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Original Research Article

Pegylated niosomal nanoparticles loaded with vincristine: Characterization and in vitro evaluation

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Abstract

Purpose: To investigate the effect of pegylated niosomal vincristine (VCR) on enhanced performance, drug resistance and prolonged blood circulation time.
Methods: Pegylated niosomal VCR was synthesized by reverse phase evaporation. The mean diameter, size distribution, and zeta potential of pegylated niosomal VCR were evaluated using a Zetasizer. The half-maximal concentration (IC_{50}) values of pegylated niosomal VCR and standard VCR were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The impact of pegylated niosomal VCR on apoptosis and cell cycle of BCL1 lymphoma cancer cells were investigated.
Results: The mean diameter, size distribution and zeta potential of pegylated niosomal VCR were 220 nm, 0.4, and −18.8 mV, respectively. Cell proliferation was evaluated using the MTT assay. The IC_{50} values of pegylated niosomal VCR and standard VCR were 1.6 and 3.5 µg/mL, respectively, after a 24-h incubation. The cytotoxicity of pegylated niosomal VCR was twice that of standard VCR. Furthermore, flow cytometric analysis of the cell cycle showed that pegylated niosomal VCR induced greater mitotic arrest than did standard VCR.
Conclusions: The findings demonstrate the effective antitumor activity of pegylated niosomal VCR compared with standard VCR.

Keywords: Niosome, Anti-tumour, Polyethylene glycol, Vincristine, Encapsulation, Lymphoma

INTRODUCTION

Vincristine sulfate (VCR) is a water-soluble alkaloid that can be extracted from *Catharanthus roseus* [1]. Clinically, VCR is used to treat various cancers, including lymphoma. The molecular mechanism of the drug involves inhibition of microtubules, leading to inhibition of cell division during metaphase of mitosis [2]. However, along with its anticancer activity, VCR has severe side effects, such as neurotoxicity and peripheral sensorimotor neuropathy. Multidrug resistance has limited its clinical application. Indeed, the neuronal tubulin binding of VCR disrupts axonal microtubules, which can lead to autonomic and peripheral sensorimotor neuropathy [3].

To decrease the side effects and simultaneously increase the therapeutic activity of VCR, studies

have attempted to develop efficient drug delivery systems [4]. One effective strategy to achieve localized drug action is to entrap drugs in nanoparticles, since their small size results in enhanced drug performance and circumvents drug resistance [5]. Moreover, nanoparticle biodegradability leads to sustained release of drugs at the target site for days or weeks [6]. Various nanoparticle drug delivery systems have been reported for VCR [7].

Niosomes are vesicles consisting of cholesterol and nonionic surfactants [8] that can be used to entrap lipophilic and amphiphilic drugs [9]. Polyethylene glycol (PEG) is generally used for steric stabilization of nanocarrier-based drug delivery systems. It is a biocompatible, non-toxic, non-immunogenic polymer used to modulate the stability, solubility, plasma half-life, and clearance of different compounds. PEG can reduce carrier uptake by the reticular endothelial system, which prolongs the blood circulation time [10].

In this study, VCR was encapsulated in pegylated-niosomal nanoparticles using a reverse phase evaporation method. The nanoparticles were characterized in terms of size, size distribution, zeta potential, drug loading and encapsulation efficiencies, morphology, stability, and drug release. Finally, the efficacy of the nano-drug was evaluated according to cell cycle arrest [11] and apoptosis of the BCL1 cell line *in vitro* using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry.

EXPERIMENTAL

Materials

Fetal bovine serum (FBS), RPMI 1640 medium supplemented with glutaMAX, minimum essential medium containing glutamine and trypsin/ethylenediamine tetraacetic acid (1× solution), and penicillin/streptomycin solution were obtained from Gibco-BRL. The BCL1 murine lymphoma cell line was supplied by the National Cell Bank, Pasteur Institute of Iran. Polyethylene glycol 3000 (PEG 3000), MTT solution, Span 60, and vincristine were procured from Sigma-Aldrich USA. Ethanol and isopropanol were purchased from Merck, Germany. All other reagents used were of analytical grade.

Preparation of pegylated niosomal VCR

Niosomes were prepared by a reverse phase evaporation method. Briefly, Span 60, cholesterol, PEG 3000, and VCR (7:3:1:1 molar ratio) were dissolved in 15 mL 96 % ethanol and stirred for 45 min at 300 rpm and room temperature. The solvent was evaporated on a rotary evaporator (Heidolph, Germany) at 90 rpm and 45 °C in a vacuum. The pegylated niosomal vesicles were formed after introducing normal saline (10 mL). The final concentrations of Span 60, cholesterol, PEG 3000 and VCR were 12.5, 5.3, 0.9, and 0.9 mM, respectively. The formulation was sonicated at 60 Hz in a bath sonicator (Bandelin Sonorex Digitec) for 10 min. Pegylated nanoparticles devoid of drug were prepared using this method.

Characterization of nanoparticles

The mean size, size distribution, zeta potential, shape, and probable crystallization of the pegylated nano-niosomal particles and pegylated nano-niosomal particles devoid of VCR were evaluated using the Zetasizer Nano Zen 3600 (Malvern Instruments, UK) and electron microscope (Nikon, Tokyo, Japan). The morphologies of both lyophilized nanoparticles were assessed by scanning electron microscopy (SEM). To assess the entrapment efficiency, the unentrapped drug was removed by gel filtration from pegylated niosomal VCR through a Sephadex G-10 column and eluted with phosphate-buffered saline (PBS; pH 7.4). Subsequently, the vesicles were disrupted thoroughly using 0.1 % Triton X-100 and analyzed spectrophotometrically (UV-1601 PC, Shimadzu) at λ = 297 nm. Encapsulation efficiency (EE%) was calculated using Eq 1.

$$EE\ (\%) = \{(D_t - D)/D_t\}100 \quad \ldots\ldots\ldots\ldots\ldots\ (1)$$

where D_t is the total amount of drug used to prepare the nanoparticles, and D is the amount of unentrapped drug

In vitro release study

The rate of vincristine release from pegylated nano-niosomes was determined using a membrane diffusion technique. For this purpose, a pegylated niosomal VCR suspension was centrifuged (14,000 rpm, 4 °C, for 1 h) to remove the unentrapped drug in the supernatant. Then, the pellet was resuspended in PBS (pH 7.4). The vesicle suspension was added to dialysis tubing (10,000 Da cutoff, Sigma-Aldrich) and immersed in 50 mL PBS (pH 7.4, 37 °C) with constant shaking (150 rpm) using a magnetic stirrer. At different time intervals, the drug content of the buffer was analyzed at λ = 297 nm, and the percentage of drug release was determined with respect to the total amount of drug entrapped using a standard curve.

Evaluation of cytotoxic activity

The cytotoxic effects of standard and pegylated niosomal VCR were evaluated by MTT assay. The BCL1 cells were cultured in 96-well plates (1×10^4/well) and incubated under 5 % CO_2 at 37 °C in RPMI 1640 medium containing 10 % FBS and 1 % penicillin/streptomycin for 24 h to promote their adhesion to the bottom of the plate. Then, the culture medium was replaced with medium containing standard VCR, pegylated niosomal VCR, or blank niosomal nanoparticles and then incubated for 24 or 48 h. After incubation, the formulations were replaced with tetrazolium salt (MTT solution) dissolved in PBS (0.5 mg/mL PBS) for 3 h. The MTT solution was then removed, and the formazan crystals that had deposited after oxidation were dissolved in isopropanol. The solubilized formazan was quantified using a conventional ELISA plate reader (BioTek Instruments , VT, USA) at λ = 540 nm. The IC_{50} was determined using Pharm-PCS software.

Cell cycle analysis

Cells were seeded at a density of 1.0×10^6/well in six-well plates and cultured in RPMI 1640 medium, as described above. After 24 h, the medium was replaced with medium containing standard or entrapped VCR, and the cells were incubated for 24 h. Subsequently, the cells were collected, washed, suspended in cold 1× PBS, fixed in 75 % ethanol, and stained with propidium iodide. The cell cycle distribution was then determined using the BD FACSCalibur flow cytometer. FCS Express ver. 3.0 software was used to analyze the data.

Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analyzed by SPSS ver. 13.0. $P < 0.05$ was considered significant.

RESULTS

Characteristics of the nanoparticles

The mean diameter, size distribution, and zeta potential of pegylated niosomal VCR were estimated to be 220 nm, 0.4, and −18.8 mV, respectively. Light microscopy demonstrated that the nanoparticles were in the form of unilamellar vesicles (ULVs), with spherical to ellipsoid hollow forms dispersed throughout the matrix (Figure 1). Crystallization was not apparent in the nanoparticles. The SEM results confirmed the preparation of nanoparticles with smooth spherical surfaces (Figure 1). The EE of the pegylated niosomal VCR was 81 %.
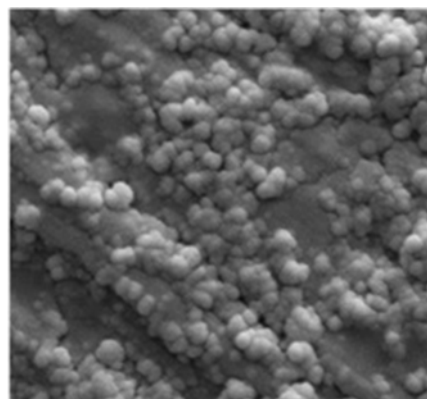
Figure 1: SEM micrograph of pegylated niosomal VCR nanoparticles (magnification: 2000×)

In vitro drug release

Pegylated niosomal VCR showed a drug release burst during the first 3 h, during which 21 % of the total release occurred (Figure 2). This was followed by sustained drug release until the end of the investigation. The amount of encapsulated VCR in pegylated niosomal nanoparticles released after 36 h was 69 %.
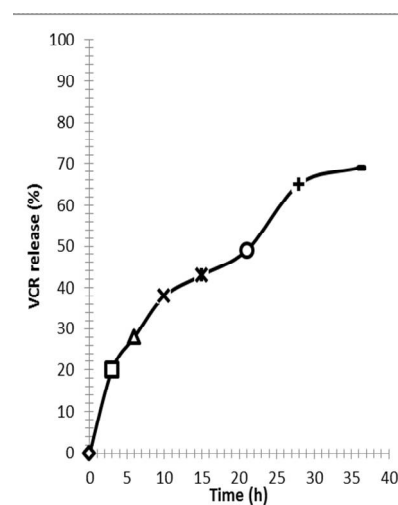
Figure 2: Profile of the release of pegylated niosomal VCR in PBS buffer from niosomal VCR, shown as a percentage, at different times. A drug release burst occurred during the first 3 h. Values are the average of three independent experiments + 5% error.

In vitro cytotoxicity

The antitumor activity of standard VCR and pegylated niosomal VCR was evaluated by MTT assay using the BCL-1 murine lymphoma cell line. The cytotoxicities of standard VCR and pegylated niosomal VCR were evaluated after 24 and 48 h and found to be independent of the

dose. However, the cytotoxic effect was more remarkable when the drug was associated with nanoparticles. The IC_{50} values for standard and pegylated niosomal VCR were 3.5 and 1.6 µg/mL after 24 h, respectively, and these values decreased to 2.8 and 1.2 µg/mL after 48 h, respectively.

## Encapsulated VCR stability

The physical characteristics of pegylated niosomal VCR did not change significantly after storage at 4 °C for 4 months. The particle size increased from 220 nm to 255 nm after storage. The zeta potential of the pegylated niosomal VCR decreased after storage, from 18.8 to 13.6.

Cell cycle

VCR depolymerizes tubulin, causing G2/M arrest. Flow cytometric analysis showed an increase in the proportion of cells arrested in G2/M after treatment with pegylated niosomal VCR compared with standard VCR after 24 h. The pegylated niosomal VCR was more efficient at arresting cells in the G2/M phase (Figure 3).

DISCUSSION

A novel formulation for delivery of greater amounts of VCR was developed as an alternative to standard VCR, which has several side effects, including peripheral neuropathy and temporary blindness [12]. The results showed that reverse phase evaporation is suitable for preparing pegylated niosomal VCR nanoparticles [13], considering the high VCR entrapment efficiency. This process used lipophilic non-ionic surfactant (Span 60) and cholesterol to form spherical vesicles. PEG 3000 was introduced to produce PEG-coated niosomes to prolong their blood circulation time. PEG can inhibit niosome opsonization by preventing RES uptake [14]. The size, size distribution, and zeta potential of the nanoparticles were satisfactory. The size distribution value of 0.4 indicated that the nanocarriers containing VCR were relatively homogenous [15]. The zeta potential of pegylated niosomal VCR was 18.8 mV, indicating the stability of the particles. Light microscopy suggested that the ULVs resulted from sonication. Similarly, SEM indicated that the prepared nanoparticles were spherical with smooth surfaces. The cytotoxicity analyses revealed localization of the nanoparticles at the cell cytoplasm and a higher intracellular concentration of pegylated niosomal VCR compared with standard VCR.
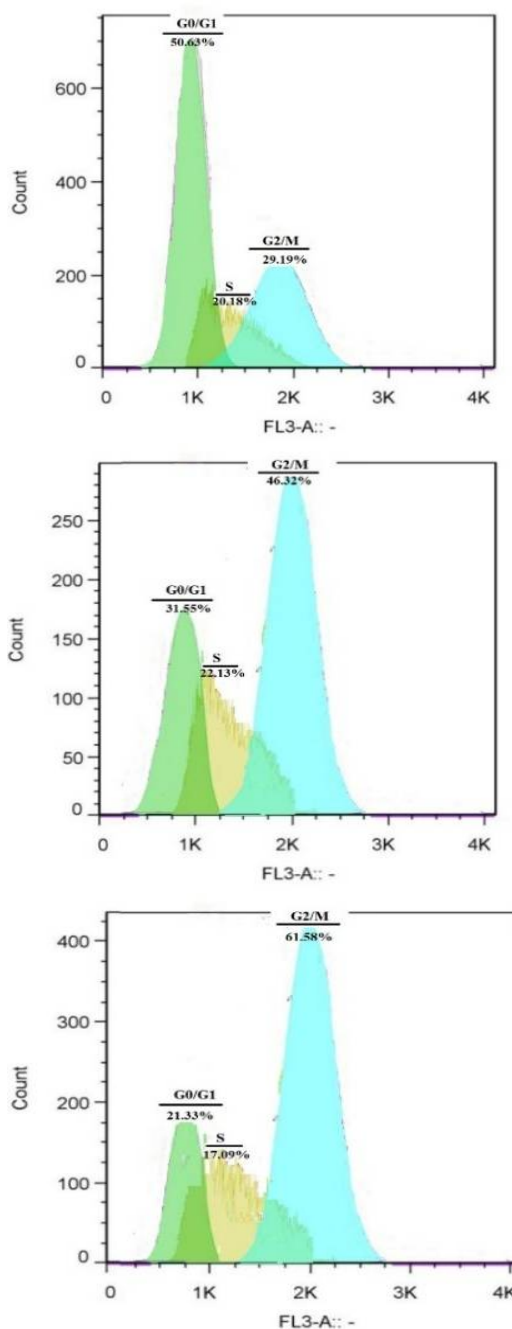
Figure 3: Changes in the BCL-1 cell cycle distribution induced by VCR. BCL-1 cells were treated with standard or pegylated niosomal VCR for 24 h, and the cell cycle was analyzed by flow cytometry. Representative figures show the percentages of cells in each phase of the cell cycle

The burst release of VCR occurred during the first 3 h. The observed burst release during the initial phase of the release profile may have resulted from VCR molecules adsorbed to PEG moieties on the vesicle surface [16].

The pegylated niosomal nanoparticles were capable of potentiating the cytotoxic effects of

VCR. The increase in cytotoxicity indicated a sustained VCR release from nanoparticles, resulting from increased penetration of pegylated niosomal VCR into cells, compared with the standard drug [17]. Nanoparticles devoid of VCR had no cytotoxic effect at the ranges evaluated. The flow cytometry results are in agreement with the fact that the capsulated formulation delivered more of the drug into lymphoma cells and induced a greater percentage of cell cycle arrest in the G2/M phase [18].

CONCLUSION

Reverse phase evaporation technique is suitable for preparing pegylated niosomal VCR nanoparticles. Spherical nanoscale ULVs had smooth surfaces and high entrapment efficiency, and were sufficiently stable and more effective than standard VCR. Furthermore, they significantly increased the cytotoxic effect of VCR against lymphoma cell line. This is probably due to the interaction between the vesicular carrier and lymphoma cancer cells, causing increased drug penetration. The findings also showed that pegylated niosome is an appropriate carrier for VCR delivery to lymphoma cancer cells.

DECLARATIONS

Acknowledgement

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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