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Effect of injection routes on pharmacokinetics and lactone/carboxylate equilibrium of 9-Nitrocamptothecin in rats

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Abstract

Pharmacokinetics and lactone/carboxylate equilibrium of 9-Nitrocamptothecin (9-NC) were compared after intravenous (i.v.) and intramuscular (i.m.) injection at a dose of 1.5 mg/kg 9-NC solution. The concentrations of three different forms of 9-NC, namely lactone, carboxylate and total 9-NC, were measured by HPLC analysis. Injection routes were demonstrated to have significant effect on pharmacokinetics of 9-NC. Compared with i.v. injection route, mean residence time (MRT) of 9-NC three forms was significantly prolonged following i.m. route ($p < 0.05$). The $AUC_{0-\infty}$ ratios of i.m. to i.v. route were calculated to be $102 \pm 43\%$, $273 \pm 221\%$ and $150 \pm 62\%$ for lactone, carboxylate and total 9-NC, respectively. Compared with i.v. injection route, although $AUC_{0-\infty}$ was barely changed, MRT of lactone 9-NC was dramatically prolonged 4.5-fold after i.m. injection, which may account for the reported improved antitumor efficacy. However, the results of the present study also demonstrated that i.m. injection route increased both $AUC_{0-\infty}$ and MRT of carboxylate 9-NC more significantly. Since the carboxylate form of CPT analogs including 9-NC is associated with their unwanted toxicity, i.m. injection route might lead to severe toxicity compared with i.v. route. Lactone/carboxylate equilibrium was also significantly influenced by injection routes. Based on the $AUC_{0-\infty}$ measurements, the lactone 9-NC constituted $50 \pm 8\%$ and $32 \pm 7\%$ of circulating total 9-NC after i.v. or i.m. administration, respectively ($p < 0.01$).

Keywords: 9-Nitrocamptothecin; Pharmacokinetics; Rats; Lactone; Intravenous injection; Intramuscular injection

1. Introduction

Camptothecin (CPT) analogues and derivatives appear to exert their antitumor activity by binding to topoisomerase I and have shown significant activity against a broad range of tumors. The intact lactone ring of CPTs has been demonstrated to be the most critical structural feature with respect to antitumor activity. However, the α-hydroxylactone group in the lactone ring is highly susceptible to facile ring opening and converting into the open-ring carboxylate form. In early clinical trials, due to the poor water solubility of the lactone form of CPT (lactone CPT), the water-soluble carboxylate form of CPT (carboxylate CPT) was selected, produced limited activity and severe toxicity. Further studies have supported the view that the open-ring carboxylate form possesses minimal antitumor activity relative to the closed-ring lactone CPTs (Ulukan and Swaan, 2002). For example, carboxylate CPT has only about 1/10 the antitumor potency of lactone CPT (Hertzberg et al., 1989).

Because lactone and carboxylate CPTs differ dramatically in the antitumor activity, there is an increasing interest in the study of lactone stability, i.e. lactone/carboxylate equilibrium of CPTs. However, the lactone form of CPTs is unstable. Under physiological pH conditions, the lactone ring is easily opened by a nucleophilic hydrolysis reaction catalysed by OH^- ions. Furthermore, because human serum albumin preferentially binds the carboxylate form with 150-fold affinity than the lactone form, the lactone/carboxyalte equilibrium of CPT was observed to shift to the latter one and lactone CPT was rapidly and completely converted into the carboxylate form (0.2% lactone at equilibrium) in human plasma *in vitro* (Mi and Burke, 1994).

9-Nitrocamptothecin (9-NC, Rubitecan), a new analog of CPT, has been identified to be a very promising antitumor drug

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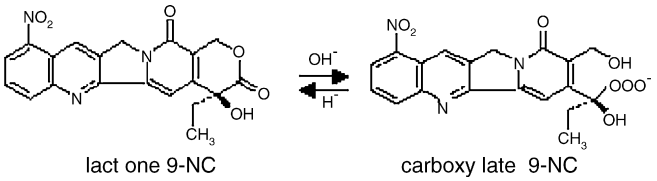

Fig. 1. Lactone/carboxylate equilibrium of 9-Nitrocamptothecin (9-NC).

with high potency against a wide spectrum of human tumors in preclinical evaluation (Giovanella et al., 2002). In addition, 9-NC has also been found to inhibit HIV-1 replication and has potential clinical utility for HIV-infection/AIDS (Hung et al., 2001). 9-NC has been proved to be efficacious as first-line therapy in the treatment of advanced pancreatic cancer (Stehlin et al., 1999). The results of *in vitro* studies have revealed that the lactone stability of 9-NC is poor and the lactone percentage at equilibrium in rat plasma *in vitro* is lower than 2.5% (Chen et al., 2005). However, the lactone/carboxylate equilibrium of 9-NC (Fig. 1) *in vivo* is significantly different from that *in vitro*. And lactone percentage based on AUC calculation is about 50% following i.v. administration (Chen et al., 2005, 2006).

CPT has been tested against human cancer xenograft carried by immunodeficient mice through both i.v. and i.m. injection routes. It was found that the i.m. injection route was significantly better than the i.v. route (Giovanella et al., 1991). 9-NC is similar to CPT in that the curative dose of i.m. injection route was also significantly lower than i.v. route (Chow et al., 2000). It appears that the i.m. injection route is better than the i.v. route with respect to antitumor activity of CPTs. Since the lactone form of CPTs is associated with antitumor potency, it is highly possible that the i.m. injection route might improve the antitumor activity by the agency of changing the pharmacokinetics of the lactone form. However, the question as to whether injection routes have significant effect on pharmacokinetics and lactone/carboxylate equilibrium of CPTs has yet to be determined.

In our previous paper, the effects of the dose and administration route (i.v. versus oral route) were studied in rats (Chen et al., 2006). It had been found that the pharmacokinetics and lactone/carboxylate equilibrium of 9-NC after i.v. administration were both independent of the dose. In addition, the drug was poorly absorbed and the absolute oral bioavailability of lactone 9-NC was calculated to be only 23.4%. Based on the $AUC_{0-\infty}$ measurements, the lactone 9-NC constituted $55 \pm 6\%$ and $60 \pm 14\%$ of the circulating total 9-NC in rats after i.v. and oral administration of 6 mg/kg 9-NC solution, respectively ($p > 0.05$). It was obvious that the lactone/carboxylate equilibrium of 9-NC was not influenced by the change of administration routes from the oral to the i.v. administration.

The objectives of this paper were two-folds: firstly, to compare the pharmacokinetics of lactone, carboxylate and total (lactone plus carboxylate forms) 9-NC in rats with i.v. or i.m. injection routes; secondly, to investigate the effect of injection routes on the lactone/carboxylate equilibrium of 9-NC in rats *in vivo*.

2. Experimental

2.1. Chemicals and reagents

9-Nitrocamptothecin 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.

2.2. Animals

Male Spargue–Dawley rats from the Laboratory Animal Centre of the Affiliated Drum Tower Hospital of Nanjing University (Nanjing, China), weighing 230–280 g, were acclimatized for at least 1 week in a 12-h light:12-h dark cycle with free access to standard chow and water. Twelve rats were divided into two groups at random.

2.3. Intravenous or intramuscular administration

9-NC solution was composed of DMSO:PEG400:ethanol: 5%glucose (pH 3.0) (3:3:2:2 by volume) (Scott et al., 1993; Chow et al., 2000; Chen et al., 2006). 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. The resulting 9-NC solution was sufficient acidic to prevent the lactone ring from opening prior to administration.

Two groups of rats were given 1.5 mg/kg 9-NC solution by i.v. (the tail vein) or i.m. (the thigh muscles) injection, respectively. Animals had free access to food and water throughout the experimentation period. 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 then immediately centrifuged at 4000 r/min for 3 min, and the plasma was separated. Two hundred microliters 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 12,000 r/min for 3 min. The supernatant was stored at −20 °C for bioanalysis.

2.4. Analysis of 9-NC in plasma

Plasma concentrations of lactone and total 9-NC were determined by validated reverse-phase high-performance liquid chromatography (HPLC) with UV detection method (Warner and Burke, 1997; Chen et al., 2006). The carboxylate form of the drug could be determined by subtracting of the lactone concentration from the total concentration. 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. Analyses were carried out with a HP1100 series model (Hewlett Packard, USA) on a reverse-phase column (Diamonsil C_{18}, 5 μm, 250 mm × 4.6 mm, Dikma, China). The mobile phase was a mixture of methanol and 1% triethylamine (adjusted to pH 6.5 with glacial acetic acid) (65:35, v/v). The

mobile phase was filtered using a vacuum filter system equipped with 0.45 μm filter and was delivered at flow-rate of 1.0 ml/min. The column temperature was 40 °C and the UV detector was set at 370 nm. The retention time of 9-NC lactone form was 7.0 min. The lower limit of quantitation of the method was 60 ng/ml and linearity in the calibration curve standards were demonstrated up to the limit of 6000 ng/ml. Within and between-day precision and accuracy determination of quality control samples were lower than 10% across the range of calibration curve.

2.5. Pharmacokinetic analysis

Non-compartmental analysis of data was performed using statistical moment theory.

The area under the plasma concentration versus time curve up to the last quantifiable time point, AUC_{0-t} was obtained by the linear trapezoidal summation. The AUC_{0-t} was extrapolated to infinity ($AUC_{0-\infty}$) by the equation of $AUC_{0-t} + C/k_e$, where C represents the last measurable time concentration and k_e represents the terminal elimination rate constant. k_e was calculated by the linear regression of the log-transformed concentrations of the last three data points in the terminal phase. The body clearance (CL) was calculated by the relationship $CL = D/AUC_{0-\infty}$ where D is the dose of the drug. The mean residence time (MRT) was calculated from the ratio of AUMC/AUC where AUMC is the area under the first moment curve. The apparent volume of distribution at steady state (V_{ss}) was calculated from the relationship: $V_{ss} = CL \times MRT$.

2.6. Statistical analysis

Pharmacokinetic parameters of intravenous and intramuscular injection routes were compared with an unpaired t-test. Statistical significance was determined at the level of $p < 0.05$. All data are expressed as mean ± S.D.

3. Results

3.1. Pharmacokinetics of 9-NC after intravenous or intramuscular administration

The mean plasma concentration–time profiles and $AUC_{0-\infty}$ of lactone, carboxylate and total 9-NC after i.m. or i.v. administration of 9-NC solution are shown in Figs. 2 and 3, respectively. The $AUC_{0-\infty}$ ratios of i.m. to i.v. route were calculated to be 102 ± 43%, 273 ± 221% and 150 ± 62% for lactone, carboxylate and total 9-NC, respectively. Concentration–time profiles of 9-NC three forms following i.v. or i.m. injection routes were completely different. After i.v. administration, the mean plasma level of the drug declined sharply with time. In contrast, absorption of 9-NC three forms from the injection site was slow following i.m. administration. Therefore, the mean plasma concentration of lactone, carboxylate and total 9-NC increased gradually, reaching a mean C_{max} of 151, 282 and 419 ng/ml at T_{max} of 0.6, 0.9 and 1.0 h, respectively. Thereafter, the concentration declined slowly. Following i.m. administration, the plasma drug level kept stable on the whole.

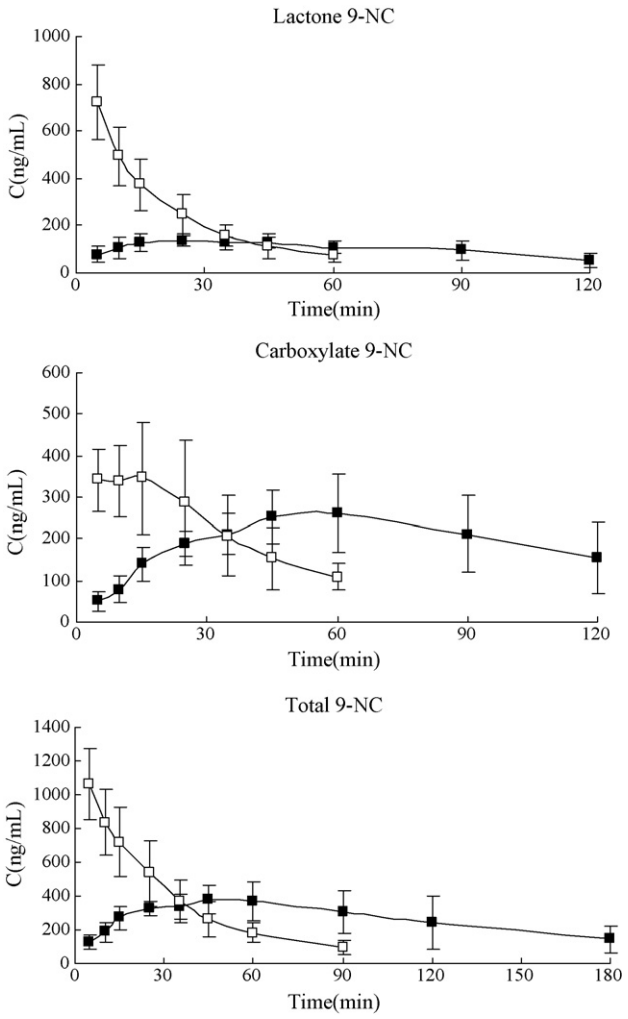

Fig. 2. Profiles of mean (±S.D.) plasma concentration of lactone, carboxylate and total 9-NC vs. time after intravenous (□)or intramuscular (■) administration of 1.5 mg/kg 9-NC solution to rats ($n = 6$).

It was obvious that the injection route had significant effect on the pharmacokinetic characteristics of 9-NC three forms, i.e. lactone, carboxylate and total 9-NC. The pharmacokinetic parameters of two injection routes are summarized in Table 1. Compared with i.v. route, MRT of i.m. route significantly

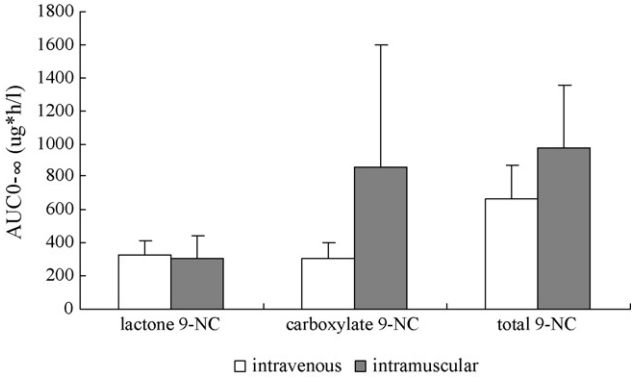

Fig. 3. $AUC_{0-\infty}$ of lactone, carboxylate and total 9-NC after intravenous (□) or intramuscular (■) administration of 1.5 mg/kg 9-NC solution to rats ($n = 6$).

Table 1
Mean (±S.D.) pharmacokinetic parameters of 9-NC after intravenous or intramuscular administration of 1.5 mg/kg 9-NC solution to rats ($n = 6$)

Parameter	Injection route	Lactone 9-NC	Carboxylate 9-NC	Total 9-NC
MRT (h)	Intravenous	0.42 ± 0.07	0.66 ± 0.08	0.60 ± 0.08
	Intramuscular	1.9 ± 1.1*	4.4 ± 3.5*	1.9 ± 0.4***
CL/F ($l\,h^{-1}\,kg^{-1}$)	Intravenous[a]	4.9 ± 1.4	5.2 ± 1.6	2.4 ± 0.7
	Intramuscular	5.5 ± 2.0	2.2 ± 1.4**	1.8 ± 0.7
V_{ss}/F ($l\,kg^{-1}$)	Intravenous[a]	2.1 ± 0.4	3.4 ± 1.1	0.7 ± 0.8
	Intramuscular	2.3 ± 0.8	1.7 ± 1.4*	1.1 ± 0.4
C_{max} ($ng\,ml^{-1}$)	Intravenous	–	–	–
	Intramuscular	151 ± 25	282 ± 75	419 ± 82
T_{max} (h)	Intravenous	–	–	–
	Intramuscular	0.6 ± 0.5	0.9 ± 0.4	1.0 ± 0.6

Control: intravenous injection route.
[a] For intravenous route, $F = 1$, i.e. CL/F = CL and $V_{ss}/F = V_{ss}$.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

increased ($p < 0.05$). For lactone 9-NC, 9-NC active form, MRT increased from 0.42 to 1.9 h. For carboxylate and total 9-NC, MRT values increased from 0.66 to 4.4 h and 0.60 to 1.9 h, respectively.

Among three forms of 9-NC, the effect of injection routes on pharmacokinetic characteristics was most significant for carboxylate 9-NC. Most pharmacokinetic parameters were significantly changed with the alteration of injection routes. Both CL and V_{ss} of carboxylate 9-NC obtained by i.m. route were decreased markedly ($p < 0.05$) relative to that observed by i.v. administration.

3.2. Lactone/carboxylate equilibrium of 9-NC after intravenous or intramuscular administration

Based on the $AUC_{0-\infty}$ measurements, the lactone 9-NC constituted 50 ± 8% and 32 ± 7% of circulating total 9-NC after intravenous or intramuscular administration of 1.5 mg/kg 9-NC solution, respectively. This means that the injection routes of administration have significant effect ($p < 0.01$) on the lactone/carboxylate equilibrium of 9-NC *in vivo*. Compared with intravenous injection route, the lactone stability of intramuscular injection route was poorer.

4. Discussion

By the comparison of pharmacokinetic parameters after intravenous administration of 1.5, 3, 6 mg/kg 9-NC solution, the kinetics of 9-NC were demonstrated to be linear because the pharmacokinetics were independent of dose and the regression analysis of the AUC-dose plots indicated good linearity ($r > 0.85$, $p < 0.01$) (Zhong et al., 2003).

Prior to the present study, i.m. injection route was shown to be the most effective route for CPT administration to immunodeficient mice bearing human tumors, while i.v. injection route had little effect (Giovanella et al., 1991). 9-NC with i.m. route also resulted in enhanced efficacy at low doses (Chow et al., 2000). The results of this study have shown that pharmacokinetic parameters of 9-NC three forms following i.m. injection differed significantly from those after i.v. injection ($p < 0.05$). In particular, pharmacokinetics of lactone 9-NC, namely the main active form of 9-NC, was significantly influenced by the injection route. Compared with i.v. injection route, although $AUC_{0-\infty}$ was barely changed, MRT of lactone 9-NC was dramatically prolonged 4.5-fold after i.m. administration, which shows the tumor is exposed to active 9-NC for much longer time. Prolonging the biological life of an active drug leads to sustained curative effect. Therefore, the results of our studies may help to explain the significant improved antitumor effect by i.m. injection route.

In the present study, it was also found that i.m. injection route influenced pharmacokinetics of the carboxylate form more significantly. Compared with i.v. route, both $AUC_{0-\infty}$ and MRT of carboxylate 9-NC via i.m. injection route increased significantly and lactone percentage decreased correspondingly. Since the carboxylate form of CPT analogs is less active and may be responsible for their unwanted toxicity (Giovanella et al., 1991; Ulukan and Swaan, 2002), i.m. injection route might be associated with higher toxicity.

Previous studies of CPT deposition in tissues after i.m. injection of CPT dissolved in DMSO showed that 60% of the injected drug was still present in the muscle tissue 1 h after injection and 20% after 2 h (Koshkina et al., 1999). After i.m. injection, a large part of 9-NC retained at the site of injection and was easily hydrolyzed under physiological pH conditions, resulting in conversion into the carboxylate form. Then the drug (most in the carboxylate form) was slowly absorbed into the blood circulation. Therefore, the detected lactone percentage was lower than that obtained intravenously. In addition, slow absorption of 9-NC from injection site to blood circulation might be responsible for significantly prolonged MRT of the drug.

In our previous paper (Chen et al., 2006), the pharmacokinetics and lactone/carboxylate equilibrium of 9-NC after i.v. administration were compared with oral administration. It was

found that pharmacokinetic parameters such as MRT were significantly different ($p < 0.05$), similar to the results of comparison between i.m. and i.v. route. It was also found that lactone percentage of 9-NC was not different between i.v. administration and oral administration ($p > 0.05$). However, in the present paper, it had been demonstrated that lactone/carboxylate equilibrium of 9-NC was significantly affected by injection routes. After i.m. injection of 9-NC solution, lactone percentage was much lower than that following i.v. injection ($p < 0.01$).

5. Conclusion

Injection routes have significant influence on pharmacokinetics and lactone/carboxylate equilibrium of 9-NC. Compared with i.v. injection route, MRT of lactone 9-NC following i.m. injection was significantly prolonged, which may account for the improved antitumor efficacy of the latter route. On the other hand, i.m. route also resulted in the more dramatically increase in $AUC_{0-\infty}$ and MRT of carboxylate 9-NC which was associated with much higher toxicity. Therefore, lactone percentage of 9-NC following i.m. injection was much lower than that with i.v. injection route. Briefly, based on the results of our studies, i.m. injection route might not be taken into account for the clinical application of CPT analogs in spite of the improved antitumor efficacy.

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