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A systematic *in vitro* investigation on poly-arginine modified nanostructured lipid carrier: Pharmaceutical characteristics, cellular uptake, mechanisms and cytotoxicity

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ABSTRACT

The aim of the present study was to develop a poly-arginine modified nanostructured lipid carrier (R-NLC) by fusion-emulsification method and to test its pharmaceutical characteristics. The influence of R-NLC on A549 cells like cellular uptake and cytotoxicity was also appraised using unmodified NLC as the controlled group. As the results revealed, R-NLC had an average diameter of about 40 nm and a positive zeta potential of about +17 mv, the entrapment efficiency decreased apparently, and no significant difference on the *in vitro* drug release was found after R8-modification. The cellular uptake and cytotoxicity increased obviously compared with unmodified NLC. The cellular uptake mechanisms of R-NLC involved energy, macropinocytosis, clathrin-mediated endocytosis, and caveolin-mediated endocytosis. The outcomes of the present study strongly support the theory that cell penetrating peptides have the ability of enhancing the cellular uptake of nanocarriers.

1. Introduction

Cancer is one of the main causes of human death, ranking second to cardiovascular diseases [1]. After constant research, scientists have known that the microenvironment of tumor tissue has different features from normal tissue [2]. One of the most important features is the vascular anomaly, including a large proportion of endothelial cell regeneration, deficiency of adventitial cell and abnormalities of the basement membrane structure, and all these lead to the increased vascular permeability, together with the inefficient drainage

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caused by the lymphatic vessel absence, making the particles between 20 and 200 nm leak and resort in tumor tissue, which is called the EPR effect [3,4]. Nanosized preparations can leak from the enlarged interspace of tumor vessel to tumor tissue according to EPR effect. Certain portion of passive targeting could be achieved by EPR effect, thus the cytotoxicity to normal tissue could be decreased somewhat compared with traditional chemotherapy [5].

Nanopreparations including liposomes, micelles, dendrimers and NLCs are liable to be modified on the surface by PEG or other targeting moiety according to their constituent and preparation method [6,7]. One of the most widely investigated subjects in recent years is their surface modification with cell penetrating peptides (CPPs). CPPs are also called the protein transduction domain (PTD), which have been proved to effectively traverse the cell membrane themselves or together with a series of therapeutic agents. Kang et al. built a novel cell penetrating peptide RIPL, modified a liposomal carrier, and increased the FITC-dextran uptake by 20- to 70- fold in Hpn (+) cells [8]. Perillo et al. used the cell penetrating peptide gH625 to deliver peglyted liposome to Hela cells, and improve the quantity of intracellular drug concentration and cytotoxicity of mitoxantrone [5]. Tang et al. modified a liposome with transferring and TAT peptide, which demonstrated a 22.17 times higher cellular uptake over PEG-liposome [9].

In the present study, we constructed the relatively short CPP, polyarginine (RRRRRRRR, R8) modified NLC (R-NLC), and pharmaceutical characteristics like average size, zeta potential, entrapment efficiency and *in vitro* drug release were investigated. The cellular uptake, cytotoxicity and the uptake mechanisms of both R8-modified and unmodified NLCs were also studied, and the results showed that the therapeutic index was significantly improved with R8 modification.

2. Materials and methods

2.1. *Materials*

Stearyl-polyarginine (SA-R8) with greater than 98% purity was synthesized by TeraBio Technology Co., Ltd., Guangzhou, China. Glycerin monostearate (GMS) and Kolliphor HS15 were generously presented by BAFS Co., Ltd., Ludwigshafen, Germany. Medium chain triglyceride (MCT) was purchased from Yuhao Chemical Co., Ltd., Hangzhou, China. Gelucire 44/14 was kindly donated by Gaffefosse Co., Ltd., France. Coumarin 6 (Cou 6) was purchased from J&K Scientific Ltd., China. Paclitaxel (PTX) was purchased from Jiangsu Hengrui Pharmaceutical Co., Ltd. Jiangsu, China.

Non-small cell lung cancer cell A549 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in DMEM-F12 1:1 culture medium (Hyclone Co., Ltd., Thermo Fisher Scientific., UK), supplemented with 10% fetal bovine serum (Sijiqing Co., Ltd., Hangzhou, China). When the cells grew to 80~90% confluence, the cells were trypsinized and passaged in the ratio of 1:3.

All other chemicals and reagents were of analytical or cell culture grade.

2.2. *Preparation of R-NLC and NLC*

Fusion-emulsification method was employed to prepare NLC and R-NLC [10]. Briefly, GMS, MCT and Kolliphor HS15 were mixed and heated up to 75 °C to get a transparent oil phase, at the same time, Gelucire 44/14 was dispersed in 3 ml water and warmed at the same temperature to get the aqueous phase. The aqueous phase was added dropwise into the oil phase with constant stirring at 75 °C to get the primary emulsion. The primary emulsion was allowed to be further emulsified for 5 min, and then cooled at 4 °C immediately to get the blank NLC. To get the R8 modified NLC, SA-R8 was added into the oil phase, and the process was as above.

PTX was chosen to be the model drug to test the cytotoxicity of PTX loaded NLC and R-NLC. PTX was mixed with the oil phase when preparing NLC and R-NLC.

A green lipophilic fluorescent dye Cou 6 was chosen to be the fluorescent probe to observe and to quantitively determine the cellular uptake of NLC and R-NLC. Like PTX, Cou 6 was added to the oil phase to get the Cou 6-loading NLC and R-NLC, and the rest of the operation was as above.

2.3. *The pharmaceutical properties of NLC and R-NLC*

2.3.1. *Size and zeta potential*

The average particle size (diameter, nm), polydispersity index (PDI) and zeta potential of the blank NLC, R-NLC; PTX loaded NLC and R-NLC, Cou 6 loaded NLC and R-NLC were determined by a Malven zeta sizer (Malvern Instruments Ltd., UK), all the samples were diluted 10 times before test, and each sample was tested 3 times.

2.3.2. *Entrapment efficiency*

1 ml methanol was added into 0.1 ml PTX loaded NLC or R-NLC; the mixture was vortexed for 30 s to break the emulsion and to extract the PTX. A centrifugation was performed at 12,000 rpm for 10 min to separate the PTX. The content of the PTX in the supernatant was analyzed by a HPLC to decide the loaded amount of the PTX. The entrapment efficiency was calculated by Eq. (1) with *Wloaded* and *Wtotal* representing the loaded PTX amount and the total amount of PTX that was added, separately.

$$EE\% = \frac{Wloaded}{Wtotal} \times 100\% \quad (1)$$

The HPLC condition was as follows: the chromato bar was Diamonsil™ (diamond) C18 (5μ 200*4.6 mm, Dikma Technologies); the mobile phase was methyl cyanides-water (55:45); the flow rate was 1 ml/min; the tested wavelength was 227 nm; the column temperature was 25 °C and the sample size was 20 μl.

2.3.3. In vitro *drug release*

100 ml PBS (pH 7.4) containing 0.2% Tween 80 was chosen to be the release medium of PTX-loaded NLC and R-NLC. NLC and R-NLC were placed into the filter bags (MW 10 KDa) separately, and the filter bags were immersed into the release medium, then 1 ml release medium was taken out at predicted time point (2, 4, 6, 8, 10, 12, 24, 48 h), and 1 ml fresh medium was supplemented. The PTX content was tested using