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Nanoparticles of 5-fluorouracil (5-FU) loaded *N*-succinyl-chitosan (Suc-Chi) for cancer chemotherapy: preparation, characterization — in-vitro drug release and anti-tumour activity

Chengyun Yan, Dawei Chen, Jiwei Gu and Jing Qin

Abstract

N-Succinyl-chitosan has favourable properties as a drug carrier, such as biocompatibility, low toxicity and long-term retention in the body. It is a good candidate for cancer chemotherapy as a polymeric drug carrier. This study describes the preparation and characterization of 5-fluorouracil-loaded *N*-succinyl-chitosan nanoparticles (5-FU-Suc-Chi/NP) by an emulsification solvent diffusion method. The influence of the initial 5-FU concentration on the nanoparticle characteristics and release behaviour in phosphate-buffered saline solution (PBS) was evaluated. The Suc-Chi nanoparticles had a particle diameter (Z-average) in the range 202~273 nm and a negative zeta-potential (approx. –27 to –18 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). The 5-FU-Suc-Chi/NP showed good anti-tumour activity against Sarcoma 180 solid tumour and mild toxicity. According to the data obtained, this Suc-Chi-based nanotechnology opens new and interesting perspectives for cancer chemotherapy.

Introduction

The efficacy of current cancer chemotherapy is mainly limited by the toxicity of the anti-cancer drugs to normal tissues. This limitation results from the fact that currently used anti-cancer drugs lack efficient selectivity towards tumour cells. For effective cancer chemotherapy, an optimal concentration of anti-cancer agent must reach the tumour tissues and remain there for the required period of time (Barichello et al 1999).

For this purpose, attempts have been focused on the development of drug delivery systems containing antineoplastic drugs. Promising results have been reported for 5-fluorouracil- (Omotosho et al 1986; Ciftci et al 1994, 1996; Nichifor et al 1997), bleomycin- (Nakamoto et al 1975), mitomycin- (Jeyanthi & Rao 1989), peptide- (Muranishi et al 1997) and pepleomycin-loaded particles (Hagiwara et al 1985; Hirao et al 1993) in clinical studies. Biodegradable polymers, such as chitosan and PLGA, have been extensively studied in controlled-release technology with respect to their biodegradability and biocompatibility.

Chitosan, the *N*-deacetylated derivative of chitin, has drawn increasing attention as a drug (Lee et al 2000; Ruel-Gariepy et al 2002) or gene (Roy et al 1999; Kim et al 2001; Koping-Hoggard et al 2001) carrier because of its advantages for biomedical applications, such as biocompatibility, biodegradability and biological activity (Hirano 1999; Molinaro et al 2002; Park et al 2003). Besides, the reactive amino groups in the backbone of chitosan make it possible to chemically conjugate various biological molecules, such as different ligands and antibodies, which may improve targeting efficiency of the drug to the site of action (Dufes et al 2000; Hejazi & Amiji 2003). The extended applications of chitosan, however, are frequently limited by its insoluble nature in a biological solution (pH 7.4). *N*-Succinyl-chitosan (Suc-Chi), which was obtained by introduction of succinyl groups into chitosan via *N*-terminals of the glucosamine units has favourable properties as a drug carrier, such as biocompatibility, low toxicity and long-term retention in the body (Song et al 1992, 1993; Kato et al 2000, 2002a, b; Onishi et al 2001). Suc-Chi has received a rising interest as

School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, 110016, R. P. China

Chengyun Yan, Dawei Chen, Jing Qin

College of Chemistry & Pharmacy, Jiamusi of University, Jiamusi, 154000, R. P. China

Chengyun Yan, Jiwei Gu, Dawei Chen, Jing Qin

Correspondence: Dawei Chen, Box 42, School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, 110016, R. P. China.
E-mail: ycyyzy@yahoo.com.cn

a novel carrier for drugs because of its good biocompatibility and solubility over a broad range of pH.

In this work, we report the preparation, characterization, in-vitro release behaviour and anti-tumour activity of 5-fluorouracil (5-FU)-loaded Suc-Chi nanoparticles by an emulsification solvent diffusion method. The objective of this study was to prepare colloidal systems of desired characteristics using 5-FU as model drug, which could improve the cancer chemotherapy.

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, 5–7 weeks old, 18–22 g, were purchased from Shen-yang Laboratory Animals Science Co. (Shen-yang, China). Mice were treated according to the ethical guidelines of Animal Center, Shenyang Pharmaceutical University. The Animal Studies Committee of Shenyang Pharmaceutical University approved the experimental protocol.

Tumours

Sarcoma 180 cells 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 tumour-bearing mice, were inoculated subcutaneously into each mouse at the axillary region. The tumours were allowed to develope for 7 days. The tumour size was measured on alternate days post treatment using a vernier calliper, and the tumour volume (V) was calculated by the following equation:

$$V = (3.14/6)\times D_1\times D_2\times D_3 \quad (1)$$

where D_1, D_2 and D_3 represent the tumour length, width and height, respectively. Further, the tumour growth ratio (GR) was calculated by the following equation:

$$GR = V(t)/V(7) \quad (2)$$

where V(t) and V(7) are the tumor volume at t days and at 7 days after the inoculation, respectively.

Preparation of 5-fluorouracil/*N*-succinyl-chitosan nanoparticles

5-FU-Suc-Chi nanoparticles were prepared by an emulsification solvent diffusion method (El-Shabouri 2002). Blank nanoparticles were obtained upon the addition of 10 mL of the Suc-Chi aqueous solution (2 mg mL^{-1}) to 100 mL of the Span-80 organic solution, which contained 20% (v/v) stirred at room temperature. The desolvation of polymeric material occurred instantaneously in the form of colloidal particles. Water was then evaporated from the colloidal suspension on a magnetic stirrer at 40°C by a vacuum-pump. Nanoparticle suspensions were centrifuged. The precipitate was washed three times with petroleum ether (60~90°C) and dispersed in 10 mL of water.

5-FU-loaded nanoparticles were obtained according to the same procedure. Variable amounts of 5-FU were incorporated into the Suc-Chi solution before the formation of nanoparticles to investigate the effect of the initial 5-FU concentration on the nanoparticle characteristics and in-vitro release profiles. Nanoparticle suspension was filtered through a 0.45-μm nitrocellulose membrane (Millipore) filter after ultrasonic treatment and concentrated to a final volume of 10 mL by removal of water under the same conditions. Mannitol 0.1% (w/v) was added to the nanoparticle suspension and it was lyophilized.

Characterization of the nanoparticles

Morphological examination of the Suc-Chi nanoparticles was performed using a transmission electron microscope (TEM) (CM12 Philips, USA). The samples were re-suspended in water and stained with 1% (w/v) phosphotungstic acid and placed on copper grids to dry for TEM analysis. The size (Z-average mean) and zeta-potential of the nanoparticles were analysed by using a Zeta PotentiaL AnaLyzer (DeLsa 440SX; Beckman Coulter).

Evaluation of 5-FU encapsulation

Prescribed 30 mg of lyophilized 5-FU-Suc-Chi/NPs were added to10 mL of cellulase (1 mg mL^{-1}) aqueous solution and heated at 40°C for digestion. After standing for 2 h, the aqueous solution was filtered through a membrane filter and the amount of free 5-FU was measured in the aqueous solution by an HPLC method. A Kromasil C18 (4.6 mm × 200 mm, 5 μm) column was used as the stationary phase and water–acetonitrile (95:5) as the mobile phase. The 5-FU was detected at a wavelength of 266 nm. The 5-FU loading capacity (LC) of the nanoparticles and their entrapment efficiency (EE) were calculated according to the following equations:

$$\%LC = 100\times[(\text{5-FU in nanoparticles})/(\text{nanoparticles weight})] \quad (3)$$

$$\%EE = 100\times[(\text{5-FU in nanoparticles})/(\text{total 5-FU})] \quad (4)$$

Evaluation of in-vitro 5-FU release

Lyophilized nanoparticles (1 mg) were re-suspended in 1 mL of PBS and incubated at 37°C under light agitation. At appropriate time intervals, individual samples were centrifuged and the amount of the 5-FU in the release medium was determined by HPLC. The calibration curve obtained from the HPLC method was linear between 0.5 and 200 μg mL^{-1}

($A = 8961.6C - 192.239$ r = 0.9996). The limit of detection was 10 ng mL^{-1}.

Anti-tumour activity

Experiments were performed on day 7 after inoculation of Sarcoma 180 cells into Balb/c mice. Mice were randomized and divided into six mice per group: I, untreated controls; II, empty Suc-Chi nanoparticles; III, 5-FU in solution (15 mg kg^{-1}); IV, Suc-Chi nanoparticles with entrapped 5-FU (15 mg kg^{-1}); V, Suc-Chi nanoparticles with entrapped 5-FU (20 mg kg^{-1}).

The treatments were repeated every week four times for a period of two weeks. The route of administration was intravenously in the tail vein of mice for all the groups. Tumour volume was measured on alternate days for a period two weeks post treatment.

Statistical analysis

In all cases, experiments were replicated six times and data represent mean ± s.d. Statistical analysis of the effect of drug loading on the various physicochemical properties was performed using one-way analysis of variance and Tukey's test. The effect of time and drug loading on drug release and the effect of time and formulation on body weight and tumour growth inhibitory effect were evaluated using a repeated measures analysis of variance and Tukey's test. $P < 0.05$ denoted significance in all cases.

Results and Discussion

Physicochemical characterization of nanoparticles

Spherical nanoparticles were formed with the narrow size distribution as observed by TEM (Figure 1) and light-scattering measurement. The particle diameter (Z-average) range was approximately 202–273 nm (Table 1).

It seems that there was no linear correlation between the 5-FU concentration and the size of the nanoparticles.

Table 1 Particle diameter and zeta-potential values of Suc-Chi nanoparticles containing different concentrations of 5-FU

Initial 5-FU concentration (μg mL^{-1})	Particle diameter (nm)	Zeta potential (mV)	%EE ($\times 10^{-5}$)	%LC
0	236.6 ± 4.7	−27.2 ± 0.2	—	—
500	231.9 ± 5.2	−23.0 ± 0.4	73.60 ± 0.46	8.21 ± 21.8
1000	219.9 ± 6.7	−21.6 ± 0.2	62.36 ± 0.74	18.98 ± 2 5
1500	202.3 ± 3.2	−18.5 ± 0.3	37.23 ± 0.66	16.15 ± 8.3
2000	273.4 ± 4.6	−18.4 ± 4.3	41.66 ± 0.72	15.67 ± 16.00

Data represent mean ± s.d., n = 6.

The nanoparticles observed by TEM displayed lower values of diameter due to drying of the sample suspension. Suc-Chi nanoparticles had a negative zeta-potential ranging from −27 to −18 mV, approximately, depending on the 5-FU content (Table 1). The %EE and %LC could not be correlated with the initial 5-FU concentration. The formulation with an initial 5-FU concentration of 1000 μg mL^{-1} provided the highest loading capacity (19%). However, regarding the particle size and %EE, larger particles were observed in higher association cases, except with the 2000 μg mL^{-1} initial 5-FU concentration where a saturation of the association seemed to occur.

In-vitro 5-FU release

Figure 2 displays the release profiles of 5-FU from the nanoparticles in PBS. An initial fast release (20–45% in 60 min) suggested that some 5-FU was localized on the surface of the nanoparticles. The highest extent of release (61% at 24 h) was observed for the formulation prepared with the 1000 μg mL^{-1} initial concentration in comparison with 50–55% at 24 h observed for formulations at 1500 and 2000 μg mL^{-1}, which showed quite similar profiles. Then the sustained release of the 5-FU from the above samples was maintained for 4 days continuously by drug diffusion from the core or as a result of Suc-Chi degradation. These results suggest that the drug that was captured in the structure was released in a controlled fashion.

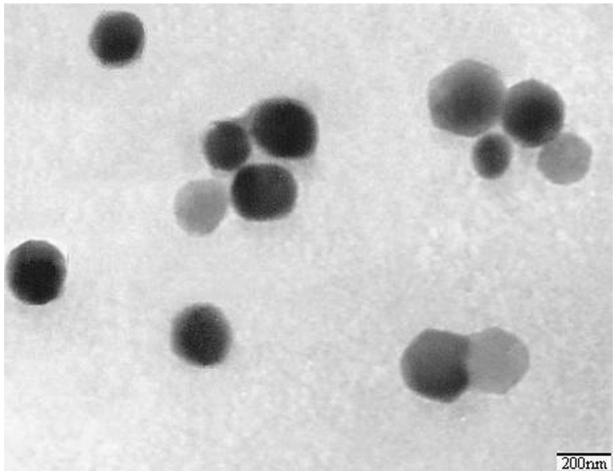

Figure 1 TEM photographs of blank Suc-Chi nanoparticles.

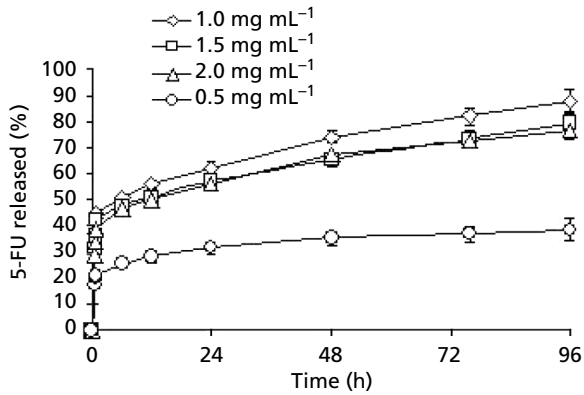

Figure 2 Release profiles of Suc-Chi nanoparticles containing different concentrations of 5-FU. Data represent mean ± s.d., n = 6.

Anti-tumour activity

In in-vivo studies in mice, a tumour was induced by the subcutaneous injection of Sarcoma 180 cells and the drugs were injected at 7 days after tumour inoculation. Compared with the initial body weights of tumour-bearing mice, a gradual increase in the body weight was found for the mice untreated and treated with Suc-Chi/NP, whereas a gradual decrease in the body weight was observed for the free-5-FU-treated mice (Figure 3), suggesting systemic toxicity of 5-FU (Son et al 2003). Irrespective of the dose we used, the intravenous injection of 5-FU-Suc-Chi/NP resulted in a gradual increase in the body weight, although the increasing rate was lower than that of the untreated mice. This may indicate that 5-FU-Suc-Chi/NP exhibits mild toxicity. With regard to the change in the tumour volume, it was evident that 5-FU-Suc-Chi/NP and 5-FU treatments effectively suppressed the tumour growth (Figure 4) (e.g., 13 days after intravenous injection, tumour volumes of the mice treated with 5-FU-Suc-Chi/NP and 5-FU were significantly smaller than those of untreated mice and tumour volumes of the mice treated with 5-FU-Suc-Chi/NP were significantly smaller than those treated with the free 5-FU ($P < 0.05$)). By comparing the tumour volumes of the mice treated with 5-FU-Suc-Chi/NP and 5-FU, there were indications that the 5-FU-Suc-Chi/NP administration was more effective than the 5-FU alone.

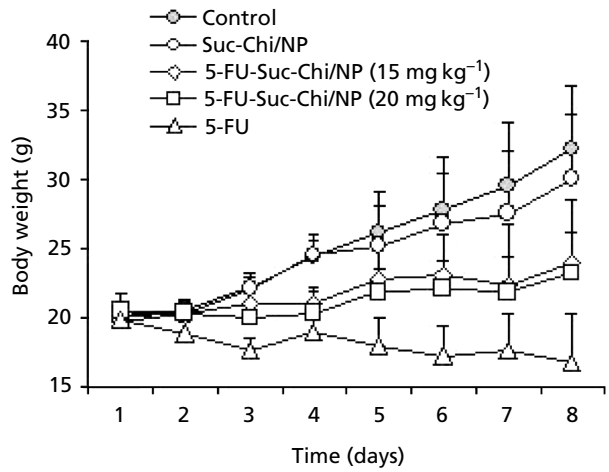

Figure 3 Body-weight change of tumour-bearing mice, untreated and after intravenous injection of Suc-Chi-NP, 5-FU-Suc-Chi-NP or 5-FU. The drug solutions were administered at 7 days after inoculation of Sarcoma 180 cells into the subcutaneous dorsa axillary region of the mice. Data represent mean ± s.d., n = 6.

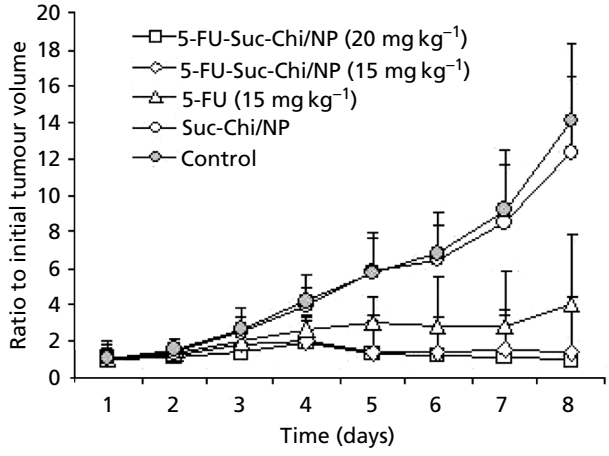

Figure 4 Tumour-growth-inhibitory effects of 5-FU-Suc-Chi/NP against Sarcoma 180 solid tumour. Data represent mean ± s.d., n = 6.

Conclusion

In this study, we have Prepared Suc-Chi nanoparticles using 5-FU as model drug, which has not yet been formulated in a drug delivery system. The emulsification solvent diffusion method was used to prepare biodegradable nanoparticles of sizes in the range of 202–273 nm by addressing the effects of processing parameters. The results of the in-vitro release of 5-FU-Suc-Chi/NP in PBS demonstrated that the drug was released by a combination of diffusion with slow and gradual erosion of the particles. Thus Suc-Chi nanoparticles offer potential for the controlled release of anti-cancer drugs. In-vivo anti-tumor activity of the Suc-Chi nanoparticles was assessed following systemic administration via the tail vein of the tumour-bearing mice. 5-FU-loaded Suc-Chi/NP showed good anti-tumour activity against Sarcoma 180 solid tumour, with mild toxicity. The results indicate that Suc-Chi-NP might be applied as a carrier in targeted cancer chemotherapy. This work can be considered as the first step for further studies on the application of Suc-Chi nanoparticles for possible targeted delivery.

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