PCSK9 Conjugated Paclitaxel Loaded Liposomes to Target the Cancer Cells

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ABSTRACT

Statement of the Problem: There is an unmet need for anti-cancer drugs to be delivered to tumors to kill cancer cells selectively. We need systems that target cancer cells to reduce side effects seen with conventional treatments. Anti-cancer drugs may be packaged within nanoparticle-based drug delivery vectors that transport them towards their therapeutic targets. In this work, we developed a Proprotein convertase subtilisin/kexin type 9 (PCSK9) conjugated Paclitaxel-loaded Liposomes (PCSK9-PTX-liposomes) for targeting cancer cells. This work is based on the hypothesis that liposomes linked with PCSK9 will recognize LDL-receptors (LDLR) on cancer cells. PCSK9 is expressed in several organs, particularly the liver and also the intestine and the kidney. PCSK9 acts on the LDLR primarily as a secreted factor and promotes the reduction of LDLR protein concentrations. Cells internalize cholesterol from circulating LDL through the LDLR on the cell surface. Increased LDLR expression to support the continuous proliferation of the cancer cells has been linked with poor patient survival. Elevated LDLR expression on tumor cells will ensure site-specific delivery of drugs via PCSK9-Linked-Liposomes. On recognition, the liposomes will deliver drugs to the cell interior where their targets lie. **Findings:** The results of *in vitro* studies indicated that PCSK9-PTX-liposomes had shown favorable cytotoxicity activity when compared with the PTX-liposomes not conjugated with PCSK-9. In comparison with PTX-liposomes, the cell treated with the PCSK9-PTX-liposomes has shown higher accumulation of the PXT and better cytotoxic effect. The cytotoxic effect of the PCSK9-PTX-liposomes was found to be lower in the non-cancerous cells when compared with the cancerous cells because of the facts that they express less LCLR as compared with the cancerous cells. **Conclusions:** These results suggest that PCSK9-PTX-liposomes would serve as a potent PTX delivery vehicle for future cancer chemotherapy and represent a suitable platform for the development of targeted liposomal PTX systems.

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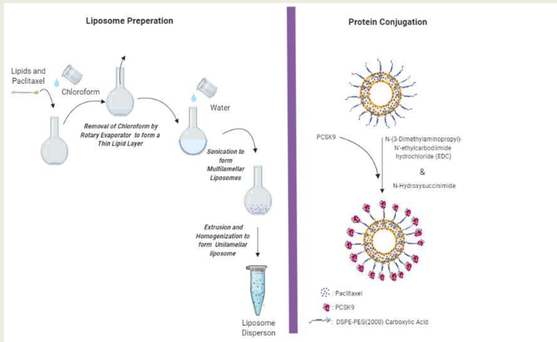
Acknowledgment : Nitin Bharat Charbe is the recipient of Conicyt Postdoctoral 2018 Fellowship (Research Project No: 3180250).

INTRODUCTION

The narrow therapeutic index, severe side effects, and unfavorable pharmaceutical characters of many anti-cancer drugs often restrict their potential effectiveness in a clinical setting. Targeted delivery of anti-cancer drugs to the cancer cells and into the tumor microenvironment is seen as a possible remedy to this problem. Nanotechnology-based systems like liposomes, micelles, linear polymers, branched polymers, and dendrimers, hold tremendous possibilities to transport drugs to the desired site. Surface modification of nanoparticles such as liposomes with targeting moieties specificto the up-regulated receptors on the surface of tumor cells represents an effective strategy. Several plasma membrane receptors have received broad interest in the drug delivery community as targets for drug delivery vectors. Low-density lipoprotein (LDL) receptors (LDLR) are usually highly expressed in hepatocytes, including cancer cells. They also offer a suitable target for drug delivery. In 2003, Seidah et al., identified the ninth member of the proprotein convertase family, proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 as a secreted protein was shown to bind to the LDLR, and both are subsequently internalized in the endolysosomal pathway. Increased LDLR expression on cancer cells is believed to meet the increased demand of the cholesterol and phospholipids to build new membranes required by the accelerated mitosis rates. In the current investigation, a novel Paclitaxel(PTX) liposomal(LIP) conjugated with PCSK9 (PCSK9-PTX-LIP) has been developed. Here the LDL-targeting properties of PCSK9 with the drug delivery properties of liposomes have been combined. This simultaneously reduces the quantity of LDLR that is utilized by the cancer cells to uptake the lipids and support the synthesis of the new cell membrane, an important hallmark of cancer. This approach, therefore, offers the dual benefit of localized delivery for the treatment of hepatocellular and other LDLR expressing carcinoma and the reduction of the critical receptor principally used by the cells for proliferation.

MHETODS AND MATERIALS

Preparation of PTX-loaded liposomes: PTX-loaded liposomes were prepared according to the thin-film hydration method described previously. Briefly, Soybean lecithin (SPC), cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), DSPE-PEG(2000)Carboxylic acid, and PTX were dissolved in chloroform. The organic solvent was removed by rotary evaporation to form the dry, thin lipid layer at the bottom. The film thus formed was hydrated in the water, and the multilaminar liposome suspension was downsized to homogeneous unilamellar liposomes using ultrasonication, and an Avanti's Extruder with 0.1 µm and 19 mm polycarbonate filter. Unilaminar liposomes thus obtained was used as the control *(CTR-PXT-LIP)* and to conjugate the PCSK9 protein *(PCSK9-PTX-LIP).*



Schematic diagram of the preparation of liposome via thin film hydration method and PCSK9 conjugation

RESULTS AND DISCUSSION

LC-MS/MS estimation of Paclitaxel: The LC-MS/MS system used was of AB Sciex(Triple Quad 4500). Calibration curve and other analysis were performed using Analyst 1.6.2 software. Paclitaxel was successfully separated using GLSciencies, Inertsil C8-3, 3 um, 4.6x 100mm column.

Zeta Potential of CTR-PTX-LIP and PCSK9-PYX-LIP Liposomes: Zeta potential values obtained were −9.31 and −2.89 mV for CTR-PXT-LIP and PCSK9-PXT-LIP, respectively. Due to the favorable zeta potential, it was therefore expected that the prepared liposomes would not only have a sustained anticancer activity, but they would be more specifically aimed at the cancer cells due to PCSK9 mediated endocytosis.

Morphological Characterization of CTR-PTX-LIP and PCSK9-PYX-LIP Liposomes: In the TEM-SEM observation (Figure 1), the *CTR-PTX-LIP* and *PCSK9-PYX-LIP* liposomes were about 130 nm in size and had a comparatively smooth spherical surface.

Drug encapsulation efficiency of liposomes: The drug encapsulation efficiency of the CTR-PTX-LIP and PCSK9-PTX-LIP was found to be 69.1% and 64.5 % respectively. The targeted liposomes showed a significant decrease in drug encapsulation efficiency. This may be because of the influence of PCSK9 (conjugate) in the formation of the liposomes that led to a disturbance in the hydrophobic membrane, which resulted in drug loss during the preparation process (Table 1).

Anticancer activity: For cell viability study the CellTiter 96® AQ_{ueous} One Solution Reagent containing novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) was used. . When the HCT116 cell was treated with CTR-PYX-LIP, PCSK9-PTX-LIP and the free drug, PCSK9 conjugated liposomes were not only found to be superior than the unconjugated (CTR-PTX-LIP) liposomes, but their cytotoxic activity was found to be better than the free drug. The difference in the activity was statistically significant after 2 hours of treatment (Figure 2).

Table 1. Particle size, polydispersity index, zeta potential and encapsulation efficiency.

Liposomes	Particle Size (nm) (mean±SD*)	Polydispersity Index (mean±SD*)	Zeta Potential (mV)	Encapsulation efficiency (%)
CTR-PTX-LIP	125±3.2	0.046	-9.31	69.1
PCSK9-PTX-LIP	149±4.0	0.136	-2.89	64.5

DISCUSSION

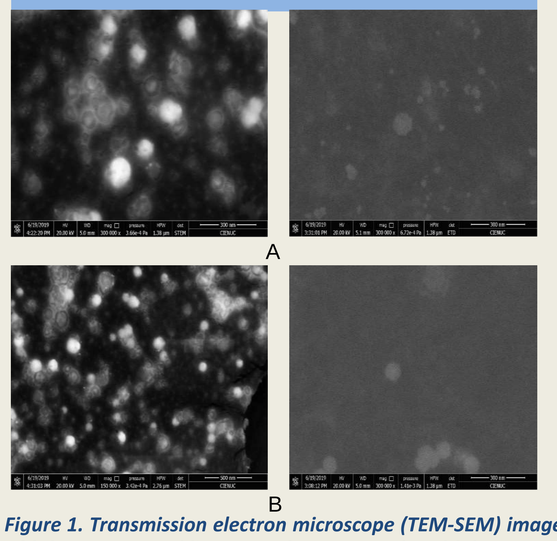


Figure 1. Transmission electron microscope (TEM-SEM) image of (A) Non-targeted liposomes (CTR-PTX-LIP) and (B) Targeted liposomes (PCSK9-PTX-LIP)

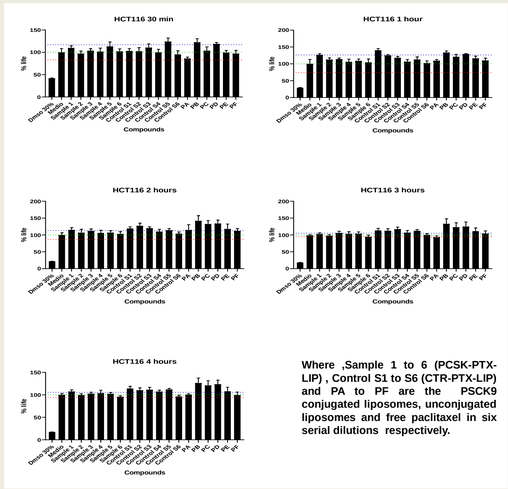


Figure 2.Cell Viability Studies using HCT116 cell line at time interval of 0.30, 1,2 ,3, and 4 hrs

CONCLUSIONS

The CTR-PTX-LIP and PCSK9-PTX-LIP liposomes containing PTX were prepared with a reproducible lipid film hydration method. The drug entrapment efficiency of unconjugated and conjugated liposomes was 69.1% and 64.5% respectively.

Considerable improvement in the drug entrapment and particle size was observed with the introduction of DSPC into the formulation.

No significant difference in the drug entrapment was observed when a 1% and 2% molar ratio PTX was loaded.

In conclusion, we successfully conjugated LDL receptor-ligand PCSK9 protein to theliposomes . Such liposomes can specifically target and deliver the drugs to the cells expressing higher than the normal levels of LDL receptors.

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