

Evaluation of Anti-Tumour Efficacy of Liposomes Loaded with Rubitecan as Potential Antitumor Drug Delivery System

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ABSTRACT

Aim: The present research objective was to characterize the prepared liposomes for *in vitro* cytotoxicity assay, cellular uptake studies of liposomes, competitive binding assay and hemolytic test. **Materials and Methods:** The prepared liposomes were assessed for the cellular cytotoxicity of Rubitecan-loaded liposomes on cancer cell linings. **Results:** The images infer that the uptake of liposomes had a significant time-dependent fusion method for cellular internalization-quantitative analysis of cellular uptake determined by flow cytometry. The fluorescence intensity profile is correlated with the results obtained in laser scanning confocal microscopic study. The results inferred that the conjugated transferrin liposomes entered the cells by surface adherence were facilitated by endocytosis mediated by the transferrin receptor. The hemolysis value of Tf-Lip/Rubitecan was less than 2%, which indicated that it had great haemo-compatibility and could be safe for intravenous injection. **Conclusion:** This work effectively created and optimized a novel transferrin liposome conjugated anti-tumor targeted drug delivery system using Rubitecan as an anticancer agent. The study's findings suggest that transferrin-targeted liposomes enhanced the delivery of Rubitecan to the tumor, potentially enhancing the drug's anticancer efficacy in clinical settings.

Keywords: Rubitecan, Transferrin, CCK-8, Cellular uptake, Cellular Internalization.

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INTRODUCTION

For the intravenous delivery of specific drugs, liposomes are flexible carriers. Numerous different kinds of liposomal formulations have been widely employed as carriers to raise the therapeutic index of cytotoxic medicines. The quick clearance from systemic circulation is the main issue in using liposomes to target tumor cells in extravascular locations. Circulating phagocytes and the liver's and spleen's macrophages quickly clear these liposomal carriers. Targeted liposome passive accumulation in tumor tissue is reduced by a short circulation lifetime. A higher circulation half-life can lead to increased accumulation within a target site because long circulation lives are needed to maintain liposomal drug accumulation at the target site, which is necessary for target cell access.

Targeted administration by receptor-mediated absorption into the cancer cells is one of the techniques that have been thoroughly investigated and proved to be effective. Transferrin (Tf) Receptors (TfR) have gained attention in this regard and have been thoroughly studied for the transport of drugs into cancer cells. A glycoprotein that is crucial for iron metabolism is Tf which is in charge of delivering ferric ions (Fe^{3+}). Iron attaches to Tf in the form of Fe^{3+} , which is produced when Fe^{2+} is oxidized and then binds to the Tf receptor. Tf also eliminates blood-borne harmful iron. Tf belongs to the family of transferrin's, which also includes melano-, ovo- and serum-Tf.

Subsequently, transferrin will bind to ferric iron and transferrin will bind to its receptor. Iron enters cells through binding to TfR. Transferrin receptors are abundantly expressed in several bodily tissues, including endothelial cells, red blood cells and even some cancerous cells. Because tumor cells and capillary endothelial cells are known to overexpress Tf receptors, this receptor system offers a promising target for targeted therapy. Tf's main job in the body is to transport iron and it does this by attaching to the TfR on the surface of cells to deliver iron to those cells. If TfRs are damaged, the body's ability to transport iron is compromised because they cannot bind Tf and carry iron into cells. Anemia



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will result from the body not having enough iron. Tf is produced poorly or is lost excessively through urine when the Tf receptor is not expressed at high levels. Reduced blood iron levels will result from this. On the other hand, if there is an increase in iron uptake and storage, this can lead to carcinogenesis and accelerate the spread of cancer.

For targeted drug delivery, an ideal liposome formulation should be stable, small (100 nm), have a zeta potential of 20 mV, have minimal drug leakage, have long *in vivo* circulating properties, interact with blood components infrequently, selectively extravasate into tumors and have a high tumor localization capability.¹

MATERIALS AND METHODS

These chemicals and solvents used were of analytical grade. Cell Lines and Cell Culture: The Chinese Academy of Sciences Cell Library provided the Human cervical cancer cell Line (HeLa) and the Human Hepatoma Cell Line (HepG2). 10% fetal bovine serum was added to Dulbecco's Modified Eagle Medium (DMEM, HyClone Thermo Fisher Scientific, UK) for cell culture (Sijiqing, Hangzhou, China).

Method of Preparation

Using a homogenous mixture of DPPC, cholesterol, DSPE-PEG2000, DSPE-PEG2000-maleimide and Rubitecan mixed in a 3:1:1 v/v chloroform/methanol solution, the film dispersion method was utilized to create Rubitecan liposomes with little leakage as shown in Figure 1. Because cholesterol makes the lipid bilayer more rigid, it affects the stability of membranes both *in vitro* and *in vivo*. A lipid film is produced once the organic solvent has been extensively combined with the lipids. Larger volumes can be achieved by rotary evaporation, which results in a thin lipid layer on the edges of a round-bottom flask that can be dried by leaving the vial or flask on a vacuum pump overnight. The organic solvent (1 mL) can be evaporated in a fume hood using a dry nitrogen or argon stream. The lipid film was hydrated for an hour by adding 3 mL of previously warmed Phosphate-Buffered Saline (PBS, 0.01 M, pH=7.4). Following hydration, the lipid film is subjected to 5-10 min of ultrasonication above the lipid's melting point in an ice bath using a probe tip ultrasonicator set to 400 W. The suspension was then extruded utilizing a lab liposome extruder. Large Unilamellar Vesicles (LUV) is often made via extrusion through filters with pores of 100 nm. LUVs have a mean diameter of 120-140 nm. The blank liposomes were prepared without the usage of rubitecan.^{4,5}

CHARACTERIZATION

Particle Size, Polydispersity Index and Zeta Potential

Using dynamic light scattering, the values of the particle size, Polydispersity Index (PDI) and zeta potential were determined. When transferrin and Rubitecan are conjugated on their surfaces,

the total particle size has grown. Using the BCA-assay method, the amount of transferrin in modified liposomes was quantified. By using TEM, it was shown that the transferrin-conjugated Rubitecan liposomes and the Rubitecan liposomes themselves had a similar appearance. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC, Shimadzu LC-20AT) was used to measure the amounts of drug entrapped in native liposomes and conjugated liposomes at a detection wavelength of 224 nm. Due to the loss of active medication during the ligand conjugation process, conjugating liposomes with transferrin marginally reduces the liposomes' encapsulation efficiency.⁶

Tumor Cell-lining Experiments

Human hepatoma (HepG2) and cervical cancer (HeLa) cell lines were grown in Dulbecco's Modified Eagle Medium with additions of 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The culture system for cells was kept at 37°C and 5% CO₂.

In vitro Cytotoxicity Assay

HeLa and HepG2 cells were used to determine the cytotoxicity of Rubitecan-loaded liposomes to evaluate their anticancer characteristics. Using the CCK-8 assay, the cellular cytotoxicity of the liposomes loaded with Rubitecan was assessed to determine its effect on the linings of cancer cells. Different potencies of free Rubitecan, Rubi-lipo and Rubi-lipo-trfrn have been administered to HeLa and HepG2 cells. A cellular density of 1×10⁴ cells per well was attained by inoculating HeLa or Hep-G2 cells in 96-well plates using 100 µL of medium. Cells were incubated for a further 24-hr period with different concentrations of free-native Rubitecan, Rubitecan-liposomes, or Rubitecan-liposomes coupled with transferrin (Rubi-Lipo-Transferrin). Each well was then filled with 10 µL of Cell Counting Kit-8 (CCK8).^{7,8} The cell linings that had undergone reagent processing were incubated for 2 hr at 37°C to continue the culture. In the proliferation and cytotoxicity tests, the Cell Counting Kit-8 provides more accurate colorimetric techniques for estimating the total number of viable cells that are actively growing and replicating. The orange-colored formazan crystals represent the absorption of chromogen produced by actively developing cells. A microplate reader was used to measure the formazan-colored complex's absorbance at 450 nm. The percentage of cell viability (% cell viability) was used to report the effect of cytotoxicity. The cells that were not given any medication were regarded as 100% viable, or total cell viability.^{9,10}

The cytotoxicity of the polymeric excipients utilized as vectors was assessed using liposomes lacking Rubitecan (Tf-Blank-Lip and Blank-Lip). HepG2 and HeLa cells were treated with blank liposomes at concentrations of 0.01 µmol/mL, 0.1 µmol/mL and 1 µmol/mL for 24 hr. As previously noted, the cytotoxicity of the medication excipients and vectors was expressed as a percentage of cell viability as shown in Figure 2.¹¹

Cellular Uptake studies

Rubitecan-D6 was encapsulated into liposomes in place of Rubitecan (DPPC: Rubitecan=100:2, w/w) to assess the cellular absorption of liposomes; the other optimized procedures remained the same as previously stated. HeLa or HepG2 cells were incubated for one day at 37°C, with coverslips holding duplicates of each cell type at a viable cellular population density of 1×10^5 cells/well in 24-well inoculation plates. Subsequently, the cells were cultured in serum-free media with 3 µg/mL of Rubitecan liposomes in both their unmodified form and in conjugation with transferrin at loading dosage concentration.¹⁵ After a specified period for incubation (120 min), the growth medium was taken out and cells were treated with cold Phosphate Buffer Solution thrice as a part of the purging or washing procedure.

The samples were impregnated with 4% (v/v) p-HCHO for 15 min at room temperature. The stained nucleus was then obtained by treating the cell nuclei for 5 min with acridine orange dye and rhodamine dye. Next, a Laser-Scanning Confocal Microscope (LSCM, Zeiss, LSM710, Germany) was used to photograph the cells.¹⁶

Blank liposomes, rubitecan liposomes and rubi-lipo-transferrin intracellular location in HeLa cells. For 24 hr the cells were exposed to 10 µM liposomes. Acridine orange was used as a counterstain for 30 min on cell nuclei. Micrometers are shown on the scale bars. The fluorescence of rhodamine was observed at a wavelength of 580 nm after being excited at 555 nm, while the fluorescence of acridine orange was identified at 650 nm after being excited at 460 nm. 400x magnification. Three distinct experiments are represented by the images (shown in Figures 3a and 3b).

The location of blank liposomes, rubitecan liposomes and rubi-lipo-transferrin within Hep-G2 cells' intracellular milieu. For 24 hr the cells were exposed to 10 µM liposomes. Acridine orange was used as a counterstain for 30 min on cell nuclei. Micrometers are shown on the scale bars. The fluorescence of rhodamine was observed at a wavelength of 580 nm after being excited at 555 nm, while the fluorescence of acridine orange was identified at 650 nm after being excited at 460 nm. 400x magnification. Three distinct experiments are represented by the images.

Quantitative cellular uptake study by Flow-Cytometry

HeLa or HepG2 cells were cultured in 6-well plates at a density of 2×10^5 cells per well for 24 hr at 37°C to quantitatively examine the cellular uptake. Then, the culture medium containing either transferrin-conjugated Rubitecan liposomes or Rubitecan liposomes including transferrin was added. For every formation, the final Rubitecan concentration was 3 µg/mL. Following a 2 hr incubation period, cells were collected and re-suspended in 500 µL of PBS after three PBS washes. As a control, cells that had been cultured in a medium were employed. The Accuri C6 flow cytometer (Becton Dickinson, USA) was used to measure the amount of Rubitecan absorbed by each group of five and each assay was performed three times.¹⁹

Competitive binding Assay

A receptor competition experiment was performed to evaluate the internalization mechanism of Transferrin modified liposomes by pre-incubation with free Transferrin. To evaluate the mechanism of cellular uptake of liposomes a competitive binding study was conducted. The remaining methods were the same as described above. However, in this procedure, Coumarin-6 (DPPC: Cou-6=50:1, w/w) was encapsulated into liposomes rather than Rubitecan. On coverslips, HeLa and HepG2 cells were multiplied and left to adhere for a whole day for 24 hr. Cells were incubated with free Transferrin (1 mg/mL), which was dissolved in a culture medium for 1 hr before the addition of Tf-Lip/Cou-6. After 2 hr of incubation with Tf-Lip/Cou-6 (Containing 3 µg/mL Cou-6) in serum-free medium, the cells were purged and drained with cold Phosphate Buffer System (PBS), fixed with 4% (v/v) paraformaldehyde and stained with DAPI as described in the cellular uptake assay. Then, the intracellular distribution of Cou-6 was imaged by the LSCM (Laser Scanning Confocal Microscopy) shown in Figure 4.^{21,22} To investigate the mechanism of receptor competition, free Transferrin (Tf) of 1 mg/mL was added to the culture medium.

Hemolysis test from human erythrocytes

The preliminary safety of Rubi-lip and Rubi-lipo-transferrin, equivalent to 500 ng/mL Rubitecan was evaluated by incubating the formulations in 2% human erythrocyte suspension for

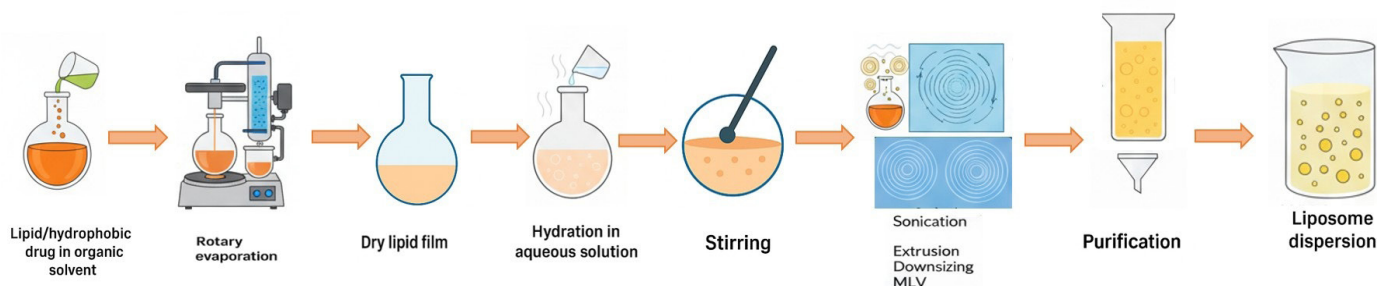


Figure 1: Preparation of liposomes by thin film hydration technique.

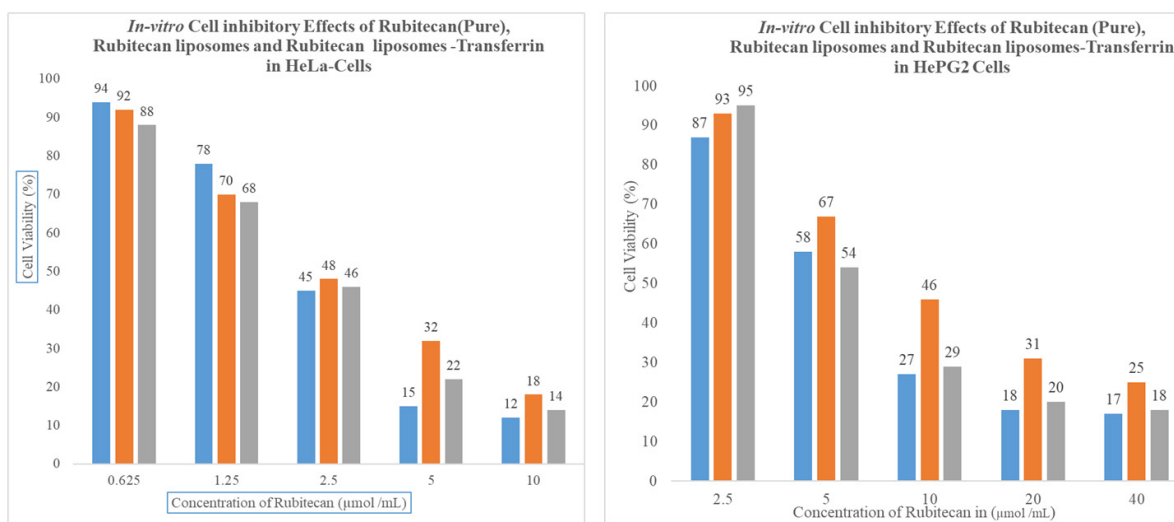


Figure 2: Illustration of *in vitro* Cell Inhibitory Effects of Rubitecan (Pure), Rubitecan-liposomes and Rubitecan-Liposomes-Transferrin (a) in HeLa-Cells & (b) HePG2-Cells.

predetermined incubation periods at 37°C. The erythrocyte suspension treated with normal saline served as the negative control and the sample treated with 1% (w/v) Triton X-100 served as the positive control for 100% hemolysis. Apheresis was used to treat venous blood taken from a healthy volunteer and RBCs were kept at 6±2°C (Blood Bank of the Red Cross Society of India). After centrifuging the whole blood for 10 min at 1,600×g, the buffy coat and supernatant were pipetted out and disposed of. The RBCs were then coarsely dispersed in 2% human erythrocyte suspension after being rinsed twice in isotonic Phosphate Buffer Solution (PBS) with a pH of 7.4. Next, 2 mL of the RBC solution was combined in triplicate with 2 mL of a buffer that contained modified Rubitecan formulations and Rubitecan liposomes.²⁴ The ultimate concentration of 500 ng/mL of Rubitecan was employed to evaluate the drug carrier's blood compatibility across all formulations. After that, each sample was incubated in a shaking water bath with 100 strokes per minute at 37°C following incubation times of 6, 12 and 24 hr lowering the temperature to (0°C) preventing the disintegration of red blood cells. To extract the analyzed RBCs, the suspensions were centrifuged at 3000×g for 10 min after incubation. To measure the amount of hemoglobin absorbed and ascertain the degree of hemolysis, the supernatants were collected for examination. Using a spectrophotometric approach, the absorbance of the supernatant was determined at a wavelength of 565 nm.²⁵ The measurements were made three times, the results of the tests were performed in duplicate and the relative hemolysis (%) was computed using the equation below:

$$\text{Hemolysis (\%)} = \frac{\text{AS-AN}}{\text{AT-AN}} \times 100 \%$$

Where AS, AN and AT are the absorbance values of the sample, normal saline and Triton X-100, respectively.

RESULTS

Particle Size, Polydispersity Index, and Zeta Potential

After conjugating with transferrin, the Polydispersity Index (PDI) of all liposomes remained constant at roughly 0.198, showing no discernible change. This suggests that transferrin did not affect the liposomes' stability. Zeta potential measurements for the transferrin-free liposomes were roughly -2.09 mV. The BCA-assay method result showed a good binding efficiency of transferrin, coming in at 44.16±0.96%. Transmission electron microscopy showed the liposomes' altered shape because of conjugation with transferrin. Both liposomes had an average diameter of 110 nm and were nearly spherical. In Rubi-Lipo and Rubi-Lipo-Tf, the encapsulation efficiencies of Rubitecan were 90.23±0.77% and 88.94±1.02%, respectively. The drug loading values of the two liposomes were discovered to be 5.11±0.07% and 4.48±0.25%, in that order.⁶

In vitro Cytotoxicity Assay

As seen in (Figure 2a), at all tested concentrations in HeLa cells, the cytotoxic impact generated by Rubi-Lipo-Transferrin was larger than that of Rubi-Lipo. In the concentration range of 5-40 μM, Rubi-Lipo-Transferrin exhibited a greater antitumor impact on HepG2 cells than Rubi-Lipo (Figure 2b).

At all working dilutions studied in HepG2 cells and concentrations of 2.5 μM, 5 μM and 10 μM in HeLa cells, plain-Rubitecan (pure API) exhibited higher cytotoxicity compared with the respective Rubitecan-loaded liposome groups. This could be attributed to the different ways that the drug enters the cells. Liposomes enter cells by active transport, whereas free drugs enter by passive diffusion.¹²

Free drug enters cells by passive diffusion, while liposomes enter by active transport. Although the free drug had higher

cytotoxicity, it has undesirable ADMET properties and a lack of specificity towards the rapidly growing cancer cells for antitumor effects *in vivo*. The CCK-8 results indicated that the Rubitecan liposome conjugation with transferrin has remarkably enhanced the antitumor property of Rubitecan-loaded liposomes. In addition, there was nearly zero cytotoxic effect of either blank liposome, indicating the safety profile of the polymer-carriers used in liposome formulation ($\mu\text{mol/mL}$).^{13,14}

Cellular Uptake studies

The Figures 3a and b shows the cellular incorporating images of Rubitecan in cells observed by laser scanning confocal microscopy. The images infer that the uptake of liposomes had a significant time-dependent fusion method for cellular internalization.¹⁶

Quantitative cellular uptake study by Flow-Cytometry

The Figure 5 displays a quantitative examination of the cellular uptake as assessed by flow cytometry. The fluorescence intensity in Transferrin conjugated Rubitecan liposomes is more than that of plain Rubitecan liposomes-in both HeLa cells and HepG2 cells after 2 hr of incubation time. The fluorescence intensity profile is in correlation with the results obtained in laser scanning confocal microscopic study (shown in Figures (3a and 3b)).²⁰

Competitive binding Assay

As shown in Figure 4, the addition of free Transferrin (Tf) could decrease the uptake of Transferrin-conjugated liposomes, which might be due to the competitive affinity between Free-Transferrin

(Tf) and Transferrin-Receptor (Tf-R). The results inferred that the conjugated transferrin liposomes entered into the cells by surface adherence were facilitated by endocytosis mediated by the transferrin receptor.²³

Hemolysis test

As shown in Figures 6a and 6b, we found that 1% Triton X-100 exhibited 100% hemolysis, while both Rubitecan-plain and modified liposomes showed less hemolytic properties. The hemolysis value of Tf-Lip/Rubitecan was less than 2%, which indicated that it had great haemo-compatibility and could be safe for intravenous injection.^{26,27}

DISCUSSION

The prior study effectively created a stable liposomal delivery system for rubitecan with a high drug entrapment efficiency, a prolonged release feature, and a tumor-specific activity. As stated in the earlier work, the liposomes that were prepared demonstrated favorable physicochemical characteristics, including consistent particle size and distribution, encapsulation effectiveness, and controlled and extended release profiles. As mentioned in the previous study, formulated liposomes showed 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 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 a stable zeta potential of

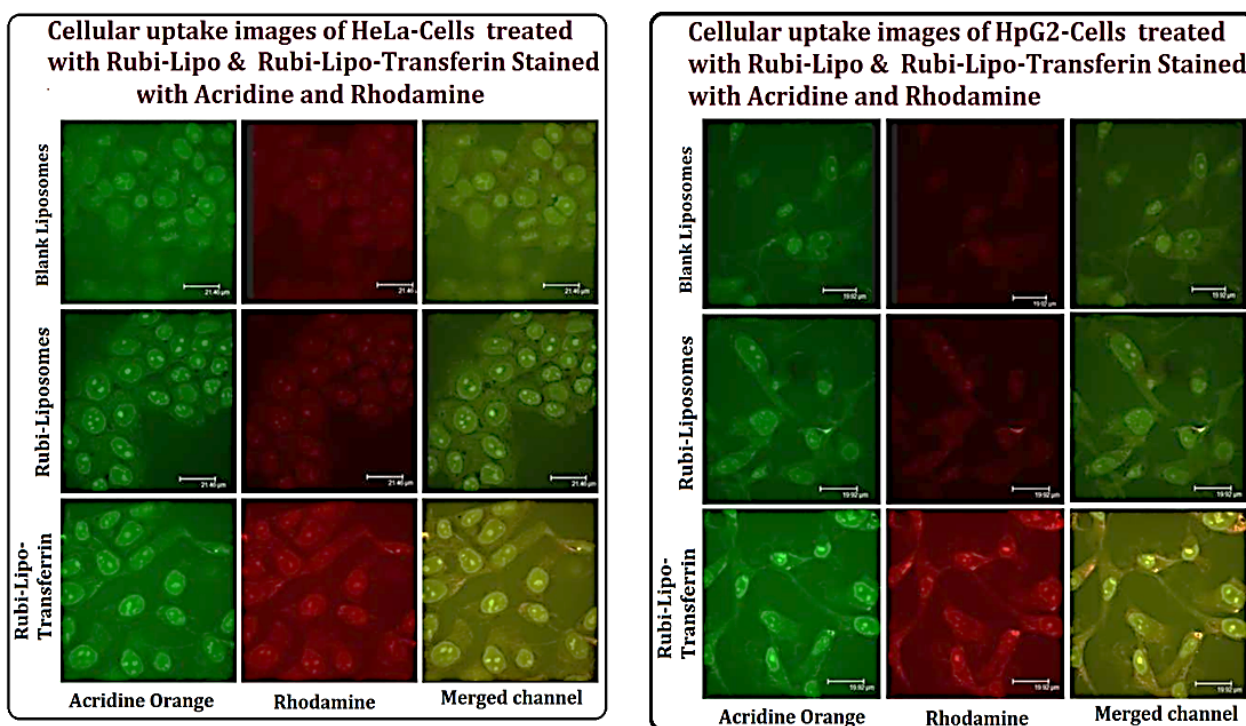


Figure 3: Confocal microscopic analysis of uptake of liposomes in (a) Human breast cancer cells & (b) Human liver cancer cells.

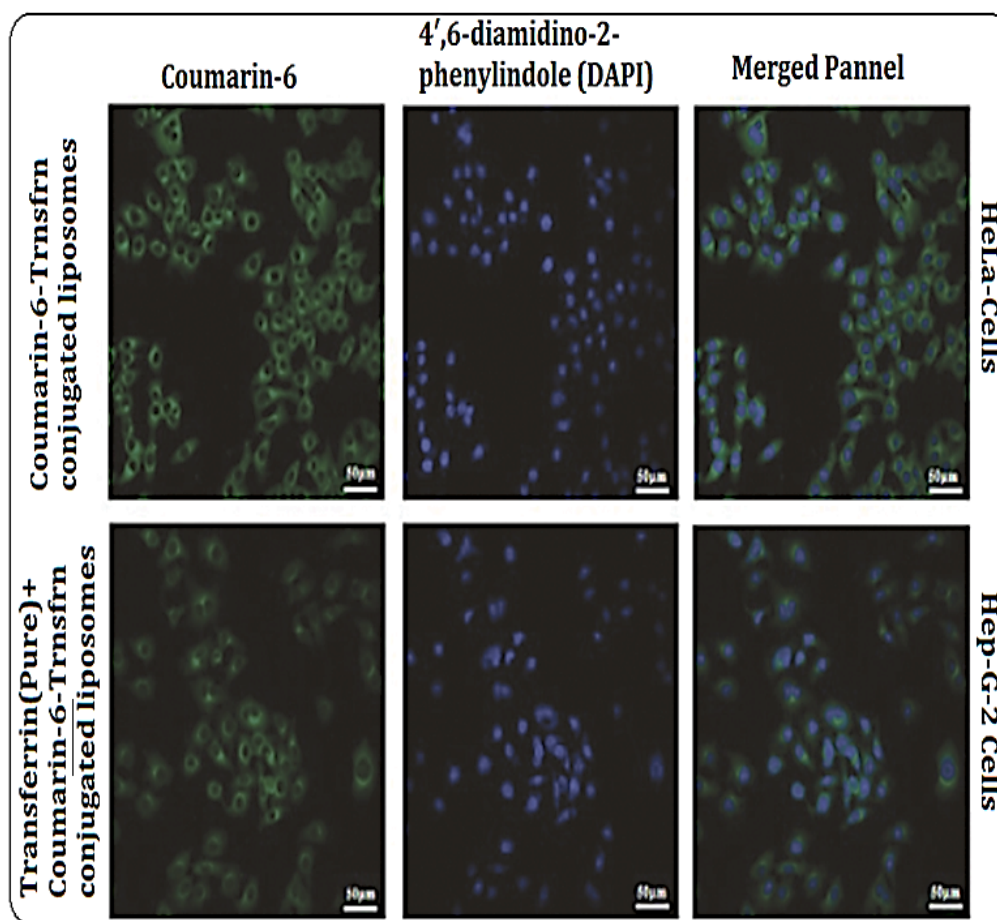


Figure 4: Competitive Binding Study.

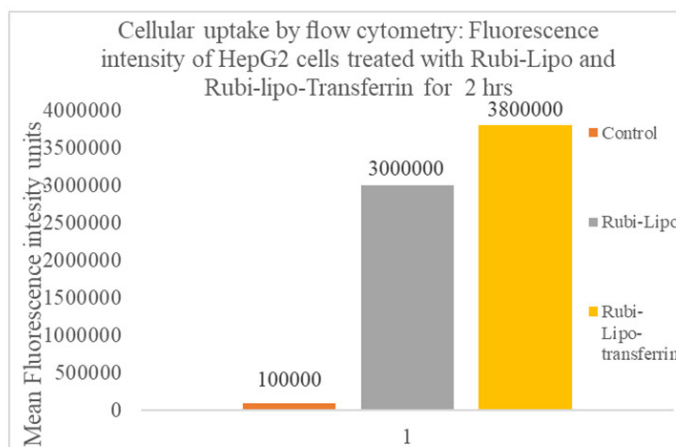
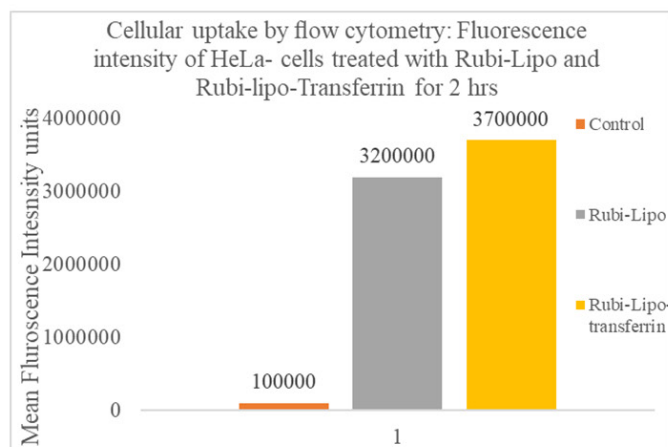


Figure 5: Cellular uptake by flow cytometry: Fluorescence intensity of cells treated with Rubi-Lipo and Rubi-lipo-Transferrin for 2 hr (a) HeLa cells and (b) HepG2 cells.²⁰

-24.2±0.38 mV. The drug Entrapment Efficiency (EE) and Drug Loading (DL) of Tf-Lip/Dio were 88.94±1.02% and 94.48±0.25%, respectively.

The liposomes' zeta potential has decreased due to transferrin conjugation. The negatively charged Transferrin molecule is thought to be the probable cause of the zeta potential drop caused by transferrin conjugation. The results for the present

study, after 72 hr at 37°C, Rubi-Lipo/Transferrin has shown a prolonged and controlled release profile of around 32% of the total Rubitecan content. Following a 24-hr drug treatment period during the incubation phase, all study groups showed a link between the Rubitecan dose and the corresponding growth inhibition, as measured by the percentage of cells that were viable (i.e., Rubitecan's dose-dependent growth suppression effects).¹¹ From the merged frames of the image, it was obvious that the

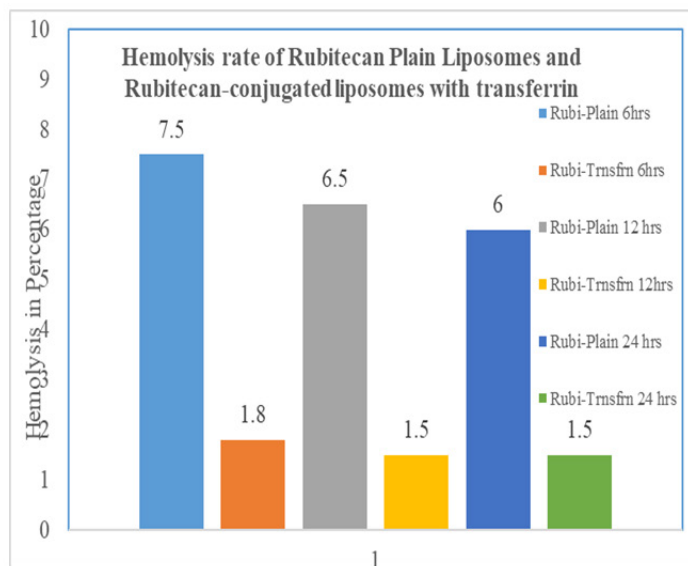
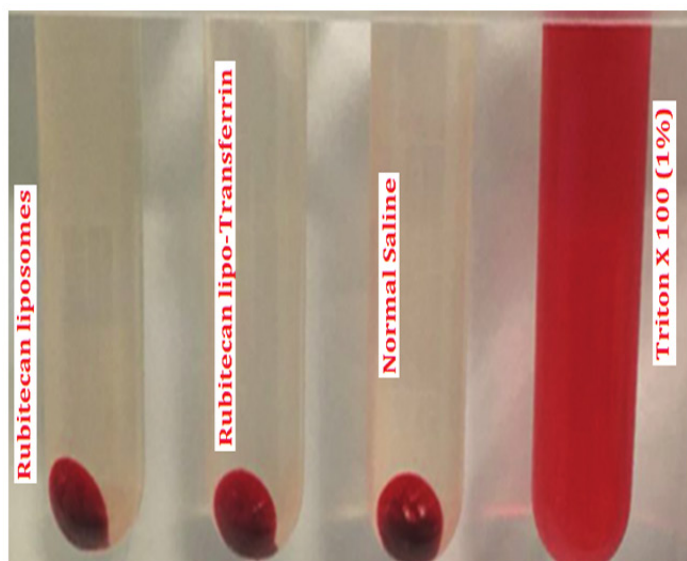


Figure 6: (a) Hemolysis test from human erythrocytes. (b) Hemolysis rate of Rubitecan Plain Liposomes and Rubitecan-conjugated liposomes with transferrin.²⁸

intracellular fluorescence intensity of Transferrin conjugated liposomes with Rubitecan was significantly higher than that of Rubitecan liposomes (plain) in both HeLa cells and HepG2 cells, and these results were in good correlation with the cytotoxicity study.¹⁷ Additionally, preliminary hemolysis studies were conducted as part of the safety protocol following intravenous delivery. Overall, this study suggests that Rubi-Lipo/Transferrin may be a useful method of delivering Rubitecan in the future for cancer treatment.

CONCLUSION

This study used the film dispersion approach to develop and manufacture novel transferrin-linked liposomes loaded with Rubitecan, a possible anticancer drug. After incubating for 24 hr in both HeLa cells and HepG2 cells, Rubi-Lipo/Transferrin has demonstrated greater cytotoxic and anticancer efficacy than in non-modified liposomes. Laser scanning confocal microscopy and flow cytometry revealed a correlation between the higher intracellular uptake and the increased anticancer property of Rubi-Lipo/Transferrin. To the best of our knowledge, this is the first instance of Rubitecan being effectively loaded into a tumor-targeted liposomal delivery system as a possible anticancer medication. This work presents the effective creation and optimization of a novel anti-tumor-targeted drug delivery system conjugated with transferrin liposomes loaded with Rubitecan as an anticancer agent. According to the study's findings, transferrin-targeted liposomes improved the ability of the drug to be delivered to the tumor, potentially enhancing its anticancer efficacy in clinical settings. The findings demonstrated that the anticancer potential of liposomes loaded with Rubitecan could be significantly increased by modifying them with transferrin.

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ABBREVIATIONS

CCK-8: Cell Counting Kit-8; **Tf-R:** Transferrin-Receptor; **Fe³⁺:** Ferric ions; **LSCM:** Laser Scanning Confocal Microscopy; **HeLa:** Human cervical cancer cell Line; **Hepg2:** Human Hepatoma Cell Line; **DMEM:** Dulbecco's Modified Eagle Medium.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

One method that has been extensively studied and shown to be successful is targeted administration via receptor-mediated absorption into the cancer cells. In this context, Transferrin Receptors (TfR) have drawn interest and have been extensively studied for their role in drug delivery into cancer cells. The present study showed that transferrin-targeted liposomes enhanced the drug's delivery to the tumor, which may have increased its anticancer effectiveness in clinical settings. The results showed that altering liposomes loaded with rubitecan with transferrin could greatly enhance their anticancer potential.

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