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Original research paper

Preparation of pegylated nano-liposomal formulation containing SN-38: *In vitro* characterization and *in vivo* biodistribution in mice

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7-Ethyl-10-hydroxy-camptothecin (SN-38), a metabolite of irinotecan × HCl, is poorly soluble in aqueous solutions and practically insoluble in most physiologically compatible and pharmaceutically acceptable solvents. Formulation of SN-38 in concentrated pharmaceutical delivery systems for parenteral administration is thus very difficult. Due to their biocompatibility and low toxicity, liposomes were considered for the delivery of SN-38. In this study, pegylated liposomes with distearoylphosphatidylcholine, distearoylphosphatidylethanolamine containing SN-38 were prepared and their characteristics, such as particle size, encapsulation efficiency, *in vitro* drug release and biodistribution, were investigated. The particle size of liposomes was in the range of 150–200 nm. The encapsulation efficiency and *in vitro* release rate of pegylated liposomes was higher than those of non-pegylated liposomes. As expected, the distribution of pegylated liposomes in body organs such as liver, kidney, spleen and lung was considerably lower than that of non-pegylated liposomes. Also, their blood concentration was at least 50 % higher than that of non-pegylated liposomes.

Keywords: nanoparticles, SN-38, pegylated liposomes, PEG, biodistribution, drug delivery

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7-Ethyl-10-hydroxy-camptothecin (SN-38) is an active metabolite of irinotecan, a derivative of camptothecin. Current research suggests that its cytotoxicity is due to double-strand DNA damage produced during DNA synthesis when replication enzymes interact with the ternary complex formed by topoisomerase I, DNA and SN-38. Irinotecan is approved for use in the treatment of recurrent metastatic colorectal cancer. Metabolic conversion of irinotecan to active SN-38 occurs in the liver *via* carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and a dipiperidine

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side-chain (1). Variability and unpredictability in the irinotecan to SN-38 metabolic conversion rates pose significant life threatening toxicity risks and complicate clinical management of patients. Previous cytotoxicity studies have shown that SN-38 is up to 1000--fold more potent than irinotecan against several tumor cell lines (2). Nonetheless, SN-38 is poorly soluble in aqueous solutions and is practically insoluble in most physiologically compatible and pharmaceutically acceptable solvents, including ethanol, polysorbate 80 and cremophor. Formulation of SN-38 in concentrated pharmaceutical delivery systems for parenteral administration is thus very difficult. In addition, SN-38 has low affinity to lipid membranes and tends to precipitate into aqueous phase, resulting in very low drug-to-liposome entrapment (3). This low lipid affinity behavior of SN-38 has made the development of a liposome-based SN-38 formulation more challenging.

Liposomes have characteristics such as biocompatibility, biodegradability and low toxicity, and can control biodistribution by changing the size, lipid composition, and physical characteristics (4–6). Furthermore, liposomes can entrap both hydrophobic and hydrophilic substrates and are able to continuously release the entrapped substrate (7), thus being useful drug carriers (8–10). On the other hand, the blood circulation and tumor accumulation of liposomes remarkably decrease as a result of entrapment in the reticuloendothelial system (RES) rich organs such as the liver and spleen (11). Polyethyleneglycol (PEG) modification on the liposomal surface is known to be effective in preventing their uptake by RES, and tumor accumulation by passive targeting associated with increased blood circulation by suppressed association of serum proteins with liposomem (12–15). Long-circulating, pegylated liposomes provide an attractive platform to improve the therapeutic index of a variety of anticancer drugs (16). Sadzuka *et al*. (17) compared some methods of preparation of the pegylated liposomal formulation of SN-38, but they did not report any *in vitro* or *in vivo* drug release result.

In this study, we developed liposomal formulations of SN-38, pegylated and conventional, and evaluated both *in vitro* behavior and *in vivo* distribution of the formulations.

EXPERIMENTAL

Materials

SN-38 was purchased from Abatra Co., China. Distearoylphosphatidylcholine (DSPC) and distearoylphosphatidylethanolamine (DSPE) were obtained from Lipoid GmbH (Germany). Methoxypolyethylene glycol succinate *N*-hydroxy succinimide ester (m-PEG) (approx. 50 %) and cholesterol (CHOL) were purchased from Sigma-Aldrich (USA). Polycarbonate filters (diameter 19 mm, pore diameter 100 nm) were obtained from Avestin Inc. (Canada). Sodium hydroxide, sodium chloride and sucrose were purchased from Merck, Germany. Deionized water was used throughout the experiments. Acetonitrile and methanol used as mobile phase in HPLC analysis were obtained from Merck. *In vitro* release measurement was carried out at pH 7.4 at 37 °C in phosphate buffered saline (PBS). All the other chemicals used were of analytical grade.

Synthesis of mPEG-DSPE conjugate

mPEG-DSPE conjugate was synthesized according to the method described by Klibanov *et al*. (18), with some modifications. Briefly, an aliquot of methoxypolyethylene glycol succinate *N*-hydroxy succinimide ester (MGS) in chloroform was added to a solution of DSPE in chloroform, followed by the addition of triethylamine (TEA) (MGS/DSPE/TEA, 3:1:3.5, *m*/*m*). The reaction mixture was incubated overnight at room temperature and $CHCl_3$ was evaporated to yield mPEG-DSPE. The formation of mPEG-DSPE was monitored and confirmed by infrared spectroscopy.

Liposome preparation

Different ratios of SN-38, DSPC, and CHOL were mixed and dissolved in 10 mL of mixture of methanol and chloroform (1:1, *V*/*V*) and dried in a rotary evaporator (Büchi Rotavapor R-124, Germany) at 65 °C for one hour to form a lipid film. Molar ratios of the mentioned materials are summarized in Table I. The molar ratio of SN-38 to lipid content of liposomes was between 1 and 4 %. The lipid film was then hydrated with 30 mL of 10 % (*m*/*V*) sucrose solution and stirred for one hour. The liposome suspension was extruded through polycarbonate filters of 100 nm pore size for 10 cycles (Liposofast® Extruder, Avestin Inc., Canada). The suspension was then freeze-dried for 48 h at –40 °C (Lyotrap Plus, LTE Scientific Limited, UK) to obtain a fine powder of liposomes.

For the preparation of pegylated liposomes, appropriate amount of DSPC/DSPE--PEG/CHOL (70/5/25) or (65/10/25) were dissolved in a mixture of methanol and chloroform (1:1, *V*/*V*) and dried in a rotary evaporator. The liposomes were prepared as described above.

Encapsulation efficiency

The concentration of SN-38 in liposomes was determined by HPLC (19). A reversed phase C_{18} column (25 × 0.46 cm, pore size 5 μm) (Teknokroma, Spain) was used. The

Table I. Physicochemical characteristics of pegylated and non-pegylated SN-38-loaded liposomes

Sample	DSPC/DSPE-mPEG /CHO/SN-38 (%)	Mean diameter (nm)[a]	Drug loading (%)[a]	Encapsulation efficiency (%)[a]	Zeta potential (mV) [a]	PI
S1	70/0/30/0	170 ± 9.5	–	–	–9.30 ± 0.21	0.239
S2	70/0/30/1	182 ± 8.9	2.02 ± 0.12	66.6 ± 2.30	–8.94 ± 0.30	0.532
S3	70/0/30/2	185 ± 8.4	4.60 ± 0.23	79.0 ± 1.72	–10.32 ± 0.25	0.119
S4	70/0/30/4	192 ± 8.7	9.51 ± 1.21	85.0 ± 4.31	–10.98 ± 0.26	0.303
S5	100/0/0/2	179 ± 9.4	1.40 ± 0.09	23.6 ± 1.98	–9.12 ± 0.12	0.263
S6	85/15/0/2	180 ± 10.3	3.72 ± 0.24	63.3 ± 3.57	–10.11 ± 0.28	0.472
S7	70/5/25/2	136 ± 4.9	2.56 ± 0.32	26.7 ± 1.34	–12.38 ± 0.45	0.123
S8	70/10/20/2	147 ± 6.4	1.53 ± 0.21	24.3 ± 0.98	–16.14 ± 0.51	0.156

[a] Mean ± SD, $n \geq 3$.

mobile phase consisted of a mixture of acetonitrile and phosphate buffer (pH 3.1, 25 mmol L^{-1}) (50:50, *V/V*) delivered at a flow rate of 1.00 mL min^{-1} with a pump (WellChrom K-1001, Knauer, Germany). Acetonitrile (1 mL) was added to 1-mL aliquots of the extruded suspension in tubes and vortexed, followed by sonication for 2 minutes. The tubes were then centrifuged at 21,000x*g* for 10 minutes. Samples (20 µL) of the supernatant were analyzed by the HPLC system. Column elute was monitored at 265 nm with a UV detector (WellChrom K-2600, Knauer). The calibration curve of SN-38 was linear over the range of standard concentrations of 0.1–20 µg mL^{-1} with $R^2 > 0.999$. Encapsulation efficiency (EE) was obtained as the ratio between the amount of drug incorporated in liposomes and that used in the liposome preparation.

Particle size and size distribution

Particle size and size distribution of liposomes in the extruded suspension were determined by laser light scattering (Zetasizer ZS, Malvern, UK). Samples were examined to determine the mean diameter, size distribution, poly-dispersity and zeta potential.

Scanning electron microscopy (SEM). – An XL 30 scanning microscope (Philips, The Netherlands) was employed to determine the shape and surface morphology of the produced liposomes. A small amount of lyophilized liposomes was stuck on a double-sided tape attached to a metallic sample stand, and coated under vacuum with a thin layer of gold before SEM.

Differential scanning calorimetry (DSC). – DSC thermograms were obtained from a Mettler Toledo DSC system (DSC-823, Mettler Toledo, Switzerland). A Mettler Stare software system, version 9.x, was used for data acquisition and indium was used to calibrate the instrument. Samples (1–2 mg) were put into DSC aluminum pans and sealed. The same volume of pure water was used as the reference. Samples were scanned from 30 to 270 °C at a rate of 10 °C min^{-1} under a 8 kPa nitrogen atmosphere. Each experiment was repeated three times.

In vitro *drug release*

Samples (10 mg) of the lyophilized liposome powder were suspended in a tube containing 10 mL of PBS (pH 7.4). The tube was then placed in a shaker bath (WB14, Memmert, Germany) at 37 °C and shaken horizontally at 90 cycles min^{-1}. At predetermined time intervals, the tubes were centrifuged (Sigma 3K30, Sigma, Germany) at 21,000x*g* for 10 min and 9-mL aliquots were taken from the supernatant and then substituted with the same amount of fresh PBS (20). Supernatant was diluted with phosphate buffer (pH 3.1) at a volume ratio of 1:1 and centrifuged at 21,000x*g* for 10 min. Samples were then taken from the supernatant and analyzed by HPLC as described above. Drug release data was normalized by converting the drug concentration in solution to a percentage of cumulative drug release. The experiments were carried out in triplicate.

Stability

Stability of the lyophilized SN-38 liposome was evaluated after storage at –20, 4 and 25 °C after 3 months of storage. The particle size distribution and EE of the samples were determined as a function of storage time.

Body distribution of SN-38 liposomes in mice

A SN-38 highly concentrated stock solution was prepared in NaOH and diluted with 0.9 % NaCl (*m*/*m*) to remove NaOH. Liposomal formulation suspensions were prepared in 0.9 % NaCl (*m*/*m*). All formulations were sterile filtered. *In vivo* biodistribution studies were performed using 13-week old female BALB/c mice (provided by the Animal Care Center, Faculty of Pharmacy, Tehran University of Medical Sciences) after a 7-day acclimatization period. Animal experiment were approved by the Ethical Committee, Tehran University of Medical Sciences (Tehran, Iran). All mice were randomly assigned into three groups (5–7 mice per group). The mice were fasted overnight but had free access to water. Each group received 200 µL of the drug solution or liposomes suspensions containing 2 mg kg^{-1} of SN-38 as either free drug, non-pegylated or pegylated liposomes *via* tail vein injection. Two hours after dosing, mice were sacrificed and major organs were collected for analysis.

Organ samples, consisting of lungs, liver, heart, kidneys, intestine and spleen, were removed, washed with NaCl (0.9 %, *m*/*m*) and accurately weighed. Organ samples were homogenized and centrifuged at 21,000x*g* for 10 min. Methanol was added to the supernatant (1:1) to precipitate the unwanted proteins and centrifuged (21,000x*g*, 10 min). The aliquots were assayed for SN-38 by HPLC to estimate the amount of SN-38 in each organ. For calculations, standard curves of SN-38 were prepared by addition of SN-38 solutions in methanol to tissues following the same treatment steps (21, 22). Blood samples were obtained by cardiac puncture in pre-weighed heparinized tubes; plasma was separated by centrifugation of the blood samples at 7,000x*g* (10 min). SN-38 was extracted from plasma by adding acetonitrile (at a ratio of 1:2, *V*/*V*), and standard curves were prepared by addition of SN-38 in plasma following the same process.

Statistical analysis

One-way analysis of variance (ANOVA) was performed on the data to assess the impact of the formulation variables on the results. All calculations were performed using a statistical software program (SPSS® 11.5, Microsoft).

RESULTS AND DISCUSSION

Physico-chemical properties of the liposomes

Formation of the PEG-DSPE conjugate was confirmed by IR spectra. The IR-spectrum of PEG-DSPE conjugate showed additionally a small peak at 1679 cm^{-1} (corresponding to a NH–C=O group) missing in the spectrum of DSPE. This peak illustrated that a

coupling reaction with probable formation of amide bonds occurred when the conjugate was made.

The SEM micrograph of liposomes is shown in Fig. 1. The liposomes prepared in this study were spherical. Drug entrapment efficiency and particle size of several SN-38 liposome formulations were investigated by using different drug-to-lipid ratios, number of extrusion cycles, type of lipids and lipid combinations on. Upon further optimization, a lead formulation of liposome-based SN-38 was developed. Basic characteristics of the formulations are summarized in Table I. In addition to the sample formulations shown in Table I, other experiments were carried out to optimize the lipid type and ratio. The simplest lipid phase was the combination of the DSPC/cholesterol. In the lead formulation, the drug-to-lipid ratio and DSPC to cholesterol molar ratio were 2:100 and 70:30, respectively. As it is seen in Table I, for non-pegylated liposomes, the particle size of liposomes was the lowest when no drug was loaded (S1). Increasing the drug-to-lipid ratio, the size of liposomes was increased. However, this increase was not significant. By lowering the amount of cholesterol, EE was also decreased (S5), and removing the cholesterol totally (S6) resulted in a significant decrease in EE ($p < 0.05$), with no significant effect on the liposome size. The size of pegylated liposomes was smaller than that of non-pegylated liposomes. The size of pegylated liposomes decreased when the amount of cholesterol was increased.

Zeta potential is another important index for the stability of liposomal formulations. High absolute value of zeta potential indicates a high electric charge on the surface of the drug-loaded liposomes, which can cause strong repellent forces among particles to prevent aggregation of the liposomes in buffer solution (23). Zeta potential of liposomes in this study was negative due to the presence of terminal carboxylic groups in the lipids (24). The zeta potential value for pegylated liposomes was more negative. PEG is typically conjugated to DSPE *via* a carbamate linkage that results in a net negative charge on the phosphate moiety at physiological pH. It is anticipated that the presence of this negative charge could have deleterious effects on liposome pharmacokinetic characteristics and increase the stability and blood circulation of liposomes (23).

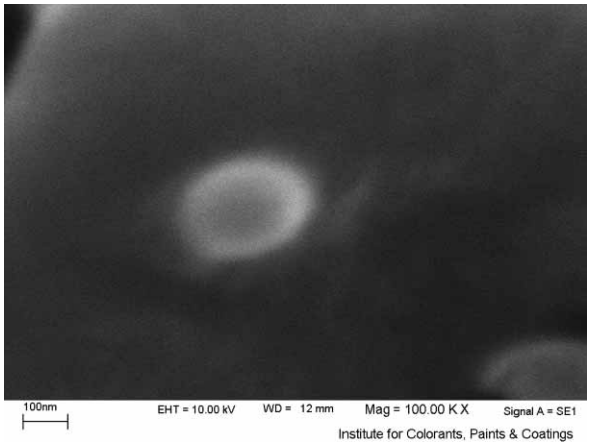

Fig. 1. SEM photograph of DSPC/CHO (70/30) liposomes containing 1 % SN-38 (S2).

DSC studies were performed to investigate the physical state of the drug in the liposomes, because this aspect could influence the *in vitro* and *in vivo* release of the drug from the system. The drug content of liposomes may be amorphous or crystalline. Moreover, a drug may be present either as dissolved or as a solid dispersion in the liposomes. The DSC thermograms of pure SN-38, pure mPEG, physical mixture of DSPC/CHOL, SN-38-loaded pegylated and non-pegylated liposomes are shown in Fig. 2. As shown in Fig. 2. lipids showed a T_g (glass to rubber transition temperature) with no T_m (melting point temperature), indicating they were amorphous. Pure SN-38 showed an endothermic melting peak (T_m) at 152 °C, which was exactly repeated in the thermogram of the physical mixture of the drug and lipids. SN-38 melting peak was depleted in the calorimetric curve of loaded liposomes, evidencing the presence of amorphous drug in the liposome samples. It might be hypothesized that the crystallization of SN-38 is inhibited during liposomal formation. Therefore, it could be concluded that SN-38 in the liposomes was in the amorphous phase of a molecular dispersion or a solid solution state in the lipid matrix after the production (25). mPEG showed a sharp T_g at 54 °C. In pegylated liposomes, this peak was maintained but it was weaker, confirming that PEG was only attached to the surface of the liposomes.

In vitro *release studies*

In vitro drug release from SN-38-loaded, pegylated and non-pegylated liposomes within 30 days is summarized in the cumulative percentage release shown in Fig. 3. In fact, both formulations produced an initial burst release in which SN-38 release was more than 25 % and 5 % for pegylated and non-pegylated liposomes, respectively, within the initial sampling time (24 h). The burst release in pegylated liposomes is related to

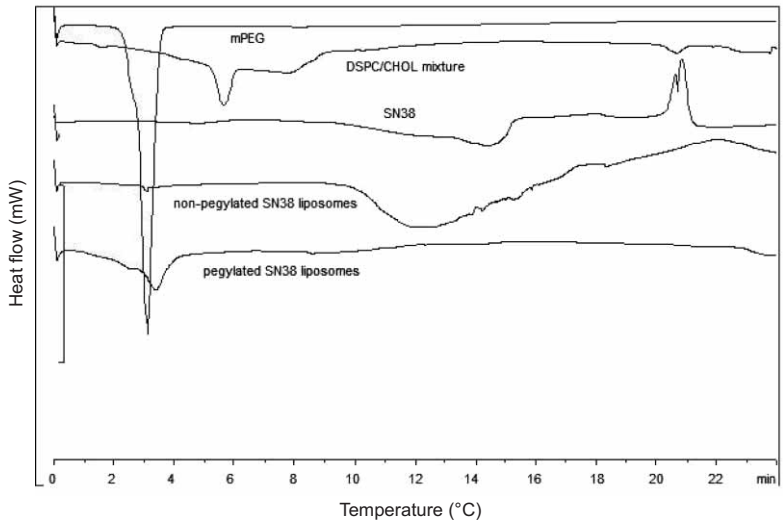

Fig. 2. DSC thermograms of pure SN-38, pure mPEG, physical mixture of DSPC/CHOL, SN-38-loaded non-pegylated and pegylated liposomes.

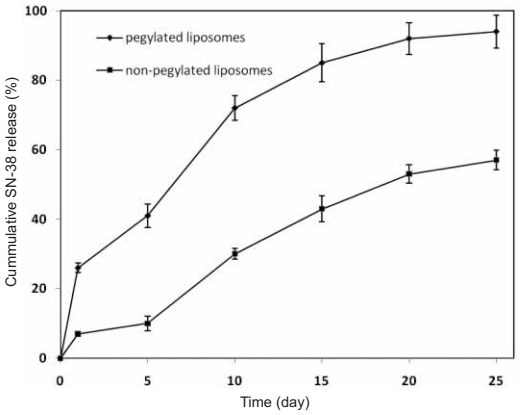

Fig. 3. *In vitro* drug release from SN-38--loaded non-pegylated and pegylated liposomes in phosphate buffer (pH 7.4). The results are expressed as the mean ± SD (n = 3).

SN-38 adsorbed on the liposome surface (26) and/or to the release of the drug encapsulated near the liposome surface. After this burst release, a constant SN-38 release was observed. After 25 days 100 and 60 % of the loaded drug was released in pegylated and non-pegylated liposomes, respectively, showing a typical sustained and prolonged drug release depending on drug diffusion and matrix erosion mechanisms (27). Faster release in pegylated liposomes may be due to the fast hydration process of PEG molecules on the surface of the particles.

Stability

The impact of storaging liposomes at different temperatures on their particle size and EE is seen in Fig. 4. SN-38 liposomes were physically and chemically stable at three different temperatures for up to 3 months. No significant changes in mean vesicle size and drug content of liposomes were observed during the course of stability study for

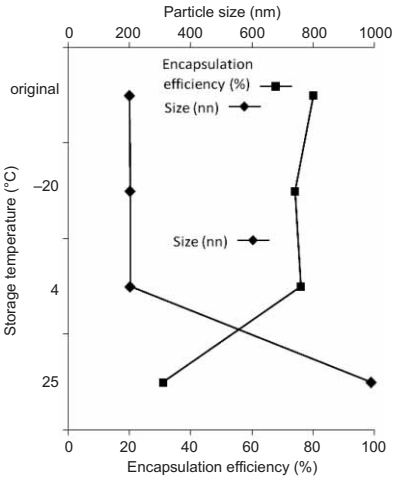

Fig. 4. The impact of 3 months of storage at –20, 4 and 25 °C on the mean particle size and encapsulation efficiency of SN-38 loaded non-pegylated and pegylated liposomes. The results are expressed as the mean ± SD (n = 3).

formulations stored at –20 or 4 °C, but there was a significant leakage of drugs from liposomes and an increase in their particle size when stored at room temperature, 25 °C ($p < 0.05$). Initially, the mean vesicle diameter was 185 nm and EE was 79 %, the mean vesicle diameter remained relatively constant at both storage temperatures of –20 or 4 °C, but the particle size increased dramatically, up to 5 folds, and EE decreased after 3 months of storage at room temperature. This extraordinary increase in the particle size of liposomes may be due to the aggregation or swelling of liposomes. Further experiments are required to explain this phenomenon.

Body distribution of SN-38-loaded liposomes in mice

The goal of the biodistribution studies was to investigate the differences in organ distribution of SN-38 entrapped in pegylated and non-pegylated liposomes compared to the SN-38 free drug. The results of the biodistribution studies are shown in Fig. 5. Only 66 % of the original dose of the free drug was found 2 h post injection in the blood, spleen, lung, liver, kidney, intestine and heart together, while this number for pegylated and non-pegylated liposomes was 100 and 84 % respectively. There was no significant difference in drug distribution of different formulations for the lung, heart and intestine. Higher concentration of SN-38 in the liver, spleen and kidney for non-pegylated liposomes compared to pegylated ones may be due to their bigger size causing their preference filtrations by these organs (22).

The SN-38 plasma level, 2 hours after *i.v.* administration of the SN-38 free drug, of non-pegylated and pegylated liposomes was 13.7, 29.0 and 41.3 % of the initial dose, respectively. As expected, the higher blood concentration of SN-38 for pegylated liposomes is due to longer blood circulation of these liposomes resulting from the presence of PEG moieties on the surface of liposomes.

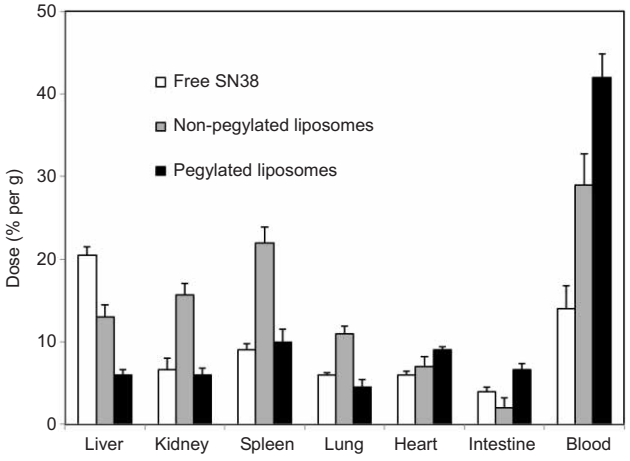

Fig. 5. Tissue distribution of SN-38 after intravenous administration of solutions of free SN-38, SN-38 loaded non-pegylated and pegylated liposomes at an equivalent dose of 2.0 mg kg^{-1} SN-38 in mice *via* tail vein injection. The results are expressed as the mean ± SD (n = 3–5).

CONCLUSIONS

Stable nano-liposome formulations of SN-38 were developed for intravenous administration. Pegylated liposomes ensured a good plasma level *in vivo* in comparison with SN-38 conventional liposomes.

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S A Ž E T A K

Priprava pegiliranih nano-liposomskih formulacija sa SN-38: *In vitro* karakterizacija i *in vivo* biodistribucija u miševa

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7-Etil-10-hidroksi-kamptotecin (SN-38), metabolit irinotekan hidroklorida, vrlo je slabo topljiv u vodenim otopinama i praktički netopljiv u većini fiziološki kompatibilnih i farmaceutski prihvatljivih otapala. Poradi toga je veoma teško formuliranje supstancije SN-38 u koncentrirane sustave za parenteralnu primjenu. Liposomi se zbog svoje biokompatibilnosti i niske toksičnosti čine pogodnim za isporuku SN-38. U ovom radu opisana je priprava pegiliranih liposoma sa SN-38 pomoću distearoilfosfatidilkolina i distearoilfosfatidiletanolamina. Pripravljenim liposomima određena je veličina čestica, sposobnost inkapsuliranja, *in vitro* oslobađanje i biodistribucija. Veličina čestica liposoma bila je u rasponu 150–200 nm. Sposobnost kapsuliranja i *in vitro* oslobađanje pegiliranih liposoma bila je veća nego nepegiliranih liposoma. Kao što se očekivalo, distribucija pegiliranih liposoma u jetri, bubregu, slezeni i plućima bila je značajno niža nego nepegiliranih liposoma. Njihova koncentracija u krvi bila je najmanje 50 % viša od nepegiliranih liposoma.

Ključne riječi: nanočestice, SN-38, pegilirani liposomi, PEG, biodistribucija, isporuka lijeka

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