Thrombosis Research xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

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

Regular Article

# A serendipitously identified novel small molecule procoagulant compound giving rise to a high-throughput screening assay based on human plasma ☆

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ARTICLE INFO

*Article history:*
Received 17 September 2012
Received in revised form 13 March 2013
Accepted 21 May 2013
Available online xxxx

*Keywords:*
Haemostasis
Haemophilia
Heparin
Thrombin

ABSTRACT

*Introduction:* Oral treatment is lacking for haemophilia, the rare bleeding disorders, and some severe forms of von Willebrand's disease. We have serendipitously identified a small molecule procoagulant compound (AZ10047130). This publication describes some characteristics of AZ10047130 and a systematic search for novel hits using a, human plasma-based, high-throughput screening (HTS) assay.
*Material and Methods:* Coagulation, thrombin generation, chromogenic assays and surface plasmon resonance (SPR) experiments were used to characterise AZ10047130. A 1536-well formatted human plasma coagulation assay for HTS was developed.
*Results:* In the plasma clot assay (re-calcified plasma with low tissue factor) AZ10047130 shortened time to coagulation with an $EC_{50}$ value of 3.9 μM (assay concentration). AZ10047130 was similarly effective in immunodepleted human and haemophilia A plasmas. SPR and chromogenic substrate experiments indicated that AZ10047130 binds to the heparin binding site of several coagulation factors. The HTS screened in excess of one million compounds. It generated some hits belonging to the same pharmacophore as AZ10047130 but also some entirely novel hits.
*Conclusion:* These novel small molecule procoagulant compounds may serve as templates for discovery of oral procoagulant drugs.

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## Introduction

Haemophilia, the rare bleeding disorder and some forms of von Willebrand Disease (vWD) are currently treated by intravenous replacement of the deficient factor. Today FVIII, FIX and FVIIa are available as recombinant factors while the other factors are plasma derived. Clinical studies and experience show that prophylactic administration of the deficient factor is highly preferable with regard to outcome [1]. However, prophylaxis is cumbersome due to the necessity of intravenous self-injections every second to third day. Furthermore, for patients in the developing world, cost, requirements on hygiene and refrigerators *etc.* are additional hurdles. Finally, 5-25% of the patients develop inhibitory antibodies towards the administered coagulation factor, a serious clinical complication. Today, only 20-30% of the estimated "haemophilia" population in the world receives adequate treatment, and only a fraction of those are given the coagulation factors as prophylaxis [2]. Thus, there is a great need for improved treatment modalities, e.g. oral prophylaxis.

The balance between haemostasis and bleeding is skewed, in the sense that there is no need to fully restore the haemostasis defect, in order to achieve a clinically meaningful effect. Persons with mild haemophilia usually do not require coagulation factor prophylaxis despite levels of FVIII or FIX between 5–40 % of normal. Currently, the only non-protein molecules with clinically proven effects give modest shifts towards haemostasis. The oral fibrinolysis inhibitors tranexamic acid and ε-amino caproic acid, stabilises the clots towards early dissolutions when used in addition to added coagulation factor [3–5]. In mild vWD desmopressin (intranasally or subcutaneously), results in transient release of von Willebrand factor and FVIII from endothelial cells [6]. Literature describes some additional non-coagulation factor approaches [7–19] to strengthen haemostasis but we are not aware of any small molecule approach that has the potential to become an oral prophylactic agent, i.e. being sufficiently potent and with a molecular

*Abbreviations:* AT, antithrombin; AZ, AstraZeneca; CAT, calibrated automated thrombogram; ETP, endogenous thrombin potential; LT, lag time; HTS, high-throughput screening; PPP, platelet poor plasma; PRP, platelet rich plasma; RU, resonance units; SPR, surface plasmon resonance; TFPI, tissue factor pathway inhibitor; ttPeak, time to peak; TF, tissue factor; vWD, von Willebrand Disease.

☆ Part of the manuscript was originally presented at the Society Biomolecular Screening (SBS) 17th Annual Conference March 27–31, 2011 Orlando, Florida, USA.

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0049-3848/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.thromres.2013.05.023

mass, physico-chemical properties and pharmacokinetic profile suitable for oral administration in the bleeding disorders.

Since its introduction, HTS has become an established process for lead discovery [20]. We had previously discovered a new small molecule haemostatic agent, AZ10047130, which reduces plasma (normal and FVIII or FIX deficient) coagulation time *in vitro* and increases thrombin generation in human plasma. To systematically search for more procoagulant compounds we have developed a novel, human plasma-based, 1536-well formatted coagulation assay for HTS. Following optimisation, this HTS assay was used to screen in excess of one million compounds. This publication describes the HTS and indicates that plasma-based HTS may be a way of identifying chemical leads for new oral agents for prophylaxis of bleeding. To understand the mechanism of action of chemical leads approaches as described for characterisation of AZ10047130 could be undertaken.

## Material and methods

### Material

Innovin, purchased from Dade Behring, Marburg GmbH, Germany, provided a stock solution of 6 nM recombinant human tissue factor (TF), according to the manufacturer. Phospholipid (PL-TGT, 0.5 mM) emulsion and BSA (20%) were purchased from Rossix, Mölndal, Sweden. HMW heparin (porcine), glycerol, CHAPS and Tween 20 were from Sigma-Aldrich, Steinheim, Germany. The NaCl solution was purchased from Fresenius Kabi, Halden, Norway. Human α-thrombin, human α-thrombin-PPACK, human antithrombin (AT) human FIXa and human FXIa were obtained from Haematologic Technologies Inc, VT, USA, human FXa, from Enzyme Research Labs, IN, USA. The chromogenic substrate L2145 was from Bachem, Bubendorf, Switzerland. The FVIII, TFPI and AT depleted plasmas were from Sekisui Diagnostics, Stamford, CT,USA and the FIX deficient plasma was from Precision Biologic, Dartmount, NS, Canada. Haemophilia A plasma (<1% FVIII) was from George King Biomedical, Overland Park, Kansas, USA. PRP-reagent (#TS42), PPP-reagent 1pM (#TS31.00), the fluorogenic thrombin substrate with $Ca^{2+}$ kit (#TS50.00) and Thrombin Calibrator (#TS20.00) were obtained from Thrombinoscope BV, Maastricht, The Netherlands. DMSO, and sodium meta-periodate were from Merck (Darmstadt, Germany), HBS-N buffer (10 mM Hepes, 0.15 M NaCl, pH 7.4), HBS-P buffer (i.e. HBS-N with 0.005% P20), 10 mM sodium acetate pH5.5 (coupling buffer) and SA chips were from GE Healthcare (Uppsala, Sweden), BioSpin 6 columns from Bio Rad laboratories (Hercules, California, USA), biotin-LC-hydrazide from Pierce Biotechnology Inc. (Rockford, IL, USA). All chemicals were of reagent grade. All solutions were made with deionised water that was further purified by reversed osmosis on an Elgastadt UHP (Elga Ltd, High Wycombe Bucks, England). All compounds tested belonged to the AZ compound collection and were provided dissolved in DMSO. All *in vitro* experiments contained DMSO and equal amounts were used in the control experiments proving no effects of DMSO at ≤2%.

### Preparation of platelet rich plasma (PRP) and platelet poor plasma (PPP)

Blood was drawn by venipuncture from healthy volunteers through an 18-gauge butterfly needle without a tourniquet. Blood (9 parts) was collected into tubes containing 1 part 0.109 M sodium citrate. For preparation of PRP, citrated whole blood was centrifuged at 240 ×g at 20 °C for 15 mins. The supernatant, containing the PRP, was transferred to new tubes, kept at 20 °C and used within 3 h. For preparation of PPP citrated whole blood was centrifuged at 2000 ×g for 20 min at 20 °C. The PPP was pooled, divided in aliquots, frozen and stored at −80 °C until use. The Göteborg Ethical Committee approved the human blood donation and use of human plasma.

### Plasma clot assays

Three related plasma clot assays were used. The 96-well clot assay served as a template for the development of the 1536-well assays described below. A long coagulation time was necessary for automation of the assay and this was achieved using the lowest tissue factor (TF) concentration, which gave a shortening of the time to clot within a reasonable observation time for a HTS assay but long enough to allow detection of a procoagulant.

In the 96-well clot assay, plates (269620, Nunc, Roskilde, Denmark) were measured continuously with regard to the optical absorbance at 405 nm, ΔA405, using a Spectramax Plus (Molecular Devices, Sunnyvale, CA, USA) plate reader at 37 °C. Time to plasma coagulation was defined as the time to the positive $V_{max}$, *i.e.* the time to the highest rate of the increase in ΔA405. In sequence 5 μl compound was added to the wells (maximal final assay concentration of DMSO, 1.5%) followed by 90 μl start cocktail ($CaCl_2$ [titrated to 1.3 mM as measured in serum], TF 1 fM, NaCl 4.05 mg/ml, Hepes buffer 0.01 M) and 5 μl phospholipids (1 μM), all final assay concentrations, and finally 100 μl pre-warmed citrated PPP. In this 96-well assay with normal re-calcified PPP the time to coagulation was 1420 ± 100 s without TF and 850 ± 57 s with 1 fM TF.

Two similar 1536-well clot assay formats were used at room temperature, for the HTS primary test and the retest (Polystyrene Shallow Flat Clear 781201, Greiner Bio-One, Frickenhausen, Germany) reading A405 in a Pherastar Plus (BMG Labtech, Offenburg, Germany). The difference between these two assay formats was that A405 was either measured as time to reach a predefined threshold in ΔA405 (time to clot format used in HTS retest) or ΔA405 was determined between two fixed time points, immediately after (t = 0 min) addition of PPP and then after 30 min (t = 30 min), *i.e.* endpoint format (used in primary HTS test). In sequence 0.12 μl compound (up to 2 mM) in 100% DMSO resulting in a final concentration up to 30 μM (1.5% DMSO) was followed by 4 μl of start cocktail (see Results) and 4 μl PPP. To define the assay window and to control for assay quality, controls with "maximal" (33 μM AZ10047130) and no procoagulant compound (DMSO) were used.

### Thrombin generation assays

We have used the commercial Calibrated Automated Thrombogram (CAT) assay in which thrombin activity is continuously monitored at 37 °C as described in [21]. First the commercial trigger solution, PPP-reagent LOW or PRP-reagent (20 μl) was added to 75 μl plasma in the assay wells of the round bottom 96 well plates, 650061 (Greiner Bio-One), followed by the test compound (5 μl, final assay concentration of DMSO 1%) and finally the fluorogenic thrombin substrate with $Ca^{2+}$(20 μl). Separate wells contained the plasmas with the commercial calibrator solution. Four different variables were analysed: Time to maximum thrombin activity (ttPeak), maximum thrombin activity (Peak), Endogenous Thrombin Potential, (ETP, the area under the thrombogram curve) and lag time (LT), time at which the thrombin activity is 1/6 of the Peak.

### The surface plasmon resonance (SPR) assays

The SPR experiments to study binding of coagulation factors to immobilised oxidised heparin and fibrin were performed on a Biacore3000 (GE Healthcare). The details of the experiments are described in the Supplement.

### Chromogenic assay

The α-thrombin antithrombin interaction was measured by monitoring the thrombin activity using a chromogenic substrate, L2145. A405 was read in a Spectramax Plus in a 384-well plate (781101, Greiner

Please cite this article as: Gustafsson D, et al, A serendipitously identified novel small molecule procoagulant compound giving rise to a high-throughput screening assay based on human plasma, Thromb Res (2013), http://dx.doi.org/10.1016/j.thromres.2013.05.023

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