—Regular Article—

In Vivo Biodistribution for Tumor Targeting of 5-Fluorouracil (5-FU) Loaded *N*-succinyl-chitosan (Suc-Chi) Nanoparticles

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5-Fluorouracil-loaded *N*-succinyl-chitosan nanoparticles (5-FU-Suc-Chi/NP) were prepared by emulsification solvent diffusion. Biodistribution and tumor targeting were evaluated after i.v. administration of 5-Fu-Suc-Chi/NPs in Sarcoma 180-bearing mice. Also, pharmacokinetic profiles were evaluated after intravenous injection of 5-Fu-Suc-Chi/NP *via* the tail vein to rats. Our experimental results showed the 5-FU-Suc-Chi/NPs could be sustained at a high level in the blood for a very long time, implying its long systemic retention in the circulation. 5-FU-Suc-Chi/NPs were distributed mainly in tumors and liver, with small quantities being found in kidney and spleen. 5-FU-Suc-Chi/NPs accumulated only slightly in the heart and lung, and lowered the toxic effect of 5-FU in the heart and lung. Pharmacokinetic analysis in plasma showed the area under plasma concentration-time curve (AUC), elimination half-life ($t_{1/2}$), and residence time (MRT) were increased 2.5-fold, 10.98-fold, and 10.8-fold for 5-FU-Suc-Chi/NP compared with that of free 5-FU, respectively. These results indicate that a long half-life in the circulation and tumor targeting of 5-FU-Suc-Chi/NPs are possible.

Key words——*N*-succinyl-chitosan; nanoparticle; biodistribution; pharmacokinetic; tumor targeting

INTRODUCTION

Nanotechnology has advanced greatly in recent years and is becoming a promising approach for cancer treatment. Owing to their small size, prolonged circulation time, and sustained drug release profile, nano-sized polymeric nanoparticles bearing anticancer drugs have received an increasing amount of attention for their ability to improve the efficacy of anticancer drugs.[1–3] The prolonged circulation time of polymeric nanoparticles allows them to extravasate and accumulate in tumor tissue, resulting in a disorganized and defective vascular architecture, which is referred to as the enhanced permeability and retention (EPR) effect in tumor tissue.[4,5] Therefore, tumor targeting of polymeric nanoparticles has been recognized as an effective strategy for passive tumor targeting in the body.

To reduce the toxicity and increase the therapeutic efficacy of anticancer drugs, Suc-Chi-NPs have been developed using an emulsification solvent diffusion method previously reported by us.[6] In this study, the distribution of the particle size, zeta potential, drug loading content, and drug loading efficiency of the prepared nanoparticles as well as their release profiles were investigated *in vitro*. Also, the antitumor activity of 5FU-Suc-Chi-NP was evaluated by measuring the change in the tumor volume. 5FU-Suc-Chi-NP showed good antitumor activity against Sarcoma 180 solid tumor and mild toxicity.

In continuation of our previous work, the aim of the present study was to evaluate the tissue distribution (blood, liver, spleen, kidney, lung, heart, and tumor) of 5-Fu-Suc-Chi/NP and tumor selectivity by examining the enhanced permeability and retention (EPR) effect in Sarcoma 180-bearing mice. Also, pharmacokinetic profiles were evaluated after i.v. administration of 5-Fu-Suc-Chi/NP in rats.

MATERIALS AND METHODS

Materials *N*-Succinyl-chitosan sodium salt (Suc-Chi; MW 3×10^5; degree of *N*-succinylation per chitosan hexosamine unit 76%) was supplied by Shenyang Pharmaceutical University (Shen-yang, China). 5-FU was purchased from Jiqi Pharmaceutical Factory (Shen-yang, China). All other chemicals were obtained commercially as reagent-grade products.

Animals Male Balb/c mice weighing approximately 18–22 g and male Wistar rats weighing approximately 180–220 g were provided by the Animal

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Experimental Center of Shenyang Pharmaceutical University. All studies were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication no. 92–93, revised in 1985), and were approved by the Department of Laboratory Animal Research at Shenyang Pharmaceutical University. The procedures with animals were reviewed and approved by the Animal Ethics Committee of Shenyang Pharmaceutical University.

Tumors Sarcoma 180 cells (solid malignant tumors) were maintained by weekly transplantation of 1×10^7 cells suspended in Hanks' balanced solution (0.1 ml) into the peritoneal cavity of each mouse. Sarcoma 180 cells (1×10^7) suspended in 0.1 ml of Hanks' balanced solution, which were obtained from the above tumor-bearing mice, were inoculated subcutaneously into each mouse at the axillary region. The tumors were allowed to develop for 7 days.

Preparation of 5-FU-Suc-Chi/NP 5-FU-Suc-Chi/NPs were prepared by a previously reported method.[6] Briefly, blank nanoparticles were obtained upon the addition of 10 ml of Suc-Chi aqueous solution (2 mg/ml) to 100 ml of the Span-80 organic solution, which contains 20% ethanol (v/v) stirred at room temperature. Water was then evaporated from the colloidal suspension with a magnetic stirrer at 40 °C by a vacuum-pump. Nanoparticle suspensions were centrifuged after ultrasonic treatment. The precipitate was washed and dispersed in 10 ml of H_2O. Mannitol 0.1% (w/v) was added to the nanoparticle suspension followed by lyophilization. 5-FU-loaded nanoparticles were obtained according to the same procedure.

Pharmacokinetic Analysis A single dose of free 5-FU or 5-FU-Suc-Chi/NP (30 mg/kg) was administered to rats. Blood samples were collected from rat veins at designated times after intravenous administration. 5-FU was extracted from plasma by mixing rat plasma with ethyl acetate and isopropyl alcohol (85/15, v/v). The samples were then dried with N_2 at 37°C and the dehydrated samples were dissolved in 400 μl of mobile phase dilutent for subsequent HPLC. Pharmacokinetic (PK) parameters were calculated by noncompartment analysis based on statistical moment theory using Microsoft Excel software. The area under the plasma concentration-time curve up to the last time (t) (AUC_{0-t}), area under curve extrapolated to infinity ($AUC_{0-\infty}$), and area under the first moment curve extrapolated to infinity ($AUMC_{0-\infty}$) were calculated using the linear trapezoidal rule. The mean residence time (MRT) was calculated as AUMC/AUC.

Biodistribution of 5-FU-Suc-Chi/NP in Tumor-bearing Mice At 7 days after subcutaneous inoculation, a single dose of free 5-FU or 5-FU-Suc-Chi/NP (30 mg/kg) was injected into the tail vein of the tumor-bearing mice. After a definite time period, blood samples were obtained from the retro-orbital plexus using a capillary. The animals were sacrificed and the heart, kidney, liver, lung, spleen, and tumor were collected. The distributed amount of 5-FU in tissues and blood was estimated by HPLC. Each tissue taken from Sarcoma 180-bearing mice was washed with phosphate-buffered saline (PBS, pH 7.4) and wiped with a filter paper. PBS was then added at a three-fold volume of the weight of the tissue, and the mixture was homogenized. After centrifugation of the homogenate, the concentrations of 5-FU in tissues samples were determined by HPLC. Consequently, the amount of 5-FU in each tissue was calculated from the concentration and tissue weight. Relative tumor tissue exposures (Re) and the ratios of peak concentrations (Ce) were calculated according to the following formula:[7,8] $100\%\times(AUC_{tumor})_{5\text{-Fu-Suc-Chi/NP}}/(AUC_{tumor})_{5\text{-FU}}$ and $100\%\times(C_{max})_{5\text{-Fu-Suc-Chi/NP}}/(C_{max})_{5\text{-FU}}$.

HPLC Assay Concentrations of 5-FU in tissues and plasma samples were determined by HPLC. The HPLC system consisted of a model LC-10AT pump (Shimadzu Corporation, Kyoto, Japan), and a model SPD-10A UV detector (Shimadzu). Separations were performed at 25°C using a 250 mm×4.6 mm column (Diamonsil C18, Dikma, USA). The mobile phase was 0.01 M KH_2PO4, which was filtered and delivered at a flow rate of 1.0 ml min^{-1}. The column was maintained at a temperature of 25°C. The eluent was detected by a UV detector at 266 nm.

Statistic Analysis The results, obtained by *in vivo* studies, were statistically analyzed using Student's *t*-test with a 95% confidence level ($p<0.05$) and are reported as the mean±standard deviation (S.D.).

RESULTS AND DISCUSSION

5-Fluorouracil-loaded *N*-succinyl-chitosan nanoparticles were prepared using a modified emulsion solvent diffusion method.[6] The resulting nanoparticles

had a mean diameter of 220–260 nm, with a mean zeta potential of approximately −26 mV. The formulation with an initial 5-FU concentration of 1000 μg ml^{-1} provided the highest loading capacity (19%) and the highest extent of release (61% at 24 h).[6]

As shown in Table 1 and Fig. 1, after i.v. administration of free 5-FU and 5-FU-Suc-Chi/NP suspensions in rats, the area under plasma concentration-time curve (AUC), elimination half-life ($t_{1/2}$), and residence time (MRT) were increased 2.5-fold, 10.98-fold, and 10.8-fold, respectively, for 5-FU-Suc-Chi/NP compared with that of free 5-FU. This indicates that when 5-FU is loaded into nanoparticles, the 5-FU has sustained-release, prolonged half-life, and increased bioavailability.

The biodistributions of the prepared nanoparticles in various organs in Sarcoma 180-bearing mice were evaluated at distinct durations after i.v. administration of 5-FU-Suc-Chi/NPs and 5-FU injection, as shown in Figs. 2, 3, and 4. At 1 hour, the amounts of 5-FU in the plasma did not show any significant differences between 5-FU-Suc-Chi-NPs and 5-FU injection in the tumor-bearing mice. At day 1 and day 3, 5-FU injection could not be found in any plasma or tissues samples. However, 5-FU-Suc-Chi/NPs could be sustained at a high level in blood for a very long time, implying its long systemic retention in the circulation. The 5-FUs were distributed mainly in the tumor and liver, with small quantities in kidney and spleen. The 5-FU scarcely accumulated in the heart and lung, and decreased the toxic effect of 5-FU in the heart and lung. These results indicate that an adequate duration in the circulation and tumor targeting of 5-FU-Suc-Chi/NPs should be achievable. The rela-

Table 1. Pharmacokinetic Parameters of 5-FU-Suc-Chi/NPs and Free 5-FU Injection in Mice after Intravenous Administration

Parameter	5-FU-Suc-Chi-NPs	5-FU injection
$T_{1/2}$ (h)	5.39±1.63**	0.45±0.12
$AUC_{0-\infty}$ (μg·h/ml)	136.41±46.18**	38.97±7.29
$AUMC_{0-\infty}$ (μg·h^2/ml)	1062.27±124.33	25.76±6.78
$MRT_{0-\infty}$ (h)	7.79±1.69**	0.66±0.14
Ke (1/h)	0.13±0.02	1.51±0.34
CL (ml/h)	43.98±9.62	353.96±38.49
V (ml)	274.90±40.15	234.53±24.51

** $p<0.01$; * $p<0.05$, compared to the corresponding parameters of free 5-FU. ($n=6$).

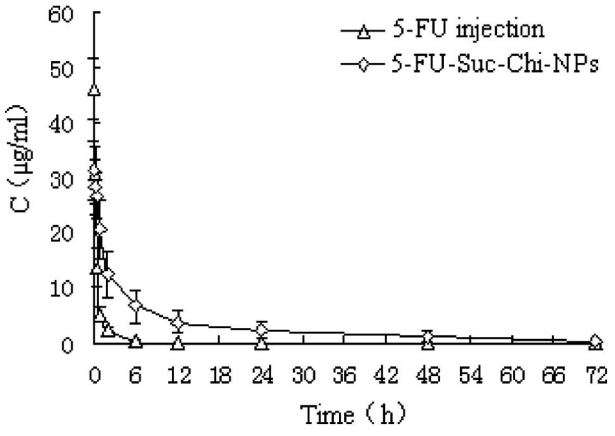

Fig. 1. Mean Plasma Concentration of 5-FU after Administration of Free 5-FU and 5-FU-Suc-Chi/NPs
Data represent mean±S.D. ($n=6$).

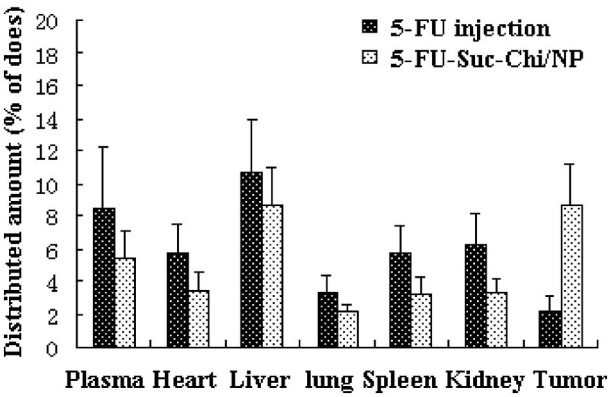

Fig. 2. Biodistributions of 5-FU at 1 Hour after I.V. Administration of 5-FU-Suc-Chi/NPs and 5-FU Injection
At 7 days after inoculation of Sarcoma 180 cells into the subcutaneous dorsa of mice, the 5-FU-Suc-Chi/NPs and 5-FU were injected into the tail vein of the tumor-bearing mice. Data represent mean±S.D. ($n=4$).

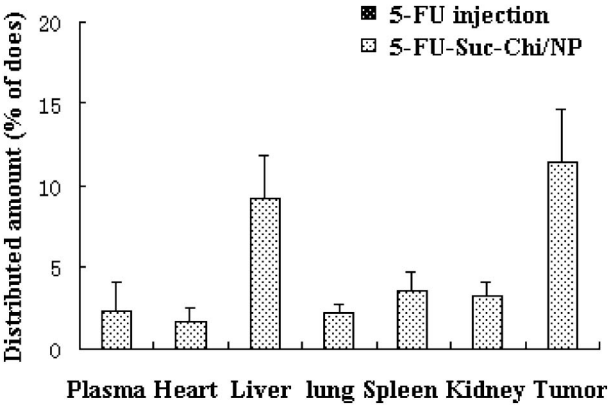

Fig. 3. Biodistributions of 5-FU at 1 Day after I.V. Administration of 5-FU-Suc-Chi/NPs and 5-FU Injection
At 7 days after inoculation of Sarcoma 180 cells into the subcutaneous dorsa of mice, the 5-FU-Suc-Chi/NPs and 5-FU were injected into the tail vein of the tumor-bearing mice. Data represent mean±S.D. ($n=4$).

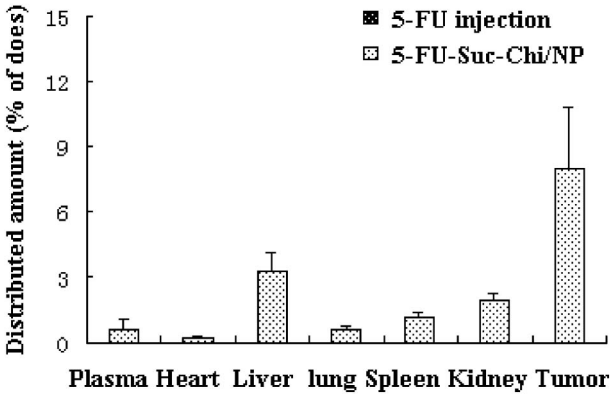

Fig. 4. Biodistributions of 5-FU at 3 Days after I.V. Administration of 5-Fu-Suc-Chi/NPs and 5-FU Injection

At 7 days after inoculation of Sarcoma 180 cells into the subcutaneous dorsa of mice, the 5-FU-Suc-Chi/NPs and 5-FU were injected into the tail vein of the tumor-bearing mice. Data represent mean±S.D. (n=4).

tive tumor tissue exposures (Re) and the ratios of peak concentrations (Ce) were 9.43±1.86 and 2.75±0.26, respectively. This showed that the tumor targeting of 5-FU-Suc-Chi/NP was increased compared with 5-FU injection.

It is now generally known that the structure of solid tumors allows an enhanced permeation and retention (EPR) effect,[9–11] the result of which is nanoparticles accumulate at the tumor site.

Our experimental results showed that 5-FU could circulate in the blood at a high level throughout the 3 days. The 5-FU was distributed mainly in the kidney and tumor, with a small quantity in the liver and spleen. This indicates that an adequate half-life of 5-FU-Suc-Chi/NP in blood circulation should be attainable, enabling the passive accumulation of 5-FU-Suc-Chi/NP into a solid tumor by the EPR effect.

In general, the size of nanoparticles, developed for site-specific delivery of drugs, must be controlled to avoid uptake by the reticuloendothelial system (RES).[12] The optimal size should be less than 100 nm in diameter. It should also be pointed out that particles (240 nm in diameter) could accumulate passively in the tumor tissue, meaning that they should escape the reticuloendothelial cell system in spite of their large particle size. This suggests that surface properties generated by 5-FU-Suc-Chi/NP and/or the size change in blood by enzymatic degradation may partially be involved.[13] Consequently, 5-FU-Suc-Chi/NP accumulated in the tumor tissue due to the EPR effect and its long-term systemic retention in the circulation.

CONCLUSION

In the present study, the biodistribution of 5-FU-Suc-Chi/NP in Sarcoma 180-bearing mice after i.v. injection was investigated. Our experimental results showed that 5-FU-Suc-Chi/NP could circulate in the blood at a high level for 4 days and the amount of 5-FU-Suc-Chi/NP that had accumulated at the tumor site was increased as the blood circulation time was increased due to the effect of EPR. These findings indicate that this nanoparticulate system, in which the circulation half-life of Suc-Chi is long, could be used as a carrier of antitumor drugs for tumor targeting.

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