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Low anticoagulant heparin oligosaccharides as inhibitors of BACE-1, the Alzheimer's β-secretase

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ABSTRACT

Heparin (HP) is a promising agent for anti-Alzheimer's disease (AD), but its anticoagulant activity limits its applications. So a low anticoagulant heparin (LAH) with anti-AD effect is needed. A novel LAH and heparan sulfate (HS) were purified from crude porcine intestinal heparin. Their structures were characterized by nuclear magnetic resonance and liquid chromatography-mass spectrometry. LAH had a relatively high degree of sulfation, but lower than that of HP. 3-*O*-Sulfated-containing glucosamine residues further confirmed the low anticoagulant activity of LAH. Sixteen oligosaccharides of LAH and HS were prepared and assigned. Evaluation of anti-BACE-1 activities suggested that their potencies were positively correlated with degree of sulfation and polymerization of oligosaccharides. Besides, LAH-derived hexa- to dodecasaccharides was promised to be administrated *in vitro* as BACE-1 inhibitors. This study presented ideal BACE-1 inhibitors, LAH-derived oligosaccharides, with virtually no anticoagulant activities, which were promised to be excellent leads for treatment of AD.

1. Introduction

Heparin (HP) is extensively used as a blood anticoagulant for surgical operation and treatment of thrombosis since it was first introduced into clinical practice nearly 100 years ago. It also possesses other pharmacological properties pharmacology functions, such as anti-inflammatory, anti-tumor and treating nephrotic syndrome (Linhardt & Toida, 2004; Capila & Linhardt, 2002). However, clinical use of HP is limited by hemorrhage and thrombocytopenia. Reduction in molecular weight or sulfation level could ameliorate these effects. As a heparin analogue, heparan sulfate (HS) possesses almost no anticoagulant activity, but it has also lower bioactivities due to its lower sulfation level. Therefore, low anticoagulant heparin (LAH), which reserves many of the biological functions of HP, is urgently needed. Unlike HP, LAH does not interact with heparin-platelet factor 4 antibodies present in patients with heparin-induced thrombocytopenia and even suppresses platelet activation in the presence of activating concentrations of HP (Rao et al., 2010). Two kinds of LAHs have been reported, including partially *O*-desulfated heparin (PDH) (Fryer et al., 1997) which has been extensively investigated, and heparin derivatives chemically generated by periodate oxidation of heparin (POH) (Fransson, 1978). To date, bioactivity studies of LAHs have mostly focused on anti-inflammatory actions. PDH can significantly attenuate activation of protease-activated receptors 1 by thrombin, and thereby prevent adverse effects of thrombin on human lung endothelial cell permeability in diseases such as acute lung injury and acute respiratory distress syndrome (Gonzales et al., 2014; Fryer et al., 1997). POH has been revealed to disrupt rosettes and cytoadherence in animal models and majority of fresh isolates from Cameroonian children with malaria, and has been successfully evaluated in a finalized Phase I study as adjunct therapy in severe malaria (Vogt et al., 2006; Leitgeb et al., 2011).

Alzheimer's disease (AD) is the most common form of dementia, characterized by progressive neurodegeneration with a clinical phenotype of cognitive impairment. A histopathological hallmark of AD is cerebral deposition of neurofibrillary tangles and β-amyloid (Aβ) (Hardy & Selkoe, 2002), which is the main component of amyloid plaques. The pathological Aβ peptides composed of 40 or 42 amino acids are products of sequential cleavage of the amyloid β precursor protein (APP) by β-secretase (β-site APP cleaving enzyme 1: BACE1, a membrane-bound aspartyl protease) (Cai et al., 2001) and γ-secretase (Bergmans & Strooper, 2010). Aβ deposition is attributed to excessive accumulation and aggregation of Aβ in the

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brain (Zhang, Zhang, Wang, & Li, 2014). However, initial cleavage of APP by BACE-1 is the first and rate-limiting step in generating the neurotoxic Aβ, and increased susceptibility of APP to BACE-1 cleavage has been found in many pathological mutations of APP (Axelman, Basun, Winblad, & Lannfelt, 1994; Howlett, Simmons, Dingwall, & Christie, 2000; Stenh et al., 2002). Consequently, the regulation of BACE-1 activity has become a key pharmaceutical target for therapeutics to alleviate symptoms of AD.

Previous studies on the effects of glycosaminoglycans (GAGs) in amyloidogenic pathways have concentrated on the direct interaction of GAGs with the amyloid proteins and peptides, rather than the enzymes involved in the pathway (Bergamaschini et al., 2004, 2002; Dudas, Rose, Cornelli, Pavlovich, & Hanin, 2008). HP, HS and their derivatives were found to inhibit BACE-1 activity and the speculative mechanism was blocking access to the enzyme active site (Scholefield et al., 2003). To date, an increasing number of studies have focused on the relationship between the detailed structure and activities of HP. Use of HP as a therapeutic agent against BACE-1 would doubtless lead to an increased risk of internal bleeding and impaired blood clotting mechanisms, which precluded heparin's clinical application in therapy of AD (Patey, Edwards, Yates, & Turnbull, 2006). Low molecular weight heparin (LMWH), such as enoxaparin sodium (ES) and dalteparin sodium, possess higher bioavailability, longer half-life, and fewer side effects compared with HP (Linhardt, 2003), but they still increase risk of bleeding (Büller et al., 2004; Spinler et al., 2003) and are not suitable for therapy of AD.

In this study, a novel and natural low anticoagulant heparin along with heparan sulfate prepared from crude porcine mucosal heparin. Detailed structural information was characterized by disaccharide composition and nuclear magnetic resonance (NMR) analysis. A library of LAH and HS oligosaccharides was prepared based on gel chromatography separation following digestion of heparin lyase I and III, respectively. Furthermore, BACE-1 inhibitory activities of poly- and oligosaccharides were examined, and they showed potential as leading compounds for treatment of AD by reducing Aβ peptide levels.

2. Materials and methods

2.1. Materials and chemicals

Crude heparin sodium salt from porcine mucosa was provided by the Tianlong Animal By-Products Co., Ltd. (Nantong, China). Expression and purification of heparin lyase I (EC 4.2.2.7), heparin lyase II (no EC assigned), heparin lyase III (EC 4.2.2.8), and chondroitin lyase ABC (EC 4.2.2.5) in recombinant *E. coli* strains provided by Professor Jian Liu (University of North Carolina, College of Pharmacy, Chapel Hill, North Carolina) were performed in our laboratory as described (Chen, Ones, & Liu, 2007). HS and HP from porcine intestinal mucosa were purchased from Celsus Laboratory, Inc. (Cincinnati, OH, USA). Q-Sepharose Fast Flow resin was purchased from GE Healthcare (Uppsala, Sweden). Antithrombin III (ATIII), bovine FXa, human thrombin (FIIa), FXa substrate and thrombin substrate were purchased from Hyphen BioMed (Neuville-sur-Oise, France). BACE-1 fluorescent resonance energy transfer Assay Kit was purchased from Thermo Fisher Scientific (New York, USA). All the other chemicals and reagents were of chromatographic grade.

2.2. Refining and fractionating the heparin components from crude heparin

Crude porcine heparin sodium salt (200 g) was treated with chondroitin lyase ABC (50 units, 50 mM ammonium acetate, pH 7.0, containing 2 mM calcium chloride) at 35 °C for 8 h to completely remove the galactosaminoglycans. After chondroitin lyase ABC digestion, the reactions were terminated by heating in a boiling water bath for 5 min, treated with 1 M HCl, and then centrifuged to remove enzyme and nucleic acid contaminants. The supernatant was neutralized with 1 M NaOH. The reaction mixture was dialyzed (MW cutoff 7000 Da) and freeze-dried. The refined heparin was loaded into a pre-activated anion exchange resin (Q-Sepharose Fast Flow) that had been equilibrated with water. Next, four fractions were eluted with 0.5, 0.7, 0.9 and 1.3 M sodium chloride solution and dialyzed overnight. The retentates were freeze-dried and designated HF1-HF4.

2.3. Anticoagulant activity assay

Factor Xa and IIa inactivation was used to assess anticoagulant activities of the heparin fractions using a Heparin Assay Kit adapted to a 96-well plate format and reading A_{405nm}. Factor Xa/IIa and antithrombin III were all present in excess; inhibition of factor Xa/IIa was directly proportional to the limiting concentration of GAGs. Thus, residual factor Xa/IIa activity, measured with factor Xa/IIa-specific chromogenic substrate, was inversely proportional to GAGs concentration (Brito et al., 2014; Fu et al., 2014). Anti-Xa and anti-IIa activities were calculated using a standard curve of different concentrations of heparin. The data indicated that HF3 was a novel low anticoagulant component, which was named LAH. LAH was further investigated as detailed in the following sections.

2.4. Molecular weight and chemical composition analysis

2.4.1. Disaccharide composition analysis

Unsaturated disaccharide standards and samples generated by heparin lyase I/II/III digestion were determined by strong anion exchange (SAX)-high performance liquid chromatography (HPLC) with UV detection (Trehy, Reepmeyer, Kolinski, Westenberger, & Buhse, 2009). An Ultimate XB-SAX column (4.6 × 250 mm, 3 μm, Welch, USA) was used with a flow rate of 0.6 mL min^{-1} at 40 °C. Disaccharide signals were detected at 232 nm. Solvent A was composed of 2 mM sodium phosphate buffer (pH 3.0) and solvent B was composed of 2 mM sodium phosphate and 1.14 M sodium perchlorate (pH 3.0). The gradient was used from 3% B to 35% B in 20 min, and then raised to 100% B from 20 to 50 min.

2.4.2. Determination of molecular weight

Molecular weight (Mw) and molecular weight distribution of LAH were determined by high performance gel permeation chromatography (HPGPC) coupled with eighteen-angle laser scattering (MALLs) (Hintze et al., 2014). This assay was performed on an Agilent 1260 LC system equipped with a Shodex Ohpak SB HQ 802.5 (8.0 × 300 mm, 6 μm, Showa Denko, Japan) and a Shodex Ohpak SB HQ 804 column (8.0 × 300 mm, 6 μm, Showa Denko, Japan) at 30 °C with a flow rate of 0.6 mL min^{-1}. The signals were detected by refractive index detector together with eighteen-angle laser scattering.

2.4.3. Analysis of 3-O-sulfo group-containing tetrasaccharides by hydrophilic interaction liquid chromatography-Fourier transform mass spectrometry (HILIC-FTMS)

Disaccharides or 3-*O*-sulfo group-containing tetrasaccharides were generated by heparin lyase II digestion and analyzed by HILIC-FTMS (Li et al., 2014). A Luna HILIC column (2.0 × 50 mm, 200 Å, Phenomenex, USA) was used to separate oligosaccharides. Mobile phase A was 5 mM ammonium acetate prepared in 98% HPLC grade acetonitrile with 2% HPLC grade water. Mobile B was 5 mM ammonium acetate prepared with HPLC grade water. The elution program