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Characterization, blood profile and biodistribution properties of surface modified PLGA nanoparticles of SN-38

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ABSTRACT

SN-38, the active metabolite of irinotecan, poses a challenge in terms of drug delivery due to its low solubility and labile lactone ring. The aim of this study was to develop a SN-38 nanoparticulate delivery system to evaluate the *in vivo* blood profile and biodistribution properties of nanoparticles (NPs).

Poly lactide-co-glycolide (PLGA) NPs that were covalently bound to polyethylene glycol-folate (PEG-FOL) were prepared, and their *in vivo* biodistribution in rats was investigated. Either the SN-38 solution or SN-38 NP suspension was administered intravenously into the tail vein at a dose of 2 mg SN-38 eq./kg. As expected, SN-38 NPs showed a higher plasma concentration *in vivo* when compared with free SN-38 during a 24 h period. Compared with the SN-38 solution, both folate targeted and non-targeted NPs exhibited superior drug concentration in body organs such as the liver, spleen, and lung at 1 and 8 h post-administration.

1. Introduction

Camptothecin (CPT), a plant alkaloid extracted from *Camptotheca acuminate* (Onishi et al., 2003; Kunii et al., 2008), and its related analogues are a group of anticancer agents that show great therapeutic potential; their unique mechanism of action is based on targeting the nuclear enzyme topoisomerase I (Yoshizawa et al., 2005). An important chemical feature of camptothecins and related analogues is the rapid and reversible hydrolysis from a closed lactone ring to the carboxylate form. Several studies have demonstrated that the lactone form is a more potent inhibitor of topoisomerase I, in addition to being a much more effective anti-tumour agent with lower toxic effects (Oguma, 2001; Sano et al., 2003). The use of the lactone form of CPT is complicated by its poor water solubility. Therefore, many attempts to improve the characteristics of CPT analogues *in vitro* and *in vivo* have been made. Among its analogues, irinotecan hydrochloride (CPT-11) has been shown to exhibit potent cytotoxic activity against colorectal, lung, and ovarian cancers (Mu et al., 2005). CPT-11 is a prodrug that is converted to 7-ethyl-10-hydroxy-CPT (SN-38), a biologically active metabolite of the compound, by carboxylesterases. A schematic of the structure and the conversion of both lactone and carboxyl forms of CPT, CPT-11, and SN-38 is shown in Fig. 1. The *in vitro* cytotoxic activity of SN-38 against various cancer cells has proven to be up to 1000-fold more efficacious than CPT-11. Although CPT-11 is transformed to SN-38 in both the liver and tumour tissues, the metabolic conversion rate is typically less than 10% of the original volume of CPT-11 administered (Carbonero and Supko, 2002; Peikov et al., 2004). Moreover, the conversion of CPT-11 to SN-38 depends on the genetic interindividual variability of carboxylesterase activity (Koizumi et al., 2006). Consequently, the direct utilisation of SN-38 might be of considerable benefit for cancer therapy. As SN-38 is a water-insoluble compound, however, it is essential to develop a soluble form for use in clinical applications. Improvements in the manufacturing technology of NPs might make it possible to use SN-38 in *in vivo* experiments and could ultimately enable clinical use of the compound. Poly DL-lactic acid-co-glycolic acid (PLGA) has previously been used to achieve a controlled-release of various drugs (Esmaeili et al., 2007, 2008; Zhao and Yung, 2008; Ebrahimnejad et al., 2009a). These polymers and their derivatives have been used

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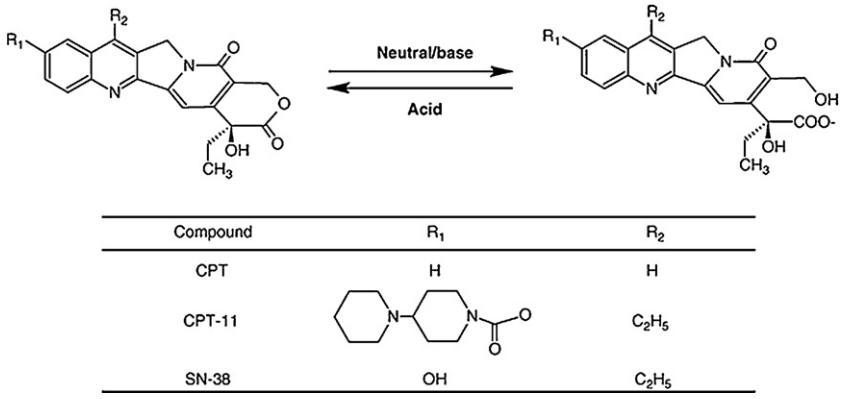

Fig. 1. Conversion of the lactone and carboxyl forms of CPT, CPT-11, and SN-38.

in the development of nanoparticulate dosage forms of various compounds (Higaki et al., 2005; Zhao and Yung, 2008; Vergoni et al., 2009; Chakravarthi et al., 2010). In previous studies, CPT-11-containing NPs were prepared using poly DL-lactic acid (PLA) and poly ethylene glycol-block–poly propylene glycol-block–poly ethylene glycol (PEG–PPG–PEG) to improve the efficacy of CPT-11 (Onishi et al., 2003). The generated NPs had a diameter of 100–300 nm and exhibited a longer systemic retention than CPT-11. Furthermore, the NPs exhibited substantial antitumour activity against a subcutaneous sarcoma 180 (S-180) solid tumour and M5076 liver metastasis in mice in comparison with CPT-11 solution (Machida et al., 2003; Onishi et al., 2003). It has been reported that NPs that have diameters of up to 400 nm and hydrophilic molecules on their surface exhibit long plasma residence times and can easily move into diseased tissues (such as solid tumours) due to their enhanced permeability and retention effects (EPR effect) (Maeda et al., 2000; Kunii et al., 2008). It has been reported that a liposome-based SN-38 formulation has shown promising results in pre-clinical studies in terms of increased cytotoxicity against tumour cell lines and/or improved therapeutic effects in xenograft mouse models in comparison to irinotecan (Zhang et al., 2004; Kunii et al., 2008; Atyabi et al., 2009). In addition, SN-38-incorporating polymeric micelles have been shown to exhibit significantly potent antitumour effects against HT-29 cells in comparison to CPT-11 (Koizumi et al., 2006).

As a targeting ligand, folate (FOL) has advantages over macromolecules such as monoclonal antibodies. These advantages include: a smaller molecule size; the lower possibility of immunogenicity; its wide availability and low price; its conjugation chemistry which is both simple and specific; and its high receptor affinity and the absence or limited number of normal tissue receptor expression, which consequently ensures its high tumour tissue specificity. In addition, the receptor–ligand complex can be prompted to internalise via endocytosis, a process that may promote the cytosolic delivery of drugs (Zhao and Yung, 2008).

In our previous work, we developed polymeric nanoparticulate drug delivery systems for SN-38 using an emulsification solvent evaporation method (Ebrahimnejad et al., 2009a). Cell culture studies indicated that treatment of HT-29 cells with drug loaded NPs resulted in lower cell viability (or equivalently, higher cytotoxicity) versus free SN-38 after a 48 h incubation when compared with the control. Targeted nanoparticles (TNPs) demonstrated significant advantages in achieving a lower levels of cell viability, or equivalently, higher cytotoxicity versus non-targeted nanoparticles (NNPs) (Ebrahimnejad et al., 2010). The goals of the present study were to evaluate the *in vivo* biodistribution properties and blood profile of PEG-FOL-decorated NPs in rats and to determine the effects of modifying the surface of NPs on *in vivo* test results.

2. Materials and methods

2.1. Materials

Folate and 3-(4,5-dimethylthiaol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). RPMI-1640 modified medium, were obtained from Gibco Invitrogen (Carlsbad, California).

PLGA (50:50; Resomer® RG 504 H, MW 48,000) was purchased from Boehringer Ingelheim (Ingelheim, Germany). SN-38 was obtained from ABATRA Co. (Shaanxi, China). Acetonitrile, methanol, and dimethylsulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Deionised water was used in all experiments. All other chemicals used were of reagent grade.

2.2. PLGA–PEG-FOL synthesis

PLGA–PEG-FOL conjugate was prepared according to the method developed by Yoo and Park (2004), but with minor modifications. NMR was employed to confirm the conjugation process. Based on the molar ratio, the conjugation percentage of FOL-PLGA–PEG was found to be 35.5%. A known amount of dried PLGA–PEG-FOL was dissolved in DMSO, and the UV absorbance value at 256 nm was measured to determine the concentration of conjugated folic acid. Serially diluted concentrations of folic acid in DMSO were used to construct a calibration curve (Zhao and Yung, 2008).

2.3. Preparation and characterisation of NPs

The SN-38-loaded NPs were fabricated using the emulsification/solvent evaporation method and characterised according to our previous studies (Ebrahimnejad et al., 2009a). SN-38 entrapment efficiency was determined in triplicate by HPLC (Ebrahimnejad et al., 2009b). Scanning electron microscopy (SEM) was used for the morphological characterisation of nanoparticles. Nanoparticles were characterised with respect to size and zeta potential (ζ) using a Zetasizer system (Malvern, UK). The release pattern of SN-38 from the nanoparticles was determined in triplicate in phosphate-buffered saline (PBS, pH 7.4) under sink conditions. 10 mg of lyophilized SN-38-loaded NPs were transferred in screw-capped tubes and dispersed in 10 ml PBS. Then the tubes were placed in an orbital shaker bath (WB14; Memmert, Schwabach, Germany), maintained at 37 °C and shaken horizontally at 90 cycles/min. At given time intervals, the tubes were taken out of the water bath and centrifuged at 21,000 × *g* for 30 min, and the entire supernatant was collected for further SN-38 analysis by HPLC. Then the precipitated particles were resuspended in 10 ml fresh buffer (to maintain sink conditions) and put back into

Table 1
The effect of the formulation parameters on physiochemical characteristics of the nanoparticles ($n = 3$).

Nanoparticles	Entrapment efficiency (%) ± S.D.	Size ± S.D. (nm)	PdI	Zeta potential ± S.D. (mV)
SN-38 loaded PLGA nanoparticles	77.1 ± 6.5	173 ± 13	0.107	−10.8 ± 0.2
SN-38 loaded PLGA–PEG-Folate nanoparticles	89.1 ± 9.2	221 ± 15	0.217	−6.5 ± 0.3

the shaker to continue the release measurement (Ebrahimnejad et al., 2010). Drug release data were normalized by converting drug concentration in solution to a percentage of the cumulative drug release. The HPLC method used was the same as that described earlier (Ebrahimnejad et al., 2009b).

A Knauer liquid chromatograph (Smart line; Knauer, Berlin, Germany) equipped with an ultraviolet detector (Wellchrom, K-2600; Knauer) and a reverse-phase C18 column (Nucleosil H.P. 25 cm × 0.46 cm internal diameter, pore size 5 mm; Knauer) were used. The mobile phase consisted of a mixture of acetonitrile, buffer solution (13.6 g KH_2PO_4 dissolved in 500 ml water), and ion-pair solution (1.2 g octane-1-sulfonic acid sodium salt dissolved in 500 ml water) at 4:3:3 (v/v) ratio, at a flow rate of 1.00 ml/min with a pump (Wellchrom, K-10001; Knauer). Each sample was injected into the column at a constant volume of 20 μl. The column effluent was detected at 265 nm.

2.4. Blood concentration profile of SN-38

Wistar rats with body weights between 200 and 250 g were used for this investigation. The rats were fasted overnight but had free access to water. Rats were randomly distributed into three groups that received free SN-38, NNPs, or TNPs. The rats were anesthetised by IV injection of pentobarbital at 25 mg/kg (4 ml/kg in saline), and then were secured on their backs. The SN-38 solution was prepared using a mixture of saline and DMSO (1:20, v/v) as the solvent. The NNP and NNP suspensions were prepared by diluting NPs with saline. Either the SN-38 solution or the NP suspension was administered intravenously into the tail vein at a dose equivalent of 2 mg SN-38 eq. per kg of rat body weight.

Three animals from each of the three groups were injected with one of the three samples. Blood samples (0.4 ml) were taken using a heparinised syringe at 0.25, 1, 8, 12, and 24 h after drug administration; immediately prior to each blood sampling, light anaesthesia was induced into the animals, along with a small amount of pentobarbital.

Plasma was obtained by immediate centrifugation at 5000 × g for 10 min at 4 °C. Plasma proteins were then precipitated by adding 200 μl methanol and acetonitrile (1:1, v/v) to a 100 μl aliquot of the plasma. After rigorous vortex-mixing for 1 min, the mixtures were centrifuged at 5000 × g for 10 min at 4 °C. A 100-μl aliquot of supernatant was then transferred to a fresh polypropylene tube, and 20 μl of the solution was injected into the HPLC system for analysis. HPLC methodology was the same for all studies.

2.5. Biodistribution study

For the biodistribution study, either SN-38 solution or NP suspension was administered intravenously as described above. The animals were then euthanized at 1 and 8 h post-administration via the inhalation of ether. The lungs, liver, kidneys, and spleens were removed; washed with saline; and weighed prior to homogenisation in saline. Tissue samples were cooled on ice after the homogenisation procedure; the homogenate was then centrifuged at 21,000 × g for 10 min.

Methanol and acetonitrile were added to the supernatant (1:1) to precipitate unwanted proteins; samples were centrifuged (21,000 × g, 10 min) as described above. The aliquots were assayed for SN-38 levels using HPLC to estimate the total amount of SN-38 in each organ. For SN-38 concentration calculations, standard curves were prepared by the addition of SN-38 in plasma and each tissue following the same process.

Recovery experiments were performed by adding specified amounts of free SN-38 or NPs to fresh plasma or organs, preparing the samples in the same manner as was done with the test samples, and then measuring the final drug concentrations in the supernatant. The amounts calculated from the concentration of the supernatant were directly used as the distributed amounts; this was done immediately because the recovery levels of free SN-38 or NPs in plasma and the organs tested were almost complete. The recovery ratio was obtained as a ratio of the observed and calculated (ideal) SN-38 concentrations.

The animal study was approved by the ethical committee of the Pharmaceutical Research Centre, Faculty of Pharmacy, Tehran University of Medical Sciences.

2.6. Statistical analysis

Comparisons were performed using the unpaired *t*-test, with *P*-values of less than 0.05 interoperated as representing statistical significance.

3. Results

NPs were fabricated as had been described in our previous study (Ebrahimnejad et al., 2009a). The mean particle size was 173 ± 13 nm, with an entrapment efficiency (EE) of 77.1 ± 6.5% for NNPs and a mean particle size of 221 ± 15 nm with an EE of 89.1 ± 9.2% for TNPs (see Table 1). The size distribution ranged from 100 to 500 nm and was primarily distributed between 100 and 300 nm for both NNP and TNP complexes. We also assessed particle size stability in PBS at 37 °C for up to 24 h. However, the particle aggregation appeared to increase gradually; the NPs' typical size range was maintained, though some larger particles (which resulted from aggregation) were observed at 24 h (data not shown).

SEM analysis revealed that the NPs were all characterised by a spherical shape with a relatively monodispersed size distribution. The nanoparticles exhibited low zeta potential values: −10.8 ± 0.2 mV for non-targeted NPs and −6.5 ± 0.3 mV for FOL-targeted NPs. The release profile of SN-38 from the nanoparticles under sink conditions showed an initial burst of release during the first day, greater than 10% and 14% for TNP and NNP, respectively, followed by a constant and gradual SN-38 release of 23.1 ± 2.0% for TNPs and 30.0 ± 2.0% for NNPs after 240 h (Fig. 2).

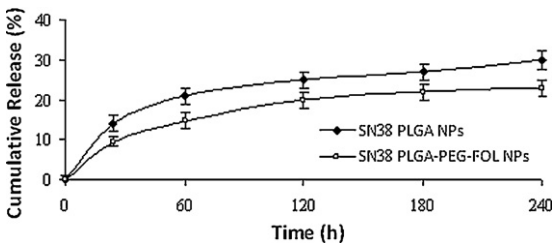

Fig. 2. Cumulative release of SN-38 TNPs and NNPs in PBS ($n = 3$).

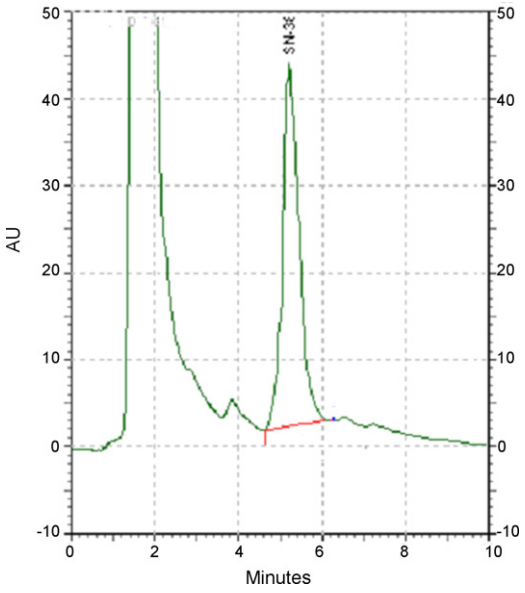

Fig. 3. Representative chromatogram of the lactone form of SN-38 NPs in plasma.

SN-38 concentrations in the blood and tissues were measured. Representative chromatograms of the lactone form of SN-38 NPs in plasma are shown in Fig. 3.

The blood concentrations of SN-38 after administration of free SN-38, NNPs, and TNPs are shown in Fig. 4. Lactone concentrations were measured 24 h after injection. The circulating levels of SN-38 for in the blood of TNP and NNP administered rats were similar for 15 min after dosing. After 15 min, the free drug in the SN-38 injections had been largely removed from the circulation. After 8 h the concentration decreased from 40.02 to 0.2 μg/ml.

In contrast, for both TNP and NNP injections a noticeably delayed blood-clearance was observed with concentration of 83.3 and 72.9 μg/ml after 15 min and 2.16 and 0.96 μg/ml after 8 h. The levels of SN-38 observed after TNP and NNP injections remained higher at 24 h than those observed after injection of SN-38 with concentration of 0.79 and 0.4 μg/ml. Injection of NP suspensions resulted in a prolonged presence of the drug in the blood, with appreciable levels of drug still present in the circulation 24 h after administration. When administered as a free drug, SN-38 is rapidly eliminated from the circulation (Williams et al., 2003; Yoshizawa et al., 2005).

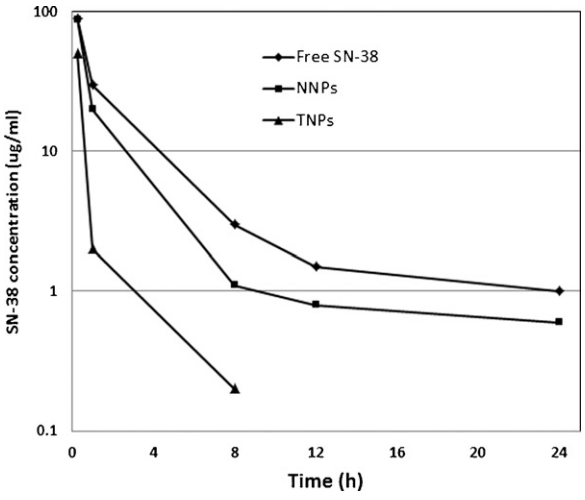

Fig. 4. SN-38 concentration in blood after i.v. administration of SN-38 solution, SN-38-loaded PLGA, and SN-38-loaded PLGA–PEG-FOL nanoparticles in rats at various time points ($n = 3$).

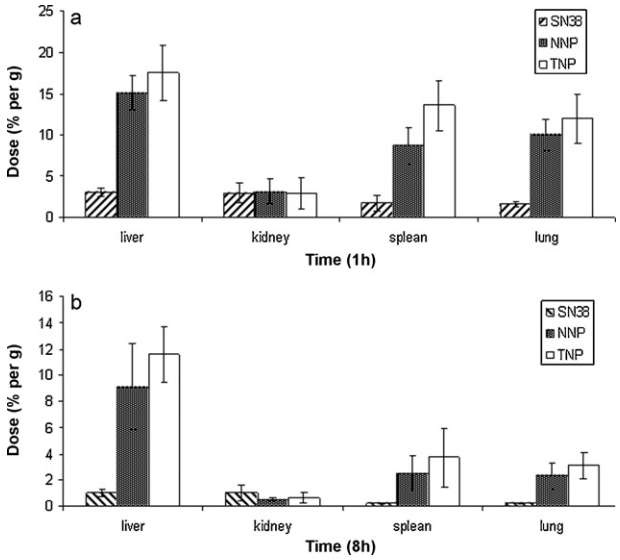

Fig. 5. Body distribution of SN-38, after i.v. administration 1 h (a) and 8 h (b) of SN-38 solution, SN-38-loaded PLGA, and SN-38-loaded PLGA–PEG-Folate nanoparticles in rats ($n = 3$).

In addition, injection of TNPs resulted in higher SN-38 concentrations in comparison to injection of NNPs 24 h after administration ($P < 0.05$). The distribution profile of SN-38 for free drug, NNPs, and TNPs injections in rats after intravenous administration is shown in Fig. 5a and b. It can be seen that, when compared with the SN-38 injection group, the biodistribution of SN-38 was remarkably different for the NPs groups. But the results Shown there were not significant difference between NNPs and TNPs in biodistribution study.

As shown in Fig. 5a., free SN-38 was chiefly distributed to the liver (3.7 ± 0.5 Dose%/g), spleen (1.7 ± 1 Dose%/g), lung (1.6 ± 0.4 Dose%/g), and kidney (3 ± 1.2 Dose%/g) after 1 h, whereas for NNP and TNP groups it predominantly accumulated in liver (15.1 ± 3.3 and 17.5 ± 2.1 Dose%/g), lung (10 ± 3 and 12 ± 1.1 Dose%/g), and spleen (8.75 ± 3 and 13.56 ± 2.2 Dose%/g). In addition, the observed SN-38 concentrations in these organs after the injection of NP formulations was approximately 4–5-fold higher than those levels observed in animals injected with a free SN-38 solution, but there was no significant differences between NNPs and TNPs.

At 8 h post-injection, free SN-38 was largely absent from all tissues (Fig. 5b). However, for TNP and NNP groups it accumulated in normal tissues to an extent different than that of free SN-38. The plasma levels of SN-38 after the injection of the drug loaded TNPs and NNPs were clearly higher than those observed after the injection of free SN-38. TNPs and NNPs exhibited higher levels of distribution in the liver, spleen, and lung in comparison to SN-38 solution, whereas the distribution profiles in the kidney were not clearly different between animals injected with SN-38-loaded NPs and SN-38 solution ($P > 0.05$). When free SN-38 solution was injected into animals, the SN-38 level in each tissue decreased relatively rapidly. In all tissues tested (except the kidney and liver), the concentration of SN-38 decreased to less than 0.3 μg/g within 8 h of injection.

4. Discussion

In the experiments described in this report, we studied the biodistribution and blood profiles of NPs in rats. One of the key questions of this study was whether delivery of SN-38 in NP formulations could circumvent the previously discussed SN-38 delivery problems.

SN-38 was successfully formulated into NPs with a particle size small enough to be suitable for IV injection; injection of these NPs demonstrated an increased solubility of SN-38 in whole animals. In a previous study, we found that NPs demonstrated stable dispersion states, and were characterised by both high absolute zeta potential values and negative surface charges (Ebrahimnejad et al., 2009a, 2010).

NPs can notably change the biodistributions of loaded drugs. Optimised biodistribution leads to improved drug efficacy and, at the same time, a reduction of side effects (Moghimi et al., 2001; Brigger et al., 2002; Vergoni et al., 2009). Therefore, this biodistribution study was performed to enhance our understanding of the *in vivo* behaviour of SN-38-loaded NPs, and to verify whether the released SN-38 was still pharmacologically active. Whereas SN-38 has poor solubility in water due to its relative hydrophobicity, it was shown in this study that high levels of incorporation into NPs with sustained-release properties from these NPs could be achieved. SN-38-loaded PLGA NPs showed improved lactone ring stability *in vivo*. This might be due to the fact that NPs exclude water and prohibit access of serum proteins, resulting in an enhanced stability of the SN-38 lactone ring in blood and tissue samples. Recently, it was reported that stability of SN-38 in the presence of human serum albumin is increased (Williams et al., 2003).

This result supported the idea of fabricating NPs with a steric PEG barrier that would prevent the opsonisation by the mononuclear phagocyte system (MPS) and improve their circulatory half-life, as shown by previous reports (Kim et al., 2005; Park et al., 2009). Recent reports have demonstrated that the uptake of PLGA NPs by the rapid reticuloendothelial system (RES) could be significantly reduced by modifying the surface of NPs with PEG. The PEG-modified PLGA NPs, prepared by using a di-block copolymer of PLGA-b–PEG as an additive, considerably prolonged their half-life in the circulation due to the presence of highly mobile and flexible PEG chains on the surface of the NPs (Kim et al., 2005; Kunii et al., 2007; Bharali et al., 2008). The colloid nature of the SN-38-loaded NPs possibly resulted in a notably increased uptake by RES organs, such as the liver and spleen.

The concentrations of SN-38 in the kidney were not significantly different between animals injected with SN-38-loaded NPs and free SN-38 solution. As SN-38 is a typical S phase-specific drug, an extended exposure of the tumour cells to the drug is necessary to attain an acceptable antitumour efficacy (Machida et al., 2003; Kunii et al., 2008). Encouragingly, NP formulations substantially increased the retention time of SN-38 in many tissues; even at 8 h after administration, a definite concentration of SN-38 was still maintained in all of the tested organs. Furthermore, NPs entrapped within liver, spleen, and lung may act as a reservoir to gradually release the encapsulated drug (Chiannilkulchai et al., 1990; Soma et al., 2000), which also contributes to the maintenance of systemic drug levels.

Thus, taking into consideration the sustained-release characteristics of the SN-38-loaded NPs, the altered physiological distribution, combined with a prolonged retention time of SN-38 in various tissues, may lead to the an enhanced antitumour effect of SN-38-loaded NPs; furthermore, such characteristics may be advantageous in chemotherapies using CPT family drugs. The trends observed with NNPs were comparable to those observed with TNPs; the differences between them in tissues were found to be insignificant ($P > 0.05$) showing that the targeting strategy may not be validated using biodistribution studies. Hence, tumour model animals are to be used for biodistribution investigations to differentiate between targeted and nontargeted NPs.

The concentration of SN-38 in the lungs was noticeably higher in animals injected with NPs, possibly due to the filtration effect of the lung capillary bed (Moghimi et al., 2001). As the lung has typically been an organ that could not be reached by anticancer drugs through IV administration, an increased dose level or the use of aerosol is often needed to achieve efficacious levels of drugs in the lung (Koshkina et al., 2003). A previous study has shown that the addition of PEG into the PLGA polymer backbone can decrease particle clearance rates by phagocytosis in deep lung tissues (Fiegel et al., 2004). Accordingly, our results suggest that lung targeting could possibly be achieved via the IV injection of NPs, thereby enhancing the therapeutic success of lung cancer.

5. Conclusions

SN-38 appears to be an attractive drug for formulation into NPs. It is highly active, and its lactone ring is more stable than many of the other camptotheca alkaloids.

The opsonisation or removal of nanoparticulate drug carriers from the body by the RES is a major obstacle that hinders the efficiency of nanoparticulate drug delivery systems. Therefore, camouflaging or masking nanoparticles have been developed to increase their blood circulation half-life. Based on our results, the incorporation of SN-38 into NPs resulted in the production of NPs characterised by efficient drug loading and release profiles and suitable body distribution. Although NPs showed superior blood profiles and body distribution, it was observed that surface modification of NPs with folate did not affect the biodistribution of SN-38 in healthy rats.

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