The use of cationic liposomes to co-deliver docetaxel and siRNA for targeted therapy of hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is one of the major causes of death worldwide. Targeted delivery of drugs to tumor cells can be achieved by introduction of a targeting ligand onto the nanocarrier system. Simultaneous delivery of a chemotherapeutic drug and siRNA in one nanocarrier system to the tumor is a promising strategy for cancer treatment. In this study, we prepared cationic liposomes to co-deliver docetaxel (DTX) and small interfering RNA (siRNA). The liposomes were modified by a hepatocellular carcinoma specific homing peptide, SP94. Serum stability assay demonstrated that liposomes can significantly protect the siRNA against enzymatic degradation in serum. The SP94 modified liposomes showed increased cellular uptake and stronger anti-tumor effect compared with the unmodified liposomes on human HCC cells. The data indicated that the SP94 modified liposomes which co-deliver DTX and siRNA could be used for the targeted therapy of hepatocellular carcinoma.
Keywords: SP94, Hepatocellular carcinoma, Liposomes, Docetaxel, siRNA

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world[1] and is responsible for 5% of all malignant tumors in humans[2]. Though there are many available options for HCC treatment, such as curative resection, liver transplantation, radiofrequency ablation, trans-arterial chemoembolization, radio embolization and so on, the prognosis of patients with advanced HCC remains very poor[3]. It is still important to develop effective and tolerable drugs for the treatment of HCC. Recently the combination of chemotherapeutic agents and small interfering RNA (siRNA) is used as a new strategy for the effective treatment of tumors. Drugs with different mechanisms of action are co-delivered into the tumor cells by nano-based delivery system can exert synergistic effects or combined effects[4,5]. Some of these delivery systems show the potential for effective treatment of cancer[6,7].

Docetaxel (DTX), an important anticancer taxane, has been widely used for the treatment of non-small cell lung cancer, breast cancer, ovarian cancer since 1996[8]. DTX inhibits the growth of tumor cells by stabilization of tubulin[9]. DTX is a hydrophobic small molecule and its solubility in water is only 5–6 μg/mL[10]. Tween-80 and ethanol are used in the clinical product Taxotere® to increase the solubility of DTX, which results in hypotension and tachycardia[11]. The poor aqueous solubility and toxicity of DTX limit its clinical application. Therefore, the development of a new nanocarrier is needed to increase the solubility of DTX and reduce the side effects.

RNA interference (RNAi) is a remarkable gene knockdown technique[12], which could be used for the treatment of many diseases ranging from viral infections to cancers[13]. siRNA can specifically down-regulate the expression of the targeted gene. But naked siRNA is unstable in the serum, and can hardly be transfected into the cells. In addition, naked siRNA has the risk of being immunogenic[14]. Successful clinical application of siRNA depends on the efficient and safe delivery of siRNA into target cells[15,16]. Many kinds of nanocarrier systems have been designed to deliver siRNA, which consist of viral delivery systems, non-viral delivery systems and chemical modified

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siRNA. Liposomes is one of the most common non-viral delivery systems[17].

However, these delivery systems have limits due to their lack of specificity. To overcome these shortages, targeting ligand can be used to modify the nanocarrier systems to guide the drugs to the desired sites. SP94 peptide has a high affinity for unknown receptors expressed by HCC, which was identified by filamentous phage display[18,19]. Nanoparticles modified with SP94 exhibit much higher avidity for binding to HCC than to hepatocytes, endothelial cells, monocytes, or lymphocytes. Several nano-scale delivery systems modified with SP94 have been introduced, and showed positive targeting by SP94 peptide[18,20–22].

Therefore, we prepared SP94 modified cationic liposomes to efficiently co-deliver DTX and siRNA for targeted therapy of HCC. SP94 peptide conjugation onto the liposomes with a PEG chain significantly enhanced the cellular uptake and anti-tumor effect. This nanosystem could be used as a potential way for the targeted therapy of liver tumor.

2. Materials and methods

2.1. Materials and cells

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) was from Avanti Polar Lipids (Alabaster, AL, USA). Soybean phosphatidylcholine (SPC) was from Lipoid (Ludwigshafen, Germany). Cholesterol (Chol) was from Wako Ltd. (Tokyo, Japan). Docetaxel (DTX) was from Norzer Pharmaceutical Co., Ltd. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-methoxy (polyethyleneglycol, DSPE-PEG_{2000}) was purchased from NOF Corporation (Tokyo, Japan). DSPE-PEG_{2000}-SP94 was synthesized in our laboratory[23]. The FAM-labeled negative siRNA (FAM-siRNA) (antisense strand, 5′-ACGUGACACGU-UCGGAGAATT-3′) was purchased from Gene Pharma (Shanghai, China). Coumarin-6 was purchased from Sigma (St. Louis, MO, USA). Taxotere® was commercially available from the local hospital of Beijing (Aventis Pharma S.A., France). The HPLC-grade solvents used for high-performance liquid chromatography (HPLC) were provided by Sayfo (Tianjin, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA).

Modified eagle medium (MEM), Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin, trypsin and Hoechst 33258 were obtained from Macgene Technology (Beijing, China).

HepG2 (Human hepatocellular carcinoma) cells were cultured in DMEM supplemented with 1% non-essential amino acids, 10% fetal bovine serum (FBS, GIBCO, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin. Hep3B (Human hepatocellular carcinoma) cells were cultured in MEM supplemented with 1% non-essential amino acids, 10% FBS (GIBCO, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin. All cells were maintained in a 37 °C humidified incubator under 5% CO_2 atmosphere.

2.2. Preparation of liposomes

The cationic liposomes were prepared by direct hydration of a lipid film[24]. The compositions of normal liposomes (Lp) and SP94-modified liposomes (Lp-SP94) were DOTAP (25%), SPC (40%), cholesterol (30%), DSPE-PEG_{2000} (1%) and DSPE-PEG_{2000}-SP94 (1%, only for Lp-SP94) in molar ratio[7]. DTX or coumarin-6 and all lipids were dissolved with chloroform in a pear-shaped flask and were subsequently dried to form a thin lipid film by rotary evaporation under vacuum at 40 °C. The lipid film was then hydrated using siRNA solution in distilled water pretreated with diethypyrocarbonate (DEPC), vortexed for 2 min and sonicated for 5 min at 40 °C. After the lipid dispersion was filtered through a 0.22 μm membrane, the liposomes (Lp) and SP94-modified liposomes (Lp-SP94) were obtained.

2.3. Gel retardation assay

Liposomes were prepared as described above to achieve N/P ratios at the range from 2 to 20. The loading ability of liposomes for siRNA was analyzed by agarose gel electrophoresis. After addition of gel loading buffer, the samples were separated by electrophoresis on a 1% (w/v) agarose gel containing 0.5 μg/mL ethidium bromide (EtBr) in TBE buffer for 15 min at 100 V. Thereafter, the gels were visualized with a UV illuminator.

2.4. Size distribution and zeta-potential

The particle size, polydispersity index (PDI) and zeta potential of liposomes were determined by dynamic light scattering (DLS) using the Malvern Zetasizer Nano ZS (Zetasizer 3000HS, Malvern, Worcestershire, UK). The samples were diluted with distilled water.

2.5. The entrapment efficiency of DTX

The free DTX in the formulation was separated by the ultrafiltration method[25]. The Lp/DTX was placed in the upper chamber of a centrifuge tube equipped with an ultrafilter (MWCO 30 kDa). Then the tube was centrifuged at 14 000 r/min for 30 min at 4 °C. The solution in the tube contains the free DTX not encapsulated in the liposomes.

The concentration of DTX was measured by HPLC (LC-20AT Pump, SPD-20A UV detector, Shimadzu, Japan). The mobile phase was consisted of acetonitrile –water (60:40, v/v) at a flow rate of 1 mL/min. The detector was set at 230 nm, and the sample was loaded onto an RP-18 column (4.6 mm×250 mm, 5 μm, Diamonsil). After the ultrafiltration, the liposomes were disrupted and the DTX was diluted with methanol. The entrapment efficiency (EE%) of DTX was calculated using the following equation:

$$EE\% = \frac{W_{total} - W_{free}}{W_{total}} \times 100\%$$

where W_{free} and W_{total} represent the amount of unencapsulated DTX and the total amount of DTX in the formulation, respectively.

2.6. The stability of siRNA in liposomes in serum

Free siRNA and Lp/DTX/siRNA were incubated with FBS for 24 h at 37 °C[26]. The concentration of FBS was 50% (v/v) and the concentration of siRNA was 1 nmol/mL. The samples were analyzed by gel retardation assay as described above at different time point. The siRNA in gels were visualized with EtBr staining with a UV illuminator.

2.7. Cellular uptake by human hepatocellular carcinoma cells

The amounts of Coumarin-6 or FAM-siRNA taken into HepG2 and Hep3B cells were determined by flow cytometry. HepG2 and Hep3B cells (5×10^4 cells/well and 3×10^4 cells/well, respectively) were seeded into 6-well plates containing 2 mL of complete DMEM medium and MEM medium respectively. The plates were placed in the 37 °C humidified incubator under 5% CO_2 atmosphere for 24 h as the attachment period. The liposomes were diluted ten times by serum-free medium for using. The cells were rinsed with phosphate buffer solution (PBS) and incubated with different liposomal formulations containing Coumarin-6 (for 2 h) or FAM-siRNA (for 4 h) at 37 °C. The concentration of Coumarin-6 and FAM-siRNA was 100 ng/mL and 100 nM, respectively. Then the cells were trypsinized and washed three times with cold PBS containing heparin (125 U/mL). The samples were centrifuged and resuspended, and then subjected to flow cytometric analysis utilizing a BD FACSCalibur flow cytometer immediately.

2.8. In vitro antiproliferation study

The antiproliferation effect of liposomes against HepG2 cells was assessed by MTT assay[27]. Briefly, HepG2 cells in exponential growth phase were seeded in 96-well plates at a density of 6000 cells/well and allowed to adhere for 24 h. The cells were exposed to different concentrations of Lp/DTX and Lp-SP94/DTX at 37 °C for 48 h. Subsequently, 20 μL of MTT indicator dye (5 mg/mL in PBS) was added into each well, and the mixtures were incubated for another 4 h at 37 °C in the dark. The medium was removed and replaced with 200 μL DMSO. The plates were placed on a shaker to help dissolve the formazan crystals sufficiently for 10 min. The absorption of each well was measured at 570 nm using an iMark microplate absorbance reader (Bio-Rad Laboratories, USA).

2.9. Statistical analysis

Statistical analysis of the samples was performed using the one-way analyses of variance (ANOVA), and $P<0.05$ was considered statistically significant. All data are presented as the means±standard deviation (SD) of three or more samples.

3. Results and discussion

3.1. Preparation and characterization of liposomes

The liposomes containing siRNA at different N/P ratios were prepared by the membrane-ultrasonic method. The positive charges of cationic lipid (DOTAP) interact electrostatically with the negative charges of siRNA, resulting in the condensation and entrapment of siRNA by the liposomes[28]. When there are enough positive charges, the siRNA can be completely loaded in the liposomes. The binding abilities of the liposomes were

investigated by gel retardation assay. EtBr staining reveals high fluorescence after intercalating into siRNA base pairs, and the fluorescence would decrease because of the entrapment of siRNA into the cationic liposomes[29]. Figure 1 shows that liposomes at N/P ratio exceeded 8 induced the complete retardance of siRNA migration, indicating sufficient siRNA loading ability. Condensation of siRNA into liposomes with proper size and zeta potential is critical for efficient cellular uptake. The size and zeta potential can be affected by the N/P ratio. DLS assay was utilized to measure the size, PDI and zeta potential of the liposomes at varying N/P ratios from 2 to 20. As shown in Figure 2, the apparent sizes were very large when the N/P ratio was 2 and reached a relatively constant value when the N/P ratio was larger than 6. With the increase of N/P ratio, the zeta potential was increased and stabilized at N/P ratio of 8, which was consistent with the electrophoretic gel retardation results (Fig. 1). Based on these data, the most suitable N/P ratio of the siRNA-loaded liposomes was determined to be 8 in this study. Under the optimized preparation condition, the hydrodynamic diameter and zeta potential of the liposomes was approximately (142.1±53.6) nm and (30.8±4.6) mV, respectively, and the weight ratio of lipids/DTX was 20:1 (w/w) with a high entrapment efficiency of about 87.75%.

3.2. The stability of siRNA in liposomes exposed to serum

In order to achieve significant gene silencing efficiency in vivo, it is important to ensure that the siRNA delivery system is sufficiently stable in the circulatory system. The stability of siRNA in liposomes was evaluated in FBS. The free siRNA was degraded and the band of free siRNA could not be detected after 6 h. However, the band of siRNA in the liposomes was still clear and bright after 24 h incubation. It shows that the liposomes can significantly protect the siRNA against enzymatic degradation in serum.

3.3. Cellular uptake of liposomes in two types of human HCC cells

Targeted delivery of a drug to the carcinoma cells can be achieved by conjugation of ligand on the nanocarrier system[30]. The SP94 peptide was identified as a targeting ligand that has high affinity for HCC[19]. The cellular uptake is expected to be enhanced due to the SP94 modification on the liposomes[18,22].

DTX is a hydrophobic chemotherapeutic agent, which shows no detectable fluorescence intensity by flow cytometry. So Coumarin-6 was encapsulated into the liposomes as a hydrophobic probe[31] to monitor the behavior of DTX. After HepG2 cells were incubated with free Coumarin-6 and Coumarin-6-loaded liposomes (Lp/Coumarin-6 and Lp-SP94/Coumarin-6) for 2 h, the cellular uptake of Lp-SP94/Coumarin-6 was increased compared with Lp/Coumarin-6, but the difference was not statistically significant (P>0.05, Fig. 3). The free

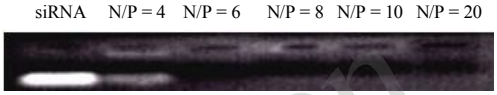

Figure 1. Entrapment ability of siRNA in the liposomes analyzed by agarose gel electrophoresis.

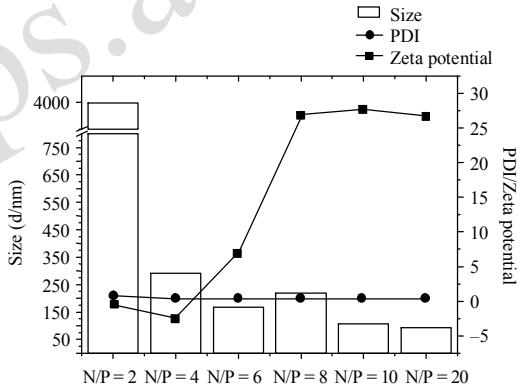

Figure 2. Hydrodynamic diameter and zeta potential of Lp/siRNA at different N/P ratios measured by dynamic light scattering (DLS).

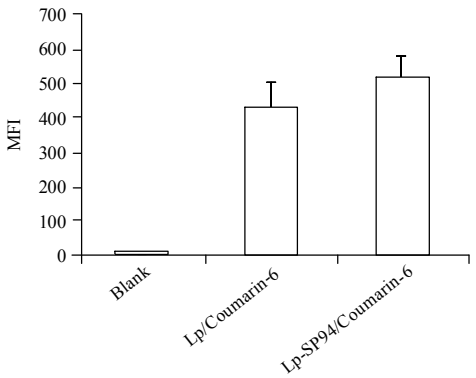

Figure 3. Cellular uptake of liposomal formulations loaded with Coumarin-6 in HepG2 cells after incubated with different liposomes at 37 °C for 2 h. The concentration of Coumarin-6 was 100 ng/mL. Results are expressed as mean±SD (n = 3).

Coumarin-6 can enter the HepG2 cells by passive diffusion, so the mean fluorescence index of free Coumarin-6 was very high (data not shown). The Coumarin-6 leaked from the liposomes could have affected the results. More experiments should be conducted to evaluate the function of SP94 peptide.

siRNA is a negatively charged hydrophilic biological macromolecule which can hardly be transfected into cells[32]. Liposomal nanocarriers have shown effectiveness in delivering siRNA with high transfection efficiency[33]. After HepG2 cells and Hep3B cells were treated with free FAM-siRNA and FAM-siRNA-loaded liposomes (Lp/FAM-siRNA and Lp-SP94/FAM-siRNA) for 4 h, all liposomes showed significant improvement of cellular uptake compared with free FAM-siRNA (P<0.01). Moreover, the cellular uptake of Lp-sp94/FAM-siRNA was obviously higher than that of Lp/FAM-siRNA in both cells (P<0.01, Fig. 4). These results indicated that the liposomes can increase the ability of siRNA to translocate into cells and the SP94 peptide modified liposomes can enhance the cellular uptake.

Overall, these results supported our hypothesis that the SP94 peptide can strongly enhance the internalization of siRNA. And modification of liposomes with SP94 peptide can improve the accumulation of siRNA in HepG2 and Hep3B, due to the interaction between the targeted peptide and corresponding cells.

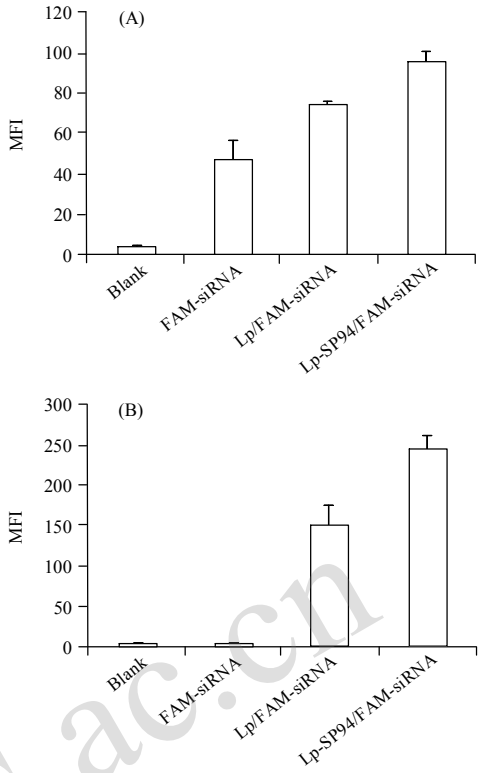

Figure 4. Cellular uptake of liposomal formulations loaded with FAM-siRNA in HepG2 (A) and Hep3B (B) cells after incubated with different liposomes at 37 °C for 4 h. The concentration of FAM-siRNA was 100 nM. Results are expressed as mean±SD (n = 3).

3.4. Antiproliferation study

In vitro antiproliferation of Lp/DTX and Lp-SP94/DTX was evaluated by MTT assay. The cell viability was decreased with a higher DTX dose. And Lp-SP94/DTX exhibited stronger antiproliferative effect on HepG2 after incubation for 48 h, as compared with Lp/DTX (Fig. 5). It showed that SP94 peptide on the liposome promoted antiproliferative activities in HepG2, which have a high affinity with the SP94 peptide. The results were consistent with previous cellular uptake studies. Above all, the antiproliferation enhancement is due to SP94 targeting effects on the cancer cells.

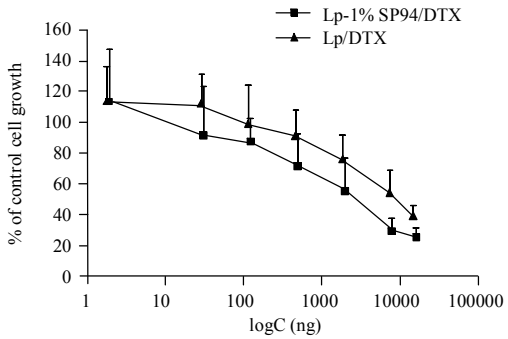

Figure 5. Cell viability of HepG2 cells cultured with various DTX-loaded liposomes after 48 h. Results are expressed as mean±SD (n = 6).

4. Conclusion

In this study, we successfully prepared SP94 modified liposomes for the co-delivery of DTX and siRNA by the membrane-ultrasonic method. When the N/P≥8, siRNA and DTX can be fully entrapped in the liposomes. The hydrodynamic diameter and zeta potential of the liposome was approximately (142.1±53.6) nm and (30.8±4.6) mV. Serum stability assay demonstrated that liposomes can significantly protect the siRNA against enzymatic degradation in serum. The SP94 modified liposomes showed increased cellular uptake and stronger anti-tumor activity compared with the unmodified liposomes on human HCC cells. Altogether, the data

indicated that the co-delivery of DTX and siRNA by SP94 modified liposomes could be potentially used for the targeted therapy of liver tumor.

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肝癌靶向的阳离子脂质体共递送多西他赛和siRNA的研究

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摘要: 肝细胞癌是全世界最常见的恶性肿瘤之一, 居癌症相关死亡原因第三位。通过在纳米载体系统上修饰靶向配体可将药物主动靶向至肿瘤细胞。随着药物制剂学的发展, 采用纳米给药系统共递送化疗药物与基因药物成为了治疗肿瘤的一种手段。本研究通过薄膜超声法制备了共包载多西他赛(DTX)和小干扰RNA(siRNA)的脂质体, 并用SP94对其进行修饰。血清稳定性试验表明, 脂质体能较好地保护siRNA免受血清中核酸酶的降解。与未修饰的阳离子脂质体相比, SP94修饰的阳离子脂质体显著增强了制剂的在肿瘤细胞内的摄取和对肿瘤细胞的抗增殖作用, 表明了SP94的主动靶向作用。本课题成功构建了主动靶向肝癌的共包载DTX和siRNA的阳离子纳米粒给药系统, 为肝细胞癌的治疗提供了新的研究思路。

关键词: SP94; 肝癌; 脂质体; 多西他赛; 小干扰RNA