ORIGINAL RESEARCH

Liposomal codelivery of an SN38 prodrug and a survivin siRNA for tumor therapy

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Purpose: A liposome-based siRNA–drug combination was evaluated as a potential therapeutic strategy to improve the curative effect.

Methods: A topoisomerase inhibitor SN38 prodrug was combined with a survivin siRNA through codelivery using transferrin (Tf)-L-SN38/P/siRNA. In this combination, SN38 was conjugated to the cell penetrating peptide TAT through a polyethylene glycol (PEG) linker to synthesize TAT-PEG-SN38. The amphiphilic TAT-PEG-SN38 was used as an ingredient of liposomes to improve the cellular uptake. Protamine was added to form an electrostatic complex with siRNA in the core of the liposomes. Tf was introduced to enable tumor cell targeting of liposomes (Tf-L-SN38/P/siRNA).

Results: Tf-L-SN38/P/siRNA exhibited a particle size of 148 nm and a ζ-potential of +7.8 mV. The cellular uptake and antitumor activity were dependent on Tf receptor targeting, TAT-PEG-SN38, and siRNA codelivery. Tf-L-SN38/P/siRNA was shown to be considerably more effective than liposomes carrying individual components. This combination induced potent tumor inhibition (76.8%) in HeLa cell xenograft tumor-bearing nude mice.

Conclusion: These data indicated that Tf-L-SN38/P/siRNA was an effective system for codelivery of SN38 and a survivin siRNA and that its therapeutic potential deserved further evaluation.

Keywords: SN38, survivin, siRNA, transferrin, prodrug

Introduction

Chemotherapy is routinely used in cancer therapy. SN38 is a topoisomerase I inhibitor with broad-spectrum antitumor activity.[1] SN38 is the active metabolite of irinotecan (CPT-11), which is a first-line agent. However, SN38 has poor water solubility, is sensitive to drug resistance mechanisms, and is associated with adverse side effects that limit its clinical application.[2] SN38 cannot be efficiently loaded into liposomes.[3] Therefore, we developed an amphipathic cationic SN38 prodrug to enhance the SN38 loading capacity and cellular penetration.

Survivin is upregulated in almost all human tumors and is largely absent in normal tissues.[4] It inhibits apoptosis, promotes angiogenesis, and enhances proliferation of tumor cells.[5] siRNA can be used to knock out oncogenes to induce apoptosis.[6] Although the survivin siRNA by itself does not exhibit antitumor activity, it has been shown to improve the sensitivity of tumor cells to chemotherapeutical agents.[7] Protamine-containing liposomes have been employed as effective siRNA carriers through the formation of virus-like structures, with the siRNA being in the core of liposomes.[8] SN38 and the survivin siRNA can potentially act synergistically. TAT-SN38 codelivers with siRNA as a cationic material in the liposomes to enhance cellular uptake and antitumor activity.

Both SN38 and the survivin siRNA can benefit from a delivery system that uses tumor cell-targeted liposomes. Transferrin (Tf) receptor is overexpressed on tumor cells.

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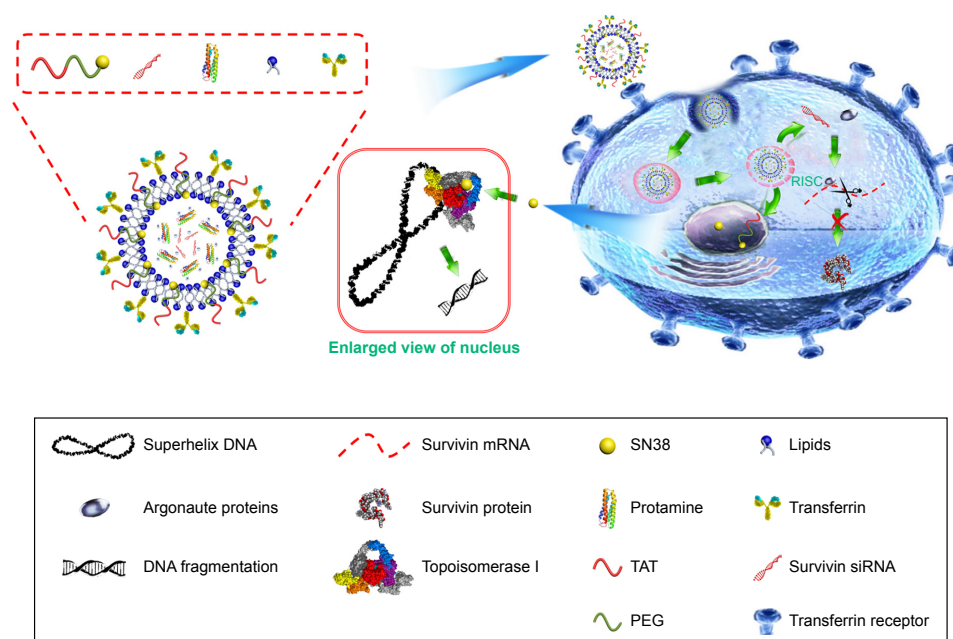Email tenglesheng@jlu.edu.cn; xiejing@jlu.edu.cn

Figure 1 The liposomes structure and effect mechanisms of Tf-L-SN38/P/siRNA.

Notes: Protamine couples siRNA to form the liposomes core, lipids and TAT-PEG-SN38 are used as skeleton of liposomes, Tf could provide targeting capability. They together constitute the liposomes delivery system of Tf-L-SN38/P/siRNA to co-deliver SN38 prodrug and siRNA. Tf-L-SN38/P/siRNA mainly enters cells by Tf mediated endocytosis, TAT-PEG-SN38 and survivin siRNA then induce cells apoptosis by inhibiting topoisomerase I and RNA interference.

Abbreviations: RISC, RNA-induced silencing complex; Tf, transferrin.

Tf-modified liposomes can be used to selectively target the delivery of SN38 and the survivin siRNA to tumor cells.[7]

In this study, Tf-liposomes coloaded with TAT-PEG-SN38 and the survivin siRNA were prepared and evaluated both in vitro and in vivo for antitumor activity. The structure and brief anticancer mechanisms of codelivery liposomes are shown in Figure 1.

Materials and methods
Materials
SN38 and CPT-11 were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, People's Republic of China). Survivin siRNA (5′-mGCAGGUUCCUmUAUCUGUCAdTd T-3′, 5′-UGAmCAGAmUAAGGAACCUGmCdTdT-3′)[9] and Cy3-labeled siRNA were synthesized by Ribo Biochemistry (Guangzhou, People's Republic of China). Soya lecithin for injection (SPC) and cholesterol (chol) were obtained from Shanghai AVT Pharmaceutical Technology Co., Ltd. (Shanghai, People's Republic of China). Holo-Tf was purchased from Sigma-Aldrich (St Louis, MO, USA). OPSS-PEG2000-OH was obtained from Yare Bio-Technology Co., Ltd. (Shanghai, People's Republic of China). The HeLa cell line was purchased from American Type Culture Collection (Manassas, VA, USA).

Animals
Female nude mice (4 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd

(license no SCXK[Jing]2016-0011). All the animal experiment protocols were reviewed and approved by the Institution Animal Ethics Committee (Jilin University) (no 201712022). The Guidelines on Humane Treatment to Lab Animals (published in 2009) were followed for the welfare of the animals.

Synthesis of SN38 prodrug
A peptide based on HIV TAT plus a cysteine,[10] GRKKRRQRRRQC, and a FITC-labeled TAT were synthesized by standard Fmoc-chemistry. The synthetic strategy of an SN38 prodrug (TAT-PEG-SN38) is shown in Figure 2.[11–13] Briefly, SN38, triphosgene, and pyridine were reacted in dichloromethane for 30 minutes at a molar ratio of 3:1:6. Next, 1× OPSS-PEG2000-OH was added to SN38, and the reaction proceeded for another 6 hours. The reaction mixture was kept anhydrous and under nitrogen. The reaction product was dried and combined with the above TAT peptide in 50/50 water/ethanol overnight. The product was purified by dialysis, freeze-dried, and characterized by 1H–nuclear magnetic resonance (NMR) spectroscopy at 300 MHz.

Liposome preparation and characterization
Conventional Tf-liposomes (Tf-L-SN38) without protamine were prepared by an ethanol injection method. DSPE-PEG
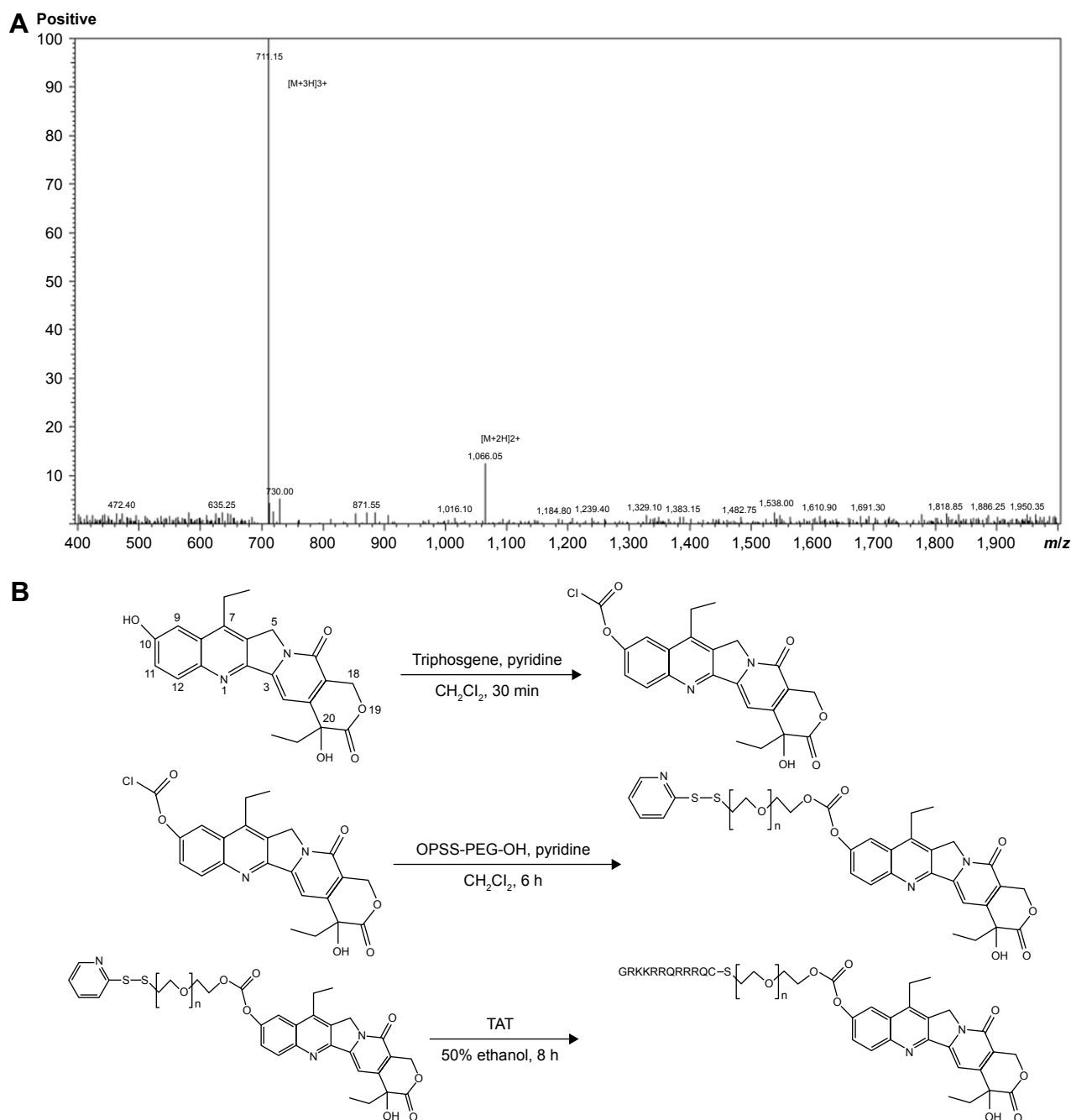
Figure 2 Synthesis scheme of TAT-PEG-SN38.
Notes: (**A**) Mass spectrum of FITC-labeled TAT (ESI source, probe bias: −3.5 kV, DL temp: 250°C, block temp: 200°C). (**B**) Synthetic strategy of TAT-PEG-SN38, sequence of TAT was GRKKRRQRRRQ and the adding cysteine (**C**) was used as a linker.
Abbreviations: DL, desolvation; ESI, electron spray ionization.

conjugated Tf (DSPE-PEG-Tf) was synthesized by a previously described method.[14] Briefly, holo-Tf reacted with Traut's reagent to yield holo-Tf-SH and later combined with Mal-PEG$_{2000}$-DSPE (at a Tf/lipid molar ratio of 1:10) to obtain DSPE-PEG-Tf. SPC and chol were dissolved in ethanol at a mass ratio of 4:1. TAT-PEG-SN38 was later added. The ethanol solution was subsequently injected into diethylpyrocarbonate-treated water with vortex mixing at a volume ratio of 1:5 to form SN38 liposomes. DSPE-PEG-Tf at a

molar ratio of DSPE-PEG-Tf/lipid of 1:200 was added to the liposomes at 37°C to prepare Tf-L-SN38. The conventional liposomes (L-SN38) without Tf were also prepared by same method. TAT-PEG-SN38 is cationic and has a high affinity to siRNA. The survivin siRNA was incubated with Tf-L-SN38 (Tf-L-SN38/siRNA) at a range of charge ratios (from 1:1 to 20:1) to evaluate the siRNA loading ability of TAT-PEG-SN38 by agarose gel electrophoresis. Free siRNA was used as the control. Sample loading of siRNA was 0.05 nmol/well.

The stripe was stained by SYBR Gold nucleic acid gel stain and visualized by the gel imaging system.

Protamine-containing Tf-liposomes (Tf-L-SN38/P/siRNA) were established using a similar method.[9] SPC and chol were dissolved in ethanol at a mass ratio of 4:1. Different amounts of TAT-PEG-SN38 were then added. Protamine was dissolved in a citrate buffer (20 mM, pH 4) and added to the lipids at a mass ratio (lipid/protamine) of 10:1 and volume ratio (ethanol/water) of 2:1. The survivin siRNA was dissolved in a citrate buffer (20 mM, pH 4) and the lipid/protamine solution was injected into the siRNA solution with vortex mixing at a mass ratio (protamine/siRNA) of 1/0.3. The complexes were dialyzed in pH 7.4 HEPES buffered saline. Next, the complexes were incubated with DSPE-PEG-Tf to form targeted liposomes (Tf-L-SN38/P/siRNA). The protamine-containing liposomes (L-SN38/P/siRNA) and the liposomes for the siRNA loading without TAT-PEG-SN38 (L-P/siRNA) were prepared as described above. All compositions of liposome preparations are shown in Table 1.

The particle size and ζ-potential of Tf-L-SN38/P/siRNA were determined using Zetasizer Nano ZS 90 from Malvern Instruments, Ltd. (Malvern, UK). The surface morphology determination of Tf-L-SN38/siRNA was performed by scanning electron microscopy (SEM) (JEOL, Ltd. Tokyo, Japan) using the settings: SEI model and 3.0 kV.

In vitro cytotoxicity and apoptosis analysis

The cytotoxicity of Tf-L-SN38/P/siRNA to HeLa cells was evaluated by MTT assay.[15,16] Cells were seeded in 96-well plates at a density of 4×10^3 cells/well and cultured for 24 hours.[17] SN38, L-P/siRNA, L-SN38/P/siRNA, Tf-L-SN38/P/siRNA, or Tf-L-SN38 was added into the basic medium and incubated with the cells for 48 hours. Then, 10 µL of 5 mg/mL MTT was added and the cells were incubated for another 4 hours. The medium was then replaced by 150 µL DMSO to dissolve formazan from the MTT conversion. Absorbance was then measured on a microplate reader at 490 nm.

Table 1 Composition of liposome preparations

Formulation	Components
TAT-PEG-SN38	TAT/PEG/SN38
Tf-L-SN38/siRNA	SPC/chol/TAT-PEG-SN38/siRNA/Tf
L-P/siRNA	SPC/chol/protamine/siRNA
L-SN38	SPC/chol/TAT-PEG-SN38
Tf-L-SN38	SPC/chol/TAT-PEG-SN38/Tf
L-SN38/P/siRNA	SPC/chol/TAT-PEG-SN38/protamine/siRNA
Tf-L-SN38/P/siRNA	SPC/chol/TAT-PEG-SN38/protamine/siRNA/Tf

Abbreviations: chol, cholesterol; SPC, soya lecithin.

HeLa cells were seeded in 6-well plates at a density of 4×10^3 cells/well and cultured for 24 hours. Then, the cells were treated with CPT-11, L-P/siRNA, L-SN38/P/siRNA, Tf-L-SN38/P/siRNA, or Tf-L-SN38/siRNA for 48 hours (2.5 µM of CPT-11, TAT-PEG-SN38 and 100 nM of siRNA). Finally, the apoptosis of cells was analyzed using a Muse™ Annexin V & Dead Cell Kit (Billerica, MA, USA) according to the manufacturer's instructions.

Internalization of Tf-L-SN38/P/siRNA

Tf-L-SN38/P/siRNA delivery of siRNA was evaluated using a confocal microscope from Carl Zeiss (Jena, Germany). Tf-L-SN38/P/siRNA was double labeled with FITC and Cy3 to visualize cell internalization. The HeLa cells were seeded in a glass-bottomed culture dish at a density of 1×10^5 cells/well and cultured for 24 hours. The medium was replaced by a fresh basic medium. Fluorescence-labeled L-P/siRNA, L-SN38, Tf-L-SN38, L-SN38/P/siRNA, or Tf-L-SN38/P/siRNA was added into culture dish and incubated for 4 hours. Then, cells were washed and fixed in 350 µL of 4% paraformaldehyde for 10 minutes. Nuclei were stained by DAPI dye (2 µg/mL) after washing. Finally, stained cells were observed using microscopy.

Uptake inhibition of Tf-L-SN38/P/siRNA by inhibitors

The cellular uptake of Tf-L-SN38/P/siRNA was analyzed by a flow cytometer. The HeLa cells were seeded into 6-well plates (2×10^5 cells/well) and cultured for 24 hours. The medium was replaced by a fresh basic medium. The internalization pathways were blocked by 5 µM of cytochalasin D, 50 µM of nystatin, 0.4 M of sucrose, or 100 µM free holo-Tf for 1 hour.[18] Next, cells were incubated with fluorescence-labeled L-P/siRNA, L-SN38/P/siRNA, or Tf-L-SN38/P/siRNA for another 4 hours. Finally, cells were harvested using 150 µL trypsin and fixed with 350 µL of 4% formaldehyde solution. The mean fluorescence intensity of cellular uptake was measured on an EPICS XL flow cytometer (Beckman Coulter Corp., Brea, CA, USA).

Gene silencing efficiency of Tf-L-SN38/P/siRNA

The HeLa cells (1×10^5 cells/well) were seeded into 6-well plates and cultured for 24 hours. Next, cells were treated with L-P/siRNA, L-SN38/P/siRNA, Tf-L-SN38/P/siRNA, or Tf-L-SN38 for 48 hours. The cells were lysed to collect proteins on ice. The survivin protein expression levels of HeLa cells after treatment were measured by Western blot analysis after the total protein was adjusted. Fifty microgram

total protein samples were loaded per lane. GAPDH was used as an internal control to normalize the intensity of the survivin protein.

Antitumor activity evaluation and in vivo distribution

The animal experiments were supervised by the Institution of Animal Ethics Committee (Jilin University) to ensure animal welfare. Female BALB/c nude mice (17 g, 4 weeks) were acclimated for 4 days. Nude mice bearing xenografts of HeLa cells were used to study the in vivo distribution and antitumor activity of Tf-L-SN38/P/siRNA. The xenografts were established by injecting tumor cells on the right backside of nude mice. When the tumor volume reached approximately 120 mm^3, nude mice were randomly separated into 6 groups. The mice received a single dose of saline, CPT-11, L-P/siRNA, L-SN38/P/siRNA, Tf-L-SN38/P/siRNA, or Tf-L-SN38 (5 mg/kg SN38-equivalent dose, 2.5 mg/kg of siRNA) through intravenous injection every 3 days for 5 total treatments. The mice were observed for another 15 days and later sacrificed.

The tumor dimensions were measured using a Vernier caliper and calculated according to the equation:

$$V = \frac{W^2L}{2},$$

where W and L were the minor and major axes of the tumor.

In additional, nude mice were respectively administered Tf-L-SN38/P/siRNA or L-SN38/P/siRNA via tail vein injection. At predetermined time points, the mice were narcotized to observe the fluorescence distribution using Caliper IVIS Lumina II (Caliper Life Sciences; Palo Alto, CA, USA).

Statistical analysis

The data are expressed as the mean ± SD. The statistical significance of the data was analyzed using a one-way analysis of variance model. $P < 0.05$ was considered to be statistically significant.

Results and discussion
Synthesis of SN38 prodrug

SN38 has low solubility and can be converted into an inactive carboxylate form under physiological pH conditions.[19,20] The low lipid solubility of SN38 limits its loading efficiency into liposomes.[3,21] In contrast, TAT-PEG-SN38 can be dissolved in ethanol along with lipids to form liposomes. Furthermore, TAT was used to enhance the cell penetrating ability of SN38. TAT-PEG-SN38 was synthesized in 3 steps. First,

an extra cysteine at the terminus of TAT was used to link it to OPSS-PEG. The molecular weight of FITC-labeled TAT, shown in Figure 2, was 2,130.5, matching the theoretical value. Second, C$_{10}$-OH of SN38 was transformed into an acyl chloride to enable conjugation to PEG by triphosgene. PEG was connected with SN38 through an ester bond. Finally, TAT was conjugated to the other end of PEG through a disulfide bond.

TAT-PEG-SN38 was dissolved in dimethyl sulfoxide-d$_6$ and characterized by 1H-NMR spectroscopy at 300 MHz (Figure 3F). Chemical shifts (δ, ppm) of SN38-C$_{10}$-OH (s, 1H, 10.3) disappeared after synthesis; exclusive chemical shifts of SN38 were always found 0.88 (t, 3H, CH$_3$), 1.18 (t, 3H, CH$_3$), 1.53 (s, 2H, CH$_2$), 3.11 (s, 2H, CH$_2$), 5.33 (s, 2H, CH$_2$), 5.426 (s, 2H, CH$_2$), 6.86 (s, 1H, C$_{20}$-OH), 7.251 (s, 1H, ArH), 7.411 (s, 1H, ArH), 7.432 (s, 1H, ArH), and 8.167 (t, 1H, ArH). The specific chemical shift of PEG was 3.50 (s, 2H, CH$_2$O), and the chemical shifts of TAT were 1.06 (t, 2H, CH$_2$), 2.594 (s, 1H, CH), and 7.3 (s, 2H, NH$_2$). 1H-NMR results of TAT-PEG-SN38 suggest that the SN38 prodrug was successfully synthesized.

Liposome preparation and characterization

Tf was conjugated to DSPE-PEG to facilitate liposome incorporation by postinsertion. First, a variety of liposome preparations were prepared. The electrostatic complexation of TAT-PEG-SN38 to siRNA was assessed by agarose gel electrophoresis. siRNA was bound to Tf-L-SN38 at ± charge ratio of 1:1, 2:1, 5:1, 8:1, 10:1, 15:1, and 20:1. siRNA was partially complexed to Tf-L-SN38 when ± charge ratio was 5:1 and 8:1. The blurry strip in the middle of the siRNA was caused by the breakage of the disulfide linkage between TAT and PEG during the electrophoresis. siRNA was completely complexed to Tf-L-SN38 at ± charge ratio of 10:1 (Figure 3G). The siRNA loading ability of TAT-PEG-SN38 was limited. When the concentration of TAT-PEG-SN38 was low, Tf-L-SN38 could not load a large number of siRNA. Therefore, we developed Tf-L-SN38/P/siRNA to improve the delivery ability of siRNA.

In Figure 3H, Tf-L-SN38/P/siRNA efficiently incorporated siRNA at a mass ratio of (protamine/siRNA) of 1/0.3, regardless of the amount of TAT-PEG-SN38 present in liposomes. The protamine complexed siRNA forms a virus-like structure inside of a lipid membrane.[8] The characterization of liposome preparations is shown in Figure 3 and Table 2. The particle size range of L-SN38 was 105–125 nm after the Tf incorporation, the particle size had only risen slightly to 127 nm after protamine/siRNA was loaded to the
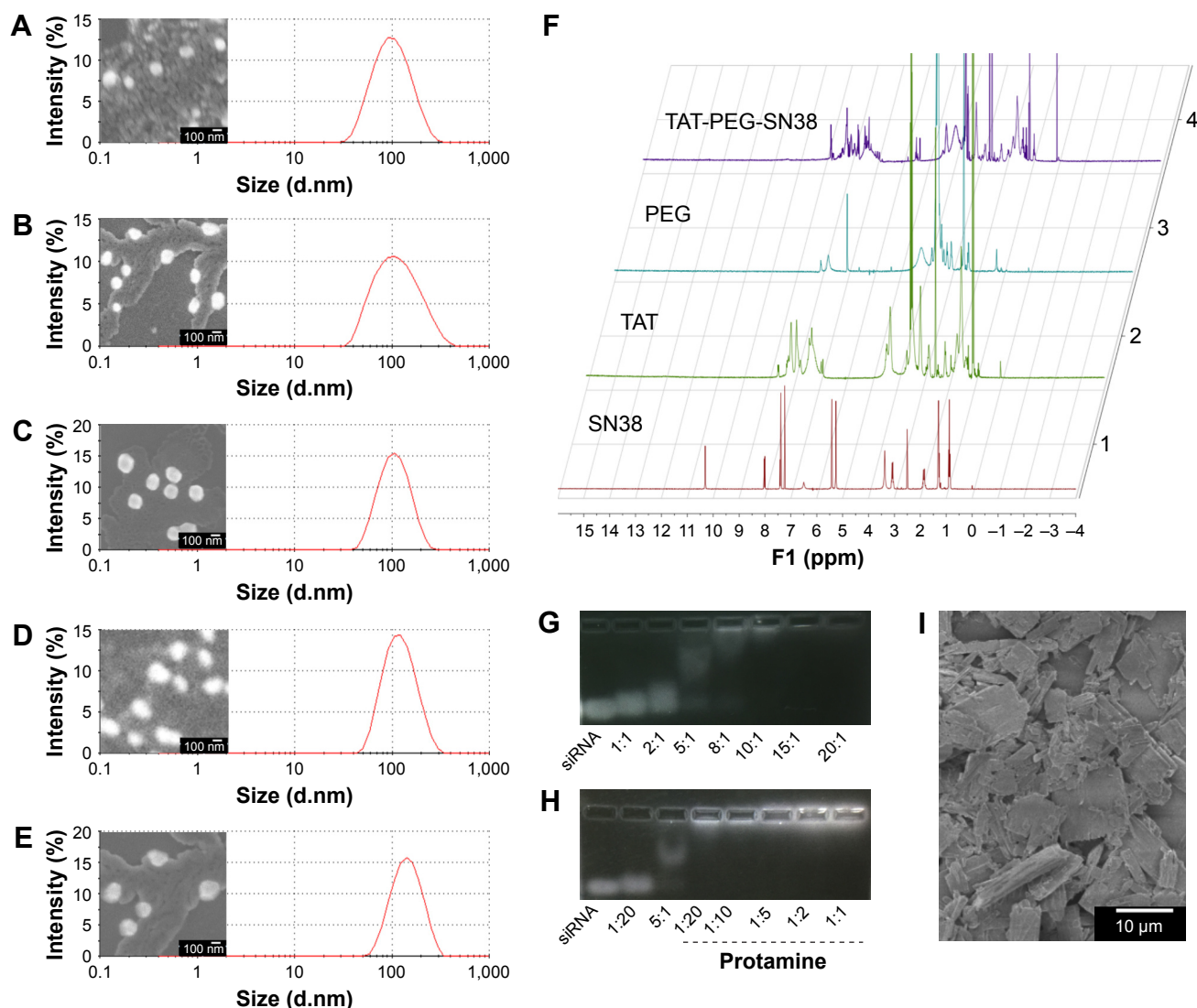
Figure 3 Characterization of a series of liposome preparations.
Notes: (**A–E**) Particle size and SEM (SEI model, 3.0 kV) of (**A**) L-SN38; (**B**) Tf-L-SN38; (**C**) L-P/siRNA; (**D**) L-SN38/P/siRNA; (**E**) Tf-L-SN38/P/siRNA. (**F**) 1H-NMR of SN38, TAT, PEG, and TAT-PEG-SN38 dissolved in DMSO-d_6 and tested at 300 Hz. (**G**) Agarose gel electrophoresis of Tf-L-SN38 loading siRNA with different charge ratios (±), siRNA was 0.05 nmol/well. (**H**) Loading siRNA ability of Tf-L-SN38/P/siRNA with different charge ratios (±) when the protamine/siRNA core existed, siRNA concentration was 0.05 nmol/well. (**I**) SEM of SN38 (SEI model, 3.0 kV).
Abbreviations: NMR, nuclear magnetic resonance; PEG, polyethylene glycol; SEM, scanning electron microscopy.

liposome core. The particle size of L-SN38/P/siRNA had a small increase to 148 nm after the Tf incorporation. A stable and appropriate particle size was beneficial to the subsequent study. The particle size of all liposomes was below 200 nm, enabling accumulation in the tumor by an enhanced permeability and retention effect. TAT has positive charges due to arginine residues, but the charge of TAT-PEG-SN38 was

partly blocked due to PEG modification.[22] ζ-potential of Tf-L-SN38 was +4.7 mV. The ζ-potential was increased only slightly to +7.8 mV compared with Tf-L-SN38/P/siRNA after loading protamine core. The SEM image of SN38 showed that SN38 had an irregular, flaky, and granular structure (Figure 3I). The liposome preparations exhibited different SEM structures from SN38. They were all approximately

Table 2 Particle size and ζ-potential of liposome preparations (n=3)

RUN	L-SN38	Tf-L-SN38	L-P/siRNA	L-SN38/P/siRNA	Tf-L-SN38/P/siRNA
Particle size (nm)	105.4±3.7	124.5±3.1	115.3±4.3	127.2±5.1	148.1±4.8
ζ-potential (mV)	6.52±0.2	4.67±0.4	3.74±0.4	13.83±1.1	7.78±0.4

spherical with a uniform size and smooth surface morphology (Figure 3A–E), and the particle sizes matched those determined by light scattering on a Zetasizer.

In vitro cytotoxicity and apoptosis analysis

The cytotoxicity of L-P/siRNA was evaluated by an MTT assay. The results are shown in Figure 4A. The cytotoxicity increased slightly when the concentration of siRNA changed from 10 to 100 nM in HeLa cells, but overall was not highly potent. The cytotoxicity of liposomes codelivered with TAT-PEG-SN38 and siRNA is shown in Figure 4B. SN38 showed a half-maximal inhibitory concentration (IC_{50}) value of 2.55 μM. However, IC_{50} of Tf-L-SN38 was lowered to 2.03 μM, which may be attributed to the improved stability of SN38 by liposomes. Tf-L-SN38/P/siRNA possessed the most cytotoxicity when different concentrations of TAT-PEG-SN38 were codelivered with siRNA. Considering that the cytotoxicity of Tf-L-SN38/P/siRNA (IC_{50}: 175 nM) was significantly greater than Tf-L-SN38 ($P<0.01$), an improved treatment effect was due to the siRNA effect. Tf-L-SN38/P/siRNA was able to deliver siRNA into tumor cells against survivin to kill cells. L-SN38/P/siRNA (IC_{50}: 491 nM) exhibited a weaker cytotoxicity compared to Tf-L-SN38/P/siRNA. The enhanced cytotoxicity of Tf-L-SN38/P/siRNA was attributed to the target effect of Tf.

Although siRNA had a weaker toxicity for tumor cells, the cytotoxicity of Tf-L-SN38/P/siRNA was increased when 100 nM siRNA and TAT-PEG-SN38 were codelivered. In addition, Tf and TAT-modified liposomes can increase cellular uptake of Tf-L-SN38/siRNA to improve cytotoxicity. The SN38 prodrug, siRNA, and Tf receptor targeting were all conducive to tumor cell death.

Apoptosis analysis was used to determine the mechanism of antitumor effects observed.

A fluorescence-activated cell sorting-based analysis was performed. Both early and late apoptosis cells were separated from live cells. The apoptosis of HeLa cells induced by codelivery of liposomes is shown in Figure 5. All treated groups could efficiently induce late apoptosis in a huge proportion of cells after 48 hours incubation, especially Tf-L-SN38/P/siRNA, which induced the highest late apoptosis ratio.

Internalization of Tf-L-SN38/P/siRNA

TAT-PEG-SN38 and siRNA were labeled with FITC and Cy3, respectively. Confocal microscopy was used to evaluate the uptake of Tf-L-SN38/P/siRNA (Figure 6). The control group was treated with empty liposomes, which were not fluorescently labeled. L-SN38-treated cells exhibited green fluorescence after 4 hours. Tf-L-SN38 exhibited increased fluorescent intensity compared to L-SN38 in cells due to Tf receptor targeting. Conversely, although L-P/siRNA was used to deliver siRNA, the result was not satisfactory. The HeLa cells after treatment with Tf-L-SN38/P/siRNA (5 μM of FITC-labeled TAT-PEG-SN38, and 100 nM of Cy3 labeled siRNA) for 4 hours showed the highest green
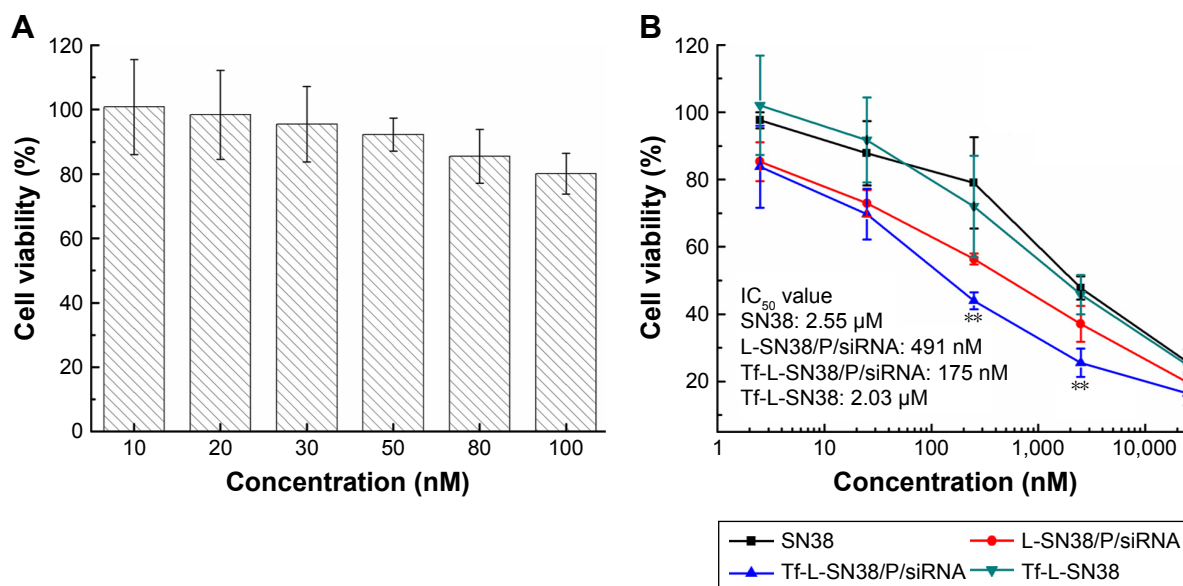

Figure 4 Cell viability for various liposome preparations by MTT assay in vitro.
Notes: HeLa cells were incubated with liposome preparations for 48 hours. (**A**) Cytotoxicity of L-P/siRNA at different concentrations. (**B**) Cytotoxicity of SN38, L-SN38/P/siRNA, Tf-L-SN38/P/siRNA or Tf-L-SN38 (significantly different at **$P<0.01$, Tf-L-SN38/P/siRNA vs L-SN38/P/siRNA).
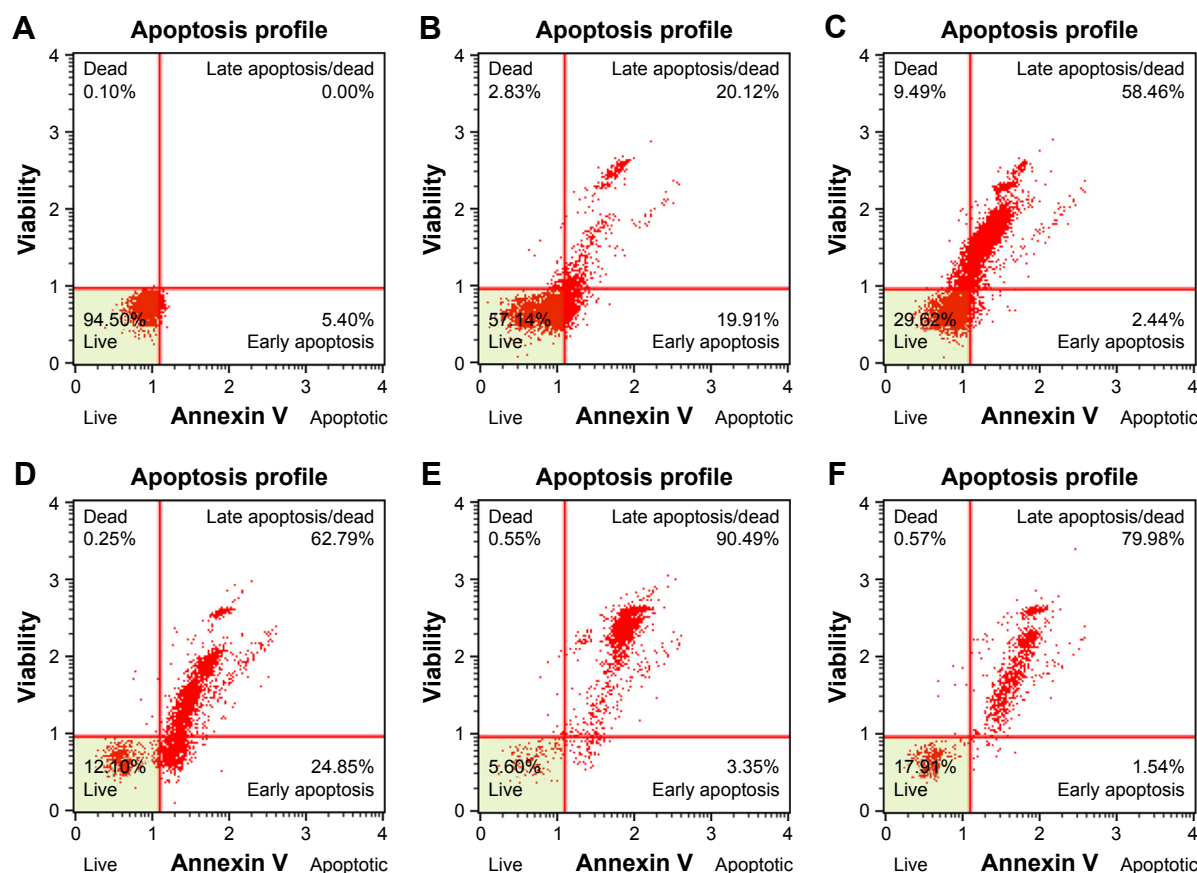
Figure 5 Cell apoptosis was analyzed by FACS.
Notes: (**A**) Untreated, (**B**) CPT-11, (**C**) L-P/siRNA, (**D**) L-SN38/P/siRNA, (**E**) Tf-L-SN38/P/siRNA, (**F**) Tf-L-SN38.
Abbreviations: FACS, fluorescence-activated cell sorter; CPT-11, irinotecan.

and red fluorescent intensity of all treatment preparations. This suggested that Tf-L-SN38/P/siRNA was a promising carrier to deliver siRNA to tumor cells. TAT-PEG-SN38 and siRNA were synchronously absorbed by tumor cells. L-SN38/P/siRNA had a relatively weaker fluorescent intensity than Tf-L-SN38/P/siRNA. The targeting effect of Tf could significantly improve the uptake of liposomes. L-SN38/P/siRNA-treated cells had a higher red fluorescence intensity than those treated with L-siRNA. The incorporation of cationic TAT-PEG-SN38 was beneficial to uptake of siRNA. Green and red fluorescence were evenly distributed in the cytoplasm. This indicated that Tf-L-SN38/P/siRNA could promote TAT-PEG-SN38 and siRNA escape from the endosome. In conclusion, Tf-L-SN38/P/siRNA can be efficiently endocytosed by HeLa cells.

Uptake of Tf-L-SN38/P/siRNA and treatment with inhibitors

TAT is internalized into cells through 3 endocytosis pathways (macropinocytosis, clathrin-mediated endocytosis,

and caveolae/lipid raft-mediated endocytosis). The relative importance of specific pathways depends on its attached cargo.[23] Cytochalasin D, nystatin, sucrose, and free holo-Tf were used to block the macropinocytosis, caveolae-mediated endocytosis, clathrin-mediated endocytosis, and Tf receptor-mediated endocytosis pathways. These reagents were used to explore the mechanism of interaction between Tf-L-SN38/P/siRNA and HeLa cells. Flow cytometry was used to quantitatively analyze the uptake of L-P/siRNA, L-SN38/P/siRNA, and Tf-L-SN38/P/siRNA (Figure 7C–F). The fluorescence of FITC and Cy3 were absorbed in concert, FITC and Cy3 were codelivered into HeLa cells by carrier. The cellular fluorescent uptake of Tf-L-SN38/P/siRNA was 2.06 and 1.95 times that of L-SN38/P/siRNA at FITC and Cy3, respectively. The uptake of Cy3 for L-P/siRNA was blocked 54.7%, 67.8%, and 70.6% by cytochalasin D, nystatin, and sucrose, respectively. Holo-Tf had no significant inhibition on Cy3 uptake for L-P/siRNA (only 10.3%) (Figure 7A). For L-SN38/P/siRNA, the uptake of FITC/Cy3 was blocked (25.0/27.6%, 49.6/38.1%, 59.3/46.4%,
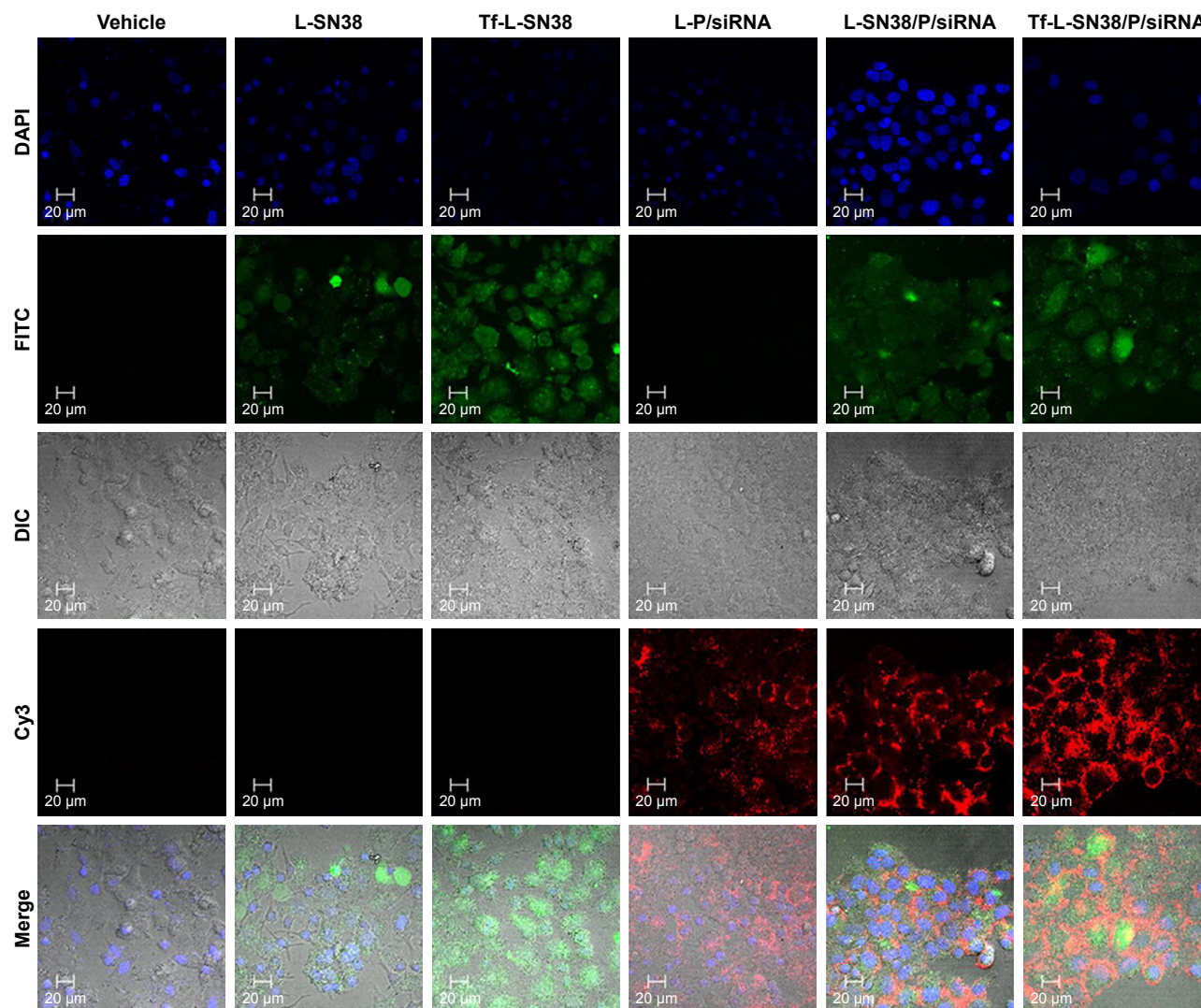
Figure 6 Cellular uptake of liposome preparations.
Notes: FITC- and Cy3-labeled series of liposome preparations were incubated with HeLa cells for 4 hours. Empty (unlabeled) liposomes were used as the control. The internalization was observed by a confocal microscope.
Abbreviation: DIC, differential interfering contrast.

and 29.1/17.2%) by cytochalasin D, nystatin, sucrose, and free holo-Tf, respectively. For Tf-L-SN38/P/siRNA, the uptake of FITC/Cy3 was blocked (37.1/28.1%, 58.9/48.6%, 66.8/60.4%, and 74.4/73.0%) by cytochalasin D, nystatin, sucrose, and free holo-Tf, respectively (Figure 7A and B). Cytochalasin D, nystatin, sucrose, and free holo-Tf all had an influence in cellular uptake of liposome preparations. Tf-modified liposomes were primarily internalized through the Tf receptor. The inhibition uptake ability of cytochalasin D, nystatin, and sucrose for Tf-L-SN38/P/siRNA changed in the following manner: hypertonic sucrose > nystatin > cytochalasin D. Tf-L-SN38/P/siRNA secondarily entered into the cells through clathrin-mediated endocytosis. Three endocytosis inhibitors more effectively blocked

Tf-L-SN38/P/siRNA uptake. This suggested that Tf made a partial contribution to the endocytosis of Tf-L-SN38/P/siRNA.

Gene silencing efficiency of Tf-L-SN38/siRNA

Western blot analysis was used to measure the survivin protein to evaluate the silencing efficiency of liposome preparations. Tf-L-SN38 was used as a positive control (Figure 8). L-P/siRNA was ineffective in survivin inhibition. The expression levels of survivin in HeLa after Tf-L-SN38/P/siRNA treatment were the lowest in all experiment groups. L-SN38/P/siRNA had a slight inhibition effect of survivin expression compared to Tf-L-SN38. The expression levels of the survivin protein correlated to the siRNA delivery.
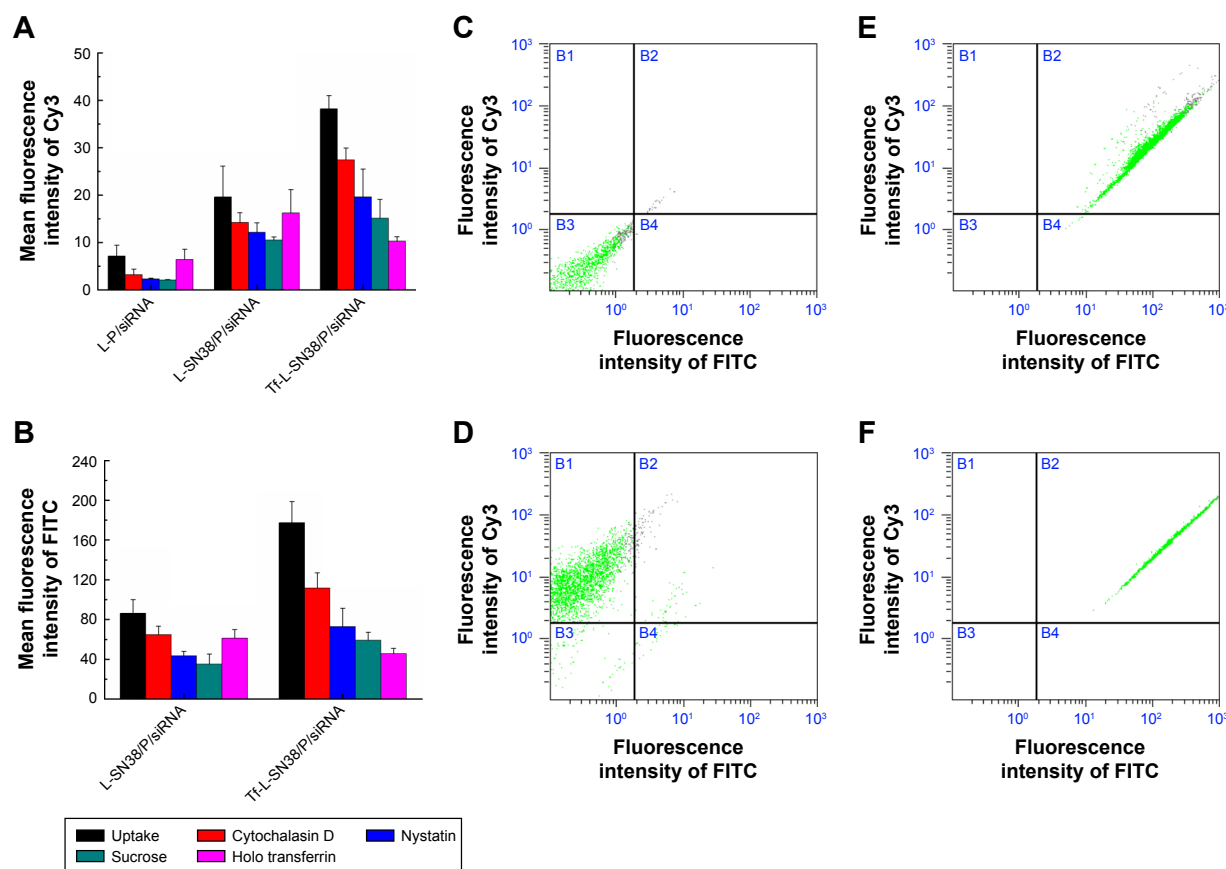
Figure 7 Cellular uptake influence by internalization inhibitors.
Notes: (**A**) Cy3 fluorescence intensity change of L-P/siRNA, L-SN38/P/siRNA, or Tf-L-SN38/P/siRNA after treatment with endocytosis inhibitors (n=3). (**B**) FITC fluorescence intensity change of L-SN38/P/siRNA, or Tf-L-SN38/P/siRNA after treatment with inhibitors (n=3). (**C**) untreated. (**D**) L-P/siRNA. (**E**) L-SN38/P/siRNA. (**F**) Tf-L-SN38/P/siRNA.

The siRNA delivery efficiency of different carriers was diverse. It can be interpreted by the uptake ability of a variety of carriers. The result of Western blot analysis was consistent with the uptake analysis by flow cytometry.

Antitumor activity evaluation and in vivo distribution

A nude mouse model bearing HeLa cells was established to evaluate the anticancer efficacy of Tf-L-SN38/P/siRNA.
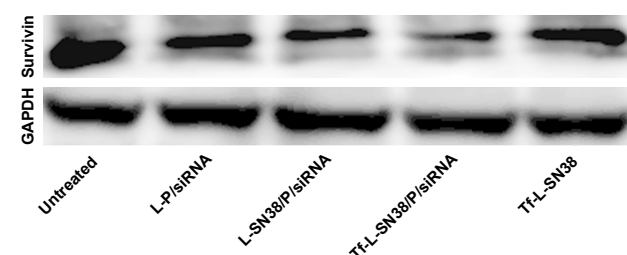
Figure 8 Expression level of the survivin protein after treatment with liposome preparations.
Notes: The amount of the survivin protein was measured by Western blot analysis after incubation with liposome preparations for 48 hours. GAPDH was used as an internal control.

The treatment agents were given intravenously every 3 days for a total of 5 times (Figure 9). The antitumor effect of the combination of the SN38 prodrug and siRNA was evaluated by the tumor volume. The side effect of liposomes was appraised using body weight loss. CPT-11 was an effective clinical anticancer drug that was used as the positive control.

The tumor images and weights are shown in Figure 9C and D, after the nude mice were sacrificed. The mean tumor volume followed the order vehicle > L-P/siRNA > CPT-11 > L-SN38/P/siRNA > Tf-L-SN38 > Tf-L-SN38/P/siRNA. The antitumor effect of L-P/siRNA was limited, with the tumor attaining 931 mm³, compared to vehicle (1,259 mm³, $P<0.05$) (Figure 9A). The tumor volumes grew slowly after CPT-11, L-SN38/P/siRNA, Tf-L-SN38/P/siRNA, and Tf-L-SN38 during the initial 9 days and were indistinguishable from each other. Tf-L-SN38/P/siRNA (292 mm³) had the most effective antitumor effect with a 76.8% inhibition compared with the vehicle. Tf-L-SN38/P/siRNA inhibited 33.8% of the tumor growth after codelivering siRNA, compared with Tf-L-SN38. Tf-L-SN38/P/siRNA reduced 39.1% of the tumor volume after Tf targeting modified liposomes,
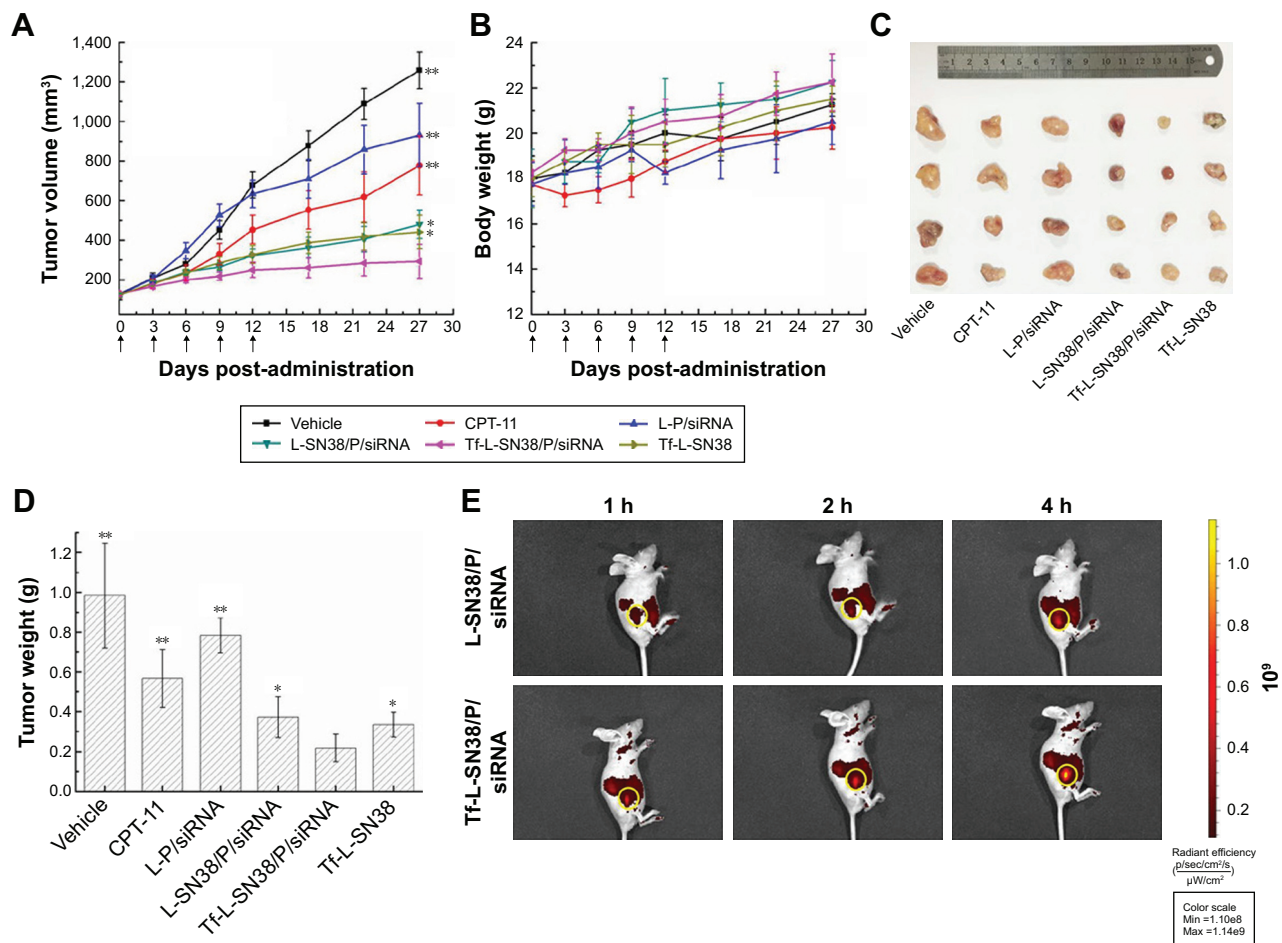
Figure 9 Antitumor activity evaluation and in vivo distribution (n=4).
Notes: (**A**) Tumor volume and (**B**) body weight were recorded at designated time points. Significantly different (**P<0.01, Tf-L-SN38/P/siRNA vs vehicle, CPT-11 or L-P/siRNA; *P<0.05, Tf-L-SN38/P/siRNA vs L-SN38/P/siRNA or Tf-L-SN38). (**C**) Tumor images of nude mice after saline, CPT-11, L-P/siRNA, L-SN38/P/siRNA, Tf-L-SN38/P/siRNA, or Tf-L-SN38 treatment. (**D**) Tumor weight was measured after nude mice were sacrificed. Significantly different (**P<0.01, Tf-L-SN38/P/siRNA vs vehicle, CPT-11 or L-P/siRNA; *P<0.05, Tf-L-SN38/P/siRNA vs L-SN38/P/siRNA or Tf-L-SN38). (**E**) IVIS image of HeLa cell xenograft tumor-bearing nude mice after administrationTf-L-SN38/P/siRNA and L-SN38/P/siRNA, siRNA was labeled by Cy5.
Abbreviation: CPT-11, irinotecan.

compared with L-SN38/P/siRNA. In addition, L-SN38/P/siRNA and Tf-L-SN38 also showed an advantage on antitumor effect over CPT-11. The body weight of all experimental groups was stable (Figure 9B). The results indicated that targeted codelivery of the SN38 prodrug and the survivin siRNA can improve antitumor activity on HeLa cells.

IVIS was used to further evaluate the targeting efficiency of Tf-L-SN38/P/siRNA in vivo, and L-SN38/P/siRNA was used as a control. The fluorescence of Tf-L-SN38/P/siRNA was primarily localized in the tumor tissue, whereas L-SN38/P/siRNA had a weaker tumor fluorescence intensity than Tf-L-SN38/P/siRNA after intravenous administration (Figure 9E).

Conclusion

Combination therapy is routinely used in clinical practice for treatment of cancer. Liposomal delivery of drug combinations

has been shown to be an effective strategy to promote efficacy between therapeutic agents, as indicated by the recent approval of Vyxeos (Dublin, Ireland) (liposomes coloaded with daunorubicin and cytarabine at a fixed ratio) for acute myelogenous leukemia therapy. Survivin overexpression has been shown to be a key factor in tumor cell resistance to chemotherapy. The survivin siRNA can therefore be used to sensitize cancer cells to chemotherapy such as SN38. The data from this study showed that codelivery of SN38 and the survivin siRNA was more effective in tumor cell growth inhibition than single agents. In addition, liposomal delivery overcame the low stability, poor solubility, and/or delivery barrier of these antitumor agents. Tf receptor targeting was able to enhance the HeLa cellular uptake of both the SN38 prodrug and the survivin siRNA, improving selective cytotoxicity. Tf-L-SN38/P/siRNA exhibited 76.8% tumor inhibition and therefore was highly active therapeutically.

These data suggest that Tf-L-SN38/P/siRNA warrants further investigation.

Disclosure

The authors report no conflicts of interest in this work.

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