European Journal of Medicinal Chemistry 114 (2016) 33–40

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Anticoagulant and antithrombotic activities of low-molecular-weight propylene glycol alginate sodium sulfate (PSS)

Meng Xin, Li Ren, Yang Sun, Hai-hua Li, Hua-Shi Guan, Xiao-Xi He**, Chun-Xia Li*

Key Laboratory of Marine Drugs of Ministry of Education, and Shandong Provincial Key Laboratory of Glycoscience and Glycoengineering, School of Medicine and Pharmacy, Ocean University of China, Qingdao, 266003, PR China

ARTICLE INFO

Article history:
Received 12 November 2015
Received in revised form
25 February 2016
Accepted 25 February 2016
Available online 2 March 2016

Keywords:
Propylene glycol alginate sodium sulfate
Antithrombotic
Anticoagulant
Oxidative reduction
Molecular weight

ABSTRACT

Propylene glycol alginate sodium sulfate (PSS), a sulfated polysaccharide derivative, has been used as a heparinoid drug to prevent and treat hyperlipidemia and ischemic cardio-cerebrovascular diseases in China for nearly 30 years. To extend the applications of PSS, a series of low-molecular-weight PSSs (named FPs) were prepared by oxidative-reductive depolymerization, and the antithrombotic activities were investigated thoroughly *in vitro* and *in vivo*. The bioactivity evaluation demonstrated a positive correlation between the molecular weight and the anticoagulant and antithrombotic activities of FPs. FPs could prolong the APTT and clotting time and reduce platelet aggregation significantly. FPs could also effectively inhibit factor IIa in the presence of AT-III and HC-II. FPs decreased the wet weights and lengths of the thrombus and increased occlusion times *in vivo*. FP-6k, a PSS fragment with a molecular weight of 6 kDa, is an optimal antithrombotic candidate for further study and showed little chance for hemorrhagic action.

1. Introduction

Thrombosis is a leading common pathology underlying ischemic heart disease, ischemic stroke, and venous thromboembolism. The Global Burden of Disease Study 2010 (GBD2010) reported that ischemic heart disease and stroke collectively caused one in four deaths worldwide [1]. Heparin has been used successfully for the treatment of thrombotic diseases for decades. Low-molecular-weight heparins (LMWHs) derived from unfractionated heparin (UFH) through different depolymerization methods have some advantages over heparin in terms of pharmacokinetics, convenience of administration, and reduced side effects [2]. Heparin and LMWHs exert their antithrombotic and anticoagulant activities principally via two coagulation enzymes: factor-Xa and thrombin (factor IIa) [3]. However, the structure variability, yield limitation and safety issues arising from heparin and LMWHs of animal origin [4] still concern the public. Heparins have been confirmed to have a number of adverse side effects, such as the development of thrombocytopenia, arterial embolism, and bleeding complications, which have even resulted in death [5]. Thus, there is a real need to consider new antithrombotic agents of non-animal origin to mimic some of the pharmaceutical actions of heparin.

The alginate extracted from brown seaweeds is a linear polysaccharide composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) (Fig. 1A) [6]. Propylene glycol alginate sodium sulfate (PSS) is the sulfated derivative of alginate that has some degree of sulfation substitution (from 0.87 to 1.53) at the hydroxyls of C-2 and C-3 and has propylene glycol groups partially attached to C-6 of the hexuronic residues (Fig. 1B). PSS had been used as a heparinoid drug for preventing and treating hyperlipidemia and ischemic cardio-cerebrovascular diseases. PSS also exhibits many important bioactivities, such as anticoagulation activity, hypotensive activity, and blood viscosity reduction [7]. However, bleeding is a strong safety concern for PSS [8]. We speculated that the bleeding was associated with the molecular weight of PSS. Previously, we obtained four PSS fractions (Mw of 5.4 kDa, 11.8 kDa, 25.7 kDa and 51.9 kDa) to investigate the relationship between molecular weight and anticoagulant activity [9]. The results demonstrated that PSS mainly inhibited FIIa mediated by AT-III and HC-II by interfering with the coagulation cascade at several stages, and the anticoagulant activity declined with the decrease in molecular weight. Of

* Corresponding author.
** Corresponding author.
E-mail addresses: hexiaoxi@ouc.edu.cn (X.-X. He), lchunxia@ouc.edu.cn (C.-X. Li).

http://dx.doi.org/10.1016/j.ejmech.2016.02.063
0223-5234/

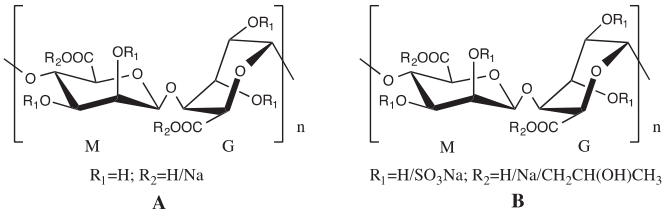

Fig. 1. Structures of alginate (A) and PSS (B).

these PSS fractions, those with Mw of 5.4 kDa and 11.8 kDa still showed moderate anticoagulant activity. Given the higher safety of LMWHs compared with heparin in thrombosis treatment, PSSs with lower molecular weights (3–10 kDa) should be evaluated comprehensively for anticoagulant activity and the corresponding bleeding risks to find an optimal fragment for a candidate antithrombotic agent.

To clarify these aspects, four fragments of PSS with lower molecular weight (3–10 kDa) were prepared by oxidative-reductive depolymerization [10]. The anticoagulant, platelet aggregation, and antithrombotic activities were tested *in vitro* and *in vivo*. The results indicated that the anticoagulant, platelet aggregation and antithrombotic effects of the fragments of PSS (FPs) increased in parallel with the molecular size. Moreover, the hemorrhagic action in mice exhibited a positive correlation with the molecular weight. The fragments of PSS inhibited factor IIa in the presence of AT-III and HC-II. Of these fragments, FP-6k is a potential antithrombotic agent with lower bleeding risks.

2. Materials and methods

2.1. Animals

Male Kunming mice and Wistar rats were housed at 23 ± 2 °C under 12 h light and dark cycles and were provided access to food and water ad libitum. The experiments were performed in accordance with the Guidelines of Animal Ethics Committee of Ocean University of China.

2.2. Materials

PSS with a molecular weight (Mw) of 20.0 kDa was provided by Dalian Tianyu Pharmaceuticals Co., Ltd (Dalian, China). The dextran standards were purchased from the National Institute for Food and Drug Control (Beijing, China). LMWH was obtained from Vetter Pharma-Fertigung GmbH & Co., KG (Germany). Anti-thrombin III (AT-III), thrombin (FIIa), coagulation factor Xa (FXa), chromogenic substrates for thrombin and FXa, type I collagen and ADP were purchased from Sigma (St. Louis, MO, USA). Adrenaline hydrochloride for injection was from Jinyao Amino Acid Co., Ltd (Tianjin, China). All other chemicals and reagents were analytical grade.

2.3. Depolymerization of PSS (FPs)

The oxidative-reductive depolymerization of PSS was based on the procedure reported by Nagasawa [10] with some modifications. Briefly, PSS was dissolved in distilled water to a final concentration of 7.5% in a flask, and then the solution was stirred and heated to 60 °C in a water bath. Subsequently, 0.1 M ferrous sulfate and 30% hydrogen peroxide were added to the solution and stirred at 60 °C for 0.5–1 h. The reaction was interrupted by addition of vitamin C, and the raw product was precipitated with 3 vol of 95% ethanol. Then the precipitate was collected by centrifugation, and freeze-dried to generate different fragments of PSS (FPs) with different molecular weights.

2.4. Analysis of structure

Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet Nexus 470 IR spectrometer using the KBr pellet.

For NMR analysis, each sample (30 mg) was dissolved in 1 ml D_2O (99.9%) and freeze-dried three times to remove all exchangeable protons before the sample was dissolved in 0.5 ml D_2O (99.96%) for analysis. All NMR spectra were obtained using a JEOL JNM-ECP600 spectrometer at 298 K with acetone-d_6 as the internal standard.

The weight-averaged molecular weight (Mw) and the distribution width of the molecular weight (D) were determined by a high performance gel permeation chromatography (HPGPC) on an Agilent 1100 chromatographic instrument. The samples were dissolved in 0.1 M Na_2SO_4 elution solvent at a concentration of 5 mg/ml. Then, 20 μl of the sample was applied to a TSK gel G3000PWxl column (exclusion limit of Mw 60 kDa, 7.8 × 300 mm, Japan) with a flow rate of 0.5 ml/min. The temperature of the column was maintained at 35 °C, and the signal was detected using a G1362A refractive index detector (Agilent, Germany). The HPGPC system was calibrated with a series of dextran standards. All data were recorded and processed using the Agilent GPC software [11].

The sulfur content (S%) was determined by an oxygen flask combustion method as reported previously [9]. The degree of sulfate substitution (DS), which was the average number of *O*-sulfate groups on each alginate acid residue, was calculated by the following formula:

$$\mathrm{DS}\left(\mathrm{SO}_3^-\right) = \frac{220S}{3200 - 102S}$$

where the carboxyl groups of alginate acid residues were postulated to be sodium salts.

2.5. Assessment of anticoagulant action and platelet aggregation

2.5.1. Anticoagulant action measured by the activated partial thromboplastin time (APTT)

An APTT clotting assay was carried out according to the manufacturer's instructions using citrated rat plasma. In this assay, 295 μl of the rat plasma was mixed with 5 μl of the sample solutions (0.9% NaCl) at various concentrations and incubated at 37 °C for 60 s. Then, 100 μl of pre-warmed APTT reagent was added to 100 μl of mixture and allowed to incubate for 5 min at 37 °C. Thereafter, pre-warmed 0.025 M $CaCl_2$ (100 μl) was added, and the time of clot formation was recorded by a coagulometer. Saline was used as a control.

2.5.2. Effects of FPs on the thrombin and factor Xa activities mediated by AT-III or HC-II

The assays were carried out in 96-well plates. The final concentrations of reactants included 50 nM AT-III or 68 nM HC-II, 15 nM thrombin or factor Xa and 10^{-2} -10^{2} μg/ml samples in 40 μl of TS/PEG buffer (0.02 M Tris–HCl, 0.15 M NaCl, and 1.0 M polyethylene glycol, pH 7.4). Thrombin or factor Xa was added last to initiate the reaction. After 120 s of incubation at 37 °C, 25 μl of chromogenic substrate (S-2238 for thrombin or S-2765 for factor Xa) was added, and the absorbance at 405 nm was recorded for 360 s [12]. The rate of change of absorbance was proportional to the activity of the thrombin or factor Xa activity remaining in the incubated mixture. Saline was used as a control.