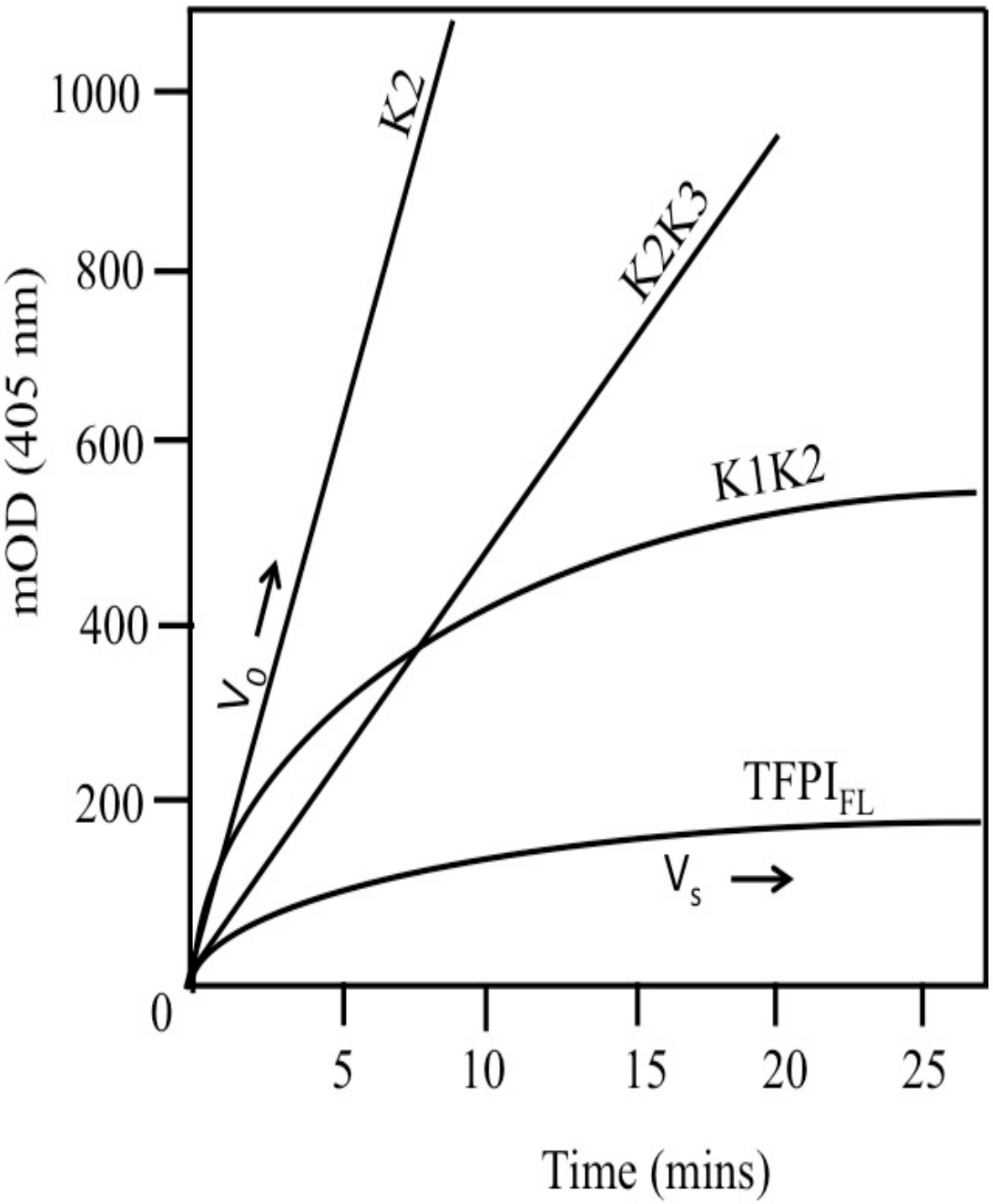

Studies on Components of Blood & Their Functions

Chapter 4

TFPI (Tissue Factor Pathway Inhibitor)

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1. Coagulation Pathway

Blood coagulation is initiated as a response to vascular damage. Vascular injury exposes the integral membrane protein tissue factor (TF) [1,2] which binds the circulating coagulation factor VIIa (FVIIa) [3] and forms the TF-FVIIa complex. This complex activates FX [4,5] to FXa, which subsequently assembles with its cofactor factor Va (FVa) in presence of calcium ions and an appropriate phospholipid surface into the so-called prothrombinase complex. The prothrombinase complex converts the zymogen prothrombin into its active form thrombin. Thrombin is a serine protease with several functions in hemostasis, which amongst others catalyzes the conversion of fibrinogen to fibrin and induces the aggregation of blood platelets resulting in the formation of stable thrombus consisting of aggregated platelets consolidated by a network of fibrin fibers.

Blood coagulation is carefully regulated by the positive and negative feedback loops that balance the clot formation (Fig. 1). The initial thrombin molecules that are formed by extrinsic coagulation pathway activate the coagulation factors FV, FVIII and FXI leading to propagation of coagulation cascade [6-8]. FVIIIa is a cofactor of activated FIX (FIXa) a serine protease that is formed by activation of FIX *via* either FXIa [9,10] or TF-FVIIa [5,11,12].

The hemostasis is regulated by the anticoagulant pathways, by ensuring to limit the thrombin formation and hemostatic plug at the site of vessel damage. The major anticoagulant proteins identified in plasma are tissue factor pathway inhibitor (TFPI), protein C, protein S, antithrombin (AT), α_2-macroglobulin, and the protein Z-dependent protease inhibitor. The complete absence of the major anticoagulant systems (TFPI, protein C/protein S or AT) leads to extensive fibrin deposition as well as bleeding due to consumptive coagulopathy and is not compatible with life [13-15]. Out of all, one of the most important anticoagulant proteins is

TFPI [16], a protease inhibitor that regulates at the initiation of blood coagulation by binding and inhibiting FXa [17] and TF-FVIIa [18] thereby shutting down the extrinsic coagulation pathway.

2. Tissue Factor Pathway Inhibitor

The TFPI gene is localized on chromosome 2 in region 2q32 [19,20]. The translated product of the *tfpi* gene (304 a.a. long) is subjected to removal of signal sequence (28 a.a) to give a mature 276 amino acid long TFPI protein called TFPIα [16,21]. Alternative splicing of TFPI mRNA results in TFPIβ, an isoform of TFPI [22,23].

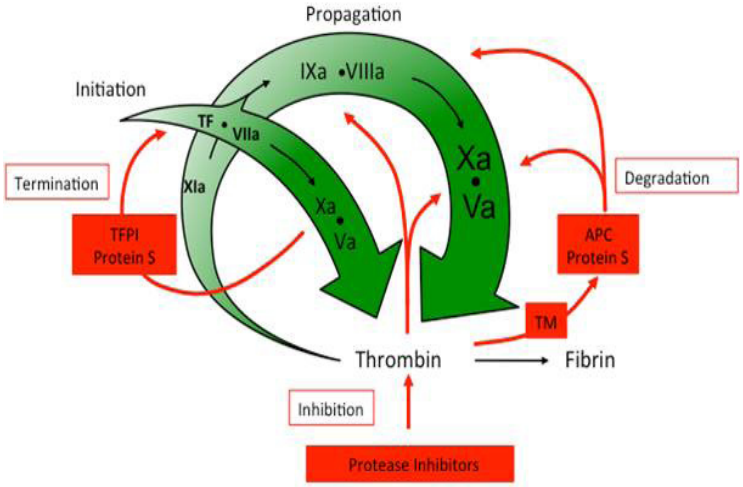

Figure 1: Blood coagulation cascade: Initiation, propagation, termination and degradation.

TFPIα is an endogenous Kunitz type serine protease inhibitor with an acidic amino terminus followed by three tandem Kunitz domains (K1-Asp13-Arg78, K2-Glu92-Gly150, K3-Glu182-Lys241), each containing 3 disulphide bonds that pair in the pattern 1 to 6, 2 to 4, 3 to 5 and a basic carboxy terminus [16]. The molecular mass of TFPIα is 32 kDa but post-translational modifications result in an increase of mass of 43 kDa [24]. The post-translational modifications of TFPIα do not seem to be important for the inhibition of FXa or the TF-FVIIa complex, but they may influence its plasma clearance and cell binding properties [25,26]. TFPIβ lacks KD3 and has a different C-terminus. A GPI anchor is attached to this C-terminus that covalently associates TFPIβ to the endothelium [27].

TFPI expression and distribution: TFPIα, which is subject of this chapter and which we will call TFPI from here on, is produced constitutively by microvascular endothelial cells [28], vascular smooth muscle cells [29], expressed by liver and monocytes/macrophages [30]. It is distributed in the endothelium (50–80%), plasma (10–50%) and platelets (less than 2.5%) [31,32]. Normal human plasma contains full length and variably truncated carboxy-terminal forms of TFPI [33]. *In vivo*, approximately 80% of plasma TFPI circulates in complex with plasma lipoproteins [18,34]. Unlike plasma TFPI, platelet TFPI is exclusively full length. It is known that local TFPI concentration increases at the site of vascular injury. This TFPI released by platelets accumulated within the thrombus that makes platelet TFPI a potent anticoagulant and prevents unnecessary thrombus formation [35]. TFPI has a rather short half-life of 60–120

min [36,37]. The total TFPI concentration in normal human plasma, which includes the variably truncated form of TFPI, is about 1.0–2.5 nM [34,38] and the full length TFPI concentration in plasma is 0.25-0.5 nM [31]. Castoldi and coworkers reported that at least part of the full length TFPI present in plasma circulates in complex with protein S [39] and FV [40].

TFPI anticoagulant function: The anticoagulant function of TFPI involves binding to and inhibition of FXa which can directly contribute to the down-regulation of coagulation [41]. TFPI can also bind and inhibit TF-FVIIa, a reaction that is greatly stimulated by FXa and that results in the rapid formation of a tight quaternary TFPI-FXa-TF-FVIIa complex [17,42]. Detailed kinetic studies indicated that inhibition of TF-FVIIa actually occurs after binding of TFPI to the ternary TF-FVIIa-FXa complex that is generated during FX activation [43]. By targeting TF-FVIIa and FXa, TFPI directly and efficiently inhibits the initiation of coagulation. It is well established that the K2 domain binds to FXa, K1 binds to FVIIa [18] and that the K3 domain is essential for binding to its cofactor protein S which in the presence of phospholipids and Ca^{2+}-ions enhances FXa inhibition by TFPI reported by Hackeng *et al* [44]. The binding of TFPI to FV [40], which results from an interaction between the C-terminus of TFPI [45] and an acidic region in the C-terminus of the B-domain of FV [46-48] may also contribute to the anticoagulant activity of TFPI in plasma because Wood *et al*. [48] reported that binding of TFPI to FVa that was activated by FXa is associated with inhibition of prothrombin activation.

Biochemistry of inhibition: TFPI inhibits FXa *via* a two-step mechanism known as slow-tight inhibition. Slow-tight inhibitors are characterized by the rapid formation of an initial encounter complex (FXa•TFPI) followed by a slow conversion into a tight FXa•TFPI*complex (Fig. 2) [42,49,50]. Both initial encounter complex and tight complex formation are dependent on the concentration of TFPI and when the TFPI concentration increases both the rate and extent of FXa inhibition increases. The shape of progress curves of inhibition obtained with the slow-tight binding inhibitors is biphasic [42,50].

Inhibition of FXa by TFPI is modulated by several components that bind to either FXa or TFPI or both. Table 1 summarizes the effects of major modulators on the K_i and K_i^* of FXa inhibition by TFPI.

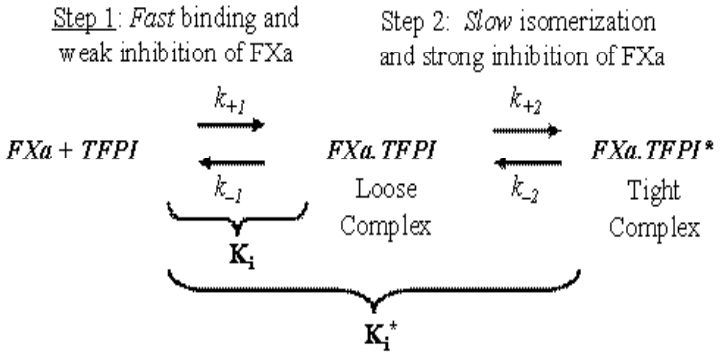

Figure 2: Slow-tight binding mechanism for inhibition of FXa by TFPI.

Table 1: Effects of Ca^{2+}, phospholipids and protein S on K_i and K_i^* of FXa inhibition by TFPI (Kinetic constants for FXa inhibition by TFPI).

Addition	K_i (nM)	K_i^* (nM)
None	1.24[a]	0.026[a]
Ca^{2+}	42.7[a]	0.085[a]
Ca^{2+} + PL	4.4[b], 14.6[a]	0.05[b], 0.018[a]
Ca^{2+} + PL + protein S	0.5[b]	0.02[b]

[a](49); [b](44)

3. Role of Different Kunitz Domains of TFPI in Inhibiting FXa

As described above K2 binds to FXa, K1 binds to FVIIa and K3 had no function apart from binding to its cofactor. However, Peraramelli *et al.*[51] investigated the role of the three Kunitz domains (K1, K2 and K3) and the C-terminal domain of TFPI in FXa inhibition and showed that $TFPI_{FL}$ (TFPI full length), $TFPI_{1–161}$ (truncated at 161 a.a) and K1K2 had biphasic curves of FXa inhibition indicative for the two-step slow-tight inhibition mechanism, whereas K2 and K2K3 of TFPI rapidly but less effectively inhibited FXa in a monophasic manner (Fig. 3). Since $TFPI_{FL}$, $TFPI_{1–161}$ and K1K2 contain the K1 domain and K2 and K2K3 both lack K1, this indicates that the K1 domain of TFPI not only functions in FVIIa inhibition [52], but also supports the slow transition from the weak encounter to the tight FXa•TFPI* complex. However, $TFPI_{1–161}$ and K1K2 inhibited FXa with a considerably higher K_i value (> 40 nM), than $TFPI_{FL}$ (4.2 nM) emphasizing the role of the K3 + C terminus domain in the formation of initial encounter complex. Interestingly, K2K3 was a much better FXa inhibitor (K_i = 3.6 nM) than K2 alone (K_i = 21 nM), which suggests that the K3 domain promotes the formation of the initial encounter complex. Therefore, all the kunitz domains are essential for efficient inhibition of FXa.

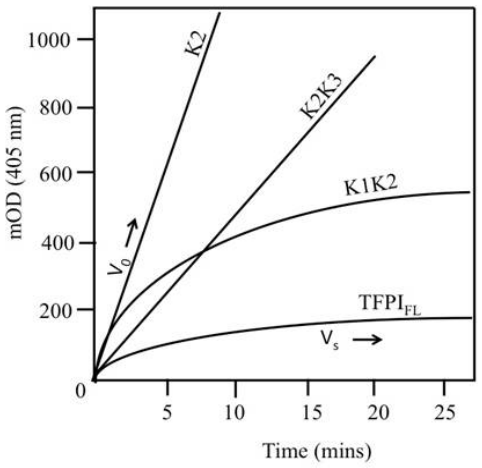

Figure 3: Pictorial representation of FXa inhibition by different kunitz domains of TFPI. V_0 indicates the initial encounter complex formation and Vs indicates the steady state tight complex formation.

4. TFPI as Target in Hemophilia Treatment

TFPI is not only associated with an increased risk of VT, but also plays role in bleeding. Considering its important role in maintaining the hemostatic 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.

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 [53] or Kunitz domain 2 [54]. As discussed above 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. The Kunitz 3 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 isomerization of the loose encounter into the tight FXa-TFPI complex. These observations provide important information for developing TFPI antagonists that efficiently can block TFPI. For instance the Kunitz 1 requirement for isomerization step explains why a peptide that is directed against the Kunitz 1 domain of TFPI [53] is a partial TFPI inhibitor, which allows rapid formation of the encounter complex and only prevents the isomerization 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. These observations support the notion that detailed knowledge of the mechanism of action of TFPI is important for the design of tailor-made TFPI antagonists for the treatment of bleeding disorders.

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