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Lactosylated gramicidin-based lipid nanoparticles (Lac-GLN) for targeted delivery of anti-miR-155 to hepatocellular carcinoma

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ABSTRACT

Lactosylated gramicidin-containing lipid nanoparticles (Lac-GLN) were developed for delivery of anti-microRNA-155 (anti-miR-155) to hepatocellular carcinoma (HCC) cells. MiR-155 is an oncomiR frequently elevated in HCC. The Lac-GLN formulation contained N-lactobionyl-dioleoyl phosphatidylethanolamine (Lac-DOPE), a ligand for the asialoglycoprotein receptor (ASGR), and an antibiotic peptide gramicidin A. The nanoparticles exhibited a mean particle diameter of 73 nm, zeta potential of +3.5 mV, anti-miR encapsulation efficiency of 88%, and excellent colloidal stability at 4 °C. Lac-GLN effectively delivered anti-miR-155 to HCC cells with a 16.1- and 4.1-fold up-regulation of miR-155 targets C/EBPβ and FOXP3 genes, respectively, and exhibited significant greater efficiency over Lipofectamine 2000. In mice, intravenous injection of Lac-GLN containing Cy3-anti-miR-155 led to preferential accumulation of the anti-miR-155 in hepatocytes. Intravenous administration of 1.5 mg/kg anti-miR-155 loaded Lac-GLN resulted in up-regulation of C/EBPβ and FOXP3 by 6.9- and 2.2-fold, respectively. These results suggest potential application of Lac-GLN as a liver-specific delivery vehicle for anti-miR therapy.

1. Introduction

Hepatocellular carcinoma (HCC) accounts for a majority of liver cancers. It is one of the fast growing causes of cancer deaths, affecting more than 500,000 people each year [1]. MicroRNAs (miRs) are non-coding RNAs that regulate gene expression by translational repression through the RNA-induced silencing complex (RISC). Aberrant expression of miRs, either a reduction or an elevation, has been shown to be involved in various diseases, such as inflammation [8], cardiovascular disease [9], and cancers [10–12]. MiRs and anti-miRs have emerged as potential therapeutic agents. MiR-155, a hepatic oncogenic miR (oncomiR), is induced by a broad range of inflammatory mediators including proinflammatory cytokines, and is trans-activated by nuclear factor kappa B (NF-κB) [1]. Increased expression of miR-155 has been shown in human inflammatory cells and tumors [2–6]. Recent studies have shown that there is a correlation between the expression level of miR-155 and histopathological changes in choline-deficient and amino acid-defined (CDAA) diet-induced HCC and in primary human HCC. This suggests miR-155 as a potential therapeutic target in early stages of tumorigenesis [7].

The function of miRs can be inhibited by anti-miRs, which are oligonucleotides with complimentary sequences. Recently, the anti-miR strategy has been validated against several oncomiRs [13–17]. In this study, an anti-miR-155 was designed and synthesized specifically to inhibit miR-155 function. Free anti-miRs are unstable in the plasma and face barriers such as nucleases and renal clearance. Non-specific tissue uptake as well as inefficient delivery limit their potential application as a therapeutic agent. Lipid nanoparticles (LNs) have been shown to be effective for nucleic acid delivery [18–23].

HCC cells are derived from hepatic parenchymal cells with expression of the asialoglycoprotein receptor (ASGR) [24,25]. ASGR is capable of recognizing galactose-terminated glycoproteins and glycoconjugates, therefore, is useful as a cellular marker for targeted delivery to hepatocytes and to HCC cells [26–29].

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In the present study, a lipophilic ASGR targeting ligand, composed of lactobionic acid (LA), bearing a galactose moiety and linked to a phospholipid, was synthesized and incorporated into LNs for liver-specific delivery of anti-miR-155. In addition, gramicidin A, a hydrophobic peptide, was incorporated to facilitate endosomal release of the anti-miR following endocytosis [30–32]. This formulation was named lactosylated gramicidin-based LN (Lac-GLN). Its potential on hepatocyte targeting was evaluated in HepG2 cells and in mice. The physicochemical properties, cellular uptake, *in vitro* and *in vivo* delivery efficacy were investigated.

2. Materials and methods

2.1. Chemicals and reagents

1,2-Dioleoyl-3-dimethylammonium-propane (DODAP), and L-α-dioleoyl phosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL); 1, 2-dimyristoyl-sn-glycerol and methoxypolyethylene glycol (DMG-PEG) were purchased from NOF America Corporation (Elysian, MN); 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were from Thermo Scientific (Rockford, IL). Monomethoxy polyethylene glycol 2000-distearoyl phosphatidylethanolamine (mPEG-DSPE) was obtained from Genzyme Pharmaceuticals (Cambridge, MA). Cholesterol, lactobionic acid, gramicidin A and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) without further purification. Firefly Luciferase (GL2 + GL3) siRNA (Luci-siRNA) (AM 4629), negative scrambled control (AM 17010), and Lipofectamine 2000 were purchased from Invitrogen (Grand Island, NY). Anti-miR-155 (sequence: 5′-A*C*CCCUA UCACGAUUAGCAUU*A*A-3′), containing phosphorothioate linkages (*) and 2′-O-Methylation, Cy3-labeled anti-miR-155 (Cy3-anti-miR-155), and Cy5.5-labeled anti-miR-155 (Cy5.5-anti-miR-155) were synthesized by Alpha DNA (Montreal, Canada). The Taqman kits for real-time RT-PCR assay of miR-155 (002623) and RNU6B (001093) were purchased from Applied Biosystems (Carlsbad, CA).

2.2. Preparation of anti-miR-155 containing Lac-GLN

The targeting ligand was synthesized as described previously [33]. Briefly, lactobionic acid was activated by EDC and converted to its NHS ester, which is then reacted with DOPE to yield n-lactobionyl-DOPE (Lac-DOPE). The product was characterized by Fourier transform infrared (FTIR) spectrometry on a Nexus 470 FTIR Spectrometer (Thermo Scientific, Rockford, IL).

Lac-GLNs were prepared by the ethanol injection method. The lipid mixture, composed of DODAP, Lac-DOPE, DOPE, DMG-PEG and gramicidin A at a molar ratio of 50:10:28:2:10, was dissolved in ethanol, and rapidly injected into RNAse- and DNAse-free HEPES buffered solution (20 mM, pH 7.4). The resulting lipid nanoparticles were sonicated for 2 min by a bath sonicator and dialyzed against RNAse- and DNAse-free water for 4 h at room temperature to remove ethanol using a molecular weight cut-off (MWCO) 10,000 Dalton Float-A-Lyzer (Spectrum Laboratories Inc., Ranco Dominguz, CA).

The anti-miR-155 containing Lac-GLN was prepared by adding an equal volume of anti-miR-155 dissolved in RNAse- and DNAse-free HEPES buffer to Lac-GLN, followed by brief vortexing for 10 s and incubation at room temperature for 10 min. The weight ratio of lipids: anti-miR was fixed at 10: 1, and the concentration of anti-miR-155 was 1 μg/mL. The resulting nanoparticles were sterilized using 0.22 μm filters (Fisher Scientific, Pittsburgh, PA). Control formulations were prepared by the same method.

2.3. Size, surface charge, and encapsulation efficiency measurements

The particle size of anti-miR-155 containing Lac-GLN was determined by dynamic light scattering on a Model 370 NICOMP Submicron Particle Sizer (NICOMP, Santa Barbara, CA) in the volume-weighted distribution mode. Particles were dispersed in cell culture medium. The morphology of Lac-GLN was examined by a FEI Tecnai G2 Bio TWIN transmission electron microscope (FEI Company, OR, USA). Briefly, samples were prepared as described above. A drop of the sample was negatively stained with uranyl acetate for 1 min on a perforated carbon grid for analysis. Images were recorded using a Gatan 791 MultiScan CCS camera and processed by the Digital Micrograph 3.1 software package.

The zeta potential of anti-miR-155 containing Lac-GLN was examined in 20 mM HEPES buffer using ZetaPALS zeta potential analyzer (Brookhaven Instruments Corp., Holtsville, NY).

Encapsulation efficiency of Lac-GLN was determined by Quant-iT™ RiboGreen RNA Kit (Invitrogen, Grand Island, NY) following the manufacturer's protocol, and the fluorescence intensity (FI) was determined using a luminescence spectrometer (KS 54B, Perkin Elmer, UK) at an excitation of 480 nm and an emission of 520 nm. The encapsulation efficiency was calculated by the following equation.

$$\text{Encapsulation efficiency } (\%) = \left(1 - \frac{\text{FIwithout Triton X} - 100}{\text{FIwith Triton X} - 100}\right) \times 100\%$$

2.4. Colloidal and serum stability of Lac-GLN

The colloidal stability of anti-miR-155 containing Lac-GLN was determined by monitoring changes in its particle size over a 30-day period during storage at 4 °C or 25 °C. A serum stability test was performed to investigate the ability of Lac-GLN to protect anti-miR from serum nuclease degradation. Briefly, anti-miR-155-lac-GLN and free anti-miR-155 were exposed to 50% fetal bovine serum (FBS) and incubated at 37 °C for various time periods. Aliquots of each sample were then loaded onto a 1.5% (w/v) agarose gel containing ethidium bromide.

2.5. Cell culture and in vitro transfection studies

Human HCC SK-Hep-1 and HepG2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO_2.

For Luci-siRNA transfection, 2×10^4 SK-Hep-1 cells stably expressing luciferase, were seeded per well in 800 μl culture medium in 48-well plates and allowed to grow overnight at 37 °C under 5% CO_2 atmosphere. Next day, the culture medium was replaced with medium containing 0%, 10% and 20% FBS, and cells were transfected with Luci-siRNA containing Lac-GLN and various control formulations at 100 nM for 4 h. After transfection, the medium was replaced with fresh medium containing 10 % FBS. At 48 h post transfection cells were washed with PBS and luciferase activity in cell lysates was determined using Luciferase Assay Kit (Promega, Madison, WI) following manufacturer's instruction. Briefly, the total amount of protein of each well was determined using BCA Assay Kit (Pierce, Rockford, IL), and luciferase activity was determined by normalization to the total amount of protein. Luciferase down-regulation was then calculated as a relative value compared to the untreated negative control.

For anti-miR-155 transfection, HepG2 cells were plated at 2×10^5 cells per well in 6-well plates with 2 ml cultured medium, and incubated overnight at 37 °C under 5% CO_2 atmosphere. The culture medium was then replaced with fresh medium, and cells were transfected with 100 nM anti-miR-155 using Lipofectamine 2000, Lac-GLN, and control formulations and after 4 h incubation, the medium was replaced with fresh medium. Cells were incubated for an additional 48 h at 37 °C, then miR-155 and its target gene expression level was determined by real time RT-PCR analysis. As a positive control, cells transfected with Luc-siRNA and anti-miR-155 using Lipofectamine 2000 were performed following manufacturer's protocol. Untreated cells and empty Lac-GLN were used as negative controls.