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Full Length Article

Discovery and assessment of water soluble coumarins as inhibitors of the coagulation contact pathway

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ABSTRACT

Over the last decade, the coagulation factor XIIa (FXIIa) has seen renewed interest as a therapeutic target. Indeed, its inhibition could offer a protection against thrombosis without increasing the risk of bleeding. Moreover, it could answer the need for a safe prevention of blood-contacting medical devices-related thrombosis. Among the FXII and FXIIa inhibitors already described in literature, organic small-molecular-weight inhibitors are rather left behind. In this study, we were focused on the discovery and assessment of water soluble small molecules. First, a search within our library of compounds flagged two promising hits. Indeed, enzymes and plasma assays suggested they have a greater activity on the contact factors (FXIa, plasma kallikrein and FXIIa) than on the TF pathway. Then, simple pharmacomodulations were undertaken with the aim to design more selective FXIIa inhibitors. This afforded compounds having different degrees of selectivity. All compounds were finally screened in whole blood using an 8-channel microfluidic model and thromboelastometry measurements. Interestingly, all molecules interfered with the thrombus formation and one of them could be considered as a small organic contact inhibitor.

1. Introduction

Tackling thrombotic disorders without affecting the hemostatic capacity remains a challenge in medicine. The non-vitamin K direct oral anticoagulants (DOACs) come closer to this goal but severe bleeding can still be reported [1]. Therefore, the search for safer antithrombotic strategies is ongoing [2]. These include a better understanding of the pathogenesis of thrombosis, an improved management of existing drugs, but also the identification of novel targets such as coagulation factor XII (FXII) [2].

FXII is a trypsin-like serine protease belonging to the contact system of coagulation together with factor XI (FXI), high molecular weight kininogen (HMWK) and plasma prekallikrein (PPK). This system is activated when FXII comes into contact with negatively charged surfaces in a process called "contact activation". Small amounts of FXIIa will be formed and in the presence of the cofactor HMWK, FXIIa activates PPK in plasma kallikrein (PK) which reciprocally activates FXII creating an amplification loop of FXII activation [3]. Following the activation of FXII, several pathways can be triggered including the intrinsic pathway of coagulation via activation of FXI by FXIIa [4,5].

The interest for FXIIa as a therapeutic target was revived a decade ago when it was suggested that FXIIa plays a critical role in thrombosis while being dispensable for hemostasis [6,7]. So far, numerous studies with different animal models of thrombosis have been conducted and they unanimously suggest that the inhibition of FXII or FXIIa is an opportunity to develop anticoagulants devoid of a bleeding risk [8,9]. In addition, anti-FXII directed therapies could answer unmet medical needs such as the safe prevention of thrombosis in patients exposed to blood-contacting medical devices including catheters [10–12], circuits of extracorporeal circulation [13], mechanical heart valves (MHV) [14] and ventricular assisted devices (VAD) [15]. Beside this advantage in the field of thrombosis, the FXIIa inhibition also rises as a therapeutic strategy to interfere with excessive vascular leakage in

Abbreviations: CTI, corn trypsin inhibitor; FXII(a), (activated) coagulation factor XII; FXI(a), (activated) coagulation factor XI; PK, plasma kallikrein; PPACK, D-Phenylalanyl-prolyl-arginyl chloromethyl ketone; TF, tissue factor; THR, thrombin.

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patients suffering from hereditary angioedema [16] (CSL312, anti-FXIIa antibody in phase I – trial ACTRN12616001438448).

The FXII or FXIIa inhibitors currently under development include peptides [17,18], proteins [19,20], antibodies [13,21] and RNA-based technologies [10,22]. In contrast, we have only few data regarding the design of synthetic small molecular-weight inhibitors of FXIIa [23,24]. Organic small molecules could complete the panel of inhibitors since they would be cheaper to produce and could be designed to be administered orally (lipophilic structures) or parenterally (soluble structures). Moreover, they could also be useful for diagnostic assays where the activation of the contact system may flaw the results [25,26].

The challenge for medicinal chemists is to design a potent and selective chemical structure. Usually, the existence of structurally characterized inhibitors as well as the availability of a three-dimensional co-crystal structure depicting the interactions between a potent inhibitor and the target are precious assets to turn a weakly active molecule into a drug candidate. Regarding FXIIa, such information is limited. Indeed, there are only four recent studies using FXIIa 3D models to propose the structure-activity relationships of their inhibitors [17,18,20,23]. This may explain why the development of small synthetic FXIIa inhibitors is a step behind the other strategies.

Coumarin derivatives were previously described as small-molecular weight FXIIa inhibitors [23,24]. The elaboration of these lipophilic molecules was prompted by the need to afford agents that could be taken orally for long-term indications (e.g. patients with MHV or VAD). In this study, we put the emphasis on the discovery and evaluation of potential water soluble small FXIIa inhibitors since such design remains clinically relevant.

2. Material and methods

2.1. Enzyme assays

All compounds were prepared in DMSO. For the IC_{50} determination, nine concentrations ranging from 1 μM to 500 μM (in the final mixture) were used and compounds were assayed in duplicates. Procedures on thrombin, plasma kallikrein, FXa and TF/FVIIa were performed as previously described [23]. Protocols applied on FXIIa (Enzyme Research Laboratories, Swansea, UK) and FXIa (Enzyme Research Laboratories, Swansea, UK) are described in the supplementary material.

2.2. Clotting time assays

Clotting time assays (aPTT, PT, dPT, TT and dRVVT) were performed on normal pooled plasma (NPP) using a KC10 coagulometer (Amelung, Germany) or the automated coagulometers STA-R® or STA-Max® (Stago, France). Preparation of the NPP and procedures followed for the assays are described in the supplementary material. The FXI antibodies 14E11 and 1OA6 were gifts from Dr. Erik Tucker (Aronora Inc., USA).

2.3. Microfluidic assay

Blood was obtained via venipuncture into CTI (0 μg/mL or 40 μg/mL) or PPACK (100 μg/mL) from healthy donors who self-reported to be free of alcohol use and medication for at least 72 h prior to blood collection. All donors provided informed consent under approval of University of Pennsylvania Institutional Review Board.

Stock solutions of coumarin compounds were prepared in DMSO. The compounds were tested at 50 μM or 100 μM (final concentration in blood) and the DMSO final concentration in blood reached 0.1%.

The experiments followed the instructions described in [27] with some adaptations. Indeed, during the preparation of surfaces, glass slides were treated with sigmacote® (Sigma) instead of ethanol before being rinsed with deionized water and dried with filtered air. Experiments were done at least in triplicates. Difference between control and treated groups was analyzed with Student's *t*-test. The difference was considered significant when *p*-value is smaller than 0.05.

2.4. Thromboelastometry measurements

2.4.1. Reagents

The experiments were performed on a ROTEM® thromboelastometer (TEM International, Munich, Germany) using standard assays according to the manufacturer's instructions. Inducers In-tem®, ex-tem® and star-tem® reagents were from TEM International, Munich, Germany. The stock solution of t-PA is Actilyse® (1 mg/mL). This stock solution is diluted with HN-BSA buffer (HEPES 25 mM, NaCl 150 mM, BSA 5 mg/mL, pH 7.5 buffer) to generate the working solution of 11.1 μg/mL. The final concentration of t-PA in blood was 0.16 μg/mL. Difference between control and treated groups was analyzed with Student's *t*-test. The difference was considered significant when *p*-value is smaller than 0.05.

2.4.2. Blood collection and blood samples preparation

Blood was obtained from healthy volunteers ($n = 2$) and were collected in 3.2% sodium citrate as anticoagulant. Immediately upon blood drawing, blood samples were prepared and ROTEM® experiments were started. The study was approved by the local institutional Ethics Committee and was conducted according to the principles of the Declaration of Helsinki. Blood samples were prepared as follows. Coumarin working solution (or vehicle) was spiked into blood in order to reach a concentration of 500 μM and a sample volume of 400 μL (DMSO kept at 1%). The mixture is then incubated for 5 min at 37 °C. Following the incubation step, 300 μL of the mixture are taken for the ROTEM® experiments.

2.4.3. ROTEM® procedure

Introduce 300 μL of treated blood sample in a ROTEM® cuvette. To start the thrombus formation, a combination of 20 μL In-tem®, 20 μL Star-tem® and 5 μL tPA working solution or a combination of 20 μL Ex-tem®, 20 μL Star-tem® and 5 μL tPA working solution were added to the cuvette. Curves raw data and parameters are given by the software. The evaluated parameters were derived from curves by the software. We studied the clotting time (CT), the clot lysis time (CLT), the maximal clot formation (MCF) and maximal rate of clot formation (α-angle). The two latter inform on the maximal elastic clot strength and the rate of fibrin strand formation, respectively [28].

2.5. Aqueous solubility assessment

The aqueous solubility was measured in PBS (KH_2PO_4 144 mg/L, Na_2HPO_4 795 mg/mL, NaCl 9.0 g/L, pH 7.4, bought from Lonza 17-516) using Multiscreen HTS-PCF filter plates (MSSLBPC10 from Millipore) and following a protocol adapted from [29]. Briefly, 10 μL of stock solution (10 mM in DMSO) and 190 μL PBS were mixed at room temperature during 30 min. Then, the mixture was filtered on the filter plate and the filtrate was prepared for analysis by mixing 160 μL of filtrate with 40 μL methanol. The analyses were performed on an Agilent 1100 series LC-UV system (UV detection at 254 nm) equipped with a C18 Zorbax SB column (100 mm × 3 mm, 3.5 μm) and following a gradient (flow rate of 0.5 mL/min) of acetonitrile in aqueous acetic acid 0.1% (v/v): from 5% to 95% of acetonitrile in 5 min, holding for 3 min, then reversing to 5% of acetonitrile in 0.1 min and holding for an additional 5.4 min. For each tested compound, the solubility was calculated thanks to a calibration curve. The calibration curves were realized in a methanol/water/DMSO (50/45/5%) solution at three concentrations (at 50, 200 and 500 μM for compounds 1–3; at 12.5, 50 and 200 μM for compound 4 and at 3.13, 12.5 and 50 μM for the previously described coumarin (compound 23 in reference [23])).