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Oral delivery of taurocholic acid linked heparin–docetaxel conjugates for cancer therapy

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ABSTRACT

We have synthesized taurocholic acid (TCA) linked heparin–docetaxel (DTX) conjugates for oral delivery of anticancer drug. The ternary biomolecular conjugates formed self-assembly nanoparticles where docetaxel was located inside the core and taurocholic acid was located on the surface of the nanoparticles. The coupled taurocholic acid in the nanoparticles had enhanced oral absorption, presumably through the stimulation of a bile acid transporter of the small intestine. The oral absorption profile demonstrated that the concentration of the conjugates in plasma is about 6 fold higher than heparin alone. An anti-tumor study in MDA-MB231 and KB tumor bearing mice showed significant tumor growth inhibition activity by the ternary biomolecular conjugates. Ki-67 histology study also showed evidence of anticancer activity of the nanoparticles. Finally, non-invasive imaging using a Kodak Molecular Imaging System demonstrated that the nanoparticles were accumulated efficiently in tumors. Thus, this approach for oral delivery using taurocholic acid in the ternary biomolecular conjugates is promising for treatment of various types of cancer.

1. Introduction

Oral delivery is the easiest route of drug administration and improves patient compliance [1]. However, large molecules such as heparin [2], proteins [3] and some specific drugs such as docetaxel [4] and paclitaxel cannot be administered orally at the present time [5]. In fact, one of the most difficult challenges in drug delivery is the development of those and other drugs as orally administered formulations. Some major challenges in oral delivery of drugs are: degradation of drugs by high acid content of the stomach and by digestive enzymes [6], poor absorption through the epithelial membrane, and transformation of drugs to forms which are insoluble at physiological pH [7].

To overcome these challenges, better carrier needs to be developed to protect drugs from degradation after oral administration. Additionally, an absorption enhancer could be used to enhance epithelial absorption and a solubilizer could increase the solubility of drug molecules at physiological pH [8,9]. Exploiting the intestinal bile acid transporter has been suggested as a strategy for the development of oral drug formulations [10–14]. Several articles have reported that drug molecules which have been conjugated with a bile acid interact with bile acid transporters of the small intestinal membrane [15,16]. Interaction between the formulation and the bile acid transporter facilitates uptake of the drug molecule [17]. In particular, Byun *et al.* reported that conjugation of a bile acid with large drugs facilitates uptake through the epithelial membrane of the small intestine [18,19]. They also reported that the same strategy could also be applied for oral delivery of insulin, and determined a notable increase in bioavailability of insulin by this mean [20]. Our group has reported that conjugation of a bile acid enhances the oral absorption of heparin, thus facilitate oral absorption of an optical imaging agent [21]. However, there is a notable limitation in the use of hydrophobic bile acids such as deoxycholic acid as absorption enhancers, because the conjugated hydrophobic bile acid is located within the core of micelles formed in water [22].

Docetaxel (DTX) is widely used in treating a broad range of human cancers, including refractory ovarian and breast cancer, non-small-cell lung carcinoma, head and neck carcinoma and leukemia [23–26]. However, docetaxel shows very low rates of oral absorption and bioavailability (less than 3%) due to both its low aqueous solubility and pre-systemic intestinal metabolism [27]. In the last few decades, numerous publications have reported regarding the oral delivery of DTX, as well as its sustained release through oral administration [28]. Kim *et al.* have reported the prospects for oral delivery of paclitaxel conjugated heparin derivatives, and another research group has reported the oral delivery of DTX [29]. However, both of the reports had the notable absence of absorption enhancer, and as a result, did not show the desired therapeutic effects. As a result, direct interaction between conjugated bile acid and bile acid transporter of the small intestine cannot occur, and the efficiency of drug absorption is likely reduced due to location of the bile acid within the core of the micelles.

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We suspect that a hydrophilic bile acid could overcome these limitations, enhancing absorption through direct interaction between the bile acid transporter and the formulation, since a hydrophilic bile acid is expected to be located on the surface of micelles when dissolve in hydrophilic solution. The bile acid conjugated drug molecules absorb through either transcellular or paracellular or through both pathways as reported earlier elsewhere [30]. Heparin is a long chain polysaccharide considered as an anticoagulant agent. It is also known as a effective agent for anti-thrombosis and anti angiogenesis treatment [15,17].

In this study, we use hydrophilic taurocholic acid (TCA) as an absorption enhancing agent to increase the bioavailability of heparin derivatives through direct interaction with the bile acid transporter of the small intestine. We first provided adequate evidence regarding conjugation, particle formation and epithelial absorption of the taurocholic acid linked heparin–docetaxel conjugates (HDTA) through bile acid transporter of small intestine and *in vivo* anti-cancer activity. This strategy could be applied to the development of new oral delivery system for anticancer drugs in the future.

2. Materials and methods

2.1. Chemicals

Low molecular weight heparin (LMWH, average MW 5000 kDa) was obtained from Mediplex Co., Ltd (Seoul, Korea). Taurocholic acid sodium salt (TCA), Docetaxel (DTX), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDAC), 4-nitrophenyl chloroformate (4-NPC), triethylamine (TEA), N-hydroxysuccinimide (HOSu), 4-methylmorpholine (MMP), 1,4-dioxane, 2% ninhydrin reagent and trypsin-EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). N,N-dimethylformamide (DMF), ethylenediamine, formamide and acetone were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Preparation of HDTA

To obtain activated TCA, 1 mol of taurocholic acid (TCA) sodium salt was dissolved in DMF (4.6 mL) at 0 °C, and then TEA (6 mol) and 4-NPC (5 mol) were added to the flask. This solution was reacted for 1 h at the same condition and was then stirred for 6 h at room temperature. The reacted solution was then centrifuged and extracted by separation funnel with absolute ethanol (EtOH) (20 mL) and DI water (20 mL), the process was repeated three times. The separated solution was placed in a rotary evaporator to evaporate organic solvent and was finally freeze dried for 48 h to get TCA-NPC powder. TCA-NPC (1 mol) was dissolved in DMF (5 mL) and 4-MMP (2 mol) were added. This reaction was continued for 1 h at 50 °C. After 1 h, EDA (100 mol) was added drop-by-drop to the solution and stirring was continued for 16 h at room temperature. The crystallized part was filtered and was dried by vacuum dryer. To synthesize the HTA conjugate, 1 mol heparin was dissolved in distilled water with gentle heat and 0.1 M of HCl was added to maintain the pH condition in the range of 5.5–6. EDC (5 mol) was added to the heparin solution, which was stirred for 5 min, and then NHS (7 mol) was added, again stirring for 30 min. Afterwards, TCA-NH_2 was added to the solution which was stirred for 12 h at room temperature. The feed molar ratio of TCA-NH_2 was controlled to get different coupling amount of TCA with heparin. Finally, the solution was dialyzed (MWCO: 1000) against water for 24 h to remove the free EDC and NHS from the solution. To obtain a final product, HDTA and DTX were dissolved in DMSO solution, which was then stirred until the solution became clear. TEA and 4-NPC were added and were stirred for 12 h at room temperature. After 12 h of reaction, the free/un-conjugated TEA and NPC were removed by extraction with methanol and hexane solution and the process was repeated three times. MMP was added to the activated DTX containing methanol solution and this was stirred for 1 h at room temperature, followed by the addition of EDA (Table 1) and stirring was continued for more 12 h at same condition. Hexane was added to the solution and was extracted to remove free MMP and EDA from the reactant solution. The solution was then rotary evaporated for 30 min to evaporate hexane from the solution. One mole of HTA conjugates were dissolved in distilled water and 10 mol of EDC and 12 mol of NHS were both added, and the solution was stirred for 30 min. Afterwards, aminated docetaxel solution was added to the reacted solution, which was stirred for 12 h at RT. The feed molar ratio of aminated docetaxel was controlled in order to get different coupling amount of DTX with HTA. Finally, the solution was dialyzed (MWCO-1000) against DI water to remove the un-conjugated DTX, EDC and NHS. Finally the entire solution was dried for 48 h by freeze dryer to get a powder form of HDTA conjugates.

2.3. Characterization of HDTA

The HDTA conjugates were confirmed by an amide bond between the carboxylic group of heparin and the amine group of TCA or DTX, using FT-IR and ^{1}H NMR. For FT-IR spectrum analysis, HDTA conjugates were placed in the sample hole and were scanned as a solid powder. For ^{1}H NMR analysis, the conjugates were dissolved in DMSO solvent and were scanned up to 10 ppm. The coupling ratio of TCA and DTX with heparin was analyzed by the sulfuric acid method [15]. Briefly, 140 μL of heparin or HDTA (60 mg/mL) in water was mixed with 360 μL of sulfuric acid at 80 °C for 3 min. The solution was cooled at room temperature and absorbance was determined at 420 nm against a blank using Microplate Reader (Varioskan flash, Thermo Fisher Scientific, NY). The thermal stability was studied using a TA-Q50 thermo-gravimetric analyzer (TGA) (TA, state). Each sample (heparin, TCA and HDTA) was heated from room temperature to 500 °C with a heating rate of 10 °C/min under a nitrogen atmosphere. The CMC (critical micelle concentration) of HDTA conjugates was determined using pyrene. Pyrene, a nonpolar polyaromatic molecule, preferentially partitions to the hydrophobic core of the micelles, with a synchronous change in its fluorescent properties such as vibrational changes in the emission spectrum and red shift in the excitation spectrum. In brief, HDTA nanoparticles (HDTA3 and HDTA4) were dissolved in water. The solution of 10^{-7} M pyrene and HDTA nanoparticles was allowed to react overnight at room temperature. The excitation intensity was measured at two excitation wavelengths, at 374 and 390 nm, for each solution by the micro-plate reader Varioskan flash (Thermo Fisher Scientific, NY). The excitation intensity ratio of the two wavelengths (I_{390}/I_{374}) was plotted as a conjugation concentration, and the CMC was determined from the first point of inflexion in the ascending portion of the sigmoid curve. Size distribution and zeta potential of HDTA nanoparticles were measured with FE-SEM (JEOL, Japan) and ELS-8000 (Photal, Osaka, Japan), respectively. The FE-SEM samples were prepared by dilution of HDTA nanoparticles. The ELS and zeta potential samples were diluted with HEPES-buffered saline (pH 7.4).

2.4. In vitro stability test

The stability of HDTA was examined by monitoring the change in size under various environmental conditions. First, the stability of the HDTA3 and HDTA4 was studied at three different pH values (1.5, 5, 7 and 9) in 0.1 M Tris–HCl buffer. The stability of HDTA3 and HDTA4 in the presence of serum (10% (v/v) in PBS) for 30 days was also tested. To minimize interference by large molecules in FBS, the serum

Table 1
Characteristics of HDTA. Particle size of HDTA varies regarding to conjugation number of DTX.

Sample no.	Heparin:TCA (coupling mole)	HTA:DTX (coupling mole)	Particle size (nm)	PDI
1	1:1.0 ± 0.1	N/A	N/A	N/A
2	1: 3.0 ± 0.5	1: 4.0 ± 0.7	124 ± 48	0.24 ± 0.1
3	1: 4.7 ± 0.3	1: 3.0 ± 0.6	115 ± 46	0.25 ± 0.3