The role of the different Kunitz domains of TFPI in the down-regulation of the extrinsic coagulation pathway

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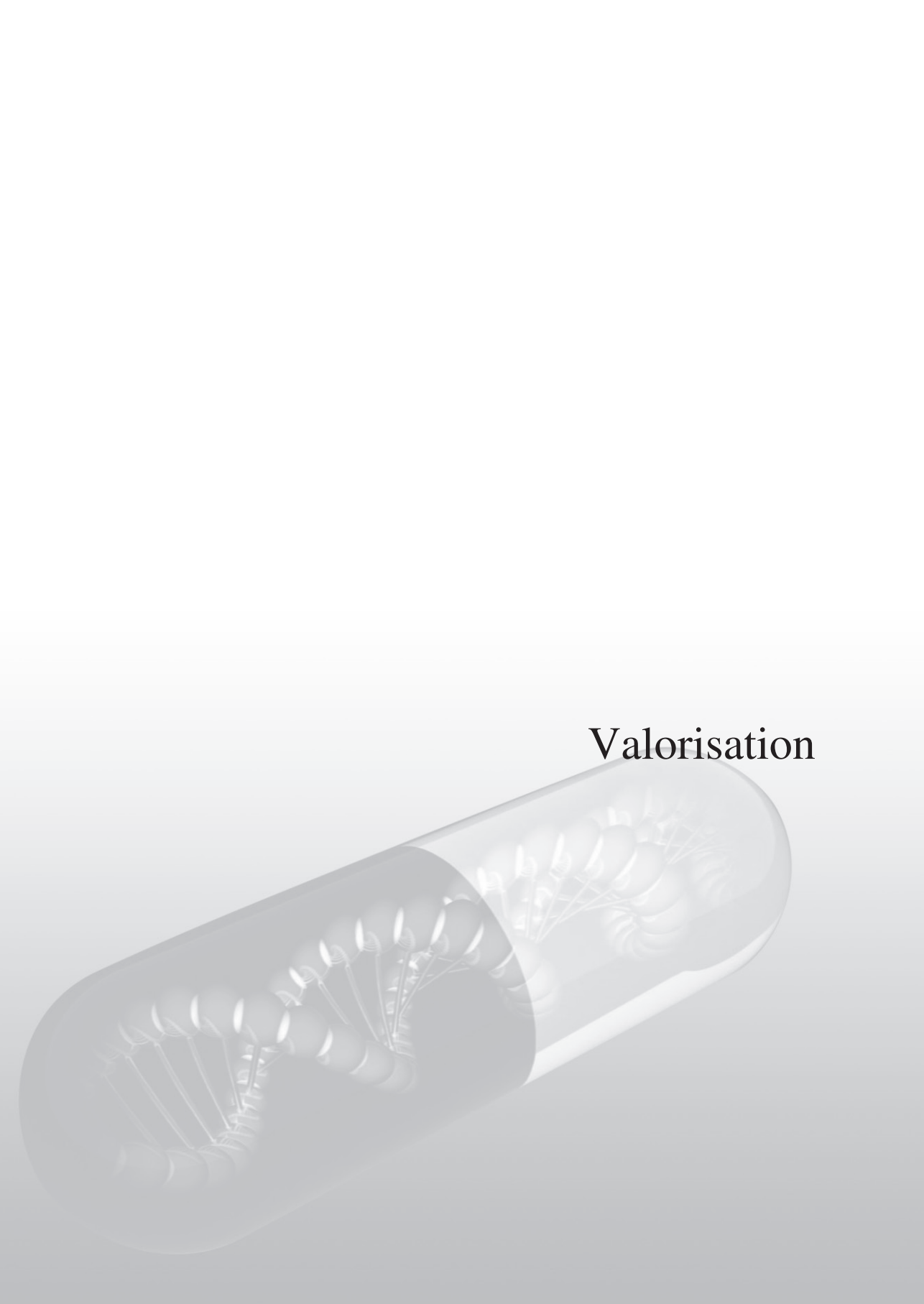

TFPI levels and risks of venous thrombosis and bleeding disorders.

Tissue factor pathway inhibitor (TFPI) is a multivalent Kunitz-type protease inhibitor which down-regulates the extrinsic coagulation pathway by inhibiting FXa via its Kunitz 2 domain and FVIIa via its Kunitz1 domain. In *in vitro* experiments TFPI is a powerful regulator of thrombin generation both in the absence and in the presence of APC (1) which together with the observation that mice lacking the TFPI gene died *in utero* (2) is indicative for an important physiological function of TFPI. Therefore it is not surprising that low levels of TFPI are associated with an increased risk of venous thrombosis (3) and that high levels of TFPI are associated with a moderate to severe bleeding disorder (4, 5).

Implications of the findings presented in this thesis for the association between low TFPI levels and risk of venous thrombosis

Venous thromboembolism (VTE) is a disease that includes both deep vein thrombosis (DVT) and pulmonary embolism (PE). It is a common disorder with a recurring tendency which may result in long-term complications like post-thrombotic syndrome. Venous thromboembolism often results from a combination of hereditary and acquired risk factors. Venous thromboembolism is the third most common cardiovascular illness after blockage of coronary artery (known as acute coronary syndrome) and stroke (6). About 30% of the of patients with symptomatic VTE develop pulmonary embolism (PE), whereas some 70% deep vein thrombosis

(DVT). VTE recurs in about 7% patients, 6 months after the initial event notwithstanding the anticoagulant therapy. Some 6% of the patients with DVT and 12% of the patients with PE die within 1 month of diagnosis (7).

According to literature, a low level of TFPI is associated with an increased risk both of a first VTE (3, 8, 9) and recurrent VTE (9). To establish the risk of developing VTE, these studies measured the plasma levels of free TFPI antigen or total TFPI antigen with ELISA's or the TFPI activity with functional tests that quantify total TFPI (full length and truncated TFPI) (10) or only full length TFPI (11). Despite the fact that TFPI is a major determinant of *in vitro* thrombin generation both in the absence and presence of APC (12, 13) and that TFPI levels in plasma vary widely (*e.g.* total TFPI varies between 10 and 65 ng/mL (14)) low TFPI levels are a mild risk factor of VT that only becomes significant at TFPI levels below the 5th percentile (3). A possible explanation for this low risk is that the assays used to quantify the plasma TFPI levels in epidemiological studies do not reflect the TFPI level that regulates thrombus formation. For instance, in addition to the various forms of TFPI present in plasma, TFPI is present in platelets (15) and in/on the endothelium (16, 17), which may play an important role in the down-regulation of thrombin formation. The fact that these activities of TFPI do not contribute to the TFPI assays used in epidemiological studies may explain the relatively low VT risk associated with low plasma TFPI levels. In addition, our observations that truncated forms of TFPI express rather low activities in the

inhibition of FXa (chapter 3), TF:FVIIa (chapter 4) and TF:FVIIa-catalysed FIX and FX activation (chapter 5) while they contribute to the TFPI levels used to quantify TFPI in plasma in epidemiological studies may have reduced the power of the risk analysis. Since the *in vivo* functional activity of TFPI is modulated by several plasma components (chapter 6) assays of TFPI that do not involve the effect of these modulators (antigen assays and functional TFPI total tests (11)) will not be the first choice tests for establishing the VTE risk of TFPI in epidemiological studies. Assays, the outcome of which include the effects of modulators like protein S and FV may give more reliable odds ratios for the VTE risk of low TFPI levels. One such assay is the thrombin generation-based functional TFPI test developed in our laboratory in Maastricht (18, 19) in which thrombin generation is determined at a low TF concentration in the absence and presence of anti-TFPI antibodies.

Effect of heparin on the anticoagulant activity of TFPI

In chapter 6, we described the effects of both unfractionated and low molecular weight heparin on the inhibition of FXa and TF:FVIIa by TFPI. Very low concentrations of heparin (0.01 U/ml) considerably enhanced FXa inhibition by TFPI and optimum stimulation was attained at 0.2 U/ml heparin. High concentrations (>0.5 U/ml) of heparin counteracted the anticoagulant activity of TFPI making it a less efficient inhibitor of FXa. In chapter 6 we show that TF-

FVIIa inhibition by TFPI is also stimulated in presence of heparin. Administration of heparin is an important therapy in the prevention and treatment of VTE (20, 21). Our study confirms that the anticoagulant effect of heparin is not limited to enhancement of the inhibition of activated coagulation factors by antithrombin (22), but that stimulation of the inhibition of FXa and TF-FVIIa by TFPI and the heparin-induced release of TFPI from the endothelium also contribute to the therapeutic effect of heparin during the treatment of patients with venous thrombosis (23).

TFPI as target in haemophilia treatment:

TFPI is not only associated with an increased risk of VT, but also plays role in bleeding. This is illustrated by a recent publication in which the so-called east Texas bleeding disorder is described [4]. This disorder is associated with a point mutation in FV gene (A2440G), which results in the enhanced expression of a FV molecule with a reduced molecular weight (FV-short). FV-short lacks a large part of the B domain and has an acidic region exposed that, compared to full length FV, binds TFPI with a 10-fold higher affinity and which can explain the 10-fold increase of the plasma level of TFPI (4, 5, 24). This elevated level of TFPI increases the bleeding risk in patients with the A2440G mutation.

Considering its important role in maintaining the haemostatic balance, TFPI may also be a target for the treatment of patients with congenital bleeding disorders like

haemophilia A or haemophilia B. Haemophilia A or B are caused by a deficiency or complete absence of coagulation factor VIII (FVIII) or factor IX (FIX), respectively. Both haemophilia A and haemophilia B are X-linked disorders which represent the large majority of inherited deficiencies of coagulation factors, occurring in ~0.02% and 0.002% of male population, respectively, without any racial predisposition (25). An indication of severe haemophilia is bleeding in soft tissue and joints leading to joint damage despite on-going treatment. Prophylaxis, that is infusion of clotting factors, has been used for treating haemophilia for a long time but is not universally implemented because these treatments are expensive; require intravenous infusion and formation of inhibitors is typical. Alternative therapies, like targeting and inhibiting natural anticoagulants such as TFPI (26), activated protein C (27, 28) or antithrombin (29) may have the potential advantages of lower cost, oral administration, and absence of inhibitor formation.

Inhibition of TFPI as a potential treatment for haemophilia was reported for the first time by Erhardtsen et al. (26) who showed that antibodies against TFPI reduced the bleeding time in haemophilic rabbits. Since then several studies were published in which molecules were investigated which target and abrogate the anticoagulant activity of TFPI. These compounds include:

1) non-anticoagulant sulfated polysaccharides (NASP), which like heparin are sulfated polysaccharides, a subset of which (pentosanpolysulphate, PPS and fucoidans) were shown to inhibit TFPI and improve haemostasis in haemophilic

mice (30, 31). Recent studies by Zhang et al (32) on fucoidan show the charge density and sugar units required to find the optimal pro and anticoagulant nature.

2) an aptamer called ARC19499 (BAX499) that binds to multiple domains (Kunitz 1, Kunitz 3 and C-terminus) of TFPI and that impairs but not fully prevents the inhibition of FXa by TFPI (33). Protein S was shown to compete and in its presence the efficacy of BAX 499 peptide was reduced.

3) a monoclonal antibody against TFPI (mAb 2021) that binds with a high-affinity to the Kunitz 2 domain TFPI thereby preventing inhibition of FXa and reducing bleeding in a rabbit haemophilia model (34)

4) a small peptide that binds with a high affinity to the Kunitz 1 domain of TFPI and that impairs inhibition of FXa and TF:FVIIa by TFPI and enhances thrombin generation in plasma (35).

It is interesting to note that amongst the TFPI antagonists that are currently in development for haemophilia treatment there are compounds that target different Kunitz domains of TFPI *i.e.* Kunitz domain 1 (35) or Kunitz domain 2 (33). In chapter 3, 4 and 5 of this thesis it is shown that the role of the different Kunitz domains of TFPI in the inhibition of FXa and TF-FVIIa is rather complex and not limited to inhibition of FVIIa by Kunitz 1 and FXa by Kunitz 2. This thesis shows that all Kunitz domains contribute to the inhibition of FXa (chapter 3) and FVIIa (chapter 4 and 5) and that several plasma components that bind to TFPI and/or FXa modulate the anticoagulant activity of TFPI (chapter 6). We showed that in the

two-step mechanism of inhibition of FXa by TFPI, the Kunitz3 domain promotes the formation of the initial encounter complex and is required for the expression of protein S cofactor function and that the Kunitz 1 domain is necessary for isomerisation of the loose encounter into the tight FXa-TFPI complex. Our observations provide important information for developing TFPI antagonists that efficiently can block TFPI. For instance the Kunitz 1 requirement for isomerisation step explains why a peptide that is directed against the Kunitz 1 domain of TFPI (35) is a partial TFPI inhibitor which allows rapid formation of the encounter complex and only prevents the isomerisation to the tight complex. Considering the two-step mechanism of FXa inhibition by TFPI one would predict that TFPI antagonists that knock out the function of both the Kunitz 1 and Kunitz 2 domains would be more effective TFPI inhibitors. Indeed, Dockal et al[1] recently reported at the 56th ASH meeting in San Francisco, CA, a synergistic effect of a fusion peptide which consisted of a linear peptide that binds to the N-terminus and Kunitz 1 domain of TFPI and that was linked to a cyclic peptide that binds to a construct composed of the Kunitz 1 and Kunitz 2 domains of TFPI. This fusion peptide effectively neutralised the anticoagulant activity of TFPI even at concentrations that were some 40-fold higher than the TFPI level in normal plasma. These observations support the notion that detailed knowledge of the mechanism of

[1]Dockal M., et al. Molecular Characterization of the Synergistic Effect on TFPI Inhibition By Fusion of Two Inhibitory Peptides. Abstract 1484, 56th ASH Annual meeting, San Francisco CA, 2015

action of TFPI is important for the design of tailor-made TFPI antagonists for the treatment of bleeding disorders.

Below table shows a comparison between all the above discussed TFPI antagonists.

Table 1: Comparison of different TFPI antagonists.

	Peptide[a]	NASP[31]	Aptamer[33]	mAb[34]
TFPI domain interaction	N terminus, Kunitz 1 and Kunitz 2	Probably binds to Kunitz 3 and C-terminus	Kunitz 1, Kunitz3 and C-terminus	Kunitz 2
Inhibition ofFXa and/or TF-FVIIa	Both	Both	Both	Both
Target of inhibition (Various TFPI forms)	Inhibits all forms of TFPI	Doesn't inhibits K3 and C-terminal truncated form	Doesn't inhibit K1-K2 form and TFPIβ, less efficiently C-terminal truncated form	Inhibits all forms of TFPI
Cross-reactivity	No cross-reaction	Interacts with selectins expressed on hematopoetic and vascular cells	No cross-reaction	No cross-reaction
Inhibitory activity in presence of protein S	Not affected	Not affected	Reduced	Not affected
Affect in activity at high dosage	No adverse effect	At high dosage shows anticoagulant proterties	No adeverse effect	No adverse effect

Mode of administration	Subcutaneous	Oral and subcutaneous	Subcutaneous	Oral and subcutaneous
Risk of antibody development	None	None	None	None
Requirmentof rFVIII for optimal activity	Not required	Required	Not required	Required
Effectivity (in vitro/in vivo)	Both in vitro and in vivo	Less efficient in vivo	Less efficient in vivo	Both in vitro and in vivo
Bioavailability	Can be synthesized	Obtained from seaweed	Can be synthesized	No proper estimate

[a] Dockal M., et al. Molecular Characterization of the Synergistic Effect on TFPI Inhibition By Fusion of Two Inhibitory Peptides. Abstract 1484, 56th ASH Annual meeting, San Francisco CA, 2015

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