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Enhanced anti-tumor effect of 9-nitro-camptothecin complexed by hydroxypropyl-β-cyclodextrin and safety evaluation

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ABSTRACT

The aim of this study was to evaluate the safety and anti-tumor effect of 9-nitro-camptothecin/hydroxypropyl-β-cyclodextrin (9-NC/HP-β-CD) complex on tumor-bearing mice. The *in vitro* anti-tumor activity was tested by MTT assay. Our study revealed that the 9-NC/HP-β-CD complex showed significant anti-tumor activity towards Skov-3, MCF-7, HeLa and S180 cell lines with IC_{50} values of 0.24 ± 0.09, 0.59 ± 0.20, 0.83 ± 0.11, and 6.30 ± 2.42 μg/ml, respectively, significantly superior to the free 9-NC. The *in vivo* therapeutic efficacy was investigated in ICR mice bearing mouse sarcoma S180. Both the high (3 mg/kg) and low (1 mg/kg) doses of 9-NC/HP-β-CD complex demonstrated high inhibition ratio of tumor growth (>75%). The subacute toxicity test was performed by measuring the body weight, histopathology, blood cell counts and clinical chemistry parameters (total bilirubin, alanine transferase, aspartate transferase, blood urea nitrogen and creatinine), and the results indicated the good safety profile of the complex. Taken together, the results suggested that the 9-NC complexed in HP-β-CD, instead of dissolved in the organic solvent, presented significant anti-tumor activity and low toxicity for the treatment of cancer.

1. Introduction

Camptothecin (CPT) is an inhibitor of the DNA-replicating enzyme topoisomerase I, which is believed to act by stabilizing a topoisomerase I-induced single strand break in the phosphodiester backbone of DNA, thereby preventing relegation (Herben et al., 1996; Hsiang et al., 1985; Liu, 1989). This leads to the production of a double-strand DNA break during replication, resulting in cell death if not repaired (Berradaa et al., 2005). 9-Nitro-camptothecin (9-NC, Rubitecan), a new analog of CPT, has been identified to be a very promising anti-tumor drug with high potency against a wide spectrum of human tumors in preclinical evaluation (Giovanella et al., 2002). In addition, 9-NC has been proved to be efficacious as first-line therapy in the treatment of advanced pancreatic cancer (Stehlin et al., 1999). However, the clinical application of 9-NC is largely hampered because of its poor solubility (lower than 5 μg/ml in distilled water, 25 °C), and stability (conversion from active lactone form to the inactive carboxylate form under physiologic conditions) (Verschraegen et al., 1998). Moreover, hematological toxicities were observed in clinical trials (Venditto and Simanek, 2010). In order to increase the solubility of 9-NC, organic solvents, such as dimethyl sulfoxide (DMSO) and ethanol were utilized to prepare the 9-NC injections in some studies on animals *in vivo* (Chen et al., 2006a,b, 2007; Chow et al., 2000; Scott et al., 1993). However, the solvents are not allowed in clinical due to the high toxicity to normal cells. Therefore, the development of a novel parenteral formulation without organic solvents should be taken into consideration.

Hydroxypropyl-β-cyclodextrin (HP-β-CD) is a hydroxyalkylated-β-CD derivative that combines relatively high water solubility with low toxicity and satisfactory inclusion ability. Several commercial formulations are composed of cyclodextrin inclusion complexes, illustrating the usefulness of this approach (Yang et al., 2009). However, to the best of our knowledge, there are no studies reported on complexation of 9-NC by HP-β-CD up to now. In the previous study of our lab, we have developed a HP-β-CD-based formulation with high solubility and stability for 9-NC using a unique but simple and highly reproducible method (Jiang et al., 2010). With complexation with HP-β-CD, the solubility of the hydrophobic 9-NC was improved dramatically. Furthermore, the freeze-dried product of the complex had excellent re-dissolution ability and displayed better dissolution performance in comparison with free drug. Pharmacokinetic studies of 9-NC formulations in rats indicated that the complex had higher bioavailability and

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ratio of active lactone form in plasma compared to free 9-NC, which suggested that the complex may exhibit better therapeutic efficacy. The main objective of this study was to evaluate the anti-tumor activity of the formulation both *in vitro* and *in vivo*, and examined the safety of the treatments.

2. Materials and methods

2.1. Materials and animals

9-NC was provided by Ai Sike Biotechnology Co. Ltd. (Shanghai, China). The purity of 9-NC was 99.6%, which was verified by HPLC. HP-β-CD (degree of substitution = 4.1–5.1) was provided by International Specialty Products Corporation as gift. All other chemicals, including buffer components, were of analytical reagent grade. 3-(4,5-Dimethyl-2-tetrazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) was purchased from Sigma (USA); cell culture medium RPMI-1640 was purchased from Gibco Co. (USA). All aqueous preparations were made using double distilled water and organic solvents used in the analysis were high-performance liquid chromatography (HPLC) grade.

ICR mice (20 ± 2 g), supplied by the Department of Experimental Animals, Fudan University (Shanghai, China), were acclimatized at a temperature of 25 ± 2 °C and a relative humidity of 70 ± 5% under natural light/dark conditions for one week before dosing.

All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University (#2011-7).

2.2. Preparation of 9-NC complex

The inclusion complex of 9-NC with HP-β-CD was prepared by the colyophilization technique as reported earlier (Jiang et al., 2010). Briefly, 9-NC and cyclodextrin were dissolved in pH 5.0 phosphate buffer solution (PBS) in an appropriate molar ratio. The system was left to equilibrate under constant stirring for 2 h at 60 °C in dark. At the end of equilibrium time, the dispersion was filtrated, and the filtrate containing soluble complex was lyophilized to obtain dry yellow powder.

2.3. In vitro cytotoxicity assay

2.3.1. Cell culture

Four tumor cell lines were used in this study, including human breast cancer line (MCF-7), human ovarian carcinoma cell line (Skov-3), human epithelial cervical cancer cell line (HeLa) and mice sarcoma 180 (S180) cells, purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All cells were grown in fresh RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 U/ml) at 37 °C under a humidified atmosphere of 5% CO_2.

2.3.2. IC_{50} values evaluation

The MTT enzyme assay was employed to determine the number of surviving cells. Briefly, cells were seeded at a density of 1×10^4 cells per well in 96-well plate. After 24 h, the medium was replaced with serial dilutions of either free 9-NC or 9-NC/HP-β-CD complex in fresh medium. The cells were then further incubated for 24 h, 48 h or 72 h at 37 °C. After incubation, 5 mg/ml MTT solution (20 μl/well) was added and cultured for 4 h, then the supernatant was discarded after centrifuged at 2000 rpm for 5 min and DMSO (100 μl/well) was added in, respectively. The suspension was placed on a microvibrator for 5 min and the absorbance was measured at 570 nm by the Model 680 Microplat Reader (Bio-Rad, USA). The growth inhibition rate (GI) was calculated as Eq. (1):

$$\text{GI}\ (\%) = 100 - \left[\frac{T-B}{C-B}\right] \times 100 \quad (1)$$

where T is the absorption value of the treatment group; C is the absorption value of the control (untreated) group; and B refers to the absorption value of the culture medium.

2.4. In vivo anti-tumor activity evaluation

In vivo anticancer activity against S180 solid tumors was evaluated in mice. The dose schedule started 24 h after tumor cell transplantation with the aim to mimic the early stage of tumor growth (Hong et al., 2010). The organic solvent of the free 9-NC solution was composed of DMSO:polyethylene glycol (PEG) 400:ethanol:5% glucose (3:3:2:2 by volume) (Chen et al., 2007). The solution was prepared by first dissolving 9-NC in DMSO followed by the addition of the other solvents and immediately administered after preparation. Complex lyophilized product was re-dissolved in 5% glucose. 24 h after subcutaneously implanting S180 cells (2×10^6 cells/0.2 ml) into right flanks, the mice were randomized into nine groups ($n = 6$) and treated with one of the following regimens: (A) normal saline (i.v.), (B) free HP-β-CD solution (40%, w/v, i.v.), (C) organic solvent of the 9-NC solution (i.m.), (D) 9-NC/HP-β-CD complex (1 mg/kg, i.v.), (E) 9-NC/HP-β-CD complex (1 mg/kg, i.m.), (F) free 9-NC solution (1 mg/kg, i.m.), (G) 9-NC/HP-β-CD complex (3 mg/kg, i.v,), (H) 9-NC/HP-β-CD complex (3 mg/kg, i.m.), and (I) free 9-NC solution (3 mg/kg, i.m.). Mice were administrated injection every other day for three times. 24 h after the last administration, the animals were sacrificed by cervical dislocation, and the tumor mass was harvested and weighed. The inhibitory rate of tumor (IRT%) was calculated according to Eq. (2):

$$\text{IRT\%} = \frac{W_{\text{control}} - W_{\text{treated}}}{W_{\text{control}}} \times 100\% \quad (2)$$

2.5. Safety evaluation

24 h after the last administration, the blood and serum samples were obtained from the tumor-bearing mice (3 mice per group) for the determination of hematological and biochemical parameters. 0.5 ml blood was collected in tubes containing EDTA-2K, and white blood cell (WBC), red blood cell (RBC), platelet (PLT) were measured by Advia 120 Automated Hematology Analyzer (Bayer Ltd., Germen). The blood samples were allowed to stand for 30 min at room temperature and then centrifuged at 3000 rpm for 15 min at 4 °C to collect serum. The serum aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (TBIL), urea nitrogen (BUN) and creatinine levels were assayed using Hitachi 7080 Chemistry Analyzer (Hitachi Ltd., Japan).

The organs were fixed with polyoxymethylene for 48 h and embedded in paraffin. Then each section was cut to 5 μm, processed for routine hematoxylin and eosin (H&E) staining, and photographed under an OLYMPUS microscope.

2.6. Statistical analysis

Statistical analysis was performed by Student's *t*-test for two groups, and one-way ANOVA for multiple groups. All results were expressed as the mean ± standard deviation (SD). A probability (p) of less than 0.05 is considered statistically significant.

Table 1
The IC_{50} values of the four kinds of tumor cells after exposing to free 9-NC and 9-NC/HP-β-CD complex solution for 24 h, 48 h and 72 h, respectively.

IC_{50} (μg/ml)	Time					
	24 h		48 h		72 h	
	Free 9-NC	Complex	Free 9-NC	Complex	Free 9-NC	Complex
Skov-3	>10	6.35 ± 1.21	1.93 ± 0.11	1.15 ± 0.43*	0.53 ± 0.10	0.24 ± 0.09*
MCF-7	>10	5.52 ± 0.72	2.43 ± 0.44	1.61 ± 0.56*	1.26 ± 0.20	0.59 ± 0.20*
HeLa	3.71 ± 0.65	1.65 ± 0.33*	1.82 ± 0.46	0.83 ± 0.11*	–	–
S180	>10	6.30 ± 2.42	–	–	–	–

All data are means ± SD ($n = 3$).
* $p < 0.05$ when compared with free 9-NC.

3. Results and discussion

3.1. In vitro cytotoxicity assay

As HP-β-CD alone may influence cell growth, it seems necessary to investigate its cytotoxicity on different cancer cell lines. Our data showed slight cytotoxicity when the concentration was higher than 1%, equal to the concentration of 9-NC was higher than 10 μg/ml. The following experiments were thus carried out in the requisition that the maximum concentration of 9-NC was 10 μg/ml to ensure that the activity of the excipient was negligible.

Three kinds of adherent tumor cell lines (Skov-3, MCF-7 and HeLa) and one kind of suspended tumor cell line (S180) were studied. The cells were incubated for different hours (24 h for S180, 48 h for HeLa and 72 h for Skov-3 and MCF-7) depending on the different growing speeds. The values of IC_{50} were displayed in Table 1. In Table 1, IC_{50} of 1, 24, 48, 72 h were all studied on MCF-7 and Skov-3, but only studied 24, 48 h on HeLa and 24 h on S180. Take S180 for example, the cells grew fast and after 48 h, parts of the cells were dead due to the lacking of nutrition and space to grow. As a result, we could not get the IC_{50} of 48 and 72 h. At the end of the incubation, the IC_{50} values of the free 9-NC against the four tested cells were 0.53 ± 0.10, 1.26 ± 0.20, 1.82 ± 0.46 μg/ml, and higher than 10 μg/ml for Skov-3, MCF-7, HeLa and S180, respectively. In contrast, the IC_{50} values of 9-NC/HP-β-CD complex were 0.24 ± 0.09, 0.59 ± 0.20, 0.83 ± 0.11, and 6.30 ± 2.42 μg/ml for Skov-3, MCF-7, HeLa and S180, respectively, which were significantly lower ($p < 0.05$).

Since free HP-β-CD showed no activity over the concentration range used in this study, the increased anti-tumor in activity of 9-NC/HP-β-CD over the free 9-NC can be attributed to the improved stability of 9-NC by complexation with HP-β-CD. This probably occurs because the complexed form of 9-NC, which is in equilibrium with the free 9-NC in solution, is less prone to hydrolysis and hence serves as a depot for continuous supply of 9-NC in its active form (Kang et al., 2002).

3.2. In vivo anti-tumor activity evaluation

The inhibitory rate of the different formulations against S180 solid tumor in mice is shown in Table 2 and Fig. 1. The negative control group injected with saline solution induced the average tumor weight of 0.79 ± 0.26 g. It was found that the injection route has significant influence on pharmacokinetics and lactone/carboxylate equilibrium of 9-NC (Chen et al., 2007). The results of other pharmacokinetics study in our lab were consistent to the report (Jiang et al., 2010). In this study, free 9-NC and 9-NC/HP-β-CD complex solution were compared via i.m. and i.v. administration. However, the tumor suppression showed no significant difference between the two routes of administration. It is probably because the $AUC_{0-\infty}$ of the active lactone form of 9-NC was nearly the same for the two routes of administration (260.14 ng h/ml for i.m. administration vs. 272.04 ng h/ml for i.v. administration), although the $AUC_{0-\infty}$ of the total 9-NC were different for the two routes of administration (Jiang et al., 2010). Because the acute toxicity of the organic solvent caused the immediately spasm and even death of the mice after i.v. administration, the free 9-NC groups were only given via i.m. For mice treated with 1 mg/kg of 9-NC, the tumor suppression was obvious with the mean tumor weight of 0.16 ± 0.04, 0.17 ± 0.03 and 0.21 ± 0.09 g for 9-NC/HP-β-CD complex via i.m., 9-NC/HP-β-CD complex via i.v. and free 9-NC via i.m., respectively, which were significantly smaller than the control ($p < 0.05$). Moreover, for mice treated with 3 mg/kg of 9-NC, the mean tumor weight was 0.16 ± 0.04, 0.17 ± 0.03 and 0.21 ± 0.09 g, for 9-NC/HP-β-CD complex via i.m., 9-NC/HP-β-CD complex via i.v. and free 9-NC via i.m., respectively, indicating a greatly superior anti-tumor efficacy to the control group ($p < 0.05$). The IRT was 73.4–79.8% for the low dosage groups and 81.9–84.9% for the high dosage groups.

We here selected S180 tumor-bearing mice as animal model because it had been one of the classical tumor models for a long time, and also introduced in the studies on CPT and analogies (Hong et al., 2009; Kunii et al., 2007). The mice were administrated 24 h after being injected the tumor cell. Interestingly, when the mice were treated with organic solvent alone, a relatively high IRT (43%) were observed, with the mean tumor weight of 0.45 ± 0.08 g. While the mice treated with HP-β-CD solution showed no difference to control group on the tumor weight and IRT. It was reported that DMSO as an anti-oxidant agents performed a good anti-tumor activity by inducing changes in specific gene expression (Bilir et al., 2004; Liu et al., 2007; Sharma et al., 1998; Yoshikawa et al., 1993). The toxicity of ethanol on the tumor cells was also reported (Datta et al., 1990; Fronio et al., 2005; Taniguchi et al., 2008). In addition, at the early stage of tumor growth, the tumor cells might be tender to the organic solvent. Therefore, it was straightforward to presume that the anti-tumor activity of the free 9-NC was the result of synergism of the 9-NC molecular and the organic solvent. On the other hand, IRT of the mice treated with the complex was similar to the free 9-NC group. Since HP-β-CD had no activity on the tumor suppression, we can conclude that HP-β-CD, instead of the organic solvent, enhanced the efficacy of 9-NC. These results were consistent with the *in vitro* cytotoxicity assay in Section 3.1. Taken together, HP-β-CD can increase the anti-tumor activity of 9-NC to a large extent, while avoiding the toxicity of the organic solvent. The underlying mechanism of enhanced *in vivo* anti-tumor activity for the complex probably involves not only the less hydrolysis discussed in the *in vitro* experiment, but also the enhanced membrane permeability and distribution of hydrophobic molecule into the cell nucleus.

3.3. Safety evaluation

3.3.1. Histopathology

The major limitation of cancer chemotherapy is the injury of normal tissue, leading to multiple organ toxicity. The main target organs for toxicity in this study are the liver and kidney. For safety purpose, we examined the important organs 24 h after the

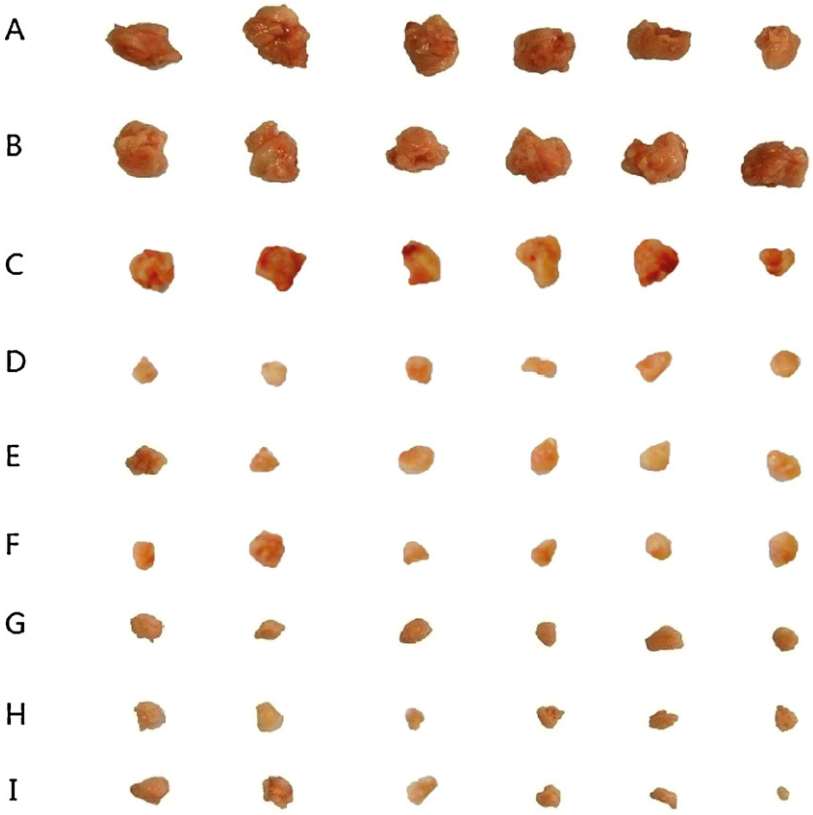

Fig. 1. Tumor growth after systemic application of different drug loaded systems. Groups (A) normal saline; (B) 40% HP-β-CD solution; (C) organic solvent; (D) 9-NC/HP-β-CD complex (1 mg/kg, i.v.); (E) 9-NC/HP-β-CD complex (1 mg/kg, i.m.); (F) free 9-NC solution (1 mg/kg, i.m.); (G) 9-NC/HP-β-CD complex (3 mg/kg, i.v.); (H) 9-NC/HP-β-CD complex (3 mg/kg, i.m.); (I) free 9-NC solution (3 mg/kg, i.m).

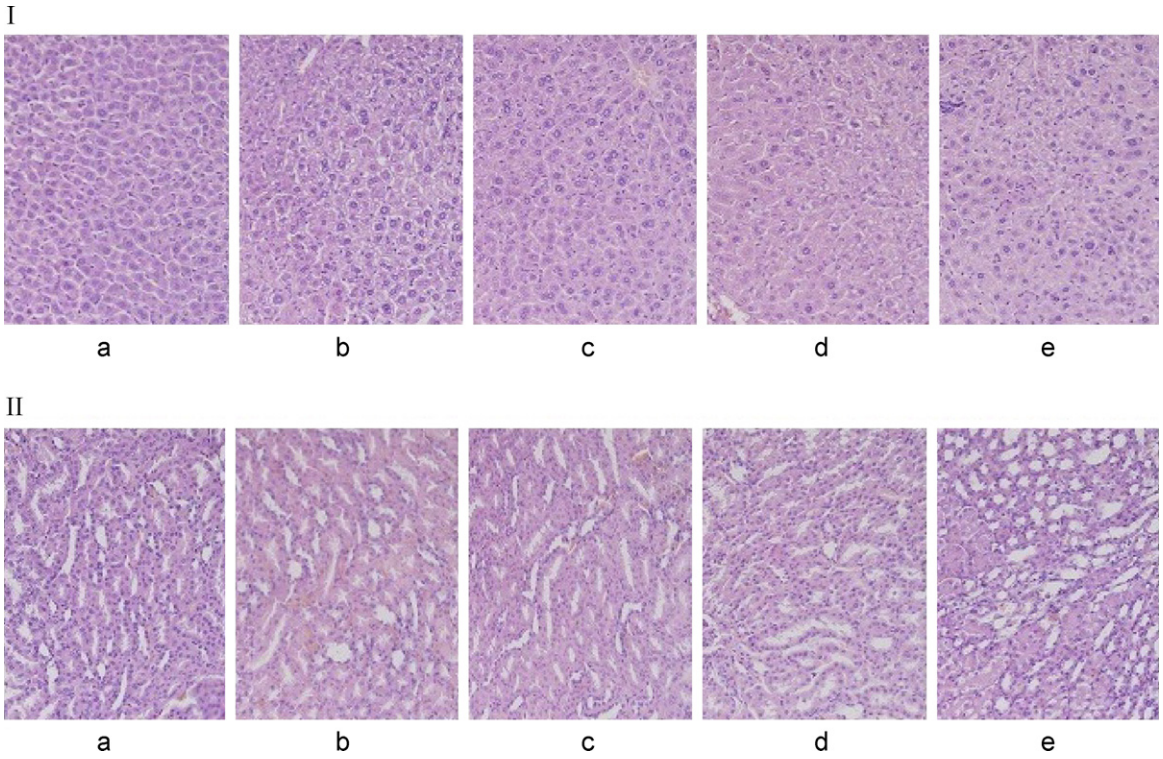

Fig. 2. Liver (I) and kidney (II) sections isolated 24 h after the last administration and stained with hematoxylin and eosin (H&E) for histopathological analysis. (a) Free 9-NC, i.m.; (b) 9-NC/HP-β-CD complex, i.m.; (c) 9-NC/HP-β-CD complex i.v.; (d) 40% HP-β-CD solution, i.v.; (e) normal saline, i.v.; images were obtained under Olympus IX 71 fluorescence microscope using a 40× objective.

Table 2
The change of the mice body weight and the inhibitory rate of tumor (IRT) of different drug loaded systems against S180 tumor in mice.

Group		Dose (mg/kg)	Body weight (g)		Tumor weight (g)	IRT%
			Initial	Final		
1	Normal saline	–	20.9 ± 1.0	25.7 ± 3.4	0.79 ± 0.26	–
2	HP-β-CD	–	21.0 ± 0.7	25.7 ± 1.4	0.78 ± 0.17	–
3	Organic solvent	–	20.9 ± 0.8	23.8 ± 2.3	0.45 ± 0.08	43.0
4	9-NC/HP-β-CD i.v.	1*	20.6 ± 0.6	22.6 ± 1.4[a]	0.16 ± 0.04[c]	79.8
5	9-NC/HP-β-CD i.m.	1*	20.9 ± 0.6	21.7 ± 1.6[a]	0.17 ± 0.03[c]	78.5
6	Free 9-NC solution i.m.	1*	21.2 ± 0.7	19.7 ± 1.7	0.21 ± 0.09[c]	73.4
7	9-NC/HP-β-CD i.v.	3*	20.6 ± 1.3	18.8 ± 0.8[b]	0.12 ± 0.06[c]	84.7
8	9-NC/HP-β-CD i.m.	3*	20.8 ± 0.6	18.1 ± 1.1[b]	0.14 ± 0.07[c]	81.9
9	Free 9-NC solution i.m.	3*	20.7 ± 0.6	14.8 ± 1.3	0.12 ± 0.07[c]	84.9

All data are means ± SD ($n = 6$).
* Calculated by the amount of 9-NC.
[a] $p < 0.05$ when compared with free 9-NC solution via i.m. as a dose of 1 mg/kg.
[b] $p < 0.05$ when compared with free 9-NC solution via i.m. as a dose of 3 mg/kg.
[c] $p < 0.05$ when compared with the normal saline.

last treatment of the high dose groups (3 mg/kg). As presented in Fig. 2(I), the liver architecture showed complete normalization and no confluent necrosis, fatty changes or hydropic degeneration was observed in the liver sections compared to the control group. Renal tissues were displayed in Fig. 2(II) and no noticeable degeneration, necrosis and congestion were showed in renal tubules. The results indicated that there were none visible lesions in the organs of treated and control groups.

3.3.2. Body weight

The change in body weight as a function of time in tumor-bearing animals was used as one of the marker of safety (Zhang et al., 2010). The body weight of the mice treated with free 9-NC kept losing weight dramatically (30% body weight losing 24 h after the last treatment), and even remained declining on the day cessative of treatment (data not shown). On the contrary, there was no serious body weight loss in the groups received HP-β-CD solution, organic solvent and 9-NC/HP-β-CD complex. Moreover, body weights of mice treated with 9-NC/HP-β-CD complex have gradually recovered after cessation of drug, which means slight systemic toxicity was found in the groups treated with the complex, suggesting better toxicity profiles of the complex than that of the free 9-NC.

3.3.3. Hematology

In order to determine whether the formulation may cause any undesirable side effects, we assayed a number of hematological parameters 24 h after the last administration. Alterations in such parameters would presumably reflect the occurrence of any abnormality in, for instance the immune system (Ishida et al., 2005).

Anticancer drugs may frequently induce host immunosuppression and symptomatic toxicities (Yoshimatsu et al., 2008). As shown in Table 3, 9-NC caused a significant decrease in WBC count and platelet in S180-bearing mice as compared to control mice ($p < 0.001$). It is reported that CPT derivatives such as Irinotecan and Topotecan changed the hematological parameters (Bülbül et al., 2008; Hijiya et al., 2008; Kato et al., 2008). However, the WBC count in S180-bearing mice receiving 9-NC/HP-β-CD complex were markedly higher than that of mice receiving free 9-NC ($p < 0.05$), and the platelet of 9-NC/HP-β-CD complex groups were also higher than that of the free 9-NC group. We can presume that the complex ameliorated the lymphocytopenia and reduced the hematological toxicity.

3.3.4. Serum biochemistry

Liver is a main tissue in the detoxification and metabolism of chemicals. This may impair its regular function due to xenobiotic modification in detoxification processes.

ALT and AST are important enzymes in liver and usually help to detect chronic liver diseases by monitoring their concentrations (Ozer et al., 2010). ALT, a better bio-indicator of liver injury than AST, catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner (Mehta et al., 2009; Willianson et al., 1996). In order to find the liver toxicity of the preparation, we tested the ALT, AST and TBIL levels of the mice. The result in Table 4 shows that AST and ALT of the mice treated with free 9-NC had significantly increases compared to the control group ($p < 0.05$), indicating the liver damage caused by 9-NC. However, there was no statistically significant difference between 9-NC/HP-β-CD groups and the control group, demonstrating that the elevated AST and ALT induced by the free 9-NC solution were prevented by HP-β-CD complexation. No significant difference on the TBIL level was found among the groups.

The marked increases of the serum BUN and creatinine concentration are markers for the kidney damage and nephrotoxicity (Cagler et al., 2002). As shown in Fig. 3, the content of serum BUN in S180-bearing mice was increased by 9-NC as compared to control mice ($p < 0.001$ for free 9-NC and $p < 0.01$ for 9-NC/HP-β-CD complex), indicating the renal toxicity due to 9-NC. The BUN were 13.4 ± 0.8, 12.3 ± 1.6, and 12.1 ± 1.4 mmol/L for free 9-NC, 9-NC/HP-β-CD i.m. and 9-NC/HP-β-CD i.v., respectively, which were marked higher than the saline (7.5 ± 0.5 mmol/L). The renal toxicity of HP-

Table 3
The hematological finding in S180-bearing mice.

	WBC ($\times 10^9$/L)	RBC ($\times 10^{12}$/L)	Platelet ($\times 10^9$/L)
Normal saline	8.20 ± 1.13	7.29 ± 0.64	1751 ± 175
HP-β-CD	8.81 ± 1.18	6.33 ± 0.83	1629 ± 127
9-NC/HP-β-CD i.v.	2.64 ± 0.42**,#	7.79 ± 0.34	1150 ± 129**
9-NC/HP-β-CD i.m.	3.19 ± 0.48**,#	7.73 ± 0.20	1182 ± 113**
Free 9-NC solution i.m.	1.69 ± 0.49***	7.93 ± 0.59	1003 ± 269**

** $p < 0.01$.
*** $p < 0.001$, when compared with normal saline.
# $p < 0.05$ when compared with free 9-NC solution via i.m.

Table 4
ALT, AST and TBIL lever in the liver of mice sacrificed 24 h after the last administration.

Groups	ALT (IU/L)	AST (IU/L)	TBIL (mg/L)
Normal saline	45.3 ± 15.5	351.0 ± 105.2	0.9 ± 0.1
HP-β-CD	55.5 ± 16.1	473.8 ± 72.6	1.0 ± 0.3
Complex i.v.	56.5 ± 7.8	472.5 ± 35.0	1.1 ± 0.2
Complex i.m.	67.5 ± 10.5	364.3 ± 72.6	1.4 ± 0.2
Free 9-NC i.v.	82.7 ± 6.7*	643.7 ± 89.3*	1.3 ± 0.2

All data are means ± SD ($n = 3$).
* $p < 0.05$ when compared with normal saline.

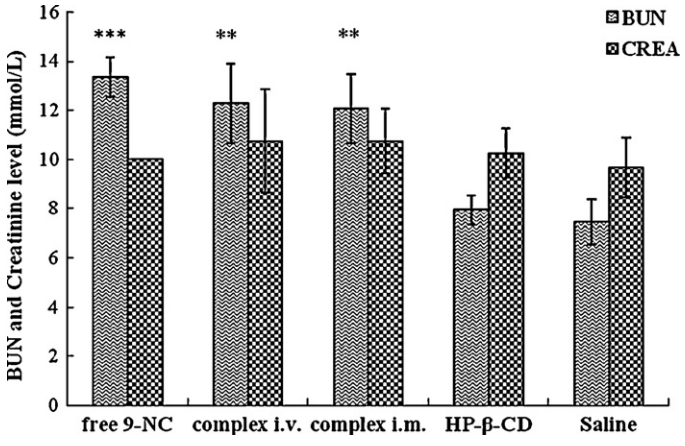

Fig. 3. BUN and CREA level in the kidneys of mice sacrificed 24 h after the last administration. All data are means ± SD ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with normal saline.

β-CD was specially tested because cyclodextrins themselves tend to be kidneytoxic, which has been shown for rabbits and dogs (Irie and Uekama, 1997). No significant increase of BUN was observed in the mice treated 40% HP-β-CD solution compared to the control group (8.0 ± 0.6 mmol/L). Clearly, HP-β-CD seems to be good tolerated cyclodextrin in the kidney and reduced the nephrotoxicity of the free 9-NC solution.

4. Conclusion

We have developed a novel HP-β-CD-based formulation for the delivery of 9-NC, using a unique but simple and highly reproducible method. As we have studied *in vitro* dissolution performance and *in vivo* pharmacokinetics as reported (Jiang et al., 2010), this studied further investigated the safety and anti-tumor activity of the complex both *in vitro* and *in vivo*. As described in this study, the 9-NC complexed in HP-β-CD, instead of dissolved in the organic solvent, presented significant anti-tumor activity and low toxicity for the treatment of cancer. Taken together, the complexation of HP-β-CD provides a promising carrier for 9-NC, and other hydrophobic drugs in the delivery.

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