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Vincristine loaded pegylated liposomal drug delivery for efficient treatment of acute lymphoblastic leukaemia

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Abstract: Vincristine sulfate (VIN) is commonly employed as a cytotoxic agent in the treatment of hematological malignancies, particularly acute lymphoblastic leukaemia (ALL). However, its maximum therapeutic benefits have been hindered due to the dose-dependent neurotoxic effects it can induce, which traditionally manifest as autonomic and peripheral sensory-motor neuropathy. The innovative approach aimed to address VIN's neurotoxic limitations while preserving its therapeutic efficacy in combating hematological malignancies, including ALL. The liposomes were prepared using the reverse-phase evaporation method. This method involved the encapsulation of VIN within liposomes through a controlled evaporation process. Secondly, PEGylated liposomes were synthesized through PEGylation. The liposomes were examined using scanning electron spectroscopy, revealing a smooth and spherical surface morphology. The particle size of the liposomes ranged from 90±0.5 to 120±0.4 nm. The encapsulation efficiency of the liposomes was found to be 77.24% and the highest drug release reached 95% over 50 hours. Cytotoxicity studies demonstrated that the liposomal formulation exhibited a non-toxic nature. Furthermore, in an *in-vivo* cellular uptake study, the PEGylated liposomes showed efficient accumulation within tumor cells. The liposomal formulation demonstrated superior effectiveness in treating ALL compared to the pure form of the drug.

Keywords: Vincristine, liposomes, acute lymphoblastic leukaemia, PEGylation, toxicity

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INTRODUCTION

Lymphoblastic leukaemia refers to a type of leukaemia that primarily affects lymphocytes, a type of white blood cell. It can be either acute lymphoblastic leukaemia (ALL) or chronic lymphoblastic leukaemia (CLL) (Terwilliger *et al*., 2017). ALL stands as the most prevalent form of childhood cancer. It emerges when errors in the DNA of bone marrow cells occur. Common indications encompass swollen lymph nodes, unexplained bruising, elevated temperature, bone discomfort, gum bleeding and recurring infections (Inaba *et al*., 2020). The prevalence of ALL is noteworthy, as it stands as one of the most prevalent types of cancer among children. This condition arises when errors in the DNA of bone marrow cells lead to uncontrolled growth of immature lymphocytes (Malik *et al*., 2022). The exact prevalence can vary by region and demographic factors, but globally, ALL is a significant concern in Pediatric oncology. Advances in medical understanding and treatment options have improved outcomes for many patients, but continued research and awareness efforts are vital in addressing this challenging disease (Bommer *et al*., 2018).

Modern ALL treatment involves personalized plans with chemotherapy, targeted therapies, stem cell transplants for high-risk cases and corticosteroids. Radiation, supportive care, and minimal residual disease monitoring are crucial. Emerging immunotherapies and clinical trials drive advancements, offering hope for effective ALL management (Inaba *et al*., 2021). The current treatment of ALL faces several challenges. The intensity of chemotherapy and other treatments can lead to severe side effects, impacting patients' quality of life. Additionally, drug resistance and relapse remain concerns, requiring constant adaptation of treatment plans (Wang *et al*., 2019). The high cost of novel therapies and limited access to advanced treatments pose barriers to optimal care. Furthermore, the complex genetic and molecular heterogeneity of ALL demands more precise and personalized approaches. Lastly, long-term effects on paediatric patients' development and overall health underscore the need for improved survivorship care (Malczewska *et al*., 2022).

Vincristine is a chemotherapy drug commonly used in the treatment of ALL. It belongs to a class of drugs called vinca alkaloids and works by disrupting the assembly of microtubules, which are essential for cell division. Vincristine's mechanism of action makes it effective in slowing down the growth and division of rapidly dividing cells, including cancer cells (Douer 2015). The use of vincristine in treating ALL presents multifaceted challenges. Foremost is its potential to induce neurotoxicity, resulting in peripheral neuropathy that affects patients' well-being and necessitates treatment adjustments (Sawalha *et al*., 2018). Achieving the right therapeutic balance while mitigating neurotoxicity's impact is complex. Patient compliance might suffer due to discomfort, potentially influencing treatment outcomes. Managing vincristine-induced neuropathy requires

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collaboration among various medical disciplines, especially in pediatric cases where balancing treatment efficacy and minimizing neurotoxicity is pivotal. Survivorship care becomes crucial due to enduring neurotoxic effects even post-treatment. Ongoing research seeks solutions, exploring alternative approaches and neuroprotective agents to lessen vincristine-induced neurotoxicity's burden (Tay *et al*., 2017).

Vincristine-loaded PEGylated liposomes show significant potential for advancing ALL treatments. By attaching polyethylene glycol (PEG) molecules to liposomes, these nanoparticles gain improved stability, prolonged circulation in the bloodstream and enhanced drug delivery precision (Wang *et al*., 2016). This approach directly addresses challenges posed by conventional vincristine use, effectively reducing its neurotoxicity by allowing controlled, prolonged release, while actively increasing drug concentration at leukaemia cells through the enhanced permeability and retention (EPR) effect (Mohamed *et al*., 2019). This results in heightened therapeutic efficacy and diminished side effects, potentially enabling optimized dosing regimens and personalized treatment strategies for improved patient outcomes (Shukla *et al*., 2023).

The objective of the present study is to reduce the dose-dependent neurotoxic effect of VIN by developing VIN-loaded PEGylated liposomes with enhanced cellular uptake and accumulation with efficient treatment in ALL.

MATERIALS AND METHODS

Materials

Vincristine sulfate (VIN) was obtained as a gift sample from Shouguang Fukang Pharmacy Factory (Shandong, China). 1, 2- Dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC) Cholesterol was purchased from Lipoid GmbH, Ludwigshafen Germany; Disodium hydrogen phosphate, Potassium dihydrogen phosphate, Sucrose and Chloroform were purchased from Shanghai Chemical Co. (Shanghai, China). All other materials were of analytical or reagent grade.

Methods

Preparation of VIN-loaded liposomes (VIN-LP) by thin-film hydration method

The film hydration methodology was used to encapsulate the VIN in liposomes. DPPC and CH were dissolved in RBF using chloroform and considered as a lipid solution. Pre-dissolved MXF at a concentration of 50mg/5ml of methanol was added to the lipidic phase. The organic solvent was evaporated from the drug-lipid phase using a rotary evaporator under reduced pressure at 40ºC to form a very thin film of the drug embedded in the lipid. The resulting dry film was hydrated with pH 7.4 saline buffer. To generate multilamellar liposomes, the suspension was physically agitated at RT for 1hour. To achieve full lipid hydration, the liposomal dispersion was kept at 40ºC overnight. Using a cooling ultracentrifuge, VIN-encapsulated liposomes were separated (30,000 RPM, -50^{0}C, time of 3 hours). The liposomal suspension was freeze-dried by the addition of trehalose at -50^{0}C for 40 hours under a vacuum. For the subsequent analysis, either liposomal solution or powder was used (Zhang 2017).

PEGylation of VIN-loaded liposomes (VIN-LP-PEG)

Following the lyophilization of the liposomes containing VIN (VIN-LP), the PEGylation process was conducted. Specifically, VIN-LP were exposed to 1500µg of PEG 400, which possesses a molecular weight in the range of 8000 to 10,000. This amount of PEG was utilized per milligram of liposomes. The next step involved diluting the mixture with PBS buffer, resulting in a final volume of 200µL. The dilution was performed to achieve the desired concentration. Subsequently, the mixture was subjected to an incubation period of 30 minutes at room temperature, during which a rotary shaker was employed to facilitate gentle agitation (Alavi *et al*., 2022).

Characterization of VIN-LP and VIN-LP-PEG

Particle size and FESEM studies

In the purified water, dried liposomes were dispersed using sonication for a minute. Using a Zetasizer, the particle size, PSD and zeta potential were measured at room temperature. Utilizing FESEM, the liposomes' surface properties were investigated. For electrical conductivity, the liposomes were coated with gold. A vacuum was kept up during the entire procedure (Paliwal *et al*., 2019).

Drug encapsulation efficiency (EE)

HPLC was used to determine non-encapsulated VIN from the supernatant recovered after centrifugation. Acetonitrile: HPLC water (80: 20) was used as the mobile phase. The samples were analysed at 240 nm wavelength (He *et al*., 2019).

$$EE\ (\%) = \frac{(Total\ drug - Free\ drug)}{Total\ drug}\ X\ 100$$

VIN release from liposomes

Dialysis tubing was used to determine the amount of VIN released from liposomes (MWCT 5 kDa). The VIN encapsulated liposomes eq. to 20mg of VIN was suspended in pH 6.8 phosphate buffer and this tube was immersed in the beaker containing buffer solution maintained at 37°C. The entire system was kept on the magnetic stirrer and stirred throughout the experiment. To maintain sink conditions, 5ml of the solution was added whenever the sample was withdrawn. The amount of VIN released from the liposomes was analysed using HPLC. The in vitro VIN release was also determined from VIN-LP-PEG (Mao *et al*., 2016).

Cell line and cytotoxicity study

The cell line Jurkat (T-cell acute lymphoblastic leukemia) was acquired from a local hospital. Before treatment, cells

were cultured for 24 hours in DMEM supplemented with 10% fetal bovine serum, 100g/mL streptomycin, and 100 IU penicillin at 37°C in a humidified environment containing 5% CO_2 at $1x10^4$ cells/well in 96-well plates. For 24 hours, the cell lines co-cultured with VIN, VIN-LP and VIN-LP-PEG at varied concentrations. The viability of the cells was studied by using the MTT assay technique. MTT (1mg/mL) was allowed to incubate with the substance under the investigation. The quantity of MTT transformed to insoluble formazan was used to confirm cell viability. The undissolved crystal was dissolved in a 1:14 (v/v) combination of 1M HCl and isopropyl alcohol and agitated for 20 minutes at room temperature (PJ RJ *et al*., 2022; Wang *et al*., 2019).

Cellular uptake studies

This study was used to determine VIN accumulation in Jurkat (T-cell acute lymphoblastic leukemia) cell lines. Quantification of MXF cellular uptake was done using the HPLC technique. In 12-well culture plates, Jurkat (T-cell acute lymphoblastic leukemia) cell lines were seeded. Both cell lines were kept at a density of 104 cells per well and incubated for 20 hours. The test chemicals (VIN, VIN-LP and VIN-LP-PEG) were given to the grown cells at doses ranging from 0.02 to 1.0M for 6 hours. The treated cells were trypsinized, washed thrice with physiological saline solution, centrifuged and treated with lysis buffer before being sonicated. Using the HPLC technique, the accumulated VIN was measured (in supernatants) (PJ RJ *et al*., 2022; Wang *et al*., 2019).

RESULTS

Characterization of VIN-loaded liposomes

The liposomes were prepared with varying molar concentrations of DPPC and cholesterol ranging from 1:0.5, 1:1, 1:1.5 and 1:2 ratios respectively (table 1). The FESEM analysis (fig. 1) of the liposomes demonstrated excellent sphericity as well as smooth characteristics of the VIN-loaded formulations. The surface characteristics also revealed very uniform and without cracks of the liposomes (Guimarães *et al*., 2021).

PDI of the liposomal formulations was also found within an acceptable range which indicated the homogeneous nature of the liposomal dispersion (Rehman *et al*., 2018). The drug loading was found to be increasing (22.20 to 39.70%) with increasing concentration of DPPC: Cholesterol ratio ($p<0.05$).

In vitro VIN release

In the pH 6.8 saline phosphate buffer, the *in vitro* VIN release from pure VIN, liposomal VIN and VIN-LP-PEG were tested. The F4 formulation and VIN-LP-PEG demonstrated nearly 20 percent initial burst release from liposomal formulation accompanied by approximately 70 percent continuous release over the 40-hour duration (fig. 2). VIN-LP-PEG shows excellent sustained release behavior as compared to other formulations.

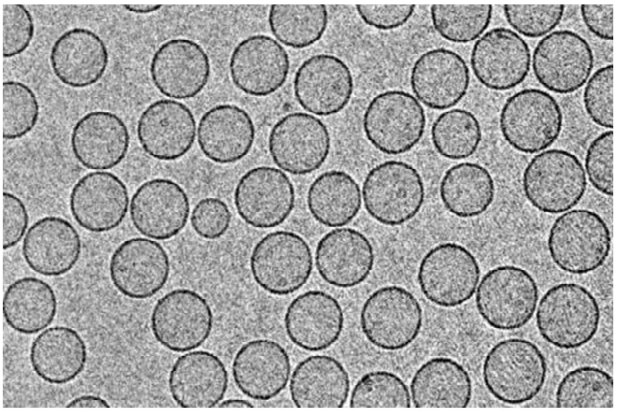


Fig. 1: SEM of VIN-loaded liposomes with spherical, smooth, and un-cracked surface

Cytotoxic assay and cellular uptake study

A direct relation was observed between the concentration and cytotoxicity against cell cultures, as shown by the dose-response curve (fig. 3). Endocytic cells were assumed to trap the liposomes and escape towards the lysosomes.

This study was performed on Jurkat (T-cell acute lymphoblastic leukaemia) cell lines. These cell lines were exposed to pure VIN, VIN-LP and VIN-LP-PEG (0.02-1.0μM) for a period of 10 hours. The cellular uptake potentials of pure VIN, VIN-LP and VIN-LP-PEG with cell lines were found to be concentration-dependent as shown in fig. 4.

DISCUSSION

The present study attempts to develop a PEGylated liposomal formulation containing vincristine sulfate that would be beneficial for the treatment of ALL. Vincristine sulfate (VIN) is commonly employed as a cytotoxic agent in the treatment of hematological malignancies, particularly acute lymphoblastic leukaemia (ALL). However, its maximum therapeutic benefits have been hindered due to the dose-dependent neurotoxic effects it can induce, which traditionally manifest as autonomic and peripheral sensory-motor neuropathy. In the current study, researchers achieved successful integration of VIN into a liposomal formulation that had been PEGylated. This innovative approach aimed to address VIN's neurotoxic limitations while preserving its therapeutic efficacy in combating hematological malignancies, including ALL (Genova *et al*., 2019; Kolimi *et al*., 2022).

The film hydration technique is successful in preparing VIN-loaded liposomes. An increase in particle size with an increase in DPPC: Cholesterol ratio was observed in liposomes (90±0.5 to 120±0.4 nm).

Table 1: Physicochemical parameters of VIN-loaded liposomes

Parameters	VIN loaded liposomes			
Formulations	F1	F2	F3	F4
DPPC: Cholesterol (mol ratio)	1:0.5	1:1	1:1.5	1:2
Solution appearance	Turbid	Turbid	Turbid	Turbid
Particle size (nm)	90	97	1112	120
PDI	0.29	0.212	0.220	0.21
Zeta potential	17.20±4.5	20.14±3.5	26.11 ±2.2	28.11 ±3.9
% DLE	22.20	30.34	35.11	39.70

Individual data shows Mean ± S.D of three determinations, VIN: Vincristine; DPPC: 1, 2- Dipalmitoyl-sn-glycerol-3-phosphocholine; PDI: Polydispersity index, DLE: Drug loading efficiency

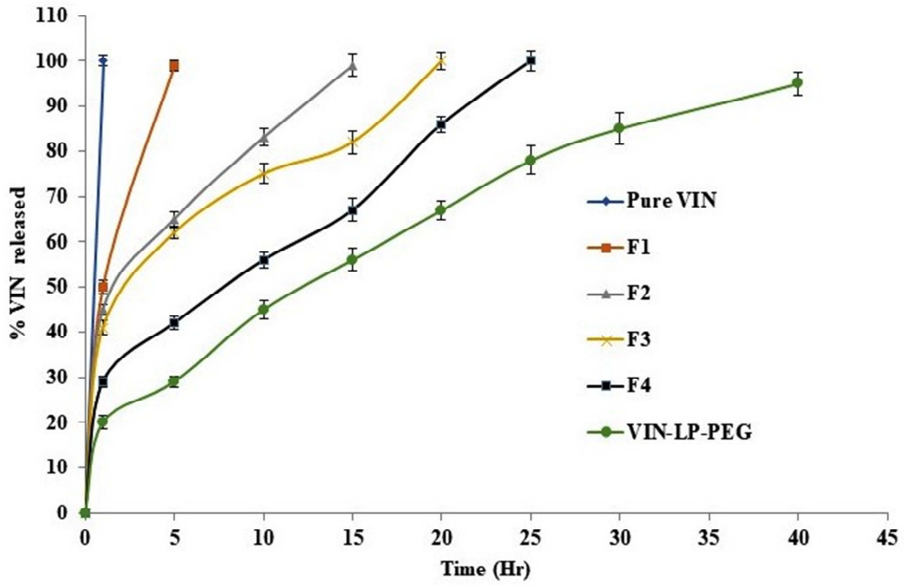
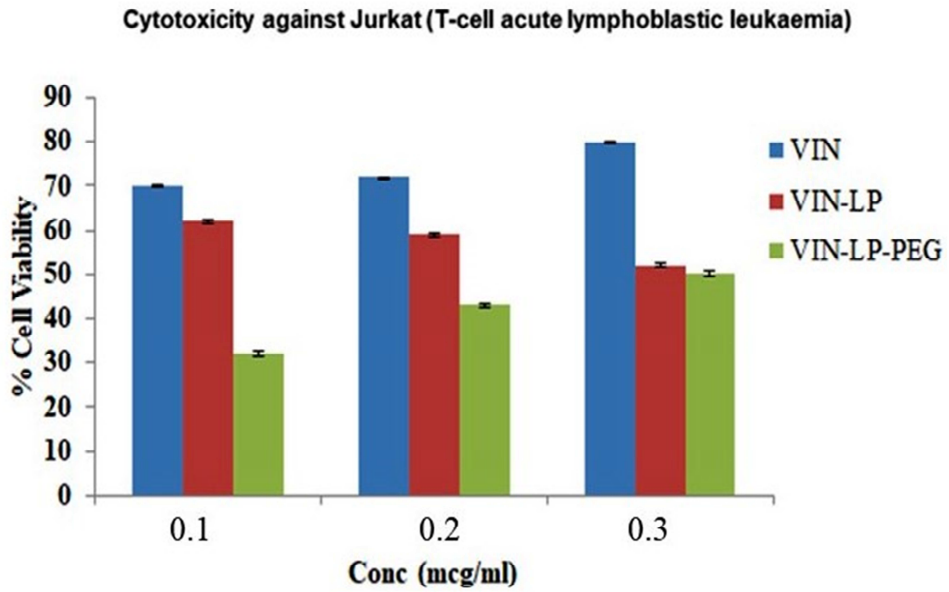


Fig. 2: Comparative *In vitro* VIN release from pure VIN, liposomal formulations (F1 to F4), and VIN-LP-PEG.



Individual data shows Mean ± S.D of three determinations, VIN: Vincristine; VIN-LP: Vincristine Liposomes; VIN-LP-PEG: Pegylated Vincristine Liposomes

Fig. 3: Cytotoxicity study against the cell lines

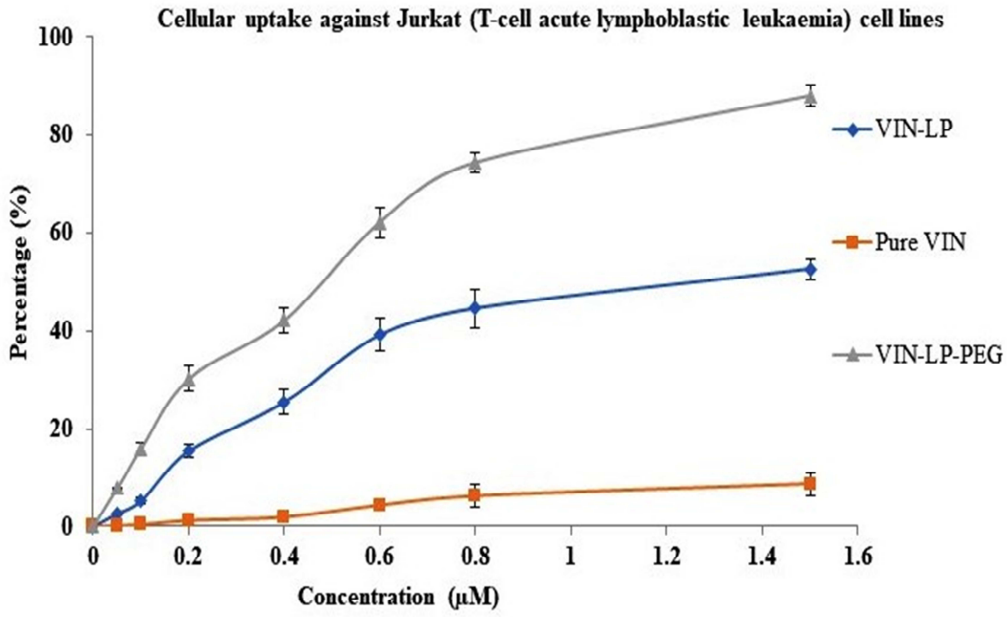


Fig. 4: Cellular uptake study against Jurkat (T-cell acute lymphoblastic leukaemia) cell lines

The zeta potential of the VIN-loaded liposomes was found within an acceptable range which was quite necessary for the stability of the liposomal formulations as well as other formulations having nano-size dimensions (Guimarães *et al*., 2021). As seen in fig. 2, formulations containing lower ratios of DPPC: cholesterol displayed a relatively faster release profile as compared to the F4 formulation ($p<0.05$). The pure VIN showed complete release within 1 hour. These observations indicated that encapsulation of VIN in lipidic vesicles sustained and retarded the release over a longer period which is quite essential in the treatment of lymphoblastic leukaemia (Lin *et al*., 2019; Shankar *et al*., 2022). Jurkat (T-cell acute lymphoblastic leukaemia) cell lines were used in the cytotoxic experiment. In this experiment, pure VIN, VIN-LP and VIN-LP-PEG were incubated with cell lines for 24 hours. However, the uptake efficiency of VIN-LP-PEG was much higher than that of pure VIN, VIN-LP produced a 4.5-fold higher cellular uptake of VIN than VIN-LP in both cell lines. The maximum uptake of VIN-LP-PEG was due to receptor-mediated endocytosis, which probably occurred due to the incorporation of the PEG moiety. The presence of the PEG on the VIN-LP-PEG surface resulted in binding with the over expressed receptors on cell lines. It has been observed very small uptake efficiencies of the VIN-LP and VIN. The nonspecific cellular adsorption as well as cellular interaction may be the reason behind these observations. Research on vincristine-loaded pegylated liposomal drug delivery for acute lymphoblastic leukemia (ALL) offers significant promise. Pegylated liposomes enhance vincristine delivery to leukemia cells, improving effectiveness and reducing systemic toxicity (Bakhshi *et al*., 2023). Encapsulation minimizes off-target effects, enhancing patient tolerance. Targeted delivery increases therapeutic efficacy against ALL cells while sparing healthy tissue, potentially improving survival rates. This approach may overcome drug resistance, advancing treatment options. Personalized medicine is facilitated by tailored drug delivery, optimizing outcomes, and reducing relapse risk.

CONCLUSION

Using the reverse phase evaporation method, we have successfully formulated PEGylated liposomes as carriers for VCR. The particle size of the liposomes ranged from 90±0.5 to 120±0.4 nm. The encapsulation efficiency of the liposomes was found to be 77.24% and the highest drug release reached 95% over 50 hours. The liposomal formulation they prepared demonstrated superior effectiveness in treating acute lymphoblastic leukaemia compared to the pure form of the drug. Overall, this research represents a significant leap in leukemia treatment, promising improved efficacy, reduced toxicity, and enhanced patient outcomes.

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