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Oral absorption mechanism and anti-angiogenesis effect of taurocholic acid-linked heparin-docetaxel conjugates

Zehedina Khatun [a], Md Nurunnabi [b,e], Kwang Jae Cho [c,*], Youngro Byun [d], You Han Bae [e], Yong-kyu Lee [a,f,**]

[a] Department of Green Bioengineering, Korea National University of Transportation, Chungju, 380–702, Republic of Korea
[b] Department of Polymer Science and Engineering, Korea National University of Transportation, Chungju, 380–702, Republic of Korea
[c] Department of Otolaryngology, Head & Neck Surgery, The Catholic University of Korea, College of Medicine Uijeongbu St. Mary's Hospital, Kyunggi-Do 480–717, Republic of Korea
[d] College of Pharmacy, Seoul National University, Seoul 151–742, Republic of Korea
[e] Department of Pharmaceutics and Pharmaceutical Chemistry, College of Pharmacy, University of Utah, Salt Lake City, 84112, USA
[f] Department of Chemical and Biological Engineering, Korea National University of Transportation, Chungju, 380–702, Republic of Korea

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ABSTRACT

Oral delivery is the preferred route to deliver therapeutics via nanoparticles due to ease of administration and patient acceptance. Here, we report on the findings of the absorption pathway of taurocholic acid (TCA)-linked heparin and docetaxel (DTX) conjugate, which we refer to as HDTA. We studied the oral absorption of HDTA using a Caco-2 cell transport system and an animal model. We have also used other absorption enhancers, such as ethylene glycol tetraacetic acid (EGTA), or inhibitors, such as sodium azide, to compare the relative permeability of HDTA conjugates. *In vivo* comparative studies were conducted using free TCA as a pre-administration and exhibited the maximum absorption site of the organ after oral administration of HDTA conjugates. HDTA was found to be absorbed mainly in the ileum and Caco-2 cell monolayer through passive diffusion and bile acid transporters. High fluorescence intensity of HDTA in mice came from the ileum, and it was eliminated from the body through colon. This novel formulation could be further investigated by clinical trials to find the prospect of oral anti-cancer drug delivery through anti-angiogenic treatment strategies.

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1. Introduction

Oral drug delivery is known as one of the easiest and most convenient routes for drug administration among the entire spectrum of possible drug administrations, such as intravenous (IV), intra muscular (IM), subcutaneous (SC), transdermal (TDS), sublingual etc. [1–5]. Hence, the vast majority of patients prefer oral administration of drugs [6–11]. Despite the advantages of oral delivery, there are several life-saving drugs, such as anti-cancer drugs, docetaxel, doxorubicin and widely used drugs such as insulin, that are not suitable for oral administration as those drugs are not absorbed or very poorly absorbed orally [12,13]. The low oral bioavailability of such drugs is due to their large molecular weight and solubility. It is most notably an obstacle for macromolecular drugs too large to pass between cells through the paracellular pathway (that is, those less than 100–200 Da) [14]. Unfortunately, many of the pharmaceutical drugs are too hydrophilic to be absorbed passively through cell membranes [15,16]. The highly acidic pH in the stomach and the proteolytic degradation in the gastrointestinal tract are other obstacles for the oral absorption of drugs [17,18].

By the last few decades, several strategies, such as oral absorption enhancer, particulate formation (nano/macro), enhancing lipophilicity, surface modification, and reducing molecular weight of long chain polysaccharides, have been considered to enhance oral absorption of those drugs [19–30]. Heparin in particular attracts attention due to its activity on anticoagulation, deep vein thrombosis, and pulmonary embolism, and nowadays, it shows anti-angiogenic and anti-cancer activity [31,32]. As a macromolecular drug, heparin itself is not absorbed orally due to its high molecular weight, negatively charged structure and very hydrophilic property [33]. In our previous studies, oral absorption of heparin was observed through conjugation with hydrophobic bile acid (deoxycholic acid, DOCA) [34–36]. We have also developed a conjugation (HDTA) of heparin, taurocholic acid (TCA, bile acid) and docetaxel (DTX). We have observed the oral absorption profile and anti-cancer activity of HDTA [37]. We have assumed that the hydrophilic TCA locates on the outer surface of the heparin backbone therefore the absorption rate becomes higher due to more interactions with intestinal bile acid transporter (IBAT). As a result, excellent bioavailability of TCA-linked DTX was observed in mice. These findings suggest that bile acid-conjugated micro/nanoparticles either become absorbed through the bile acid transporters or passive diffusion [38].

The aim of this study is to enhance oral absorption of HDTA using hydrophilic bile acid, which is located on the outer surface of the self-assembly nanoparticle. To determine the exact oral absorption rate of HDTA, both *in vitro* and *in vivo* systems were developed. Caco-2 cell

* Corresponding author. Tel.: +82 31 820 3797; fax: +82 31 847 0038.
** Correspondence to: Y. Lee, Department of Green Bioengineering, Korea National University of Transportation, Chungju, 380–702, Republic of Korea. Tel.: +82 43 841 5224; fax: +82 43 841 5220.
E-mail addresses: entckj@catholic.ac.kr (K.J. Cho), leeyk@ut.ac.kr (Y. Lee).

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monolayer was used to predict the intestinal drug permeability in various conditions, including absorption enhancer, transporter inhibitor and tight junction's opener. Competitive oral absorption profiles of TCA conjugates (Heparin-TCA and HDTA) were also obtained by *in vivo* molecular imaging system. *In vivo* biodistribution studies were also done to observe the retention and absorption site of HDTA in rats. Most importantly, the anti-angiogenic activity of HDTA shows very interesting and promising results *in vitro*. A wide range of analytical techniques, such as flow cytometry analysis, tubular formation assay, and western blotting assay, revealed that the HDTA nanoparticle is very promising for anti-angiogenesis therapy.

2. Materials and methods

2.1. Materials

Low-molecular weight heparin (heparin, average MW 5000 Da) 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, rhodamine B and trypsin-EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). N,N-dimethylformamide (DMF), ethylenediamine, formamide fluorescein-5-isothiocyanate (FITC) and tetramethyl rhodamine B isothiocyanate (TRITC)-labeled phalloidin and acetone were purchased from Sigma Chemical Co. (St. Louis, MO). Caco-2 cells were purchased from Korea Cell Bank (Seoul, Korea). MEM, HEPES, FBS, PBS and Coatest anti-Factor Xa assay kits were purchased from Invitrogen (Grand Island, NY).

2.2. Preparation of HDTA nanoparticles

To obtain activated TCA, one mole 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) was 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 (heparin–TCA) conjugate, 1 mole of 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, 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 was dissolved in distilled water and 10 mole of EDC and 12 mole of DCC 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 a 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 DCC. Finally the entire solution was dried for 48 h by freeze dryer to get a powder form of HDTA nanoparticles.

2.3. Visualization of cellular uptake of HDTA conjugates

For cellular uptake of heparin, HTA and HDTA nanoparticles, Caco-2 cells were grown for 30 days to reach monolayer and the cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2 in a MEM medium with 10% fetal calf serum. At the time of the cellular uptake study, the cells were washed with PBS twice. First, 200 μM of free TCA was introduced to the cells and cell membrane plates were incubated for 30 min. After 30 min incubation with TCA, 50 μg/mL of rhodamine B dye (Emission: 627 nm) conjugated heparin, HTA and HDTA solutions were placed inside of the cell membrane plate and incubated for 1 h at 37 °C. After that, the cell membrane plates were washed 3 times in PBS to remove the free particles from the outside of the cell membrane. Afterwards, 4% formaldehyde solution was added and the membrane plate was kept at room temperature for 5 min. Finally, the cell membrane was placed on a glass cover slip with PBS and observed by confocal laser scanning microscope (CLSM) to get clear cellular image at 20× magnification.

2.4. Transport study of HDTA conjugates using Caco-2 cell transport model

Caco-2 cells (human colon cancer cell lines) were used as a transport model due to expression of bile acid transporters [38]. The cells were cultured in MEM medium with 10% fatal bovine serum. The grown cells were collected by 0.25% trypsin–0.03% EDTA solution. Caco-2 cells (3×10^5 cells/mL) were seeded on the collagen-coated filter membranes of Transwell cell culture inserts (3.0 μm pore diameter, Costar, Cambridge, MA), and this continued for 30 days to reach the confluence. The transepithelial electrical resistance (TEER, Ω cm^2) of cultures was monitored with an EVOM epithelial voltohmmeter. The culture media were placed into the apical (0.5 mL) and basolateral (1 mL) chambers and changed every day.

Apical-to-basolateral and basolateral-to-apical permeability of HDTA was measured under various conditions. In brief, Hank's balanced salts solutions (HBSS) supplemented with 25 mM glucose was used in all studies and incubated for 30 min at 37 °C in a 5% CO_2 incubator for 30 min. After that, for evaluation of the main absorption routes, different concentrations of HDTA and HTA (0.1, 0.5 and 1 mg/mL)-containing mediums were placed in the apical or basolateral region of transwell. The culture medium in the opposite part was collected at 60, 90, 120 min after treating with HDTA and HTA derivatives. Different kinds of blockers, such as sodium taurocholate, sodium azide, and EGTA (ethylene glycol tetraacetic acid), were used to compare the absorption rate. Free sodium taurocholate, sodium azide, and EGTA were treated with Caco-2 cells for 30 min and then 0.5 mg/mL concentration of HDTA and HTA was treated for 2 h. Culture medium from the opposite part was collected for analysis and the volume of the apical of basolateral solutions was adjusted by adding fresh transport medium. All experiments were conducted at 37 °C. The amounts of HDTA and HTA that permeated through the filter were measured indirectly using anti-FXa activity chromogenic assays.

The apical and basolateral permeability was calculated by the following equation:

$$\mathrm{Papp} = \mathrm{d}Q/\mathrm{d}t \times 1/AC_0$$

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