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Liver-targeted antiviral peptide nanocomplexes as potential anti-HCV therapeutics

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ABSTRACT

Great success in HCV therapy was achieved by the development of direct-acting antivirals (DAA). However, the unsolved issues such as high cost and genotype dependency drive us to pursue additional therapeutic agents to be used instead or in combination with DAA. The cationic peptide p41 is one of such candidates displaying submicromolar anti-HCV potency. By electrostatic coupling of p41 with anionic poly(amino acid)-based block copolymers, antiviral peptide nanocomplexes (APN) platform was developed to improve peptide stability and to reduce cytotoxicity associated with positive charge. Herein, we developed a facile method to prepare galactosylated Gal-APN and tested their feasibility as liver-specific delivery system. *In vitro*, Gal-APN displayed specific internalization in hepatoma cell lines. Even though liver-targeted and non-targeted APN displayed comparable antiviral activity, Gal-APN offered prominent advantages to prevent HCV association with lipid droplets and suppress intracellular expression of HCV proteins. Moreover, *in vivo* preferential liver accumulation of Gal-APN was revealed in the biodistribution study. Altogether, this work illustrates the potential of Gal-APN as a novel liver-targeted therapy against HCV.

1. Introduction

More than 185 million people worldwide are infected with the Hepatitis C Virus (HCV) [1]. This blood-borne pathogen leads to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, and it is also the primary cause of liver transplantation in the United States [2]. In spite of being such a fatal disorder, the standard of care until 2011 was restricted to the combination of pegylated interferon-α (PEG IFN-α) and ribavirin, which exhibited limited sustained viral response and poor tolerability [3]. The emergence of direct-acting antivirals (DAA), which specifically block HCV replication, has marked a new era in HCV therapy [4]. However, the challenges such as affordability, genotype selectivity and possible long-term resistance to DAA therapy due to viral mutations [5] still necessitate the development of alternative anti-HCV drug candidates. As an option, therapeutic peptides are revolutionizing the paradigm of drug development with their prominent bioactivity [6], and, in fact, several anti-HCV peptides, targeting different stages of HCV life cycle, have been discovered [7–9]. One such, the 18-mer C5A, which is derived from the membrane anchor domain of HCV nonstructural protein NS5A and exhibits broad spectrum antiviral activity with submicromolar potency *in vitro* [10,11]. The characteristics of α-helicity and amphipathicity allow this peptide to neutralize the viral particles by destabilizing the lipid composition of viral membranes without genotype dependency [12]. Its cationic derivative, with 4 residues in C5A replaced with positively charged amino acids (lysine and arginine), which we refer to as p41, also displays equivalent antiviral potency. However, the susceptibility to proteolysis and the unfavorable toxicity profile, typical for cationic peptides, remain the obstacles in their translation to clinics.

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In an attempt to overcome these restraints, our group has successfully developed the well-defined antiviral peptide nanocomplexes (APN) through the immobilization of cationic p41 into nanoscale block ionomer complexes with oppositely charged poly(amino acid)-based block copolymers [13]. The p41 encapsulation into APN led to higher proteolytic stability, reduced cytotoxicity and unaltered antiviral potency of the peptides. The self-assembly behavior and preparation simplicity make the APN an extremely promising approach for peptide delivery. Using the APN platform, a more selective delivery of p41 to hepatocytes as primary sites for HCV replication can be achieved through targeting the asialoglycoprotein receptor (ASGP-R), which is a well-defined endocytotic receptor primarily expressed on parenchymal liver cells [14–16]. Therefore, we hypothesize that the decoration of the APN surface with the ligand β-D-galactose (Gal), which possesses high binding affinity to ASGP-R ($K_d \sim 10^{-3}$–10^{-4} M) [17,18], will target APN to the liver, thereby providing a basis for organ-specific anti-HCV therapy development.

Here, we have successfully prepared a series of APN with varying densities of the Gal ligand on the surfaces (Gal-APN) and evaluated their antiviral activities in cell culture systems. Biodistribution studies demonstrated preferential liver accumulation of Gal-decorated APN proving the effectiveness of this targeting approach for the delivery of antiviral peptides.

2. Materials and methods

2.1. Materials

Peptide p41 (SWLRRIWRWICKVLSRFK) and Cy5-labeled p41 were custom synthesized by AnaSpec (USA). α-(9-Fluorenylmethyloxycarbonyl)amino-ω-carboxy succinimidyl ester poly(ethylene glycol) (Fmoc-PEG-NHS, M_W (PEG) = 5000 g mol^{-1}, M_w/M_n = 1.02) was purchased from Jenkem Technology (China). Amberlyst® 15 hydrogen form, molecular sieve UOP type 3 Å, L-glutamic acid γ-benzyl ester, D (+)-galactose, propargylamine, L(+)-ascorbic acid sodium salt and copper (II) sulfate pentahydrate, 98+%, A.C.S. reagent were obtained from Sigma–Aldrich (U.S.). Silica gel (for chromatography, 0.03–0.200 mm, 60 Å), 2-bromoethanol, ethyl acetate, methanol, dichloromethane (DCM), dimethylformamide (DMF), tetrahydrofuran (THF) were purchased from Acros Organics (USA). S-2-(4-Isothiocyanatobenzyl)-diethylenetriamine pentaacetic acid (SCN-DTPA) was ordered from Macrocyclics (Dallas, TX, USA). Lutetium-177 trichloride was obtained from PerkinElmer (USA).

2.2. Synthesis of Gal-terminated poly(ethylene glycol)-block-poly(L-glutamic acid) copolymer (Gal-PEG-b-PLE)

Gal-PEG-*b*-PLE copolymer was synthesized using the following steps (Scheme 1):

2.2.1. Synthesis of propargyl-PEG-NH_2

Fmoc-PEG-NHS (0.5 g, 0.1 mmol) was dissolved under stirring in 5 ml of DCM at 0 °C. The propargylamine (80 μl, 1.2 mmol) dissolved in 5 ml of cold DCM was added dropwise and the solution was stirred for 24 h. The solvent was removed at reduced pressure and the product was dialyzed against distilled water (MWCO 2 kDa) followed by lyophilization to give Fmoc-PEG-propargyl. Standard 20% v/v piperidine/DCM solution (3 ml) was used to remove the Fmoc-protecting group. After stirring for 2 h at room temperature, DCM was removed by rotaevaporation, reaction mixture was redissolved in water and after filtration dialyzed against distilled water (MWCO 2 kDa) for 2 days, and then lyophilized to obtain propargyl-PEG-NH_2.

2.2.2. Synthesis of propargyl-PEG-b-PLE

The BLE-NCA (γ-benzyl L-glutamate-N-carboxyanhydride) monomer was prepared as described previously [19]. Benzyl-protected propargyl-PEG-*b*-PBLE was synthesized via ring-opening polymerization of BLE-NCA monomer using propargyl-PEG-NH_2 as a macroinitiator. Propargyl-PEG-NH_2 (20 mg, 0.004 mmol) was dissolved in 300 μl of anhydrous DMF with 3 μl of triethylamine (TEA) (5 equivalent of PEG) and then added into water-free and oxygen-free ampules. BLE-NCA (26.3 mg, 0.1 mmol, the feed molar ratio of propargyl-PEG-NH_2 to BLE-NCA was 1:25) dissolved in 250 μl of DMF was added dropwise under nitrogen. The ampule was sealed and the reaction was allowed to proceed at 40 °C for 48 h. The solvent was evaporated under vacuum and the residue was dissolved in THF followed by addition of 2 N NaOH to deprotect glutamate residues. After stirring for 4 h at 40 °C, THF was removed at reduced pressure, the residual solution was neutralized by 1 M HCl, dialyzed using a dialysis membrane (MWCO 3.5 kDa) against distilled water for 48 h, and lyophilized to obtain propargyl-PEG-*b*-PLE.

2.2.3. Synthesis of 2′-azidoethyl-O-D-galactopyranoside

The 2′-azidoethyl-O-D-galactopyranoside was synthesized according the procedure reported by Geng et al. [20]. Briefly, the Amberlyst® 15 hydrogen form (8.0 g) and molecular sieve (4.0 g) suspended in 2-bromoethanol (50 ml) were refluxed for 30 min followed by addition of D(+)-galactose (8.0 g). After another 3 h reflux, the product formation was confirmed by TLC (ethyl acetate/methanol, 7/1, v/v, R_f: 0.3), reaction mixture was filtered, and rotaevaporated. The crude 2'-bromoethyl-D-galactopyranoside was further purified by flash column chromatography on silica gel. The obtained 2'-bromoethyl-D-galactopyranoside (3.0 g, 10.5 mmol) and sodium azide (2.1 g, 21 mmol) were further dissolved in water/acetone (7.5 ml/45 ml) and refluxed for 20 h. After removal of solvents by rotaevaporation, the residue was suspended in ethanol and filtered to remove an excess of salts (sodium azide and sodium bromide). The filtrate was concentrated under vacuum to obtain the final product as a white solid. The structure was characterized by ^{1}H NMR [Bruker AVANCE III instrument (400 MHz)] and mass spectrometry (Qtof Micro electrospray ionization mass spectrometer, Waters, USA).

2.2.4. Galactosylation via click reaction

The 2′-azidoethyl-O-D-galactopyranoside (3.12 mg, 20 μmol) was reacted with propargyl-PEG-*b*-PLE (20 mg, 4 μmol) dissolved in 100 μl of a water and methanol (1:1) mixture in the presence of $CuSO_4$•$5H_2O$ (62.42 μg, 0.25 μmol), tris-(hydroxypropyltriazolylmethyl)amine (218.5 μg, 0.5 μmol) as the stabilizing agent and ascorbic acid (495.28 μg, 2.5 μmol) for 2 days at room temperature. The product was dialyzed against distilled water (MWCO 3.5 kDa) for another 2 days to remove all the unreacted small molecules and then lyophilized to produce Gal-PEG-*b*-PLE.

2.2.5. Polymer characterization

The composition of propargyl-PEG-*b*-PBLE was confirmed by ^{1}H NMR in DMSO-*d6* at 80 °C using a Bruker 400 MHz spectrometer. Gel permeation chromatography (GPC) measurements to determine the molecular weights and polydispersity (PDI = M_w/M_n) of the polymers were carried out at 40 °C using a Shimadzu liquid chromatography system equipped with TSK-GEL® column ($G4000H_{HR}$) connected to Shimadzu RI and UV/vis detectors. DMF was used as mobile phase at a flow rate of 0.6 ml/min. Poly(-ethylene glycol) standards (Agilent Technologies, USA) with a molecular weight range of 106–34,890 were used to generate the standard curve. The Gal conjugation efficiency was determined by phenol-sulfuric acid method in microplate format [21]. Briefly,