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Full Length Article

Doxorubicin and paclitaxel co-bound lactosylated albumin nanoparticles having targetability to hepatocellular carcinoma

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ABSTRACT

Anticancer drug targeting to liver asialoglycoprotein receptors (ASGPR) is viewed as a good approach for hepatocellular carcinoma (HCC) treatment. Lactose residue is a promising ASGPR ligand due to its high receptor affinity. Herein, we introduce doxorubicin and paclitaxel co-bound lactosylated albumin (Lac-BSA) nanoparticles (Dox/Pac Lac-BSA NPs) with good liver targetability. Lac-BSA was synthesized by conjugating lactobionic acid to naïve BSA then characterized by mass spectrometry. Dox/Pac Lac-BSA NPs were fabricated utilizing high-pressure homogenization and evaporation with Nab® (nanoparticle albumin bound) technology. Dox/Pac Lac-BSA NPs were spherical and well-dispersed, with a 148.7 ± 13.8 nm particle size and -54.1 ± 0.7 mV zeta potential at a 100% Lac-BSA feed ratio. Combined Dox and Pac synergistic cytotoxicity was confirmed in Hep G2 cells. Specifically, the inhibitory concentration (IC_{50}; 0.21 ± 0.02 μg/ml) for Dox/Pac Lac-BSA NPs was 3.2 time lower than plain Dox/Pac BSA NPs (IC_{50}; 0.68 ± 0.04 μg/ml). Also, Dox/Pac Lac-BSA NPs exhibited better internalizing in Hep G2 cells (61.8% vs. 14.4% for Dox) and spheroids compared to Dox/Pac BSA NPs. Finally, Dox/Pac Lac-BSA NPs displayed much greater localization into ICR mice livers compared to Dox/Pac BSA NPs. This was indicated by the presence of NP lactose residues revealed by a galactose inhibition study. Based on these results, we suggest that lactose-modified albumin-based nanoparticles fabricated with the Nab® technique can be a potential therapeutic vector for treating HCC via hepatocyte targeting.

1. Introduction

Hepatocellular carcinoma (HCC) is the major primary liver cancer type and constitutes 70–90% of all cases [1,2]. HCC is the fifth commonly occurring cancer worldwide and the second leading cancer-related death cause [3,4]. HCC presents a very poor prognosis, which leads to short median survival (6–9 months) for patients with even early tumors [3]. Furthermore, most HCCs are discovered at an advanced stage which results in a high death rate and extremely short median survival (1–2 months) [3,5]. Therefore, an efficacious, timely therapeutic treatment for HCC is required to improve the quality of life of patients and to avoid their abrupt mortality.

To achieve safe and effective antitumor therapy, drugs should be targeted directly to tumor sites [6]. This tumor targetability augments antitumor activity by increasing local drug availability while minimizing unfavorable side effects to other tissues caused by the toxic nature of chemotherapeutics. It is well documented that the asialoglycoprotein receptor (ASGPR) is exclusively expressed in hepatocytes that are well-differentiated HCC forms and minimally on extra-hepatocyte regions [7–9]. Hence, targeting anticancer drugs to ASGPRs may be a good approach for HCC treatment. For this purpose, galactose or lactose residues are promising ligand materials because these sugar moieties have high ASGPR affinity [9–11]. Actually, galactosylated polymeric micelles and lactosylated lipid nanoparticles or liposomes display prominent HCC targeting [11–13].

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Albumin has been viewed as a versatile pharmaceutical protein carrier and has been expanded for use with a wide spectrum of drugs [14,15]. Most of all, albumin is very safe and biocompatible because it is an abundant endogenous protein in human serum [16]. Furthermore, albumin has many chemical functional groups, such as amines, carboxylates or thiols, which are available for modification by various targeting ligands for specific receptors [17]. For instance, amidated albumin (+ charged) was distributed to the liver (parenchymal or non-parenchymal cells) [18], and succinylated albumin (− charged) was delivered to Kupffer cells via scavenger-receptor mediated endocytosis [19]. Also, mannose-modified albumin significantly accumulated in liver Kupffer cells and endothelial cells [7,20,21]. For these reasons, albumin has been considered an exceptionally useful material for preparing nanoparticles designed for liver cell targeting [16,17]. Furthermore, albumin is preferentially taken up by tumor endothelial cells via the gp60-mediated transcytosis pathway [22], and nano-sized particles tend to accumulate efficiently in tumors due to an enhanced permeability and retention (EPR) effect [14,22].

Previously, we developed a series of albumin-based nanoparticles that showed prominent targetability to various brain, colon or pancreatic tumors [23–26] and reported synergistic antitumor effects in terms of co-therapy using a dual-drug system [24–26]. Here, we sought to make albumin nanoparticles target hepatocellular carcinoma. For this purpose, we synthesized lactobionic acid-modified bovine serum albumin (Lac-BSA), because a lactose residue is one of the most selective asialoglycoprotein receptor ligands. This study examined Lac-BSA physicochemical properties and in vitro cytotoxicity in HepG2 cells, as well as liver cell targetability for co-bound doxorubicin (Dox) and paclitaxel (Pac) nanoparticles made of Lac-BSA (Dox/Pac Lac-BSA NPs).

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA; 66.5 kDa and ~99%) and fluorescein-NHS (ester dye were purchased from Sigma- Aldrich (St. Louis, MO, USA). Doxorubicin (Dox) hydrochloride was obtained from the Research Laboratories of Korea United Pharm. Inc., (Seoul, Korea). Paclitaxel (Pac) was obtained from JW Pharmaceutical (Dangjin, South Korea). Cy5.5-NHS ester dye was purchased from GE Healthcare (Piscataway, NJ, USA). HepG2 human liver cancer cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were obtained from Corning (Corning, NY, USA). Unless specified all other reagents and chemicals were purchased from Sigma-Aldrich.

2.2. Animals

ICR mice (males, 6 weeks old) were purchased from the Hanlim Experimental Animal Laboratory (Seoul, South Korea). Mice were cared for according to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 80-23, revised 1996). Mice were housed in groups of 6–8 under a 12-h light/dark cycle (lights on at 6 a.m.), allowed food and water ad libitum, and acclimatized for one week before use. This animal study was approved by the Ethical Committee on Animal Experimentation at Sungkyunkwan University.

2.3. Lactobionic acid-modified BSA (Lac-BSA) synthesis

Lactobionic acid (0.358 g, 1 mmol) was dissolved in 10 ml dimethyl sulfoxide (DMSO, anhydrous) in a glass tube. *N*-dicyclohexylcarbodiimide (DCC, 0.413 g, 2 mmol), *N*-hydroxysuccinimide (NHS, 0.230 g, 2 mmol) and triethylamine (TEA, 0.265 ml, 2 mmol) were added to this tube serially during gentle shaking. The reaction was allowed to continue at room temperature overnight in a dark room. The white precipitate (dicyclohexyl urea) which was the reaction byproduct, was removed by vacuum filtration. The obtained Lac-NHS was stored at −70 °C until needed. Lac-BSA was synthesized via conjugation reaction between the BSA amino groups and Lac-NHS. Briefly, BSA (1 g, 0.015 mmol) was dissolved in 200 ml 0.1 M sodium borate buffer, and a Lac-NHS (100 mM, in DMSO) aliquot (3.0, 6.0, and 15.0 ml; 20, 40, and 100 equiv., respectively) was slowly dropped into the BSA solution, followed by continuous stirring at ambient temperature for 24 h. The resulting suspension was centrifuged at 13,500 rpm for 10 min to discard the precipitant. The unreacted Lac-NHS and other byproducts were removed by passage through desalting columns containing Sephadex G-25 resin. Finally, Lac-BSA was concentrated in DW with a centrifugal concentrator (MwCO: 10 kDa, amicon ultra, Millipore, Beverly, MA, USA), and then lyophilized at −70 °C until required.

2.4. Lac-BSA characterization

Lac-BSA conjugates were characterized by matrix-assisted laser desorption and ionization time of flight (MALDI-TOF) mass spectrometer (UltrafleXtreme, Bruker, Coventry, UK). The BSA or Lac-BSA conjugates were dissolved in deionized water (DW) at a 1 mg/ml concentration. A saturated sinapinic acid solution was prepared in 0.1% trifluroacetic acid (TFA) with DW-acetonitrile (ACN) (1:1) as the matrix. BSA or Lac-BSA solutions and the matrix were mixed at a 1:4 vol ratio (1 μl each) then added to a stainless steel plate. Samples were allowed to dry at room temperature and then kept in the system for Mass analysis. Spectra were recorded in linear mode with a +25 kV acceleration voltage then analyzed using the software provided with the system. Separately, BSA and Lac-BSA were subjected to reversed-phase high performance liquid chromatography (RP-HPLC) on a PLRP-S column (150 × 4.6 mm, 8 μm, Agilent Technologies, CA, USA) equipped with a guard cartridge (5 × 3 mm, Agilent Technologies) at 40 °C. Gradient elution was carried out at a flow rate of 1.0 ml/min using solution A (0.1% trifluoroacetic acid (TFA) in DW) and solution B (0.1% TFA acetonitrile). The following gradient elution profile was used: 20–80% B over 8 min and 80–100% B over 2 min. Eluates were monitored at 220 nm.

2.5. Dox/Pac BSA NPs and Dox/Pac Lac-BSA NPs preparation

Dox free base form was converted from the Dox hydrochloride form (Dox·HCl) by using a previously described method [23,25]. Dox/Pac BSA NPs and Dox/Pac Lac-BSA NPs were prepared using nanoparticle albumin bound (nab^{TM}) technology with slight modifications [25,26]. Briefly, BSA (150 mg) or BSA/Lac-BSA (20%, 50% or 100% feed ratio of Lac-BSA vs. BSA mixtures, total quoted as 150 mg BSA equivalents) were dissolved in 15 ml deionized water (DW). Dox (7.5 mg) and Pac (7.5 mg) were separately dissolved in 0.3 ml of a 9:1 chloroform and ethanol solution. These two solutions were then mixed at low rotating speed to form a crude emulsion prior to high-pressure homogenization using an EmulsiFlex-B15 device (Avestin, Ottawa, Ontario, Canada) at 20,000 psi for nine cycles. The resulting colloids were rotary-evaporated to remove chloroform at 40 °C for 15 min under reduced pressure. The obtained NPs were filtered through a 0.22 μm membrane syringe filter, then solvent was removed by lyophilization, and the product was stored at −70 °C until required.