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Cholesterol anchored arabinogalactan for asialoglycoprotein receptor targeting: synthesis, characterization, and proof of concept of hepatospecific delivery

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ABSTRACT

Asialoglycoprotein receptors (ASGPR) are hepatocyte bound receptors, which exhibit receptor mediated endocytosis (RME) for galactose specific moieties. Arabinogalactan (AG), a liver specific high galactose containing branched polysaccharide was hydrophobized using cholesterol (CHOL) as a lipid anchor via a two step reaction process to yield the novel polysaccharide lipid conjugated ligand (CHOL-AL-AG). CHOL-AL-AG was characterized by Fourier transform infra red (FTIR) spectroscopy, ^{1}H and ^{13}C nuclear magnetic spectroscopy (NMR), size exclusion chromatography (SEC) and differential scanning calorimetry (DSC). Conventional liposomes (CL) and surface modified liposomes (SML) containing CHOL-AL-AG were prepared using reverse phase evaporation technique. Effect of CHOL-AL-AG concentration on particle size and zeta potential of SML was evaluated. Surface morphology of CL and SML was studied using cryo-transmission electron microscopy (cryo-TEM). In vitro binding affinity of SML and CL was evaluated using *Ricinus communis* agglutinin (RCA) assay. Cellular uptake of SML and CL was determined on ASGPR expressing HepG2 cell lines by confocal laser scanning microscopy technique (CLSM). FTIR spectra revealed bands at 1736 cm^{-1} and 1664 cm^{-1} corresponding to ester and carbamate functional groups, respectively. Signals at δ 0.5–2.5 corresponding to the cholestene ring and δ 3–5.5 corresponding to the carbohydrate backbone were observed in ^{1}H NMR spectrum of the product. CHOL-AL-AG possessed a mean average molecular weight of 27 KDa as determined by size exclusion chromatography. An endothermic peak at 207 °C was observed in the DSC thermogram of CHOL-AL-AG, which was not observed in thermograms of reactants and intermediate product. Synthesized CHOL-AL-AG was successfully incorporated in liposomes to yield SML. Both CL and SML possessed a mean particle size of~200 nm with polydispersity index of~0.25. The zeta potential of CLs was observed to be −17 mV whereas zeta potential of SMLs varied from −18 to −22 mV. RCA assay revealed enhanced binding of SML compared to CL confirming presence of galactose on surface of SML. CLSM studies demonstrated enhanced cellular uptake of SMLs compared to CL by HepG2 cells post 3 h administration indicating enhanced uptake by the ASGPR. Thus surface modified liposomes specific to target heptocytes demonstrate a promising approach for targeted drug delivery in liver cancer therapeutics.

1. Introduction

Therapeutic efficacy of bioactives apart from their pharmacological effectiveness largely depends upon their concentration at the desired site of action. Systemic metabolism of the bioactives requires administration of higher doses as to compensate the

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losses. Increased doses lead to adverse drug reactions, which are more common with chemotherapeutic agents due to non-selectivity between host and tumor cells. Target specific drug delivery aims at spatial as well as temporal delivery of the bioactive resulting in negligible and/or reduced exposure to the host cells and therefore reduced side effects. In cancer chemotherapy, the delivery system often aims towards targeting receptors of ligands like transferrin, folic acid, sugars, lectins and growth hormones, which are over-expressed on cell surface etc. Asialoglycoprotein receptor (ASGPR) is a selectin E type receptor present in high density[1] ($1–5\times10^5$ receptors/cell) on the surface of liver hepatocytes and is present on Hepatocellular Carcinoma (HCC) cells.[2] ASGPR exhibits receptor mediated endocytosis (RME) and shows selective uptake of moieties with terminal galactose or galactosamine residues.

Use of carbohydrates as ligands for receptor based drug targeting has achieved substantial importance in recent times. Carbohydrate/polysaccharide coated particulate carrier systems have been shown to be selectively taken up by the lungs, liver and spleen depending upon the presence of mannose, galactose or glucose as the terminal residue.[3] Apart from delivery of chemotherapeutic agents,[4] carbohydrate ligand based particulate carriers have been explored for delivery of genetic materials[5] and oral vaccines.[6] Liposomes are lipid based biocompatible particulate carrier systems, which have been extensively explored for site specific targeted drug delivery owing to ease of assimilation of cell specific ligands and feasibility of incorporation of hydrophobic and/or hydrophilic agents. Reports on use of carbohydrates/polysaccharides for coating/covalent attachment to liposome for targeting the delivery of bioactive to specific organs such as lungs and liver,[3,7] imparting physical stability[8] and increasing the plasma circulation of the carrier systems have appeared in literature. Apart from lactose[9] and galactose,[10,11] polysaccharides such as pullulan,[12] amylopectin,[13] scleroglucan,[14] xyloglucan[15] etc. have been successfully used for liposome coating. Incorporation of galactose or galactose rich polysaccharide onto the surface of liposome will render the drug delivery system hepatospecific owing to its selective uptake by the ASGPR of hepatocytes.

Early developments were focused towards physical adsorption of carbohydrate solutions on the carrier system.[16] Nonetheless, these systems showed poor physical stability and also had substantial chances of ligand being dissociated from the carrier system during or post administration in the biological system. Subsequent studies lead to the development of conjugated systems in which the ligand was covalently attached to a lipid anchor such as long chain fatty acids. Use of polysaccharides, which have numerous terminal monosaccharide units of interest, increases probability of presence of free terminal carbohydrate for receptor ligand binding.

Larch arabinogalactan (AG) is a high molecular weight branched polysaccharide composed of galactose and arabinose units with a trace of uronic acid. The molecular weights of the major fractions of AG in larch are 16,000 and 100,000 Da. Glycoside linkage analysis of AG is consistent with a highly branched structure comprising a backbone of 1,3-linked galactopyranose connected by 1,3-glycosidic linkages, comprised of 3,4,6-,3,6-, and 3,4- as well as 3-linked residues.[17] Use of AG as a drug conjugate has been reported for improving the water solubility of an *anti*-fungal agent.[18] AG is reported to have activity against INF-α and also enhancing the activity of natural killer cells.[17] However, the high aqueous solubility and poor solubility in organic solvents of AG hampers its successful use in lipid based systems.

The present work aims at design and synthesis of novel polysaccharide lipid conjugate ligand and its incorporation in drug delivery system to render them hepatocyte specificity. We propose hydrophobization of AG by its covalent attachment to a lipid anchor. Cholesterol chloroformate (CCF), a derivative of cholesterol was used as the lipid anchor to conjugate with AG via a bi-functional spacer arm β-alanine (AL).

2. Results and discussion

It is reported that ASGPR present on hepatocytes specifically bind to terminal galactose residues of carbohydrate/polysaccharides. Use of hydrophobized carbohydrates is reported for incorporation into liposomal systems intended for organ targeting. Most of these conjugations are esterification and amidation reactions involving hydroxyl/amino group of carbohydrates and carboxylic acid group of the lipid anchor.[8,19] Poor specificity of carbohydrate chemistry does not allow selective esterification unless protecting groups are introduced. This makes the reaction more complicated, cumbersome and lengthy.[20] Further, there is a fair chance of decrease in the yield of the product with increasing number of reaction steps. One strategy to overcome the above issues is to choose polysaccharides, which have multiple terminal monosaccharide units of interest for reaction.

In the present work, we propose hydrophobization of AG by its covalent attachment to a lipid anchor. AG is a dietary supplement approved by the USFDA, has a high galactose content and highly water soluble. Pharmacokinetic study of AG in rats has revealed high liver uptake postulated to be due to selective uptake by the ASGPR, which has specificity for the galactose present in AG.[17] CCF, which is a derivative of CHOL was used as the lipid anchor due to its dual advantage. CHOL is one of the components of liposome, which provides rigidity, stability and aids in controlled release of the entrapped bioactive. Acyl derivative of CHOL further provides ease of chemical reaction. AG and CCF were linked via bi-functional spacer arm β alanine (AL)—a bio-compatible amino acid.

2.1. Synthesis of CHOL-AL-AG

The two step reaction scheme is depicted in Fig. 1. The first step consisted of reaction of CCF with AL to give N-(cholest-5-ene-3β-oxycarbony alanine) [CHOL-AL] through a carbamate bond between the amino ($-NH_2$) function of AL and the acyl group of CCF. It is a base catalyzed reaction in presence of a phase transfer catalyst. Use of phase transfer catalyst provided ease of reaction since both the reactants could not be solubilised in a common solvent. Initially, AL was dissolved in 3 M NaOH so as to provide the basic condition for further addition of CCF. Nevertheless, presence of 'excess' base was avoided as CCF degrades in aqueous environment in presence of strong base resulting in formation of CHOL. Hence, simultaneous addition of CCF and NaOH was performed over a period of 30 min maintaining ice cold condition. The reaction conditions were optimized and it was observed that reaction was complete in two hours. Beyond two hours, the acyl portion of CCF was prone to degradation and resulted in formation of CHOL. The product yield was greater than 90% and thin layer chromatography (TLC) indicated a single spot using a mobile phase composed of hexane: ethyl acetate; 6:4. $R_{f\ value}$ was found to be 0.23. The product CHOL-AL was observed to have a melting point (mp) of 127–128 °C.

The second step involved conjugation of CHOL-AL with AG by forming an ester bond between the −COOH group of CHOL-AL and $-CH_2OH$ group of AG. The −COOH group of CHOL-AL was activated using carbonyldiimidazole (CDI), which is an organic soluble carbodiimide derivative. Use of CDI requires anhydrous conditions, which were maintained using calcium chloride guard tube. The reaction conditions were optimized and it was found that there was no difference in the product with regards to molecular weight and yield after carrying out the reaction for 24 h and 12 h. The product being a polysaccharide derivative did not have a sharp melting