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Oral delivery of a potent anti-angiogenic heparin conjugate by chemical conjugation and physical complexation using deoxycholic acid

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ABSTRACT

Angiogenesis, the formation of new blood vessels, plays a pivotal role in tumor progression and for this reason angiogenesis inhibitors are an important class of therapeutics for cancer treatment. Heparin-based angiogenesis inhibitors have been newly developed as one of such classes of therapeutics and possess a great promise in the clinical context. Taurocholate conjugated low molecular weight heparin derivative (LHT7) has been proven to be a potent, multi-targeting angiogenesis inhibitor against broad-spectrum angiogenic tumors. However, major limitations of LHT7 are its poor oral bioavailability, short half-life, and frequent parenteral dosing schedule. Addressing these issues, we have developed an oral formulation of LHT7 by chemically conjugating LHT7 with a tetrameric deoxycholic acid named LHTD4, and then physically complexing it with deoxycholylethylamine (DCK). The resulting LHTD4/DCK complex showed significantly enhanced oral bioavailability (34.3 ± 2.89%) and prolonged the mean residence time (7.5 ± 0.5 h). The LHTD4/DCK complex was mostly absorbed in the intestine by transcellular pathway via its interaction with apical sodium bile acid transporter. *In vitro*, the VEGF-induced sprouting of endothelial spheroids was significantly blocked by LHTD4. LHTD4/DCK complex significantly regressed the total vessel fractions of tumor (77.2 ± 3.9%), as analyzed by X-ray microCT angiography, thereby inhibiting tumor growth *in vivo*. Using the oral route of administration, we showed that LHTD4/DCK complex could be effective and chronically administered as angiogenesis inhibitor.

1. Introduction

Angiogenesis plays a key role in the tumor progression of tumor mass growth and metastasis [1,2]. Numerous angiogenesis inhibitors including bevacizumab (Avastin, Genentech/Roche), a humanized anti-VEGF monoclonal antibody, have been developed, especially targeting the VEGF signaling pathway [3–5]. However, the acquired resistance against VEGF blockers is a critical issue in anti-angiogenic therapy. The reason for such resistance has not been fully understood, but a number of clinical studies have reported that the up-regulated alternative pro-angiogenic factors are responsible for the resistance that occurs when the VEGF signaling pathway gets blocked [6–8]. Therefore, the inhibition of VEGF alone may not be sufficient for effective and sustainable inhibition of tumor angiogenesis.

In our previous studies, we reported a new non-anticoagulant heparin-based angiogenesis inhibitor, LHT7, constituting of sodium taurocholates chemically conjugated to low-molecular-weight heparin (LMWH) [9]. LHT7 showed a potent inhibition

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effect on tumor angiogenesis without anticoagulant activity because it has a lower binding affinity to antithrombin III but higher binding affinity to VEGF than LMWH does. Furthermore, we described a heparin-based anti-angiogenic therapy that could block multiple proangiogenic factors, especially VEGF and bFGF, thereby potentially circumventing resistance encountered in the single targeting anti-angiogenic therapies [10].

However, because of a relatively short half-life of LHT7, it is required that LHT7 be frequently administered in the clinical setting. Therefore, the development of an oral formulation of LHT7 as a promising second-line cancer therapy would improve patient compliance and provide a means for a long-term anti-angiogenic therapy. Furthermore, the oral delivery of anticancer drug can enable chemotherapy at home by providing daily administration on a long-term basis [11,12].

In the present study, we used the bile acid based absorption enhancing systems, which are both chemically conjugated and physically complexed bile acids with LHT7. It has been reported that deoxycholic acid (DOCA) and its derivatives possess the enhancing effect of drug absorption in the intestine due to their ability to increase hydrophobicity of the target drug and their effective interaction with the apical sodium-dependent bile acid transporter (ASBT) in the small intestine [13,14]. ASBT is a membrane transporter that exists in the small intestine (mainly ileum) of gut and moves endogenous small molecular bile acids from the apical surface of enterocytes to the portal vein [15,16]. ASBT has gained the attention in the filed of drug delivery for ASBT's capability of transporting various molecules coupled to bile acids, which are usually poorly absorbed in the intestine [17–19]. In a previous study, we had reported that *tetra*DOCA conjugated LMWH bound with ASBT in the apical membrane, followed by internalizing into the cell by forming vesicles instead of penetrating (directly) through the transporter. This newly found receptor-like transformation of ASBT, induced by *tetra*DOCA conjugated LMWH, was further utilized in the present study to develop an oral delivery system of anti-angiogenic LHT7 [14,20]. We also showed that deoxycholyl-L-lysyl-methylester (DCK), synthesized by conjugating L-lysine with deoxycholic acid (DOCA), could enhance the absorption of anioic charged drugs in the small intestine [21,22]. Bile acid based enhancers reversibly bind and form a complex with the drug, thereby delivering the drug to the other side of GI epithelia [19,23]. Physical complexation of heparin conjugates with DCK might further increase the hydrophobicity of the complex. It is hence hypothesized that, the increased hydrophobicity would improve the permeability of the complex across the intestinal wall [24].

In this study, LHTD4 was sysnthesized as an orally active anti-angiogenic drug by conjugating tetrameric DOCA (*tetra*DOCA) at the end saccharide of LHT7. LHTD4 was further formulated with DCK by physical complexation without altering the structure of LHTD4, and the physicochemical properties and oral bioavailability of the formulated LHTD4/DCK complex were evaluated. Its anti-angiogenic efficacy was also evaluated by *in vitro* and *in vivo* studies, and tumoral angiogenesis regression study was conducted in a squamous cell carcinoma (SCC7) model.

2. Materials and methods

2.1. Synthesis of LMWH-taurocholate conjugate (LHT7)

The chemical conjugate (LHT7) of low molecular weight heparin (LMWH) and taurocholate (TCA) was synthesized as previously described [9]. Briefly, a primary amine was introduced to 4-hydroxyl group of TCA by activating its hydroxyl group using 4-nitrophenylchloroformate in the presence of triethylamine, followed by adding ethylenediamine (EDA). The amine group of EDA introduced to TCA was conjugated with carboxylate group of LMWH (Fraxiparin; Glaxo Smith Kline, Genval, Belgium) by the EDAC/NHS coupling method to produce LHT7. The final product was analyzed by ^{1}H NMR (Avance, Bruker, MA) and the coupling ratio of the conjugated TCA was determined by using the quantitative detection method of bile acids [25]. Anticoagulant activity was determined by using the chromogenic FXa assay (Coatest heparin; Chromogenix, Milan, Italy) as instructed by the manufacturer.

2.2. Synthesis of LHT7-tetraDOCA conjugate (LHTD4)

LHTD4 was synthesized by coupling of LHT7 with a tetramer of deoxycholic acid (*tetra*DOCA), as shown in Fig. 1. *Tetra*DOCA was synthesized as follows; lysine dimer, BOC-CH_3O-lys(lys(boc)$_2$), was firstly synthesized by the peptide synthesis method. H-lysBOC-CH_3O·HCl (GL Biochem, China) and BOC-lysBOC-OSu in DMF were reacted overnight in dark, followed by evaporating completely under vacuum. The crude product was extracted with 5% HCl solution. After the solvent was evaporated, the product was dissolved in methanol, followed by precipitating in cold water. The precipitate was lyophilized, followed by saponifying to obtain the lysine dimer, BOC-COOH-lys(lys(boc)$_2$). The synthesized lysine dimer was reacted with equimolecular amounts of dicyclohexylcarbodiimide (DCC) and hydroxysuccinimide (HOSu) in DMF at 4 °C for 30 min, followed by filtering the precipitated urea to obtain the activated lysine dimer. After an equimolar amount of H-lysBOC-CH_3O·HCl was stirred with 4-MMP for 1 h, the activated lysine dimer was slowly added and reacted for 24 h to obtain the lysine trimer, BOC-CH_3O-lys(lys(boc)$_2$)$_2$. Finally, the synthesized lysine trimer was freeze-dried and purified by column chromatography packed with silica gel (0.04–0.06 mm) and 10% MeOH/MC as an eluent. After the synthesized BOC-CH_3O-lys(lys(boc)$_2$)$_2$ was deprotected in an acidic condition, the lysine trimer containing four amino groups and one carboxyl group was obtained.

On the other hand, DOCA was activated using the DCC/HOSu coupling method to obtain DOCA-OSu. Four amine groups of the lysine trimer were then reacted with DOCA-OSu, thereby forming *tetra*DOCA. The *tetra*DOCA was purified on a preparative column packed with silica gel and a mixture of 10% MeOH/MC as an eluent. Finally, *tetra*DOCA was reacted with EDA to obtain N-*tetra*deoxycholylethylamine (*tetra*DOCA-NH_2), followed by passing through a silica gel-packed column for the final purification. A mixture of chloroform, MeOH and NH_4OH (7.75:3:0.25) was used as eluent. The purity of each compound was checked by MALDI-TOF (Voyager-DE STR Biospectrometry Workstation, Applied Biosystems Inc., CA).

In order to conjugate the synthesized tetraDOCA-NH_2 and LHT7, LHT7 was reacted with potassium metaperiodate (KIO_4, 57.5 mg) in acetate buffer (pH 4.5) for 3 h in dark. The reaction was stopped by adding glycerol and dialyzed to remove unreacted KIO_4. Finally, the oxidized LHT7 was reacted with tetraDOCA-NH_2 to synthesize LHTD4. The oxidized LHT7, dissolved in a co-solvent of formamide and DMF, was reacted with tetraDOCA-NH_2 at 50 °C with a molar ratio of 1:5. The imine bond, formed between *tetra*DOCA and the oxidized saccharide unit of LMWH, was reduced to a secondary amine using sodium cyanoborohydride ($NaCNB_4$). The materials were extracted and purified by precipitation in cold ethanol. The residual solvents were evaporated and freeze-dried to obtain LHTD4 as white powder.

Rhodamine isothiocyanate (RITC) labeling to LHT7 and LHTD4 were conducted in aqueous condition at pH 8 by reacting for 6 h. The products were precipitated in ethanol and dialyzed, followed by purifying using size exclusion column (Bio-Rad BioGel P-30, Bio-rad, Korea).

2.3. In vitro anti-angiogenic effect of LHTD4

In vitro anti-angiogenic effect of LHTD4 was evaluated by three-dimensional HUVEC sprouting assay. HUVECs were purchased from Promocell GmBH (Germany) and used within the passage numbers between 2 and 5. The HUVEC spheroid was prepared as described in the literature [26,27]. In brief, HUVEC monolayers were trypsinized and suspended in endothelial cell growth media (ECGM) and methocel (4:1). Methocel was prepared by dissolving 6 g of methylcellulose (Sigma–Aldrich, Korea) in 500 ml of ECGM. The mixture was centrifuged and the clear supernatant was used as methocel. Endothelial cell (EC) spheroids were prepared by a hanging drop method such that 1000 ECs in a hanging drop (25 μl) were cultured overnight at 37 °C under 5% CO_2 and 100% humidity. The generated spheroids were harvested and suspended in the methocel solution containing 20% FCS (Cambrex, Verviers, Belgium). Subsequently, the ice-cold collagen solution (rat tail type I in 0.1% acidic acid) was mixed with 10% MEM 199 (10×, GIBCO; Invitrogen, CA), followed by adding 10% 0.2 N NaOH to adjust the pH to 7.4. After EC spheroids in the methocel solution was mixed at 1:1 ratio with the neutralized collagen solution, 0.9 ml of the mixed solution containing 50 EC spheroids were pipetted into individual wells of a 24-well plate to be polymerized for 30 min at 37 °C under 5% CO_2 and 100% humidity. EC spheroids were cultured in ECGM containing VEGF-A (25 ng/ml, Peprotech, Germany) alone or mixed with LHTD4. VEGF-A activates ECs, thereby inducing the formation of tube-like EC structures (sprouting EC). The plates were incubated at 37 °C under 5% CO_2 and 100% humidity for 24 h. Sprouting EC spheroids were photographed and quantitatively determined by counting the cumulative sprout branch points per spheroid using light microscope (Nikkon Ltd., Japan).

2.4. Preparation of LHTD4/DCK formulation

Deoxycholylethylamine (DCK) was synthesized as described in the previous study and used as an absorption enhancer [28]. In brief, deoxycholic acid (10 g, 23 mmol) was prepared in 50 ml acidified methanol and refluxed at 70 °C for 1 h, followed by cooling to 0 °C. The product was filtered and washed twice with cold methanol and dried in vacuum to obtain methyl deoxycholate. The prepared methyl

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