboratory) recently (Chen et al., 2006a). It was found that the 9-NC plasma levels of liposomal preparation were higher and exhibited the sustained release property compared with 9-NC solution. The liposomal encapsulation resulted in 1.8-fold and 1.9-fold increases in AUC_{0-t} of lactone and total 9-NC, respectively.

The concentration–time profiles of lactone 9-NC and total 9-NC in some of the tissues were significantly altered after the liposomal encapsulation, because liposomes could be congregated in certain tissues, namely the RES tissues such as spleen, liver and lung. For example, compared with 9-NC solution, the drug concentrations in the spleen at 60 min were increased more than fourfold by liposomal encapsulation. Moreover, whether liposomal encapsulation or not, high drug levels were found in the kidney at 10 and 30 min and in the intestine at 60 and 120 min, implying that the urinary excretion might be the major pathway of elimination in the initial phase and the bile excretion into intestine might be the major pathway of elimination thereafter (Zhang et al., 1998).

Lactone stability of 9-NC in vivo after i.v. injection of free 9-NC was extensively investigated in this study. Lactone percentage versus time profiles of 9-NC in plasma and tissues were first shown in this work. It was noteworthy that lactone stability in liver was very low so that the lactone ratio was even lower than that in plasma. In liver, only molecules with polar groups such as the carboxy group can be excreted into bile directly (Jiang, 2003). Therefore, 9-NC might be excreted into bile mainly in the carboxylate form, resulting in the shift of lactone/carboxylate equilibrium to the latter. The assumption could account for the low lactone stability in liver. After liposomal encapsulation, 9-NC cannot be excreted into bile unless released from the liposomes. Thus, the preferential excretion of carboxylate 9-NC was inhibited until the liposomes were degraded in liver. Therefore, the lactone-protecting effect in liver was clearly demonstrated in at least 30 min. As the liposomes were destructed in liver and encapsulated 9-NC was released gradually, the lactone stability no longer existed after 60 min.

In other tissues, the lactone percentages were originally higher than that in the plasma following i.v. administration of 9-NC solution. The carboxylate forms of CPT analogs bind to plasma albumin with much higher affinity than the lactone forms (Burke & Mi, 1994). Because the albumin-combined carboxylate 9-NC cannot distribute into tissues, 9-NC taken by tissues may exist mainly in the lactone form, resulting in higher lactone stability in most tissues than in plasma. Furthermore, after the preferential distribution of lactone 9-NC into tissues, the lactone/carboxylate equilibrium in plasma consequently shifted to lactone form, resulting in the phenomenon that observed that lactone stability of 9-NC in vivo was thus much higher than that in rat plasma in vitro (Chen et al., 2005b). Because the lactone-protecting effect of liposomes was not obvious in tissues except liver, it seemed that liposomal encapsulation had little effect on the preferential combination of carboxylate 9-NC with plasma albumin and subsequently preferential tissue distribution of lactone 9-NC.

The same phenomenon that the lactone form preferentially distributed into tissues was also observed for free CPT (Scott et al., 1993). After i.v. administration of 1 mg/kg CPT solution, the volume of distribution (V_{ss}) for the lactone CPT (4,727 ± 732.7 mL) was much larger than that of the carboxylate (232 ± 93.5 mL), suggesting that lactone CPT had higher tissue accessibility and binding than the carboxylate form.

CONCLUSION

In summary, the results of lactone stability studies of 9-NC in vivo showed that lactone stability in most tissues was better than in plasma and preferential tissue distribution of lactone 9-NC could account for this phenomenon. However, it was found that lactone stability in liver was the poorest in vivo. In our opinion, preferential bile excretion of carboxylate 9-NC might lead to the shift of lactone/carboxylate equilibrium to the latter in liver.

After liposomal encapsulation, both lactone and total 9-NC concentrations in plasma and RES tissues such as spleen, liver, and lung were remarkably higher than those of free 9-NC at the same dose (6 mg/kg). However, concentrations in heart, stomach, and kidney were hardly influenced by liposomal encapsulation. Therefore, liposomal encapsulation might improve the therapeutic index of intravenously administered 9-NC. Furthermore, the results of our study also showed that liposomal encapsulation could significantly improve the stability of the lactone form, main active form of 9-NC. Therefore, compared with free 9-NC, liposomal encapsulated 9-NC might be more suitable for liver cancer therapy.

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