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Liposomes for targeting hepatocellular carcinoma: Use of conjugated arabinogalactan as targeting ligand

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ABSTRACT

Present study investigates the potential of chemically modified (Shah et al., 2013) palmitoylated arabinogalactan (PAG) in guiding liposomal delivery system and targeting asialoglycoprotein receptors (ASGPR) which are expressed in hepatocellular carcinoma (HCC). PAG was incorporated in liposomes during preparation and doxorubicin hydrochloride was actively loaded in preformed liposomes with and without PAG. The liposomal systems with or without PAG were evaluated for *in vitro* release, *in vitro* cytotoxicity, *in vitro* cell uptake on $ASGPR^+$ cells, *in vivo* pharmacokinetic study, *in vivo* biodistribution study, and *in vivo* efficacy study in immunocompromised mice. The particle size for all the liposomal systems was below 200 nm with a negative zeta potential. Doxorubicin loaded PAG liposomes released significantly higher amount of doxorubicin at pH 5.5 as compared to pH 7.4, providing advantage for targeted tumor therapy. Doxorubicin in PAG liposomes showed superior cytotoxicity on $ASGPR^+$ HepG2 cells as compared to $ASGPR^-$, MCF7, A549 and HT29 cells. Superior uptake of doxorubicin loaded PAG liposomes as compared to doxorubicin loaded conventional liposomes was evident in confocal microscopy studies. Higher AUC in pharmacokinetic study and higher deposition in liver was observed for PAG liposomes compared to conventional liposomes. Significantly higher tumor suppression was noted in immunocompromised mice for mice treated with PAG liposomes as compared to the conventional liposomes. Targeting ability and superior activity of PAG liposomes is established pre-clinically suggesting potential of targeted delivery system for improved treatment of HCC.

1. Introduction

Cancer, a major cause of mortality and morbidity worldwide, has accounted for 8.2 million deaths in the year 2012 (WHO, 2014). Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third most common cause of cancer mortality causing more than one million deaths annually worldwide (Nowak et al., 2004). Higher incidence of HCC is reported in African Americans and Asians (El-Serag and Rudolph, 2007). Chemotherapy presents a second line or in some cases first line approach for treatment of HCC. The efficacy of chemotherapy is limited by number of factors which include nonspecific cytotoxicity, drug resistance, relapse of tumour, limited solubility of the chemotherapeutic agent in pharmaceutically accepted solvents, and drug stability in plasma.

Targeted tumour therapy is the most sought after option for patients undergoing chemotherapy as it provides increased therapeutic efficacy and reduced adverse effects. Targeting can

Abbreviations: PAG, palmitoylated arabinogalactan; ASGPR, asialoglycoprotein receptor; HCC, hepatocellular carcinoma; SCID mice, severely compromised immunodeficient mice; TEM, transmission electron microscopy; REV, reverse phase evaporation; PBS, phosphate buffer saline; SPECT, single photon emission computed tomography; CT, computed tomography; DMEM, Dulbecco's modified eagle medium.

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be achieved passively *via* PEGylation (Kuesters and Campbell, 2010; Takae et al., 2005) or actively by virtue of receptor ligand interaction (Arnold et al., 1990; Carafa et al., 2006; Kuesters and Campbell, 2010; Sunamoto et al., 1992; Venkatesan and Vyas, 2000). A few targeted systems in the market or under advanced clinical stage include Doxil/Caelyx-PEGylated liposomal system which is commercially available; ThermoDox-Thermo sensitive liposomal system under Phase 2 and Phase 3 clinical trial; MBP-426-Transferrin conjugated liposome under Phase 2 clinical trial (Nagarsenker et al., 2014, in press). Liposomes are one of the most preferred delivery systems for targeted tumour therapy. Liposomes are amenable to incorporation of targeting ligand in the bilayers and have ability to accommodate a range of hydrophilic and hydrophobic drugs (Jain et al., 2014).

Asialoglycoprotein receptor (ASGPR) is a membrane glycoprotein receptor that binds to terminal galactose and *N*-acetylgalactosamine residues on serum glycoproteins. ASGPR's are present on the surface of liver hepatocytes in high density and are expressed in HCC (Schwartz et al., 1981; Trere et al., 1999; Weigel, 1980). ASGPR exhibits receptor mediated endocytosis and hence is a potential target for effective drug delivery to hepatocytes. Many attempts have been made to label liposomes or carriers, like polymers, human serum albumin (Franssen et al., 1993), and recombinant high density lipoprotein (Rensen et al., 2001) with ASGPR specific ligands. Galactose, lactose, acetylgalactosamine and asialofetuin have been used to develop hepatocyte-specific carriers for drug and gene delivery. Synthetic galactose polymer ligands, such as poly-(*N*-vinylbenzyl-*O*-β-D-galactopyranosyl-[1–4]-D-gluconamide, displayed a higher degree of affinity to ASGPR than the natural ligand, asialofetuin (Watanabe et al., 2000). Larch arabinogalactan, a water soluble polysaccharide with more than 80 mol% galactose, has been reported to have affinity for asialoglycoprotein receptors (Kaneo et al., 2000) and has been reported to have anti-metastatic activity by production of natural killer cells (Arabinogalactan, 2000).

In our previous report, synthesis, characterization, and safety of PAG is described (Shah et al., 2013). PAG is a palmitoyl derivative of arabinogalactan that can be incorporated into the liposomal bilayer. PAG is buff coloured solid and is soluble in chloroform, which makes it suitable for preparation of liposomes. The *in vitro* binding ability of PAG to galactose specific Ricinus communis agglutinin is already established (Shah et al., 2013). The current investigation describes formulation and characterization of doxorubicin loaded liposomes with or without palmitoylated arabinogalactan (PAG) and it's *in vitro* and *in vivo* studies. Doxorubicin loaded liposomes with or without PAG were characterized for particle size distribution, zeta potential, entrapment efficiency, and morphology using TEM. *In vitro* release profiles of doxorubicin from PAG liposomes, conventional liposomes, and solution were evaluated. *In vitro* cytotoxicity on $ASGPR^+$ cells and $ASGPR^-$ cells was evaluated to confirm specificity of PAG liposomes. Cell uptake studies were performed to understand the extent of uptake of PAG liposomes and conventional liposomes by $ASGPR^+$ HepG2 cells. *In vivo* pharmacokinetic and *in vivo* biodistribution study of PAG liposomes and conventional liposomes was performed to understand the fate of liposomes after intravenous administration. *In vivo* efficacy study in SCID mice was performed to confirm the targeting ability of PAG liposomes.

2. Experimental

2.1. Materials

Lipoid S 100 (phosphatidylcholine from soybean) and Lipoid S PC 3 (hydrogenated phosphatidylcholine from soybean) were obtained as gift samples from Lipoid®, Germany. Cholesterol was obtained from Fischer Scientific, India. Doxorubicin hydrochloride was obtained as gift sample from Khandelwal Laboratories Pvt., Ltd., Mumbai, India. HPLC grade acetonitrile (ACN) and Methanol were purchased from Merck, Germany. Double distilled water was freshly prepared as required. All the other chemicals and reagents used were of analytical grade unless otherwise specified.

2.2. Analytical method development

2.2.1. Determination of entrapment efficiency

A reverse phase HPLC method was modified to detect doxorubicin content in liposomes (Vigevani et al., 1981). The HPLC system consisted of a Shimadzu LC 20AD with a PDA Detector SPD-M20A and chromatograms were recorded and processed using the LC Solutions Software. The method consisted of mobile phase composition of methanol: phosphate buffer (10 mM, pH 3.0) in a ratio of 70:30, stationary phase of octadecyl silane (250 mm × 4.6 mm, 5 μm particle size, Hypersil ODS) and detection wavelength of 251 nm. The flow rate was 1 mL/min and injection volume was 20 μL.

2.2.2. Determination of doxorubicin from plasma

Reverse phase HPLC system comprised of a PU2080 plus pump and FP-2020 PLUS fluorescence detector from JASCO Corporation, Japan. Mobile phase composed of buffer:MeOH:ACN in a ratio of 38:12:50 with the buffer composition of 25 mM ammonium acetate, 0.25% trifluoroacetic acid, and pH adjusted to 3 with glacial acetic acid. HPLC was performed on a thermo scientific C_{18} column (250 mm × 4.6 mm, 5 μm particle size). The flow rate was 1 mL/min and injection volume was 50 μL (Wei et al., 2008; Wu et al., 2007). Excitation and emission wavelength were set at 480 nm and 580 nm respectively with a gain of 100. daunorubicin was used as internal standard.

2.2.3. Extraction procedure of doxorubicin from plasma

Internal standard (10 μL) was added to 100 μL of plasma followed by addition of 90 μL ACN. The mixture was vortexed for 1 min followed by centrifugation at 13,000 rpm for 10 min (Minispin Centrifuge, Eppendorf, USA). The supernatant was injected into the HPLC system and analysed.

2.3. Formulation of liposomes

Doxorubicin was actively loaded into preformed liposomes prepared by reverse phase evaporation vesicle (REV) method (Szoka and Papahadjopoulos, 1978) using ammonium sulphate gradient technique (Zucker et al., 2009). The method was modified for effective size reduction (Papagiannaros et al., 2006; Shah and Nagarsenker, 2010).

2.3.1. Liposome preparation by modified REV

Lipoid S 100, Lipoid S PC 3 and Cholesterol (25:25:50 mol%) with or without PAG (at 1%, 3% or 5% w/w of total lipid) were dissolved in chloroform (5 mL) and 1 mL ammonium sulphate solution (300 mM) was added and sonicated (Trans O Sonic D 250/IH, Mumbai, India) to form w/o emulsion. The resulting emulsion was subjected to rotary evaporation (Büchi Rotavapor R 114, Switzerland) in a water bath (Büchi water bath R 480, Switzerland) at 40 °C and 100 rpm for 30 min. After evaporation of chloroform, the volume of liposomal dispersion was adjusted to phospholipid concentration of 8 μmol/mL with ammonium sulphate (300 mM) solution. The resulting liposomal dispersion was probe sonicated (Branson Sonifier 250 A, USA) at power output of 30 W for 3 cycles of 2 min each.

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