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(RESEARCH ARTICLE)

Formulation and characterization of a stable vinorelbine-PAMAM conjugate loaded liposomes

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Abstract

The cancer activity of vinorelbine is primarily due to inhibition of mitosis during metaphase of cell division process and its interaction with tubulin. The aqueous solubility of the vinorelbine is > 1000 mg/mL in distilled water. Prolonged vinorelbine exposure is resulted in improved antineoplastic effects, as evident by improved response rate during patients receiving a continuous infusion of the drug. A phase-I pharmacokinetic study of the vinorelbine liposomal injection has been reported and concluded well tolerated and exhibited more favourable pharmacokinetic profiles than free vinorelbine. Dendrimers are new class of artificial macromolecules. Dendrimer possess a well-defined topological structure, are versatile candidates as scaffolds or vehicles for nanomedicine the field of cancer diagnosis and therapy. Dendrimers can be used for conjugating of anticancer compounds and by non-covalent interactions (ionic, hydrophobic, hydrogen-bond interactions) and spacer-mediated conjugates. Also, they can be used for targeting to cancer cells, tumour tissues or abnormal vessels adjacent to the disease focus based on the molecular ‘‘hooks’’ conjugated on the surface of dendrimer through active targeting, and can be accumulated in tumours via the enhanced permeability and retention (EPR) effect of the nanosized dendrimer through passive targeting effect of the nanosized dendrimer through passive targeting. Targeting to tumour tissues & prolonged release profile can further improved by encapsulating vinorelbine-PAMAM conjugated inside liposome. In this study we formulated and characterized a stable vinorelbine-PAMAM conjugate liposomal formulation.

Keywords: Vinorelbine; PAMAM dendrimer conjugate; Cancer; Characterization; Stability; Liposomal formulation.

1. Introduction

Vinorelbine is a cytostatic antineoplastic drug. It is a semi-synthetic vinca alkaloid family that interferes with microtubule assembly. The antitumor activity of vinorelbine is due to primarily inhibition of mitosis during metaphase and its interaction with tubulin. In intact tectal plates from mouse embryos, vinorelbine, vincristine and vinblastine inhibited mitotic microtubule formation at the same concentration 2 micro mole, including a blockade of cells at metaphase. The aqueous solubility of the drug is > 1000 mg/mL in distilled water. Prolonged vinorelbine exposure is correlated with improved antineoplastic effects, as evidenced by increased response rate in patients receiving continuous infusion. Work has already been executed and published on administration of slow release pegylated liposomal vinorelbine formulation by Li CL *et al.* 2010.[1] A phase I pharmacokinetic study of the Vinorelbine liposomal injection has been reported and concluded well tolerated and exhibited more favourable pharmacokinetic profiles than free vinorelbine.[2] Dendrimers are new class of artificial macromolecules. Dendrimers possess a well-defined topological structure, are versatile candidates as scaffolds or vehicles for nanomedicine in the field of cancer diagnosis and therapy. Dendrimers consists of three parts from the interior to the surface: a central core with more than one reactive group, secondly, repeated some units that covalently attached to the central core and organized in a series of radially homocentric layers called generations. Finally, peripheral functional groups existed on the surface which

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majorly determines the physicochemical properties. Dendrimers can be used for conjugating of anticancer compounds and/or diagnostic probes by non-covalent interactions (ionic, hydrophobic, hydrogen-bond interactions), covalent bindings, and spacer-mediated conjugates. Also, they can be used for targeting to cancer cells, tumour tissues, or abnormal vessels adjacent to the disease focus based on the molecular ''hooks'' conjugated on the surface of dendrimer through active targeting, and can be accumulated in tumours via the enhanced permeability and retention (EPR) effect of the nanosized dendrimer through passive targeting effect of the nanosized dendrimer through passive targeting. Encapsulation of vinorelbine-PAMAM conjugate in to liposome may reduce the toxicity further and improve the release characteristics of the drug further. Improved release and reduction of toxicity will improve the therapeutic efficacy. In this study we developed an optimized technology to load vinorelbine-PAMAM conjugate in to liposome.

2. Material and methods

Sample of vinorelbine tartrate has been received from Dr. Reddy's Laboratories limited on request basis as free sample. Similarly, samples of lipids such as DOPC, DSPC and EPC were received from Avanti polar lipids. Cholesterol was procured from Avanti polar lipids. PANAM generation 2 was a gift sample from Sigma Aldrich. Keeping the variability and impurity in the in-house synthesis of PANAM, it was decided to take this from a commercial source. Other chemicals like methanol, sodium 1-decanesulfonate and sodium dihydrogen phosphate for HPLC method establishment were procured from Thermo Fisher. NAVELBINE (vinorelbine tartrate injection) was procured from market.

Estimation of Vinorelbine was done using optimized HPLC method for estimation of Vinorelbine was done by referring HPLC method for quantification of vinorelbine discussed in Xiao-hong *et al*. 2012. [3]

2.1. Conjugation of vinorelbine to PAMAM G2

PAMAM dendrimer G2 of 10 mg quantity was dissolved in 1 mL of DMSO. Vinorelbine was prepared at a concentration of 2 mg/mL in DMSO. The drug solution was then added drop-wise to the dendrimer solution at controlled room temperature (20-25 °C) with vigorous stirring for 24 hrs. to produce conjugates of Vinorelbine-PAMAM. Resulting vinorelbine –PAMAM conjugate was subjected to purification by dialysis method using 1000 Da dialysis membrane in distilled water, as described in Samantray *et al.* [4].

2.2. Liposomal encapsulation of vinorelbine-PAMAM

A solution of 10 mg/mL of Vinorelbine-PAMAM G2 conjugates was prepared in double distilled water. Lipid such as pegylated DSPE has been taken with cholesterol with a proportion cholesterol and lipid ratio of 75:25 in absolute ethanol. Liposome has been prepared by using film hydration method using rotary evaporator.

Vinorelbine –PAMAM conjugate prepared 10 mg/mL was re-suspend in double distilled water. Then vinorelbine – PAMAM conjugate in double distilled water was used to hydrate the dried lipid film followed by vigorous shaking to form multi lamellar vesicles (MLVs). MLVs were then subjected to sonication for 60 min and then pass through 100 nm polycarbonate membrane for extrusion using Lipofast Pneumatic extruder. The resulting unilamellar liposome suspension was centrifuged at 25,000 rpm for 30 min to remove residual dendrimers. The pellet was re-suspended in 1 mL of 8 % sucrose and lyophilized over 72 hrs. as described in Samantray *et al.* [5].

2.3. Characterization study

2.3.1. Particle size and size distribution by dynamic light scattering (DLS)

Lyophilized liposomal formulation was reconstituted with distilled water. Particle size and size distribution of the formulation was measured using Malvern Nano ZS. Malvern Nano ZS is working on the principle of light scattering technology. Liposomal loaded PAMAM conjugated drug was diluted to 10 times of the initial concentration using particle free distilled water. Distilled water was made particle free by filtering through 0.2 µ syringe filter. Post dilution, the preparation was kept 5-10 min for stabilization. Post stabilization 1 mL of the stabilized sample was transferred into the polypropylene cuvette of the instrument and particle size was measured using instrumental protocol for particle size measurement. Results were reported as the average of triplicate reading.

2.3.2. Zeta potential

Lyophilized liposomal formulation was reconstituted with distilled water. Zeta potential of the formulation was measured using Malvern Nano ZS. Malvern Nano ZS is working on the principle of migration of the liposomes towards positive or negative electrodes due to surface charge after application of electric potential and the rate of migration of the liposomes is measured by light scattering technology. Based on the migration and rate of migration of the liposomes

zeta potential was assigned. Liposomal loaded PAMAM conjugated drug is diluted to 5 times of the initial concentration using particle free double distilled water. Distilled water was made particle free by filtering through 0.2 µ syringe filter. Post dilution the preparation was kept 5-10 min for stabilization. Post stabilization 1 mL of the stabilized sample was transferred into the polypropylene cuvette of the instrument and zeta potential was measured using instrumental protocol for zeta potential measurement. Results were reported as the average of triplicate reading.

2.3.3. Particle morphology by TEM

Lyophilized liposomal formulation was reconstituted with distilled water. Morphology of liposomes was determined using transmission electron microscopy (TEM). Liposomal formulation was dissolved in double distilled water at a concentration of 1 mg/mL. 5 µL of the solution was then placed on a 300-mesh copper grid and left to dry overnight, followed by negative staining with 2% phosphotungstic acid (PTA). TEM images were acquired using a JEOL-JEM 1220 at an accelerating voltage of 80 kV.

2.3.4. Determination of free drug

Lyophilized liposomal formulation was reconstituted with distilled water. Reconstituted formulation of 2 mL was transferred into centrifugal 10 kDa filter. It was then centrifuged at 3000 RCF for 2 minutes. Due to low molecular weight free drug passed through the filter by retaining the encapsulated liposome. Filtrate was analyzed for vinorelbine.

2.3.5. In-vitro drug release

Reconstituted liposomal formulation with optimum drug to lipid ratio of 3 ml quantity (explained earlier) was transferred into dialysis membrane tubing. This was then placed into 300 mL of dialysis medium (PBS buffer, pH 7.4) at 37 ºC under constant slow stirring in dark throughout the experiment. Two milliliters of dialysis medium were withdrawn at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 20, 22, 28, and 48 hour time points of the experiment. Samples were also taken from the dialysis membrane tubing before and after the experiment. Release studies were conducted till 48 hours without any change or replacement of dialysis medium.

2.3.6. Stability of formulation

Stability indicating parameters has been identified before initiating stability study. Based on the nature of the formulation particle size distribution, zeta potential, increase in free vinorelbine and dissolution profile have been identified as critical quality attributes of the formulation for evaluation. Stability condition was selected as 40°C/75% RH for 1 month. Stability sampling intervals were selected as 7th day, 15th day, 22nd day and finally on 30th day.

3. Results and discussion

Average particle size of the liposomes presented in Table -1, was found to be 162.7 nm, which reflects a safe use of the liposomal formulation through intra-venous route. PDI of the liposome in Table-1 was very near to 1.0 which refers a better size distribution in the formulation. Zeta-potential values of (-15) mV is sufficient to provide good stability to the liposomes by strong electro static repulsion. Strong electrostatic repulsion among the liposomes will give a good stability to the formulation.

Table 1 Particle size and zeta potential of liposomal formulation

Measurement	Particle size (nm) z-average	PDI	Zeta potential (mV)
1	160.2	0.91	-15
2	165.0	0.92	-18
3	163.0	0.98	-12
Average	162.7	0.90	-15

Average particle size of the PAMAM-Vinorelbine liposomal formulation was found to be 170-180 nm from TEM analysis. The result of TEM is also supporting the data of particle size by dynamic light scattering. TEM images (not presented

here) shows spherical shape liposomes and majority are single uni-lamellar vesicles (SUVs). However, there were some bi-lamellar vesicles has been found in the field. But very less multi lamellar vesicles were observed.

Free drug has been calculated by subtracting, % of encapsulated drug concentration from the total drug concentration and presented in Table-2. It was observed that the optimized formulation was showing an encapsulated drug 98%. The free drug in the formulation was found to be 2% in the formulation. This reading was considered as initial reading and the same has also been monitored as a part of stability of liposome.

Table 2 Stability results

Stability time point	PAMAM-conjugated Liposomal vinorelbine			Liposomal Vinorelbine		
	Particle size (nm)	Zeta potential (mV)	% free drug	Particle size (nm)	Zeta potential (mV)	% free drug
Initial	162.7	-15.0	2.0	159.0	-12.0	5.0
7th day	162.7	-15.0	2.1	160.0	-12.1	5.5
15th day	166.0	-16.2	2.2	162.2	-12.5	5.8
22nd day	165.2	-18.2	2.2	165.0	-12.8	6.5
30th day	163.5	-16.0	2.3	169.0	-14.0	7.8

A comparative picture of stability profile of the formulation has been presented with initial results of the respective analysis in Table-2. It was observed that, the changes in particle size and zeta potential were within the acceptable range of analytical variation. This supports improved stability of the liposomes. Free drug has not been increased significantly. This may be due to conjugation of the drug with PAMAM.

Similarly, the *in-vitro* release profile also perfectly superimposing to the initial release profile which refers the stability of the PAMAM conjugated formulation comparable to simple encapsulated formulation as presented in Figure-1.

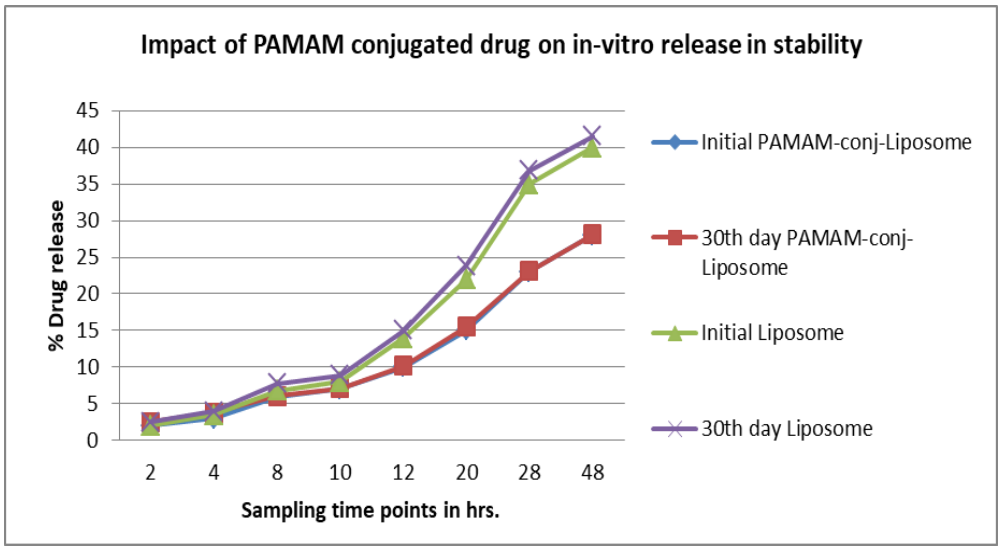

Figure 1 Impact of PAMAM conjugated drug on in-vitro release in stability

Result suggests, there was formation of liposomes and mostly single uni-lamellar vesicles (SUVs). However, there are some multi lamellar vesicles (MLVs) found in the formulation. The average particle sizes of the liposomes are in a range of 160 to 170 nm. This makes the formulation suitable for intra venous (IV) injection. Initial free drug in the formulation is significantly less in PAMAM conjugated drug compared to un-conjugated drug encapsulated in liposome. Reduction of free drug may be due to conjugation, which protecting the drug due to binding within a network like structure. Stability of the liposomal formulation has been improved conjugated liposomal formulation compared to unconjugated

liposomal formulation, which evident from the less free dug content during stability in case of drug. Release of the drug has been delayed further due to PAMAM conjugation compared to un-conjugated drug liposomal formulation.

4. Conclusion

It can summarized that, formulation, stability as well as release characteristics of the PAMAM conjugated vinorelbine is superior to the un-conjugated vinorelbine in liposomal formulation. Further, the in-vitro efficacy of the PAMAM conjugated vinorelbine supports retention of efficacy of vinorelbine after chemical conjugation process with PAMAM. PAMAM dendrimers has been proven its safety previously and has been used as for human consumption through parenteral route. US-FDA has been approved them as a diagnostic device for quantification of NTproBNP in human plasma. [6] In this article, we have presented formulation process, characterization and stability data of Vinorelbine-PAMAM conjugated liposomal formulation, which has been proven improved release characteristics and stability with less free drug content. Vinorelbine-PAMAM conjugated liposomal formulation has shown a better release than the unconjugated liposomal formulation with a comparative release profile on stability. Therapeutic dendrimer based nano particulate formulations are under approval process for human use by US-FDA. Hope the findings published in this article may enlighten the new dimensions for modified release dosage form in the field of onco therapeutics.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest exist in this research article, declared by authors.

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