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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.

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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 quantitatively 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 W_{loaded} and W_{total} representing the loaded PTX amount and the total amount of PTX that was added, separately.

$$EE\% = \frac{W_{loaded}}{W_{total}} \times 100\% \quad (1)$$

The HPLC condition was as follows: the chromatobar was Diamonsil™ (diamond) C18 (5 μ 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 μ l.

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

HPLC as described above, and the accumulative drug release was calculated.

2.4. Cellular uptake of Cou 6 loaded NLC and R-NLC

2.4.1. Quantitative analysis of cellular uptake of Cou 6 loaded NLC and R-NLC

A549 cells were seeded in the 24-well plate at the density of 1×10^5 cells/well. After the cell attachment for 24 h, the culture medium was discarded and the cells were incubated with FBS free DMEM/F12 1:1 medium containing Cou 6 loaded NLC and R-NLC with the Cou 6 concentration of 2 $\mu\text{g/ml}$ for 2 h to test the cellular uptake. After the incubation, the medium was again abandoned, and the cells were washed with PBS 3 times, then 100 μl RIPA cell lysate was added to each well to lyse the cells. 25 μl lysate was used to assay the protein content by Pierce® BCA Protein Assay Kit (Thermo Scientific). The residue was mixed with 100 μl ethanol to extract the Cou 6 internalized in the cells, and the Cou 6 concentration was calculated after read in an ELISA (Model ER-8000, Sanko Junyaku Tokyo, Japan) [11,12].

2.4.2. Cellular uptake by flow cytometry

1×10^5 A549 cells were seeded in the 24-well plate. After 24 h attachment, the culture medium was abandoned and FBS free DMEM/F12 1:1 medium containing Cou 6 loaded NLC and R-NLC at the Cou 6 concentration of 2 $\mu\text{g/ml}$ was incubated with the cells for another 2 h. The cells were rinsed with PBS 3 times after the incubation period to remove the NLC and R-NLC that were not internalized. The cells were trypsinized and harvested and the cell-associated fluorescence was quantitatively determined by flow cytometry in which 1×10^4 events were counted. Only viable cells were gated for fluorescence analysis.

2.4.3. Laser scanning confocal microscopy

A549 cells were seeded in a cover slip placed 24-well plate at the density of 1×10^5 cells/well. After the cell attachment, the medium was changed into FBS free fresh DMEM/F12 1:1 medium containing Cou 6 loaded NLC and R-NLC at the Cou 6 concentration of 2 $\mu\text{g/ml}$. After a further incubation for 2 h, the medium was discarded and the cells were rinsed 3 times with PBS. The cells were then fixed with 4% paraformaldehyde for 30 min at room temperature, and the nuclei was stained by DAPI. The cellular uptake was observed by a laser scanning confocal microscopy.

2.5. Cellular uptake mechanisms

To learn the cellular uptake mechanisms of NLC and R-NLC, a batch of cellular uptake inhibitors were employed. After the cell attachment of A549 cells on the 24-well plate at the density

of 1×10^5 cells/well, the culture medium was replaced with FBS free fresh DMEM/F12 1:1 medium containing different inhibitors including Colchicine (20 $\mu\text{g/ml}$), Chlorpromazine (10 $\mu\text{g/ml}$), Indomethacin (20 $\mu\text{g/ml}$), and NaN_3 (20 $\mu\text{g/ml}$) for 30 min. Then the Cou 6 loaded NLC and R-NLC were added at the Cou 6 concentration of 2 $\mu\text{g/ml}$. A further incubation was conducted for 2h, and then the cells were treated with the method under the session of "Quantitative analysis of cellular uptake of Cou 6 loaded NLC and R-NLC". The cellular uptake of both NLC and R-NLC with various inhibitors was calculated [10].

2.6. Cytotoxicity of PTX loaded NLC and R-NLC

A549 cells were cultured on the 96-well plate at the density of 1×10^3 cells/well, and incubated overnight to allow the cell attachment. At the second day, the medium was changed into fresh DMEM/F12 1:1 medium with 10% FBS and PTX loaded NLC and R-NLC at the PTX concentrations of 0.5, 1, and 5 $\mu\text{g/ml}$. The incubation was allowed to continue for another 6, 24 and 48 h. 10 μl MTT (5 mg/ml) was added into each well after the pre-determined time period and a further incubation of 4 h was carried out at 37 °C. Then the culture medium was abandoned and 150 μl DMSO was used to dissolve the formazan, and the absorbance of formazan was measured at 490 nm. Cells cultured without any NLCs were set to be the controlled group. And the cytotoxicity of PTX loaded NLC and R-NLC was calculated by Eq. (2).

$$\text{Inhibition ratio\%} = 1 - \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{controlled}}} \times 100\% \quad (2)$$

3. Results and discussion

3.1. Pharmaceutical characteristics

In the present study, the SA-R8 was employed to conjugate R8 with the NLC, the SA group was lipophil and mixed with the oil phase thus incorporated in the NLC, while the R8 part was hydrophilic, so it could stretch out on the surface of the NLC to form R8-modified NLC.

The average size and zeta potential of blank NLC and R-NLC, Cou 6 loaded and PTX loaded NLC and R-NLC were all tested in thrice. The results were listed in Table 1. A slight increase of average size and a strong elevation of zeta potential were found after the R8 modification, which indicated the successful incorporation of SA-R8 into the NLC [13]. No significant distinction of size and zeta potential was observed after the Cou 6 or PTX loaded. The entrapment efficiencies (EE%) of PTX

Table 1 – Size, PDI and zeta potential of various NLCs.

Parameters	Blank NLC	Blank R-NLC	Cou 6-loaded NLC	Cou 6-loaded R-NLC	PTX-loaded NLC	PTX-loaded R-NLC
Size (nm)	39.82 ± 0.57	40.36 ± 0.41	40.16 ± 0.63	42.65 ± 0.44	40.22 ± 0.39	42.34 ± 0.87
PDI	0.20 ± 0.0249	0.21 ± 0.0165	0.23 ± 0.0278	0.23 ± 0.0136	0.22 ± 0.0177	0.2 ± 0.0149
Zeta potential (mV)	-13.44 ± 1.21	16.66 ± 1.19	-12.43 ± 1.05	17.47 ± 1.31	-13.01 ± 1.05	16.84 ± 0.96

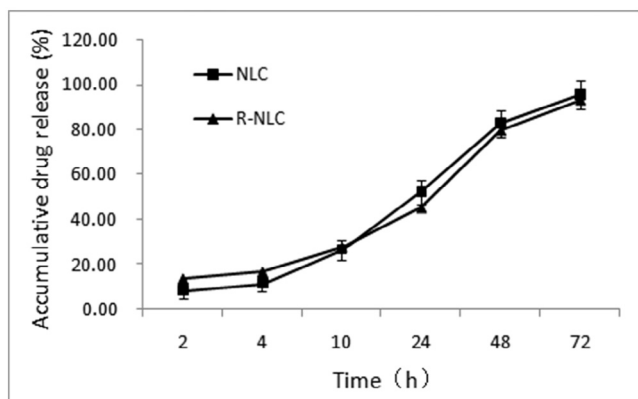


Fig. 1 – In vitro drug release of PTX loaded NLC and R-NLC.

loaded NLC and R-NLC were studied, and the results revealed that with R8 modification, the EE% of PTX decreased from 86.55% to 65.94%. As we have learned before, SA has some compatibility of PTX, but the EE% of PTX dropped when SA was added in the oil phase in the form of SA-R8. The reason to explain this phenomenon might be the steric hindrance. After SA-R8 was added in the oil phase, the R8 moiety may have affected the contact of SA and GMS with PTX, and also the dissolution of SA in GMS may have some influence on the PTX dissolution in GMS, which led to a depression of EE% in R8-modified NLC.

The in vitro drug release of the two preparations was showed in Fig. 1. As the release curve revealed, both NLC and R-NLC gave out over 90% PTX at the time point of 72 h, and a sustained drug release was achieved. A sustained release profile was very important in the antitumor drug delivery system, because it could guarantee the minimal exposure of the drug to a healthy tissue while increasing the accumulation of the therapeutic drug in the tumor site [14].

3.2. Cellular uptake

Coumarin 6 (Cou 6) was frequently used as a fluorescent tracker to observe or to measure the nanocarrier uptaken by cells. In the present study, three testing methods were employed to determine the cellular uptake of Cou 6 loaded NLC and R-NLC.

A quantitative analysis by extracting the internalized Cou 6 was conducted. The A549 cells were incubated with both two preparations at the Cou 6 concentration of 2 µg/ml for 2 h. Then the cells were rinsed and lysed, and the total protein amount was determined by Pierce® BCA Protein Assay Kit; the aim of this step was to evacuate the influence of cell quantity on cellular uptake. Meanwhile, ethanol was added to the residues to extract the Cou 6 internalized in the cells, and the concentration of Cou 6 was obtained by an ELIASA. The Cou 6 concentration was corrected by the protein content, and the standardized cellular uptakes of the two preparations were calculated and compared. As can be seen in Fig. 2, NLC and R-NLC both showed strong cellular uptake, and the internalization of R-NLC was around 4 times higher than NLC.

Besides the method above, we employed flow cytometry to demonstrate the better cellular uptake of R-NLC over NLC. Fig. 3

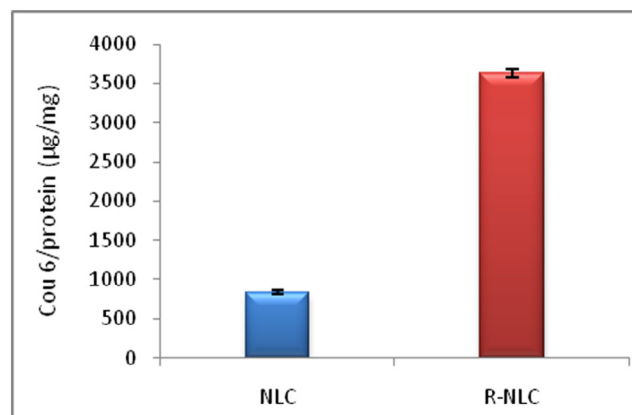


Fig. 2 – Quantitative analysis of cellular uptake of NLC and R-NLC.

gave out the result that the fluorescent intensity of R-NLC was a lot stronger than that of the NLC, which was in accordance with the extracting method.

Extracting method and flow cytometry are two methods that are widely used in the quantitative analysis of cellular uptake. Each has its advantages and disadvantages. Extracting method was used a lot before and at the beginning of the development of flow cytometry [11,12,15], and it reveals the average cellular uptake amount after a group of cells incubation with the preparations, representing the cellular uptake amount in the mass irrespective of the single cells. Besides, the outcomes of different groups could be calculated to estimate the quantity relationships. The principle of the flow cytometry was to detect the cells in the individual form [16]. In cellular uptake assay, various internalizations in individual cells could happen according to different contact angles between cells and the preparations [17], which would be reflected in the outgoing curves. So in the results of flow cytometry not only the cellular uptake trend but also the internalization variation of single cells could be found. However, there is a shortcoming of the flow cytometry which is the cell proliferation ability that may affect the appearance of the curve. In the present study, both the two methods were used, and the results were in the same tendency. The outcomes of the two methods corroborated with each other, indicated that with R8 decorated on the surface, the cellular uptake elevated noticeably. Also it was believable that both the two methods used here could represent the cellular uptake change.

The laser scanning confocal microscopy was used to visualize the cellular uptake of Cou 6 loaded NLC and R-NLC at 2 h (Fig. 4). NLC and R-NLC groups both exhibited obvious green fluorescence, indicating the localization of the two preparations. Fig. 4 showed that the NLC dispersed in the cytoplasm, and scarcely any green fluorescence emerged in the nuclei. Meanwhile the fluorescent intensity of A549 cells incubated with R-NLC was exceedingly stronger than that with NLC, and a prominent distribution in the nuclei was observed, suggesting that R8 had the ability to deliver NLC to the cell nuclei.

A similar result was presented in the research of Biswas et al. [18], that after the surface modification of CPP, the liposome visibly entered the nuclei. In fact, many groups have found that

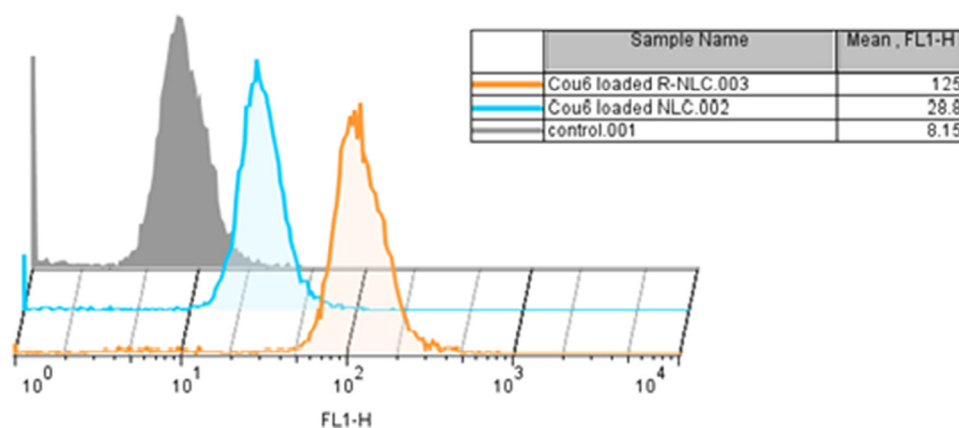


Fig. 3 – Flow cytometry analysis for the Cellular uptake of Cou 6-loaded NLC and R-NLC.

CPPs could mediate the location of cargos to the cell nuclei [19–22], and such ability has put CPPs to an important place in the delivery of RNAs, which is a popular subject in current CPPs research [23–25]. The definition of CPPs implies that they are small peptides which could penetrate the cell membrane, and the membrane here indicated not only the plasma membrane, but also other membranes like the nuclei membrane and lysosomal membrane [26].

R8 is a member of the CPP family; it is designed and synthesized according to the arginine-rich structure of the Penetratin CPP [27]. Polyarginine was uncomplicated to synthesis and its cell membrane penetrating efficiency was repeatedly proven [22,24,28,29]. R8 is an oligo-peptide containing highly positive net charges, and the surface charge turns negative to positive after the modification of R8 on NLC. It was reported that the interaction between the positively charged CPP and the negatively charged cell surface took responsibility of the faster and added cellular uptake of CPP modified nanocarrier [26]. In our research, the R-NLC well and truly entered the cells a lot more than NLC, and the result was consistent with the theory.

3.3. Cellular uptake mechanisms

We also investigated the cellular uptake of NLC and R-NLC in the presence or absence of several inhibitors. Colchicine, Chlorpromazine, Indomethacin and NaN_3 were selected as four uptake inhibitors. The cellular uptake outcomes were compared with that with no inhibitors (set as the controlled group). As shown in Fig. 5, R-NLC exhibited much higher internalization value than NLC, which was in accordance with the result of cellular uptake. In addition, the four inhibitors affected the cellular internalization of both NLC and R-NLC evidently. When Colchicine was added to the cells, the cellular uptake of NLC and R-NLC dropped to 49.11% and 77.25% of the controlled group, signifying that macropinocytosis was involved in the intake of NLC and R-NLC; when the cells were pretreated with Chlorpromazine, the NLC and R-NLC uptake decreased to 50.13% and 84.06% of the controlled group, indicating the participation of clathrin in the uptake of NLC and R-NLC. It is worth noting that, with Indomethacin appended to the cells, the cellular uptake of NLC showed no prominent difference with the controlled group, while the uptake of R-NLC dropped to 53.66% of control

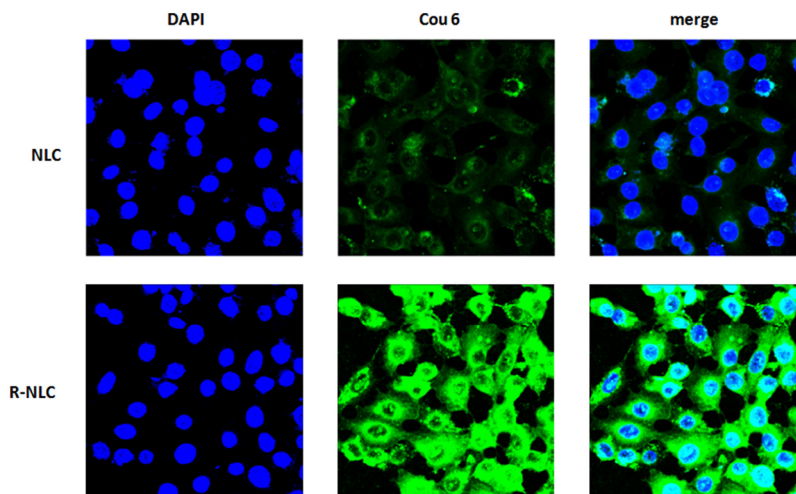


Fig. 4 – Cou 6 loaded NLC and R-NLC internalization visualized by CLSM.

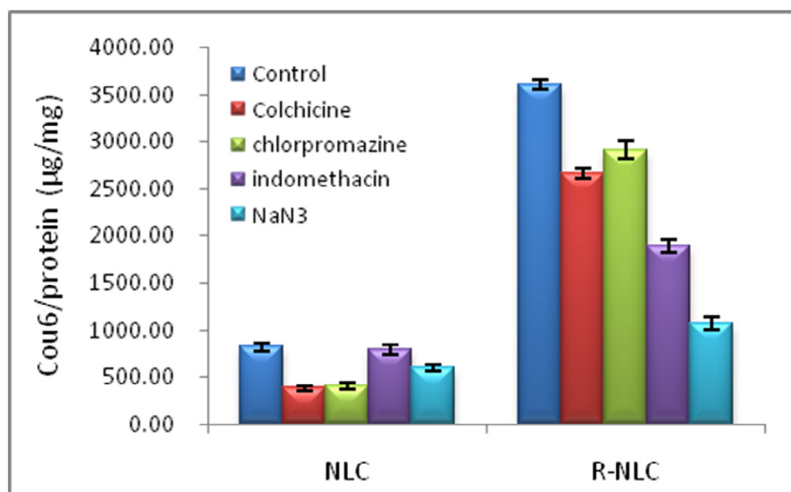


Fig. 5 – Quantitatively analysis of cellular uptake with inhibitors.

group. This phenomenon declared that with R8 modified on the surface of NLC, the cellular uptake path changed to some extent, and that caveolin took responsibility of R-NLC uptake [30]; when NaN₃ was added, the internalization of both preparations descended apparently, with NLC decreased to 73.26% and R-NLC dropped to 30.70% of the controlled group, indicating that the cellular uptake of R-NLC needed more energy than NLC [10,30].

There has been a considerable interest in exactly how CPPs enter cells from the beginning of their discovery. The more we learn about the mechanisms, the better understanding we gain about the interaction between the preparations and the cells. Vast investigations on the cellular uptake mechanisms of CPPs and CPPs-modified nanopreparations have been conducted. It's known how CPPs and CPPs-modified nanocarriers enter the cells depending not only on the type of the CPPs and their concentrations but also on the properties of the cargo that they transport, and the cell line on which they were tested, and even the density of CPPs on the cargo surface [26,27,31,32]. The

manuscript indicated that the cellular uptake mechanisms of R8-modified NLC to A549 cells involved at least with energy, macropinocytosis, clathrin-mediated endocytosis, and caveolin-mediated endocytosis.

3.4. Cytotoxicity of PTX loaded NLC and R-NLC

In this part of the research, model drug PTX was loaded in NLC and R-NLC, and incubated with A549 cells at different concentrations for different times. The cytotoxicity results were listed in Fig. 6. The cytotoxicity of PTX loaded NLC and R-NLC increased as time and dosage amplified, and when analyzed at the same time and dosage, R-NLC exhibited greater cytotoxicity over NLC. The high dosage group (5 µg/ml) revealed maximum cytotoxic difference at 6 h, with inhibition ratio of R-NLC at 65.97% and NLC at 21.21%. The results of low concentration (0.5 µg/ml) and medium concentration (1.0 µg/ml) also demonstrated the conclusion that with R8 modified, R-NLC

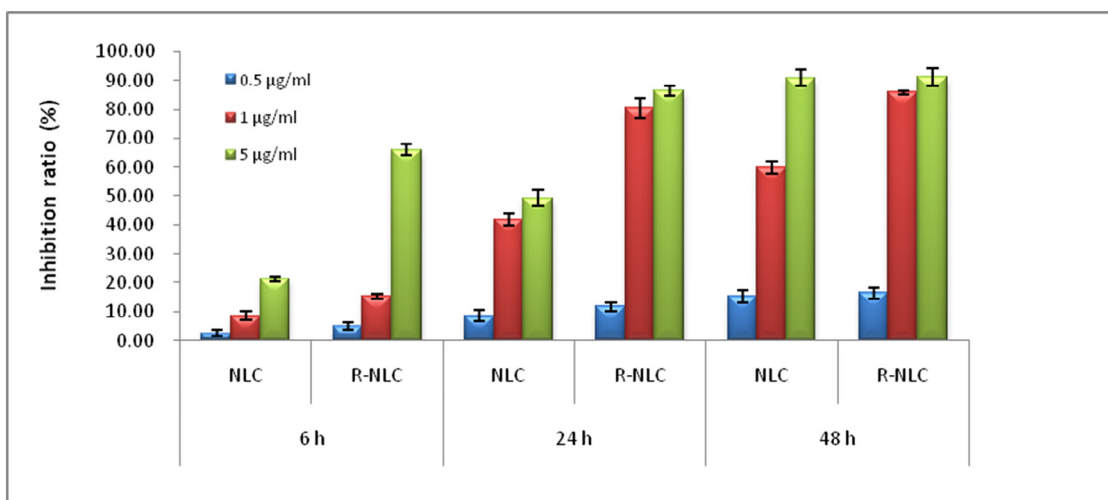


Fig. 6 – Cytotoxicity of PTX loaded NLC and R-NLC at different concentration and time.

represented stronger cytotoxicity. The consequence was consistent with the tendency of cellular uptake, more R-NLC entered the cells than NLC in a short period, and as PTX was released constantly, more cells were killed by R-NLC than NLC.

Cytotoxicity is an important index to evaluate the antitumor drugs and antitumor preparations. In the current study, we have certified that after R8 modification, R-NLC exhibited greater cytotoxicity to A549 cells.

4. Conclusion

In the present manuscript, we constructed the short cell penetrating peptide R8 modified NLC (R-NLC), the average size of which was around 40 nm, meeting the requirements of leaking to the tumor tissue by EPR effect. The *in vitro* drug release in 72 h was inspected, and a sustained release was guaranteed. A green fluorescent dye Coumarin 6 was employed to study the cellular uptake of R-NLC comparing with Cou 6 loaded NLC, and three methods were carried out to prove that the internalization of R-NLC was as high as 4 times to NLC at 2 h incubation with A549 cells. The cytotoxicity research of PTX loaded NLC and R-NLC was conducted, and the results implied that PTX loaded R-NLC exhibited much stronger cytotoxicity than PTX loaded NLC. This work has proved that with R8 decorated on the NLC surface, both the pharmaceutical characteristics (especially zeta potential) and the *in vitro* influence on A549 cells changed greatly. However, although CPPs can generate greater cellular uptake and elevate the therapeutic efficacy, CPPs have no specificity and they could not identify tumor cells, so they have some negative influence on normal tissue. The topic on how to strengthen the targeting ability of CPPs is a task worthy of research. And the topic is the next research objective of our group.

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