Journal of Controlled Release xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Prevention effect of orally active heparin conjugate on cancer-associated thrombosis

Taslim A. Al-Hilal [a], Farzana Alam [b], Jin Woo Park [c], Kwangmeyung Kim [d], Ick Chan Kwon [d], Gyu Ha Ryu [e,*], Youngro Byun [a,b,**]

[a] Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, South Korea
[b] Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, South Korea
[c] College of Pharmacy, Mokpo National University, Mokpo 534-729, South Korea
[d] Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, Seoul 136-791, South Korea
[e] Department of Molecular and Cellular Biology, School of Medicine, Sungkyunkwan University, Seoul 135-710, South Korea

ARTICLE INFO

Article history:
Received 20 March 2014
Accepted 16 May 2014
Available online xxxx

Keywords:
Heparin conjugate
Oral delivery
Anticoagulants
Cancer-associated thrombosis

ABSTRACT

Thrombogenesis is a major cause of morbidity and mortality in cancer patients. Prophylaxis with low-molecular-weight heparin (LMWH) is recommended for cancer patients, but requires non-parenteral delivery methods for long-term treatments. In this study, we sought to generate a new oligomeric-bile acid conjugate of LMWH that can be used for oral delivery. We first synthesized a tetramer of deoxycholic acid (*tetra*DOCA), which was site-specifically conjugated at the end saccharide of LMWH. When LMWH-*tetra*DOCA conjugate (LHe-*tetra*D) was orally administered at a dose of 5 mg/kg in ICR mice, the maximum anti-factor Xa level was increased up to 0.62 ± 0.05 IU/mL without any evidence of liver toxicity, gastrointestinal damage, or thrombocytopenia. The cancer-associated thrombosis was induced in tumor-bearing mice by local heat application, and the fibrin deposition in tumors was evaluated. The oral administration of LHe-*tetra*D (either a single dose or multiple daily doses for up to 10 days) in mice substantially abolished the coagulation-dependent tropism of fibrinogen in the heated tumors and significantly decreased hemorrhage, compared to the mice treated with saline or subcutaneous injection of LMWH. Thus, the anticoagulation effect of oral LHe-*tetra*D invokes the benefits of oral delivery and promises to provide an effective and convenient treatment for cancer patients at risk of thrombosis.

1. Introduction

Thromboembolic events including deep-vein thrombosis (DVT) and pulmonary embolism (PE), collectively known as venous thromboembolism (VTE), are risk factors and leading causes of death in cancer patients [1]. The pathogenesis of VTE in cancer patients is linked to thrombin activation and fibrin formation, which is triggered by the release of procoagulants directly by cancer cells or indirectly through the activation of endothelial cells [2]. Cancer patients receiving chemotherapy are prone to developing thrombosis because chemotherapy can exacerbate the pathogenesis of VTE in various ways, such as by increasing procoagulant levels, decreasing endogenous anticoagulant levels, or releasing cytokines that can activate tissue factors [3]. The treatment with anticoagulants is recommended as the first-line therapy for cancer patients who are at high risk for VTE. However, patients with malignancies inevitably have poor prognoses and suffer from bleeding complications while receiving anticoagulants [4,5]. New oral anticoagulant targeting factor Xa (FXa) or thrombin have shown great promise in treating VTE, atrial fibrillation, and acute coronary syndrome [6,7]. Synthetic oral anticoagulants, such as dabigatran for atrial fibrillation and rivaroxaban for deep vein thrombosis, are in the most advanced stages of clinical trials [8,9]. However, although these novel anticoagulants are being considered for use in cancer patients, bleeding risk and chemotherapeutic drug interactions are still serious clinical constraints. In addition, antidotes are not available for these new anticoagulants, further diminishing their therapeutic potential [10].

Low molecular weight heparin (LMWH), a highly sulfated hydrophilic molecule with an average molecular weight of about 4500 Da, is a highly potent anticoagulant agent with negligible bleeding complications [11]. There is a clear survival benefit for cancer patients who are treated with LMWH in combination with chemotherapy [12,13], and LMWH surpasses the new oral anticoagulants in terms of clinical safety and efficacy. In an investigation of thromboprophylaxis in cancer patients, LMWH was associated with a decreased bleeding rate and displayed a survival benefit comparable to rivaroxaban [14]. More

* Correspondence to: G.H. Ryu, Department of Molecular and Cellular Biology, School of Medicine, Sungkyunkwan University, Seoul 135-710, South Korea. Tel.: +82 2 3410 2283; fax: +82 2 3410 1597.
** Correspondence to: Y. Byun, Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, South Korea. Tel.: +82 2 8807866; fax: +82 2 872 7864.
E-mail addresses: gyuharyu@gmail.com (G.H. Ryu), yrbyun@snu.ac.kr (Y. Byun).

http://dx.doi.org/10.1016/j.jconrel.2014.05.027
0168-3659/

recently, semuloparin (an ultra-LMWH) significantly reduced thromboembolic events in cancer patients who received chemotherapy [15]; despite these promising results, semuloparin has not been approved by the FDA for VTE prevention in cancer patients receiving chemotherapy [16], probably because this would require a long-term subcutaneous injection. Indeed, the available delivery method is one of the major barriers limiting the long-term use of LMWH for the treatment of cancer-associated thrombosis (CAT) [17]. An oral form of heparin can allow frequent administration, and it can induce effective suppression of CAT. Therefore, this study was designed to examine the efficacy of oral heparin conjugate against CAT.

Many strategies have been developed towards the oral delivery of LMWH [18,19]. In the previous study, it was reported that tetrameric deoxycholic acid (*tetra*DOCA) conjugated LMWH (LHe-*tetra*D), where *tetra*DOCA is attached to the end saccharide unit of LMWH, was effectively absorbed in the intestine and prevented DVT [20]. Especially, LHe-*tetra*D could retain an anticoagulant bioactivity similar to that of LMWH since *tetra*DOCA was specifically conjugated to the end saccharide unit of LMWH (see Fig. 1), thereby avoiding the possibility that ATIII-binding pentasaccharide unit of LMWH is sterically hindered by *tetra*DOCA. The conjugated *tetra*DOCA strongly bound to apical sodium bile acid transporter (ASBT) in the apical epithelial cell membrane. ASBT is the major transporter for the uptake of bile acids in the intestine. Bile acids, as they bind with the hydrophobic substrate-binding pocket of ASBT, cause a conformational change between the outward- and inward-facing states of ASB [21]. The cytoplasmic channel of ASBT is thus opened to drive the substrates (small molecule bile acids) across the cell membrane, utilizing the sodium gradient. For this reason, ASBT is a promising target for improving the oral bioavailability of small molecular pharmaceutical drugs [22,23]. The challenge of transporting macromolecular drugs had remained largely unexplored, but has now been met in our previous studies: the development of high-affinity binding macromolecular substrates, LHe-*tetra*D, allows the 'receptor-like' functional transformation of ASBT and the LHe-*tetra*D/ASBT complex to be internalized into the cytoplasm by forming vesicles instead of penetrating through the transporter [24]. This unique transport mechanism could significantly enhance the absorption of LHe-*tetra*D in the small intestine.

In this study, we reported the preventive effect of orally active LHe-*tetra*D on an experimental model of CAT. In an effort to identify the efficacy of LHe-*tetra*D, a predictive model for CAT has been developed by thermally heating the tumor. Thermal heating could selectively activate the coagulation cascade in tumors [25–28]. The extent of coagulation activation was examined by evaluating fibrinogen deposition in tumors since fibrinogen is the most abundant component of blood clots [29–31].

2. Materials and methods

2.1. Materials

Low molecular weight heparin with an average molecular weight of 4500 Da was obtained from Nanjing King-Friend Biochemical Pharmaceutical Company Ltd. (Nanjing, China). Deoxycholic acid (DOCA), dicyclohexylcarbodiimide (DCC), hydroxysuccinimide (NHS), octanol, methanol (MeOH), methylene chloride (MC), tetrahydrofuran (THF), cyclohexane, acetonitrile, 1,2-ethylene diamine (EDA), formamide, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), Hoechst 33258, sodium cyanoborohydride, and 4-methyl morpholine (4-MMP) were purchased from Sigma Chemical Co. (St. Louis, MO). Polyethylene polyoxypropylene block copolymer 188 was purchased from BASF Aktiengesellschaft (Ludwigshafen, Germany), and caprylocaproyl macrogolglyceride (Labrasol®) was obtained from Gattefossé (Lyon, France). Coatest anti-Factor Xa assay kits were purchased from Chromagenix (Milan, Italy). Cy5.5-NHS was purchased from Lumiprobe (Hallandale Beach, FL). Proton nuclear magnetic resonance (^{1}H NMR, JEOL JNM-LA 300WB FT-NMR, Japan) was recorded at 500 MHz using D_2O or DMSO-d_6 as solvents (Sigma-Aldrich). Unless stated otherwise, all other materials were purchased from Sigma-Aldrich and used without further purification.

2.2. Synthesis and characterization of LMWH-tetraDOCA (LHe-tetraD)

LHe-*tetra*D conjugate was synthesized by coupling N-tetradeoxycholylethylamine (*tetra*DOCA-NH_2) with the end-saccharide unit of LMWH as described in the previous study [20]. Briefly, a lysine dimer [BOC-CH_3O-lys(lys(boc)$_2$); containing two amino groups] was generated by peptide synthesis and saponified with NaOH for 6 h at 70 °C to generate BOC-lys(lys(boc)$_2$)-COONa. The salt constituent was acidified with 1 N HCl to generate BOC-COOH-lys(lys(boc)$_2$), which was then activated by dissolution in dry DMF containing equimolecular amounts of DCC and NHS, followed by cooling to 4 °C. The mixture was reacted for 30 min, and the precipitated dicyclohexylurea was filtered out. The mixture was slowly added to a solution of desalted H-lysBOC-CH_3O•HCl and the reaction was allowed to proceed for 24 h to synthesize the lysine trimer, BOC-CH_3O-lys(lys(boc)$_2$)$_2$. The material was purified by conventional column chromatography, using 10% MeOH/MC as the eluent. The purified lysine trimer was reacted with acetyl chloride to remove the protected groups, generating a deprotected lysine trimer containing four amino groups. The product was reacted with NHS-activated DOCA to synthesize *tetra*DOCA. Finally, *tetra*DOCA was reacted with EDA to generate *tetra*DOCA-NH_2, which was further purified by silica gel-packed column chromatography.

Depolymerized LMWH by nitrous acid has a 2,5-anhymannose moiety at its reducing end, allowing direct end-saccharide conjugation. *Tetra*DOCA-NH_2 was reacted with LMWH to obtain the end-saccharide specific conjugated product, which was designated LHe-*tetra*D. Briefly, LMWH was dissolved in a co-solvent mixture of formamide and DMF, and reacted with *tetra*DOCA-NH_2 at 50 °C at a molar ratio of 1:12. *Tetra*DOCA-NH_2 was conjugated to the end-saccharide unit of heparin using sodium cyanoborohydride. The conjugated product was extracted, and the excess *tetra*DOCA-NH_2 was removed by precipitation in cold ethanol. After the residual solvents were evaporated, the solution was freeze-dried to obtain the powdered form of LHe-*tetra*D.

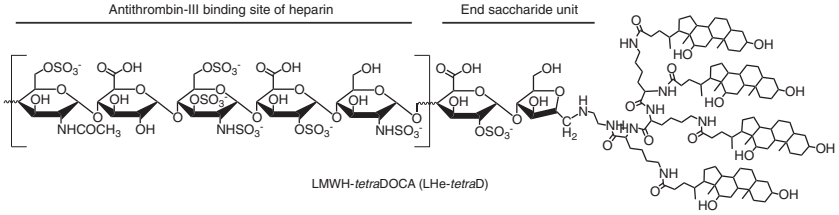

Fig. 1. Schematic diagram of *tetra*deoxycholylethylamine (*tetra*DOCA) conjugated to LMWH, resulting in an end saccharide conjugated LHe-*tetra*D derivative.

Please cite this article as: T.A. Al-Hilal, et al., Prevention effect of orally active heparin conjugate on cancer-associated thrombosis, J. Control. Release (2014), http://dx.doi.org/10.1016/j.jconrel.2014.05.027