PREPARATION AND CHARACTERIZATION BIOCOMPATIBLE TRANSFERRIN-CONJUGATED LIPOSOMES LOADED WITH RUBITECAN AS POTENTIAL ANTITUMOR DRUG DELIVERY SYSTEM

FARSIYA FATIMA[*1], M. KOMALA[2]

[1]Department of Pharmaceutics, VELS Institute of Science, Technology and Advanced Studies (VISTAS), Chennai
[2]Professor, Department of Pharmaceutics, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Chennai, India.
*Correspondence email: farsiya@gmail.com

Abstract: Rubitecanis an oral topoisomerase inhibitor, having oral absorption of about25-30% leading to low bioavailability of the drug due to low permeability and poor water solubility. The aim of the present study is toenhance the tumor targeting by conjugating drug with transferrin. It is an attractive approach to that involves the superficial modification of liposomes with specific ligands to overexpressed receptors on the surfaces of tumor cells, such as transferrin receptors (TFR's).To our knowledge, this is the first time that transferrin-conjugated liposomes for delivery of rubetican to cancer cells were investigated. In this study, we developed and characterized rubetican loaded liposomes conjugated with transferrin. The formulated liposomes showed the satisfactory physicochemical propertiesand also they had prolonged and controlled release profile of 32% after 72 hours.The transferrin decorated liposomes loaded with rubitecan (Tf-Lip/Rubi) were almost-spherical in shape with an average particle size of 139.97 ± 8.12 nm, drug entrapment efficiency (EE) and drug loading (DL) of Tf-Lip/Dio were $88.94 \pm 1.02\%$ and $4.48 \pm 0.25\%$ respectively.The results of this study have indicated that transferrin-targeted liposomes increased the ability to target the tumour to deliver the Rubitecan, which could improve its antitumor efficacy in clinical applications.

Keywords: Rubitecan; antitumour; liposomes; conjugated

1. Introduction

Recently, the performance of liposomal drug delivery systems is improved by active targeting, e.g. by using a ligand coupled to the surface which recognizes specific marker molecules or receptors on tumor cells. Among others, the transferrin receptor has been found as one of the successful target molecules[1]. Transferrin (ß-1 glycopeptide) is a hydrophilic

transport vector, controls the extracellular iron level in the body fluid by binding and sequestering. It is a serum glycoprotein that can transport ferric ions inside cells via receptor-mediated endocytosis, and the molecular weight of transferrin is 80 kDa. Transferrin receptors (Tf-Rc) are overexpressed on many malignant tumour cells when compared to normal cells[2]. This is attributed to the fact that tumour cells need more iron to support their fast growth, and thus, cancer cells typically absorb a larger amount of transferrin. Many studies have indicated, transferrin receptor expression on cancer cells can be up to 100-fold higher than the average expression in normal cells owing to their rapid proliferation rate and iron demand, thereby transferrin has been explored as a targeting ligand for nanocarriers to deliver diagnostic and therapeutic agent into cancer cells[3]. Many studies have bound Tf with liposomes to achieve tumour targeting in drug delivery in recent years. Thus, transferrin is a potential ligand in drug delivery systems to achieve tumour targeting and reduces toxicity[4].

2. Methodology

Preparation of Rubitecan Liposomes by Film-Dispersion method

Rubitecan liposomes were prepared using the film dispersion method using a homogeneous mixture of DPPC, Cholesterol, DSPE-PEG2000, DSPE-PEG2000-Maleimide and Rubitecan (15:5:0.6:0.15:1, molar ratio) were dissolved in chloroform/methanol (3:1, v/v) in a round bottom- flask. Once the lipids are thoroughly mixed in the organic solvent, the solvent is removed to yield a lipid film. The organic solvent (<1mL) may be evaporated using a dry nitrogen or argon stream in a fume hood and larger volumes by rotary evaporation yielding a thin lipid film on the sides of a round bottom flask, dried by placing the vial or flask on a vacuum pump overnight[5]. The lipid film was hydrated for 1 h with the addition of 3 mL of pre warmed phosphate buffered saline (PBS, 0.01 M, pH = 7.4). The hydration of the lipid film is followed by sonication at 400 W for 4 min in an ice bath with a probe tip ultrasonicator and sonicated for 5-10 minutes above the Tc of the lipid. Subsequently, the suspension was extruded using a laboratory liposome extruder. Extrusion through filters with 100nm pores typically yields large unilamellar vesicles (LUV) with a mean diameter of 120-140nm. The blank liposomes were prepared by using the similar method without Rubitecan[6].

Conjugation of Rubitecan Liposomes with Transferrin

The modified Rubitecan liposomes by controlled conjugation with transferrin (Rubi-Lipo-Tf) were prepared by following procedure: Traut's Reagent reacts with primary amines (-NH$_2$) to introduce sulfhydryl (-SH) groups while maintaining charge properties similar to the original amino group (Figure 1). Once added, sulfhydryl groups may be specifically targeted for reaction in a variety of useful labelling, cross-linking, and immobilization procedures.
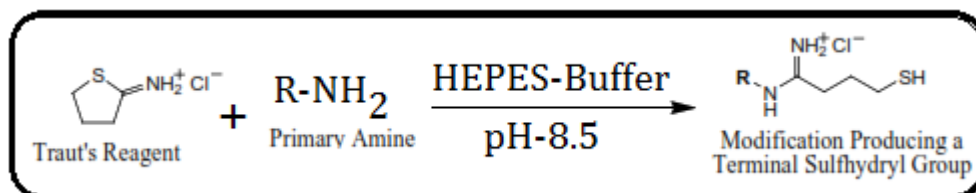
First, transferrin was thiolated with 2-iminothiolane (Traut's reagent) in HEPES buffer (10 mM HEPES with 150 mM NaCl, pH = 8.5), and the molar ratio of 2-iminothiolane to transferrin was 50:1. The mixture was shaken in the dark for 1 hour. The thiolated transferrin was then concentrated by ultrafiltration (Centricon30; Amicon, Beverly, MA) to a volume of 0.2 mL, washed with 2 mL of PBS, pH 8.0 (0.15 M sodium chloride, 0.1 M Na$_2$HPO$_4$2H$_2$O), and re-concentrated by ultrafiltration to a volume of 0.2 mL. Then, unreacted Traut's reagent was removed by Amicon Ultra 0.5 (MWCO: 30 kDa, Millipore), and the thiolated transferrin solution was concentrated. Ellman's reagent was employed to test the amount of sulfhydryl groups conjugated to transferrin. The thiolated transferrin was incubated with liposomes overnight under a nitrogen flow at room temperature and the thiolated transferrin to maleimide ratio was 1:2 (molar ratio). During incubation the hydrophobic interaction occurs between transferrin and maleimide linkers of the PEG liposomes to generate transferrin-conjugated in modified liposomes[7].

Purification of Transferrin conjugated Rubitecan liposomes:

Purification of Liposome Constructs Rubi-Lipo and Rubi-Lipo–Tf were purified on Sephacryl S-500 HR gel filtration columns (1.6 x 16 cm; Pharmacia, Uppsala, Sweden) at 4 °C. The columns were equilibrated and eluted with 0.01 M Phosphate Buffer System (pH 7.4). This method allowed us to separate Rubitecan-containing PEG liposomes from free Rubitecan API and transferrin-conjugated-Rubitecan-PEG liposomes from non-conjugated transferrin. The average percentage of transferrin conjugated to the liposomes was determined using the BCA protein assay kit.

Characterization of the Liposomes

Chromatographic Conditions for the determination of Rubitecan by RP-HPLC method from Rubitecan liposomes

Column: Phenomex (BP-C18, 4.6 mm × 250 mm, 5 µm);

Detection wavelength: 285 nm;

Mobile phase: methanol: water =78:22 (v/v) with mobile phase

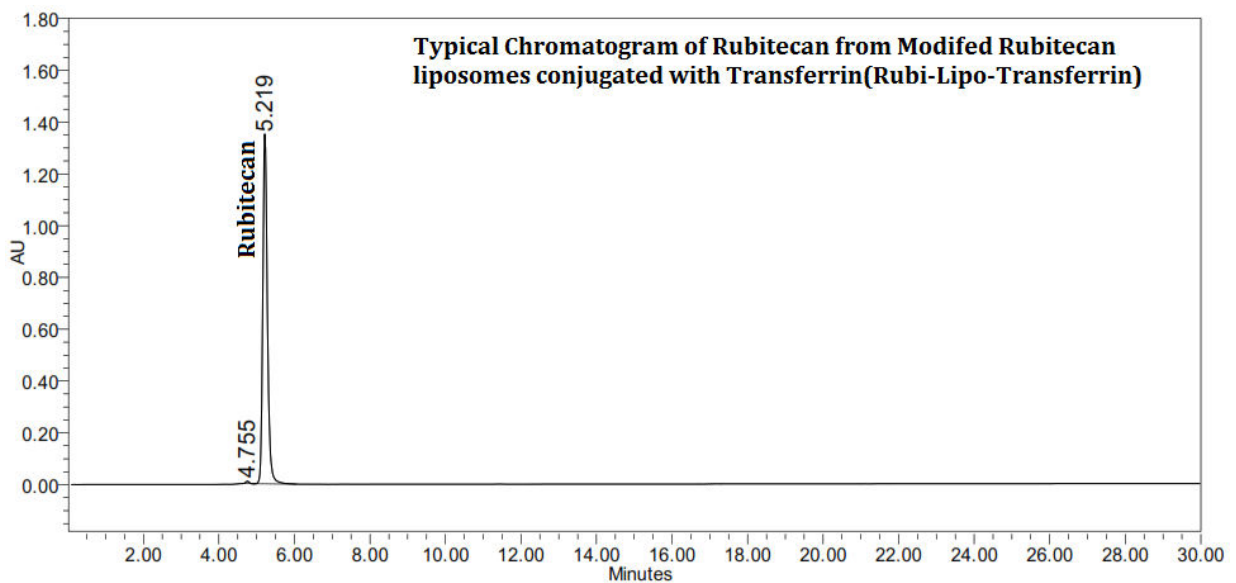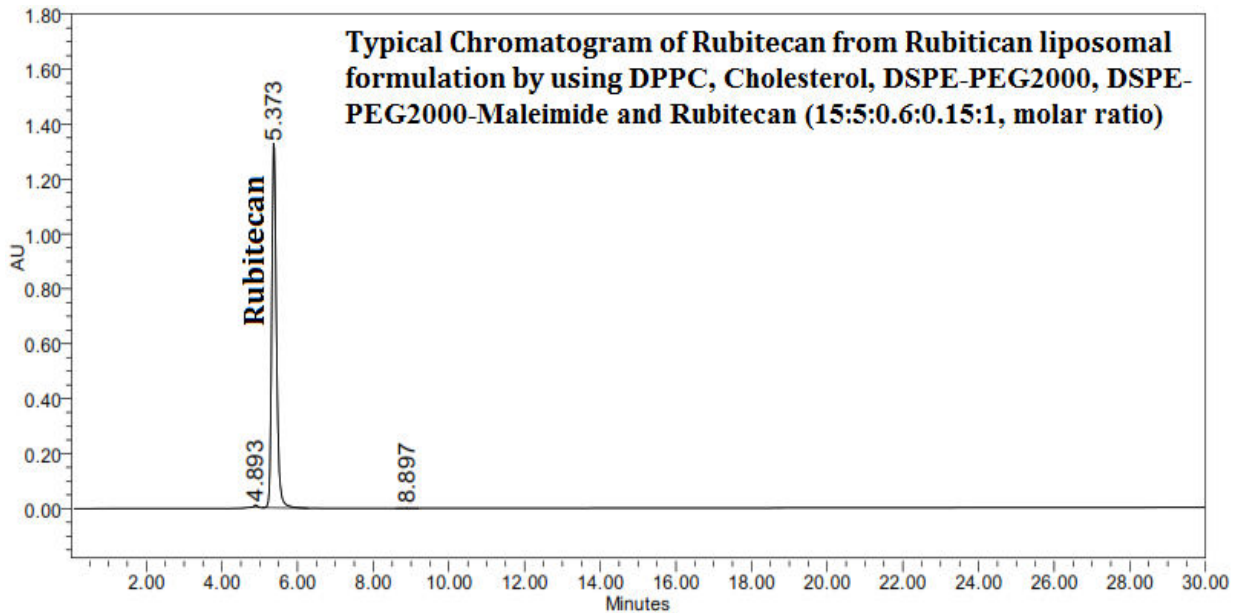pH= pH is 3.2 adjusted with o-phosphoric acid

Fow rate: 1 mL/min

Injection volume: 20 µL;

Column temperature: 20 °C.

Retention time= Rubitecan liposomes- 5.37/Rubi-Lipo-Transferrin-5.21

Runtime= 30 minutes

Diluent: Methanol

Typical Chromatogram of Rubitecan from Rubitican liposomal formulation by using DPPC, Cholesterol, DSPE-PEG2000, DSPE-PEG2000-Maleimide and Rubitecan (15:5:0.6:0.15:1, molar ratio)

Typical Chromatogram of Rubitecan from Modifed Rubitecan liposomes conjugated with Transferrin(Rubi-Lipo-Transferrin)

Standard Curve: The working dilutions are made by taking appropriate dilutions from working standard stock solutions were accurately drawn to 10 mL volumetric flask and methanol diluted to volume in the range of 20-200 µg/mL. At a wavelength of 282 nm, determination was carried out according to the chromatographic conditions above. The peak area was used as vertical axis; Rubitecan concentration was used as abscissa to plot the standard curve[8].

Calibration curve of Rubitecan standard solutions

Concentration in micrograms per mL	20	40	60	80	100	120
Peak areas	2649485	5191210	7424436	9399817	11234089	13266619
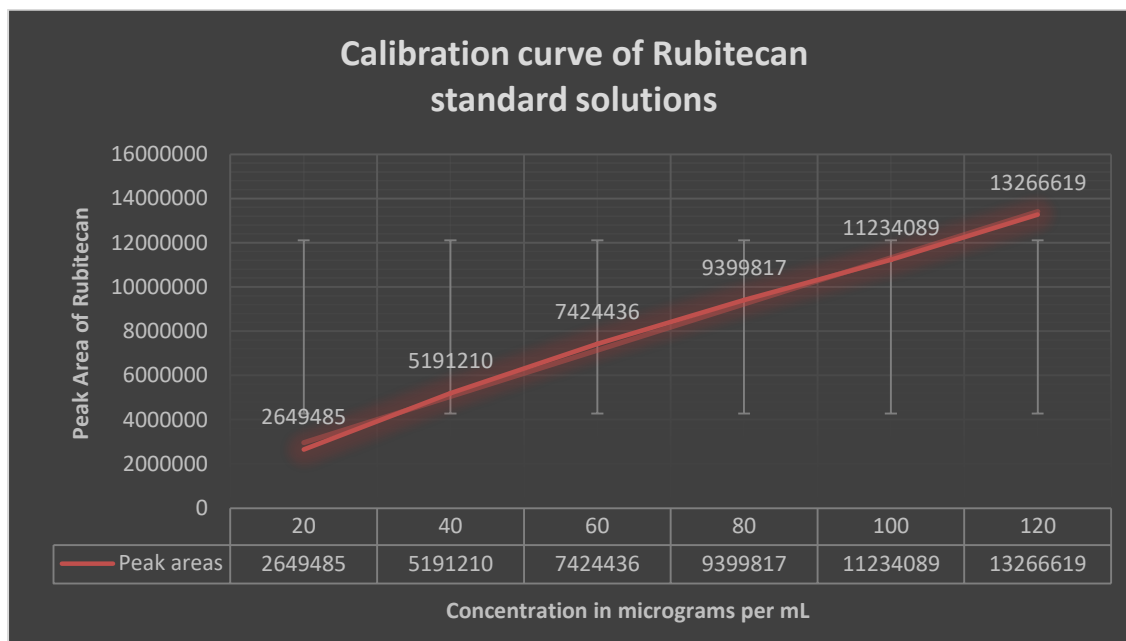
Precision Test. Rubitecan standard stock solutions 10 mL were accurately drawn and placed in 10 mL volumetric flask and diluted to the mark with methanol. High, middle and low concentration of the solution was respectively detected according to the chromatographic conditions above, five times per day for five consecutive days, the precision was then calculated.

Recovery Test: Rubitecan standard stock solutions; 0.8 mL, 1 mL, and 1.2 mL (80%, 100%, and 120%), three equal parts each, were accurately drawn and placed in 10mL volumetric flask, then spiked with a 1:1 (molar ratio) of Rubitecan-Transferrin conjugated liposomes was diluted to the mark with methanol. Solutions of high, middle and low concentration were then measured according to the above chromatographic conditions, and recovery calculated[9].
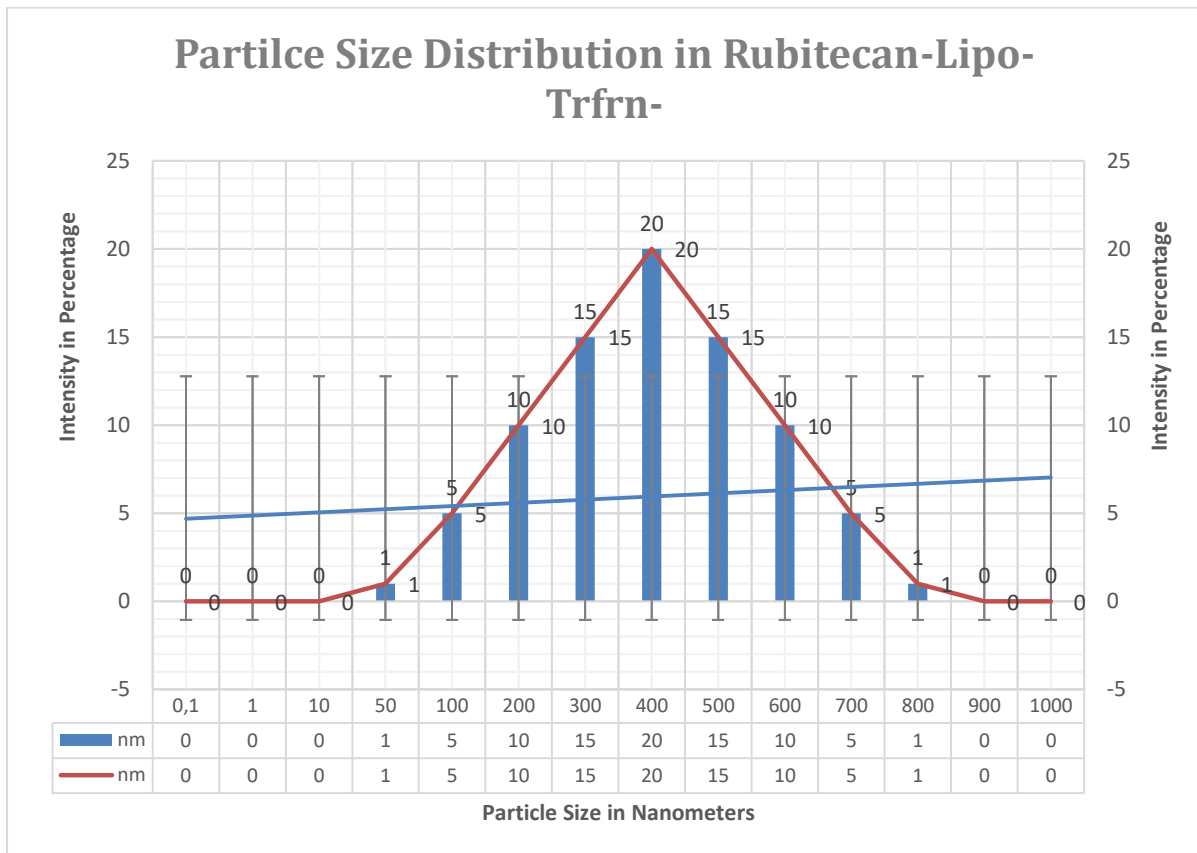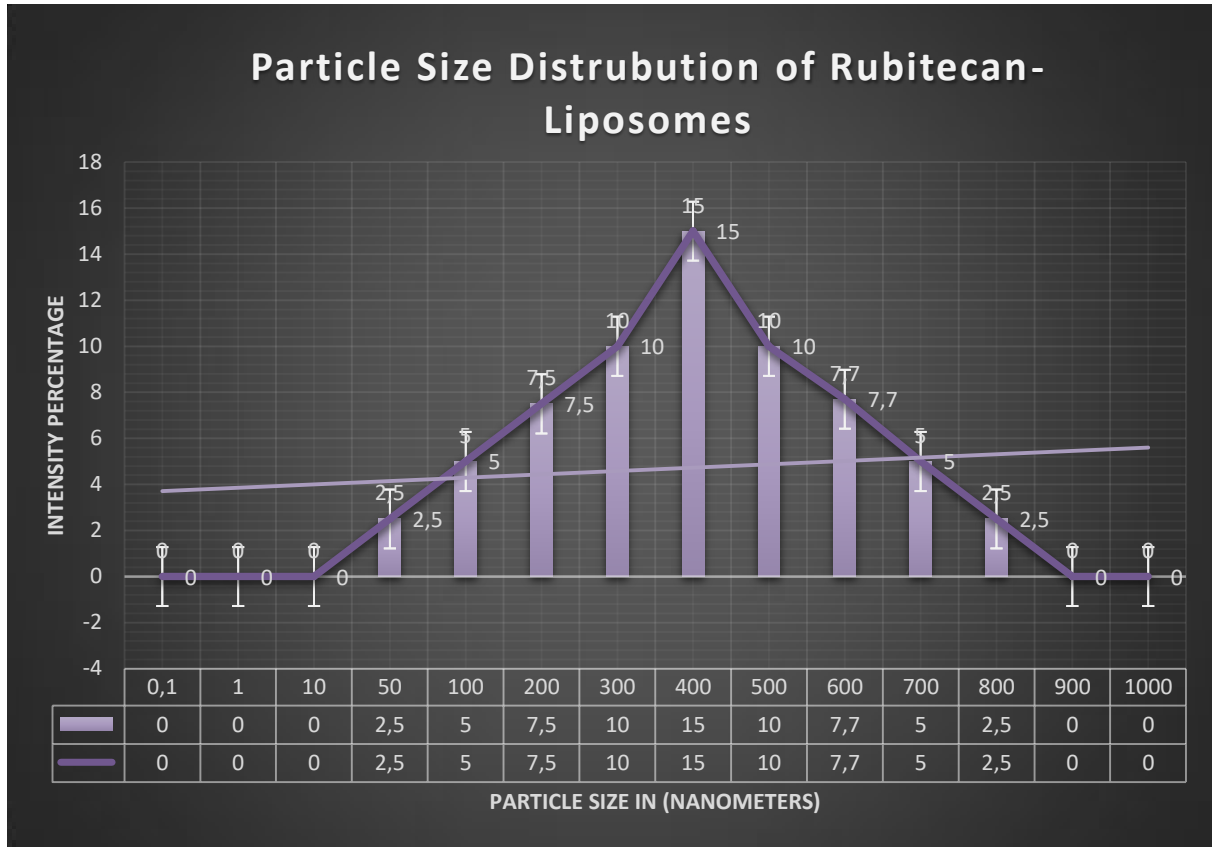
Determination of Rubitecan-from liposomes: Accurately weighed dried Rubitecan-DPPC-Complex liposomes (10 mg) was placed in 25 mL volumetric flask, dissolved in methanol, set to the volume, and determined according to the chromatographic conditions above.
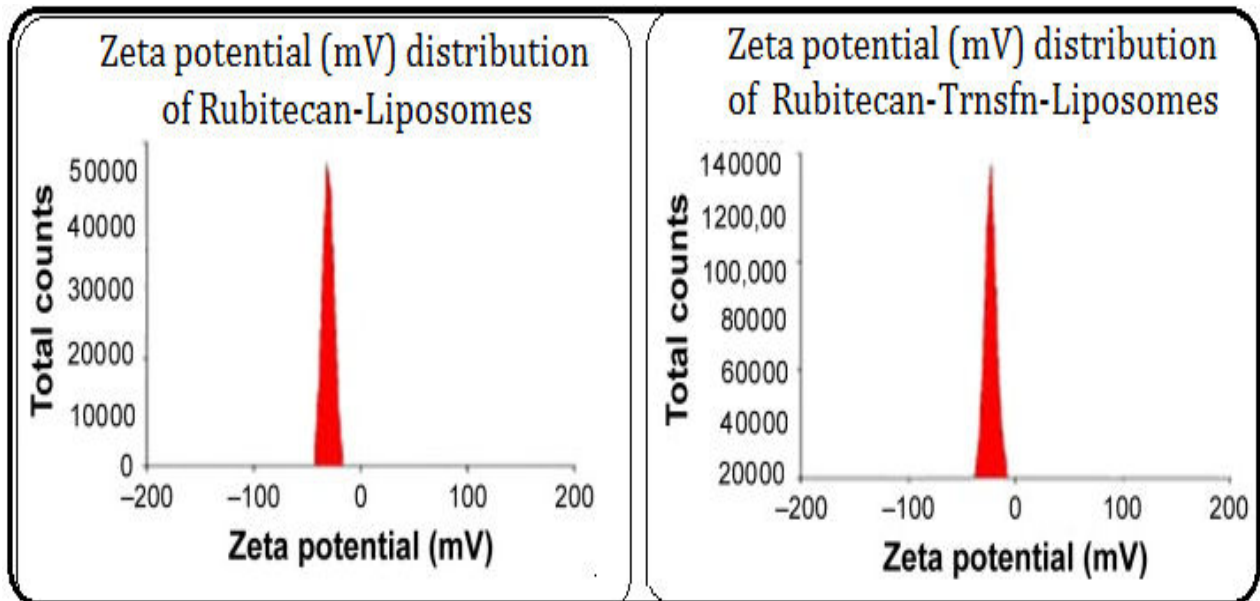
Estimation of Rubitecan- from-Transferrin conjugated liposomes: Dextran G-50 gel column was used to separate rubitecan inclusion liposomes and free RUBITECAN; 5 g

Sephadex G-50 powder was weighed, added to 100 mL distilled water and kept overnight. It was then washed repeatedly to remove particles floating on the surface, and slowly poured into the gel chromatography column while stirring and adding up to the 12 cm of column length. It was then let to natural sedimentation for 30 min[19]. Column flow rate was kept within 1 mL/min; a PBS buffer solution with pH 6.8 was used for balance for 20 min. 200 µL of Rubitecan inclusion liposome samples was then added and the elute measured according to the above chromatographic conditions[10].

Particle Size and Zeta Potential Analysis: The particle size, poly-dispersity-index (PDI) and zeta potential values were measured by dynamic light scattering (DLS, Zetasizer Nano ZS90 instrument, Malvern, UK). Each test was repeated three times. A schematic illustration of particle size distribution of transferrin modified Rubitecan-loaded liposomes and Rubitecan liposomes is shown in Figure[11]. The physical properties of the liposomes before and after conjugation with Tf are listed in Table I

Liposomes	Size in nm	PDI	Zeta (mV)
Blank-Liposomes	98.06±7.95	0.187±0.09	-2.35±0.54
Transferrin-Blank-Lip	105.72± 8.83	0.182±4.52	-2.09±0.31
Rubitecan Liposomes	132.17±5.69	0.198±0.98	-2.34±0.49
Rubitecan-Liposomes-Transferrin	139.97±8.12	0.158±0.76	-24.2±0.38

Particle Size Distrubution of Rubitecan-Liposomes

	0,1	1	10	50	100	200	300	400	500	600	700	800	900	1000
	0	0	0	2,5	5	7,5	10	15	10	7,7	5	2,5	0	0
	0	0	0	2,5	5	7,5	10	15	10	7,7	5	2,5	0	0

PARTICLE SIZE IN (NANOMETERS)

Partilce Size Distribution in Rubitecan-Lipo-Trfrn-

|	0,1	1	10	50	100	200	300	400	500	600	700	800	900	1000
nm	0	0	0	1	5	10	15	20	15	10	5	1	0	0
nm	0	0	0	1	5	10	15	20	15	10	5	1	0	0

Particle Size in Nanometers

The average particle sizes of Blank-Liposomes, Transferrin coupled-Blank-Liposomes, Rubitecan-liposomes and transferrin modified Rubitecan liposomes (Tf-Lip/Rubi) were 98.06 ± 7.95 nm, 105.72 ± 8.83 nm, 132.17 ± 5.69 nm and 139.97 ± 8.12 nm, respectively. Surface conjugation of Rubitecan with transferrin has increased the overall particle size of the liposome by 10 nanometres. It is already reported in literature that high particle sized liposomes are more frequently engulfed by phagocytes & macrophages[12]. Also the micro-sized liposomes (100 to 200 nm) can reach and assembled comfortably in malignant tissue due to the increased permeability by high vasculature and retention (EPR) effect. For all liposomes, the Polydispersity Index (PDI) was approximately 0.198, and they remain same and does not exhibit any notable deviation after the conjugation with transferrin. This indicates the stability of the liposomes was not changed by transferrin in any extent. Surface charge is of the liposomes at micro particulate stage is very important parameter for the stability of a targeted-drug-delivery-system[17]. Measures of the zeta potential were approximately -2.09 mV for the transferrin-free liposomes. The conjugation of transferrin has reduced the zeta potential of liposomes. The possible reason for reduction of zeta potential by transferrin conjugation is attributed to the negatively charged Transferrin molecule (Iso-electric point of transferrin-5.91). The quantification of transferrin in modified liposomes was done by the BCA-assay method, and the result indicated the good binding efficiency of transferrin and is found to be 44.16 ± 0.96%[12].

Transmission Electron Microscopy: The change in morphology of the liposomes due to conjugation with transferrin was observed in transmission electron microscopy. Samples were prepared by placing one drop of liposome (Rubi-Lipo or Rubi-Lipo-Tfrn) on a formvar

copper grid. Then, the samples were stained with 0.5% (w/v) Phosphotungstic acid for 30-seconds and observed by a transmission electron microscope. The accelerating voltage was set at 120 kV[13]. The images are shown in Figure, both the Rubitecan-liposomes and transferrin conjugated Rubitecan liposomes representing a similarly looking, where both liposomes were found to be almost spherical in shape and had an average diameter of 110 nm. The diameter observed by TEM was slightly smaller than that determined by Malvern, which might be because TEM imaged the morphology of static liposomes in dried state, while DLS represented the hydrodynamic diameter of liposomes[18].
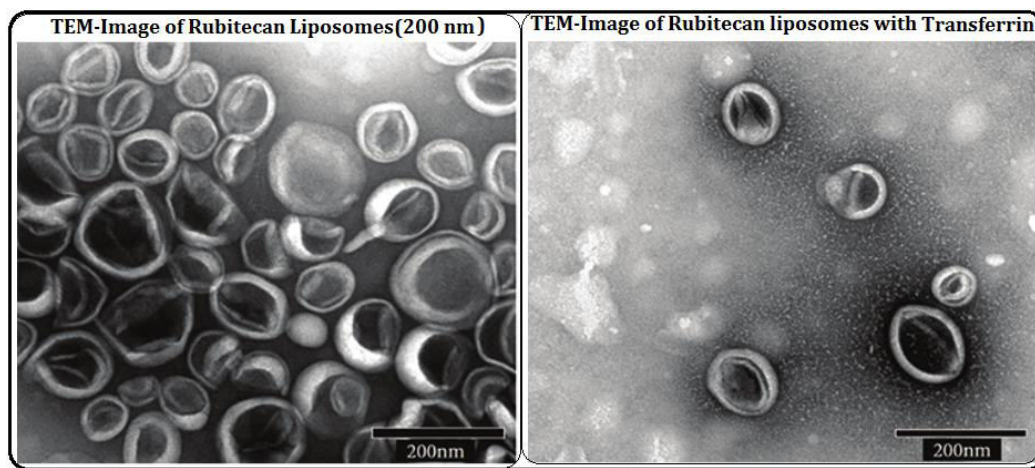
Encapsulation Efficiency and Drug Loading:

The unloaded Rubitecan was separated from Rubitecan loaded liposomes using a Sephadex G-50 column.. The separated liposomes were subjected to breakdown by hydrolysis with adequate methanol or combination polar solvents with high dielectric constant. The amounts of drug entrapped in the conjugated liposomes was native liposomes were assayed by using reverse phase high-performance liquid chromatography (RP-HPLC, Shimadzu LC-20AT) at a detection wavelength of 224 nm[14]. The concentrations of Rubitecan were calculated by comparing the peak areas obtained by Rubitecan with the standard calibration curve. The encapsulation efficiency (EE %) and drug loading (DL %) of Rubitecan loaded liposomes were calculated using the follow equations:

$$EE \% = \frac{\text{Amount of Drug entraped in liposomes}}{\text{Amount of Drug used for liposomal preparation}} \times 100 \%$$

$$DL \% = \frac{\text{Amount of drug in liposomes}}{\text{Total amount of liposomes}} \times 100 \%$$

Encapsulation Efficiency and Drug Loading results are shown in Table. As seen in data table, the encapsulation efficiencies of Rubitecan in Rubi-Lipo and Rubi-Lipo-Tf were 90.23 ± 0.77% and 88.94 ± 1.02%, respectively. The conjugation of liposomes with transferrin is slightly reduced the encapsulation efficiency of liposomes, and this is attributed to loss of active medicament during the ligand conjugation process. In addition, the drug loading measurements of both the liposomes were found to be 5.11 ± 0.07% and 4.48 ± 0.25%, respectively.

Table:

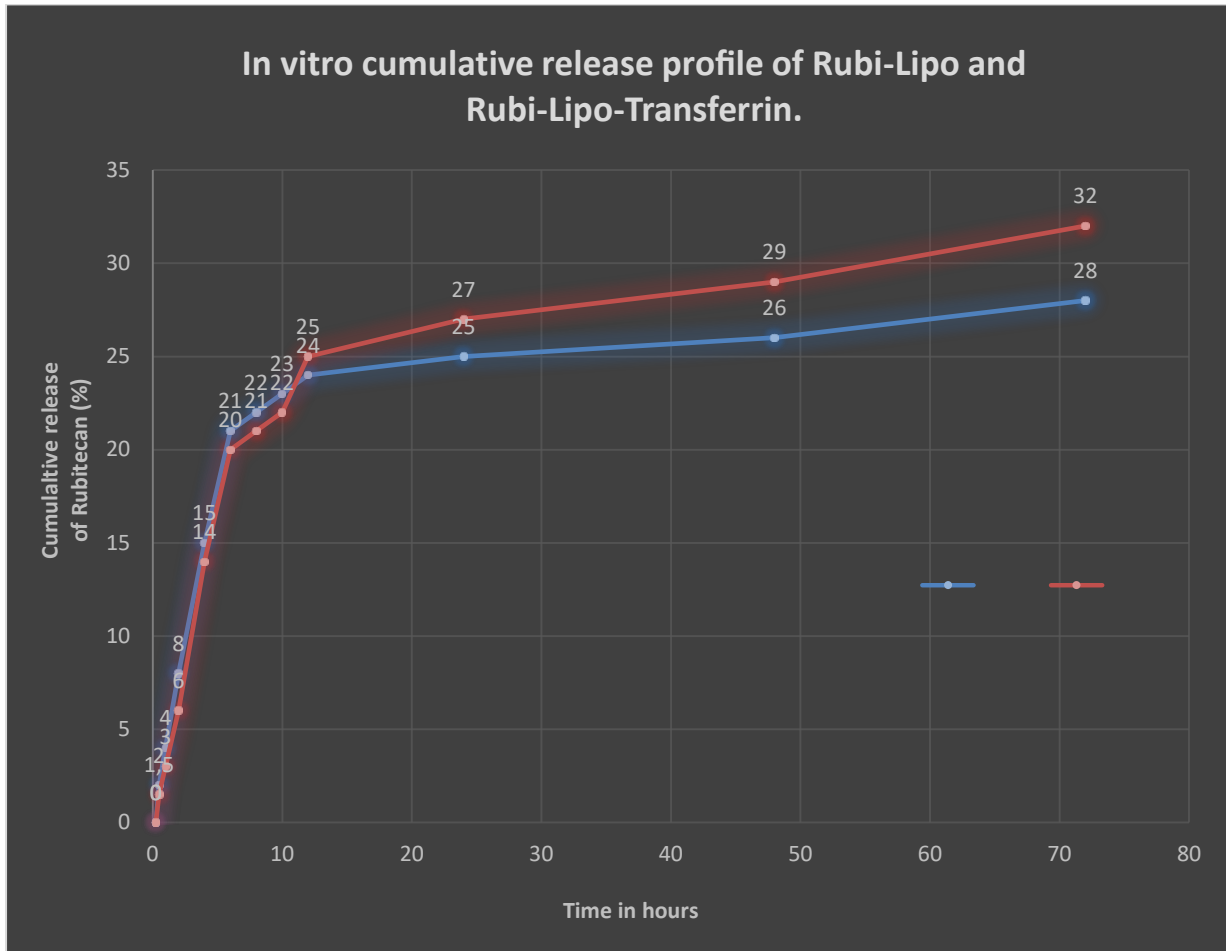| Liposomes	EE (%)	D.L (%)
Rubitecan Liposomes	90.23 ± 0.77	5.11 ± 0.07
Rubi-Liposomes-Transferrin	88.94 ± 1.02	4.48 ± 0.25

In-Vitro Drug Release:

To study the release pattern of liposomes, the release experiments of Rubi-Lipo and Rubi-Lipo-Trfrn were investigated using a sephadex G-50 column[16]. The in vitro release pattern of Rubitecan from Rubi-Lipo and Rubi-Lipo-Trfrn was assessed by measuring the retention times of Rubitecan. In brief, 100 µL of liposomes was added to 100 µL of potassium dihydrogen phosphate buffer system (PBS) (pH =7.4), and the testing samples were incubated in an orbital shaker operating at 100 rpm at 37° C. At predetermined intervals of time starting form 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 hrs, the samples were collected[15]. As shown in *Figure-*, only approximately 32% of Rubitecan was liberated from both native and conjugated liposomes during 72-hour testing period. Rubi-Lipo and Rubi-Lipo-Trfrn exhibited similar controlled release profile, which demonstrated that the conjugation of transferrin did not significantly altered the release pattern of Rubitecan loaded liposomes. The experimental data inferred that the release of Rubitecan was at slow-rate and consistent from both free and conjugated liposomes. These results also indicating that transferrin conjugation is useful in avoiding the drug leakage or sudden outbreak or rupture of liposomes in blood circulation before reaching the actual site in malignant tissue.

Figure: In-vitro cumulative release profile of Rubi-Lipo & Rubi-Lipo-Transferrin. Data are presented as mean ± SD (n = 3).

In vitro cumulative release profile of Rubi-Lipo and Rubi-Lipo-Transferrin.

Conclusion

In this study, a new transferrin conjugated liposomes loaded with rubitecan as potential antitumor agent was formulated and prepared by the film dispersion method. The formulated liposomes showed the satisfactory physicochemical properties in terms of uniform particle size and distribution along with encapsulation efficiency, and also they had prolonged and controlled release profiles. The transferrin decorated liposomes loaded with rubitecan (Tf-Lip/Rubi) were almost-spherical in shape with uniform particle size and distribution. They had an average particle size of 139.97 ± 8.12 nm, a narrow polydispersity index of <0.2 and stable zeta potential of -24.2 ± 0.38 mV. The drug entrapment efficiency (EE) and drug loading (DL) of Tf-Lip/Dio were 88.94 ± 1.02% and 4.48 ± 0.25% respectively. Rubi-Lipo/Transferrin has demonstrated a prolonged and controlled release characterization of approximately 32% of the total rubitecan concentration after 72 h at 37 $^\circ$C.Overall, this research study inferred Rubi-Lipo/Transferrin to be a promising delivery vehicle for Rubitecan in future cancer therapy.The present study was successful in design a stable

liposomal delivery carrier for Rubitecan with a suitable size, high drug entrapment efficiency, sustained release characterization and tumor-targeted activity.

References

1) Sonali, Rahul Pratap Singh et al., Transferrin liposomes of docetaxel for brain-targeted cancer applications: formulation and brain theranostics, Drug Delivery, Volume, 2016; 23(4): 1261-1271.

2) Paszko, E.; Vaz, G. M. F.; Ehrhardt, C.; Senge, M. O.Transferrin conjugation does not increase the efficiency of liposomal Foscan during in vitro photodynamic therapy of oesophageal cancer, European Journal of Pharmaceutical Sciences, 2013, 48; 202–210.

3) HongyanLi, HongzheSun,Zhong MingQian, The role of the transferrin–transferrin-receptor system in drug delivery and targeting, Trends in Pharmacological Sciences, 2002 May;23(5) :206-209.

4) Vandana Soni, D V Kohli, S K Jain, Transferrin-conjugated liposomal system for improved delivery of 5-fluorouracil to brain, Journal of Drug Targeting, 2008, 16(1); 73-78.

5) SamahAnabousi,UdoBakowsky et.at., In vitro assessment of transferrin-conjugated liposomes as drug delivery systems for inhalation therapy of lung cancer, European Journal of Pharmaceutical Sciences, 2006; 29(5):367-374.

6) Mengqiao Wang, Robert J. Lee,Ye Bi, Transferrin-conjugated liposomes loaded with novel dihydroquinoline derivatives as potential anticancer agents, journals.plos.org, 2017, 1-13.

7) Mehran Alavi, * Naser Karimi, and Mohsen Safaei, Application of Various Types of Liposomes in Drug Delivery Systems, Adv Pharm Bull. 2017 Apr; 7(1): 3–9.

8) Srinivas Lankalapalli*, V. S. Vinai Kumar Tenneti and Rajasri Adama, Preparation and evaluation of liposome formulations for poorly soluble drug itraconazole by complexation, Der Pharmacia Lettre, 2015, 7 (8):1-17.

9) Roudayna Diab, Lars P Jordheim, Ghania Degobert, Suzanne Peyrottes, Preparation, and Characterization and In Vitro Evaluation of a New Nucleotide Analogue Prodrug Cyclodextrin Inclusion Complexes, Journal of Nanoscience and Nanotechnology, 9(1):295-300.

10) Rupal K Jani*, Mansi Pandya, Hemali Rathod, Liposomes encapsulating cyclodextrin enclosed hydrophobic anti-cancer drugs: A novel drug delivery system for cancer, Journal of Drug Delivery & Therapeutics. 2019; 9(2-s):598-605.

11)   O. Das, V. M. Ghate and S. A. Lewis*, Utility of Sulfobutyl Ether beta-Cyclodextrin Inclusion Complexes in Drug Delivery: A Review, Indian J Pharm Sci 2019;81(4):589-600.

12)   Harsh Trivedi*, Shreya Chauhan, Shreyash Patel, Cyclodextrins as a Drug Delivery Carrier for Anti-Cancer Drugs, Himalayan Journal of Health Sciences, 2020; 5(4).

13)   Muhammad Hasnor Ja'far, Nik Nur Syazni Nik Mohamed Kamal, Inclusion of Curcumin in β-cyclodextrins as Potential Drug Delivery System: Preparation, Characterization and Its Preliminary Cytotoxicity Approaches, Sains Malaysiana 47(5)(2018): 977–989.

14)   Yogita P. Patil, Sameer Jadhav, Novel methods for liposome preparation, Chemistry and Physics of Lipids, 177 (2014) 8– 18.

15)   Mi-Kyung Lee,Liposomes for Enhanced Bioavailability of Water-Insoluble Drugs: In Vivo Evidence and Recent Approaches, Pharmaceutics 2020, 12, 264.

16)   Iyan Sopyan, Insan Sunan K, Dolih Gozali, A Review: A Novel of Efforts to Enhance Liposome Stability as Drug Delivery Approach, Sys Rev Pharm 2020; 11(6): 555 562.

17)   Upendra Bulbake, Sindhu Doppalapudi, Nagavendra Kommineni and Wahid Khan,Liposomal Formulations in Clinical Use: An Updated Review, Pharmaceutics 2017, 9, 12.

18)   Marwa Mohamed, Eman Alaaeldin, Amal Hussein, Hatem A. Sarhan, Liposomes and PEGylated liposomes as drug delivery systems, J. Adv. Biomed. & Pharm. Sci. 3 (2020) 80-88.

19)   Mohammed Maher Mehanna, Nouran Abd El-Kader, Magda Wadih Samaha, Liposomes as potential carriers for ketorolac ophthalmic delivery: formulation and stability issues, Braz. J. Pharm. Sci. 2017;53(2):e16127.

20)   Yan Chen, Qingqing Wu, Zhenghai Zhang, Ling Yuan, Xuan Liu and Lei Zhou, Preparation of Curcumin-Loaded Liposomes and Evaluation of their Skin Permeation and Pharmacodynamics, *Molecules* 2012, *17*, 5972-5987.

21)   Abdulrahman K Al Asmari, Zabih Ullah, Mohammad Tariq, Amal Fatani, Preparation, characterization, and in vivo evaluation of intranasally administered liposomal formulation of donepezil, Drug Design, Development and Therapy 2016:10 205–215.