Small Peptides Blocking Inhibition of Factor Xa and Tissue Factor-Factor VIIa by Tissue Factor Pathway Inhibitor (TFPI)*

Michael Dockal†, Rudolf Hartmann†, Markus Fries†, M. Christella L. G. D. Thomassen‡, Alexandra Heinzzmann‡, Hartmut Ehrlich‡, Jan Rosing‡, Frank Osterkamp‡, Thomas Polakowski‡, Ulrich Reineke‡, Andreas Griessner†, Hans Brandstetter†, and Friedrich Scheiffinger†

From †Baxter Innovations GmbH, Uferstrasse 15, A-2304 Orth/Donau, Austria, the ‡Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Universiteitssingel 50, 6229ER Maastricht, The Netherlands, §3B Pharmaceuticals GmbH, Magnusstrasse 11, 12489 Berlin, Germany, and the †Department of Molecular Biology, University of Salzburg, Billrothstrasse 11, A-5020 Salzburg, Austria

Background: Tissue factor pathway inhibitor (TFPI) inhibits coagulation factors Xa and VIIa.

**Results:** A de novo synthesized 20-mer peptide that binds to TFPI was structurally and functionally characterized.

**Conclusion:** The peptide binds to the Kunitz domain 1 of TFPI and blocks inhibition of factor Xa and factor VIIa by TFPI.

**Significance:** The peptide can potentially prevent bleeding in hemophilia patients.

Tissue factor pathway inhibitor (TFPI) is a Kunitz-type protease inhibitor that inhibits activated factor X (FXa) via a slow-tight binding mechanism and tissue factor-activated FVIIa (TF-FVIIa) via formation of a quaternary FXa-TFPI-TF-FVIIa complex. Inhibition of TFPI enhances coagulation in hemophilia models. Using a library approach, we selected and subsequently optimized peptides that bind to TFPI and block its anticoagulant activity. One peptide (termed compound 3), bound with high affinity to the Kunitz-1 (K1) domain of TFPI (Kd ~ 1 nM). We solved the crystal structure of this peptide in complex with the K1 of TFPI at 2.55-Å resolution. The structure of compound 3 can be segmented into a N-terminal anchor; an Ω-shaped loop; an intermediate segment; a tight glycine-loop; and a C-terminal α-helix that is anchored to K1 at its reactive center loop and two-stranded β-sheet. The contact surface has an overall hydrophobic character with some charged hot spots. In a model system, compound 3 blocked FXa inhibition by TFPI (EC50 = 11 nM) and inhibition of TF-FVIIa-catalyzed FX activation by TFPI (EC50 = 2 nM). The peptide prevented transition from the loose to the tight FXa-TFPI complex, but did not affect formation of the loose FXa-TFPI complex. The K1 domain of TFPI binds and inhibits FVIIa and the K2 domain similarly inhibits FXa. Because compound 3 binds to K1, our data show that K1 is not only important for FVIIa inhibition but also for FXa inhibition, i.e. for the transition of the loose to the tight FXa-TFPI complex. This mode of action translates into normalization of coagulation of hemophilia plasmas. Compound 3 thus bears potential to prevent bleeding in hemophilia patients.

**Results:**
- Tissue factor pathway inhibitor (TFPI) is a Kunitz-type protease inhibitor that down-regulates the extrinsic coagulation pathway via inhibition of activated factor X (FXa) and FVIIa.
- TFPI is a rather potent FXa inhibitor that inhibits FXa via a so-called slow-tight binding mechanism, i.e. rapid formation of a loose FXa-TFPI complex that subsequently slowly isomerizes to a tight FXa-TFPI* complex (1, 2). TFPI is a rather poor inhibitor of FVIIa. Efficient down-regulation of FVIIa by TFPI requires the presence of tissue factor (TF) and FXa, which together rapidly and efficiently form a quaternary FXa-TFPI-TF-FVIIa complex in which the activity of FXa and FVIIa are inhibited (3, 4).

After cleavage of the 29-amino acid signal peptide, the mature TFPI consists of a negatively charged N terminus, 3 Kunitz type domains designated Kunitz 1, 2, and 3 (K1, K2, and K3), and a positively charged C-terminal sequence, cf. Uniprot code P10646 (5). We follow the convention to sequentially numbering the mature protein without the signal peptide. High resolution structural studies have been carried out on K2 and K3 domains (6, 7), but not on the K1 domain. K1 has been reported to bind and inhibit FVIIa and K2 binds and inhibits FXa (4). However, the non-inhibitory parts of TFPI (K1, K3, and C terminus) also function in the inhibition of FXa and possibly FVIIa (8–12). In addition, the K3 domain was shown to play a role in the interaction between TFPI and protein S (2, 13) and the C terminus of TFPI interacts with phospholipid membranes (14). In this respect it is important to mention that both phospholipids and protein S enhance the anticoagulant activity of TFPI (1, 2, 15).

TFPI is a major physiological inhibitor of coagulation. In otherwise healthy individuals, low TFPI levels are associated with an increased risk of venous thrombosis (16, 17). However, clinical studies as well as experiments in animal models indicate...
that patients with bleeding tendency (e.g. hemophilia and FVIII-deficient patients) may benefit from low TFPI levels (18) or inhibition of TFPI (19–23). Molecules that have been used to block the anticoagulant activity of TFPI include non-anticoagulant sulfated polysaccharides, e.g. fucoidan (21, 22, 24), aptamers (20, 25), and monoclonal antibodies (26).

In the present study, we determined the crystal structure of the TFPI K1 domain in complex with an antagonistic peptide, providing a rational framework to study the effect of peptides targeted at TFPI that effectively bind to the K1 domain of TFPI and that inhibit TFPI. These peptides are the result of an iterative optimization of an initial mRNA display hit by amino acid substitutions within the peptide sequence. One of these peptides (compound 3) binds to TFPI with an affinity in the low nanomolar range. Compound 3 neutralized the anticoagulant activity of TFPI in plasma and model assay systems, i.e. blocked both inhibition of FXa and the extrinsic Xase (TF-FVIIa) complex by TFPI. This peptide is used as a tool to clarify the role of K1 in its interaction with FXa and TF-FVIIa. The data presented in this article suggest that peptides like compound 3, which bind with high affinity to TFPI and reduce its anticoagulant activity, can be useful in treatment of hemophilia patients, particularly in those who developed inhibitory antibodies in response to replacement therapy.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The chromogenic substrate CS-11(65) (N-α-benzoyloxycarbonyl-n-arginyl-l-glycyl-l-arginine-p-nitroanilide-dihydrochloride) and the fluorogenic substrate Fluo-1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Delfzijl, The Netherlands). HEPES, Tris-HCl, bovine serum albumin (BSA), ovalbumin, and plasma from patients with hemophilia A and inhibitory antibodies (49 Bethesda units/ml) were obtained from George King (Overland Park, KS). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipids (Delfzijl, The Netherlands). Phospholipid vesicles (20% DOPS, 20% DOPE, and 60% DOPC) were prepared as described (28). CaCl₂, NaCl, and EDTA were obtained from Merck Chemicals (Darmstadt, Germany). HEPES, Tris-HCl, bovine serum albumin (BSA), ovalbumin, and reagents for SDS-PAGE were obtained from Sigma. Chromatographic media for protein purification were obtained from GE Healthcare. PPP-Reagent Low, FluCa-Kit, and thrombin calibrator were obtained from Thrombinscope BV (Maastricht, The Netherlands). Normal pooled plasma, plasma from patients with severe hemophilia A and B (factor levels <1%), and plasma from patients with hemophilia A and inhibitory antibodies (49 Bethesda units/ml) were obtained from George King (Overland Park, KS).

**Expression, Purification, and Characterization of TFPI Proteins**—The cell line SK-Hep-1 (ATCC number HTB-52), which produces TFPI endogenously was used as a source for human full-length TFPI. Cells were cultured in DMEM containing 10% fetal bovine serum (Life Technologies) and full-length TFPI was purified from conditioned media by a two-step affinity purification protocol. First, total TFPI was captured by a biotinylated anti-TFPI peptide coupled to streptavidin-Sepharose and eluted with 100 mM glycine (pH 2.3), 175 mM NaCl, 0.01% polysorbate 80, and 10 mM benzamidine (Sigma). The full-length TFPI was further purified on an antibody affinity matrix using a monoclonal anti-C terminus antibody (TFPI-27, Sanquin, Amsterdam, The Netherlands). Homogeneous human full-length protein was characterized by mass spectrometry for molecular weight determination and SDS-PAGE at reducing and non-reducing conditions followed by Coomassie staining, silver staining, or immunoblotting. The active concentration of full-length TFPI was determined by titrating a known amount of bovine FXa with TFPI (29).

The TFPI constructs TFPI-(1–150) and TFPI-(1–150)-Thr, containing a thrombin cleavage site between K1 and K2 were expressed as insoluble inclusion bodies in Escherichia coli BL21(DE3)pLysS (Merck, Darmstadt, Germany) using the expression vector pET19b (Merck). Solubilized (8 M urea, 20 mM DTT, 50 mM Tris-HCl, pH 8.0) TFPI-(1–150)-Thr was folded in 50 mM Tris-HCl (pH 10.0), 1.1 mM oxidized glutathione by rapid dialysis followed by dialysis against 20 mM Tris-HCl (pH 7.0). TFPI-(1–150)-Thr was purified using a two-step purification procedure, a Q-Sepharose column, and a streptavidin affinity column with an immobilized peptide specific toward TFPI. Purified TFPI-(1–150)-Thr was digested by incubation with thrombin resulting in the generation of TFPI-(1–83)-Thr and TFPI-(90–150)-Thr. TFPI-(1–83)-Thr was purified from the digestion mixture using benzamidine-Sepharose for removal of thrombin, followed by the peptide affinity column. Purified TFPI-(1–83)-Thr was used for complex formation with compound 3 and further crystallization.

**Peptide Synthesis**—Peptides were synthesized using standard Fmoc-Solid Phase Peptide Synthesis conditions. Starting with a Fmoc-Rink-Amide resin Fmoc deprotection was done by treatment with 20% piperidine in N,N-dimethylformamide. Coupling of the individual Fmoc-amino acids (5 equivalents) was carried out by activation with O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (GL Biochem, Shanghai, China)/N,N-diisopropylxylamine (Sigma) in N,N-dimethylformamide or N-methyl-2-pyrrolidone (Sigma). The coupling steps of aminoisobutyric acid and Leu following an aminoisobutyric acid residue were performed twice. After completion of the synthesis the peptides were deprotected and cleaved from the solid support by standard trifluoroacetic acid cleavage mixtures. After precipitation the crude peptides were purified by preparative reversed phase HPLC (>90% at 230 nm) (30).

**Crystallization and Structure Determination**—Successful co-crystallization of equimolar complexes of TFPI-(1–83)-Thr and compound 3 was obtained under 100 mM MES (pH 6.5), 20% PEG 4000, 600 mM NaCl. Crystals diffracted to better than 2.5-Å resolution, albeit with some non-merohedral twinning. Diffraction data were processed to 2.5-Å resolution with iMosflm and SCALA from the CCP4 program package, revealing a monoclinic crystal form with unit cell dimensions of a = 113.67 Å, b = 69.32 Å, c = 42.37 Å, α = 90.0°, β = 92.97°, γ = 90.0°, space group C2 (31, 32). Self-rotation calculations indicated an ~2-fold non-crystallographic symmetry. Consistent
Peptides Inhibiting TFPI

herewith, two molecules were localized in the asymmetric unit related by a 170° rotation. The Patterson search was carried out using the program PHASER and a structure ensemble of the available Kunitz domain 2 crystal structures as search model (33). The unit cell contains ~64% solvent. Non-crystallographic electron density averaging, model building, and model refinement was carried out with the programs Coot, Refmac, MAIN, and CNS (34–37). The current model is completely defined for both copies of the compound 3 peptide and the interaction with the protein with current r = 0.257, R_free = 0.298, deviation from ideal geometry root mean square (bond) = 0.008 Å, root mean square (angle) = 1.8°. Crystallographic data are deposited in the Protein Data Bank (code 4bqd).

Affinity Measurement by Biacore—Biacore experiments were performed on a BIACore T200 (GE Healthcare). Full-length human TFPI was immobilized on a CM5 chip (GE Healthcare) aiming at 500 response units using amine coupling chemistry. Peptide binding to TFPI was analyzed in a single cycle kinetic analysis in the concentration range from 0.6 to 9.6 nM peptide using HBS-P buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% P20) at a flow rate of 30 μl/min at 37 °C. Biacore T200 Evaluation Software 1.0 (GE Healthcare) was used to analyze the data.

Effect of Peptides on FXa Inhibition by TFPI—Varying concentrations of TFPI were incubated in HNBSA buffer (50 mM HEPES, pH 7.7, 175 mM NaCl, 5 mg/ml of BSA) containing 125 μM of the FXa-specific chromogenic substrate CS-11(65), 1 mM EDTA or 3 mM CaCl2 and if present, varying concentrations peptide, 40 nM protein S and/or 30 μM phospholipid vesicles (20:60:20, DOPS/DOPC/DOPE) for 10 min at 37 °C. hFx was added and the increase in absorbance at 405 nm was followed in an Ultra Microplate Reader (Bio-Tek, Burlington, VT) until a steady state rate of chromogenic substrate conversion was achieved (~60 min).

Effect of Peptides on the Inhibition of TF-FVIIa-catalyzed FX Activation—Varying concentrations of TFPI were preincubated in a microtiter plate at 37 °C in 90 μl of HNBSA buffer containing FVIIa, the fluorescent substrate Fluophen Xa, and peptide. After 7 min 10 μl of TF and phospholipid vesicles (20:60:20, DOPS/DOPC/DOPE) were added and after 10 min FX activation was started by adding 25 μl of FX. The final reaction mixture contained 2 pm TF, 500 pm FVIIa, 100 nM FX, 10 μM phospholipid vesicles, 0.36 nM TFPI, 400 μM Fluophen Xa, 3 mM CaCl2 and various concentrations of peptide in HNBSA buffer (pH 7.7) and incubated at 37 °C.

FXa generation was monitored by following Fluophen Xa using a Fluoroskan Ascent FL reader (Thermo Labsystems, Helsinki, Finland) equipped with a 390/460 nm filter set and thrombin generation curves were calculated with the Thrombinscope software (Thrombinscope version 3.0). Each well contained 80 μl of plasma in a total volume of 120 μl. To prevent contact activation, all samples were measured in the presence of 41.3 μg/ml of corn trypsin inhibitor (Hematologic Technologies, Essex Junction, VT). Thrombin generation was measured in normal plasma, in normal plasma supplemented with an amount of anti-human FVIII heat-treated goat plasma (Z994) that completely abolished intrinsically triggered thrombin generation (39) and in plasmas from a hemophilia A, a hemophilia B, and a hemophilia A inhibitor patient.

TFPI ELISA—Plasma TFPI was quantified by ELISA as described in Knappe et al. (39). Full-length TFPI was detected using a monoclonal mouse antibody raised against the C terminus of human TFPI (Sanquin) as a capture antibody. The full-length TFPI protein standard (Baxter BioScience, Austria), controls, and samples were incubated at room temperature for 2 h, followed by washing. Full-length TFPI was detected by incubating a primary (polyclonal rabbit anti-human TFPI; American Diagnostica Inc.) and a secondary antibody (antirabbit whole molecule IgG peroxidase conjugate; Sigma). The peroxidase substrate Sure Blue TMB (KPL Inc.) was applied for a colorimetric reaction. The reaction was stopped with 1 M HCl and absorption was measured at 450 nm on an ELx 405 reader (Bio-Tek Instruments Inc.).

The detection of total TFPI was equivalent to full-length TFPI with the following changes: the capture antibody was a mouse monoclonal anti-human raised against the KD-2 domain (Sanquin). Human TFPI (R&D Systems) was used as the standard. The concentrations of total and full-length TFPI were quantified based on the respective standard curve. Every sample was tested in duplicate.

RESULTS

Identification and Optimization of Peptides That Neutralize TFPI—Screening of a 27-mer mRNA display library (Cosmix, Braunschweig, Germany) resulted in the identification of several peptides that bind and inhibit TFPI. Truncations and substitutional analysis of one hit (compound 1) led to the active core sequence, a 20-mer peptide (compound 2, Ac-FQSQKN-VFVDGYFERLRAKL-NH2). Surface plasmon resonance experiments showed that compound 2 binds to immobilized TFPI with a K_d of 17.7 ± 5.9 nM (Fig. 1A, closed symbol) and acts as a TFPI antagonist that blocks FXa inhibition by TFPI in kinetic experiments in a model system (Fig. 1B). Affinity and stability improvement by iterative amino acid substitutions in compound 2 resulted in an optimized peptide (compound 3, Ac-FQSQpNVHVDGYFERL-Aib-AKL-NH2) that binds to immobilized TFPI with an affinity of 0.5 ± 0.2 nM (Fig. 1A, open symbol). The affinity optimization is mainly due to an improvement of the dissociation rate characterized by 47-fold reduction.
Structural Basis for TFPI-K1 Binding Specificity—For elucidation of the atomic details of peptide-TFPI interactions, the crystal structure of compound 3 complexed to recombinant TFPI (1–83) was solved (Fig. 2). The K1 structure is defined in the electron density from Glu15 through Asp79; whereas chemically present, the N-terminal sequence (amino acids 1–14) and K1–K2 linker sequence (amino acids 80–83) are conformationally disordered in each of the two crystallographically independent copies of the molecule. As with other Kunitz domains, only ~1/3 of the structure is engaged in secondary structure elements; these are two short α-helical elements at Ser-24–Ala-27 (α1) and Leu-69–Cys-76 (α2) and a two-stranded β sheet comprising Met-39–Asn-45 (β1) and Arg-49–Ile-55 (β2) (Fig. 2, B and C). These elements form the topological framework that is stabilized by the three canonical disulfide bonds involving Cys-26–Cys-76, Cys-35–Cys-59, and Cys-51–Cys-72. Additional stabilization derives from a remarkably hydrophobic cluster formation, contributed by Phe-42–Phe-43–Phe-44. This triple aromatic residue cluster is universally conserved in all Kunitz inhibitors.

Like in related Kunitz inhibitor domains, the reactive center loop (RCL) is exposed and well accessible to interact with its target protein. The P4 to P2′ sequence is Gly-33–Pro-34–Cys-35–Lys-36–Ala-37–Ile-38. Although Cys-Lys/Arg is a rather conserved P2–P1 motif, reflecting the topological restraints in Kunitz protease inhibitors, proline at position P3 induces an additional conformational constrain to the RCL, which would not be tolerated in the narrow active site of FXa. Proline at P3 and to a lesser extent Lys rather than Arg at P1 thus represent two major specificity determinants of K1 toward FVIIa over FXa (40). Although the surface charge distribution is overall balanced, there is some negatively charged cluster surrounding the RCL, generated by Asp-31–Asp-32, Glu-60 as well as several solvent-exposed carboxyl oxygen atoms (Fig. 2, A and C).

Compound 3 Is Segmented into Structural Modules—The structure of the 20-mer peptide can be segmented into (i) the N-terminal anchor consisting of acetylated Phe-1AP–Gln-2AP; (ii) an Ω-shaped loop comprising Ser-3AP–Asn-6AP; (iii) an intermediate segment built from Val-7AP and His-8AP; (iv) a tight glycine-loop containing Val-9AP–Gly-11AP; and (v) the C-terminal α-helix comprising Tyr-12AP–Leu-20AP (Fig. 2, A and C). The subscript AP indicates the sequence numbering in the antagonistic peptide. The conformation of the α-helix is stabilized by a non-natural α-methyl alanine positioned at the center of the helix (position 17AP); a C-terminal amide allowing to complete the 1–4 hydrogen bonding pattern of the α-helix; and a stacked cluster by the aromatic side chains of His-8AP, Tyr-12AP, and Phe-13AP. These effects cooperate to stabilize the C-terminal α-helix spontaneously in solution, consistent with circular dichroism data on the peptide (supplemental Fig. S1 and Table S1). The observed aromatic side chain stacking by His-8AP, Tyr-12AP, and Phe-13AP enforces a left-handed helical turn that can be only accomplished by glycine at position 11AP, cf. Fig. 2C. Consistent with this structural constraint, replacement of Gly-11AP by any other amino acid is reflected by dramatic losses in binding affinity toward K1, as such residues would compromise the main chain trace of the antagonistic peptide (supplemental Table S2). The conformation of the N-terminal loop segment is partly stabilized by a D-proline, known to induce a tight turn conformation, and a 1–4 hydrogen bond by the carbonyl oxygen of Ser-3AP with the amide nitrogen of Asn6AP. Importantly, all ring side chains (Tyr-1AP, Pro-5AP, His-8AP, Tyr-12AP, and Phe-13AP) point in the same direction, enabling them to interact with the K1 domain of TFPI (Fig. 2A).
Note the detailed interaction of the compound 3-K1 complex in stereo representation.

steric complementarity.

The ω-shaped loop (Ser-3AP–Asn-6AP) makes few nonspecific interactions with the connecting loop between β1 and β2; most notably, the pyrrolidine ring of D-Pro-5AP contacts with Phe-47, whereas the pyrrolidine ring of L-Pro at position 5AP would be oriented away from K1 toward the solvent. The polar interaction by the side chains of Asn-6AP and Lys-29 (α1) may further stabilize the conformation of both residues.

Val-7AP confers well shielded hydrophobic contacts with Asn-45 and Phe-43, forming the entrance to the binding pocket utilized by Gln-2AP. Whereas His-8AP hardly contributes direct contacts with TFPI, it exhibits important intra-molecular stacking interaction, mostly with Tyr-11AP and partly with Phe-13AP (Fig. 2, A and C). The subsequent glycine-loop makes rather negligible interactions with the protein; the strict requirement for glycine at position 11AP can be explained by the special conformation of the loop.

The C-terminal helix is anchored to TFPI at its RCL and the two-stranded β-sheet. Important interactions are mediated by Tyr-12AP via its hydroxyl Oγ with the amide nitrogen of Ile-55, positioned on β2. Arg-15AP forms a perfect bidentate-bidentate interaction with Asp-32, positioned close to the RCL (Fig. 2C). Similarly, Lys-19AP forms important contacts, both hydrophobic and polar in nature, reaching the carbonyl oxygen of Ala-37 (RCL-P1'). Another attractive interaction for Lys-19AP seems possible via a cation trap formed by the carboxyls of Asp-32 and Gly-33 (RCL-P5-P4); this interaction is mediated by solvent molecules located at the cation trap.

Effect of Compound 3 on the Kinetics of Inhibition of FXa by TFPI—TFPI inhibits FXa via a so-called slow-tight binding mechanism that involves rapid formation of a loose FXa-TFPI complex that subsequently slowly isomerizes to a tight FXa-TFPI* complex (1, 2) (Equation 1).

\[
\text{FXa} + \text{TFPI} \overset{k_1}{\underset{k_{\text{exp}}} \rightleftharpoons} \text{FXa} \cdot \text{TFPI} \overset{k_2}{\underset{k_3} \rightleftharpoons} \text{FXa} \cdot \text{TFPI}^* \\
(\text{Eq. 1})
\]

Hence, progress curves of FXa inhibition by TFPI followed by continuous measurement of the conversion of a FXa-specific chromogenic substrate are biphasic (1, 2) with a rapid loss of part of the FXa amidolytic activity as the result of fast formation of the loose FXa-TFPI complex followed by a gradual but more pronounced loss of amidolytic activity representing the slow transformation of the loose FXa-TFPI to the tight FXa-TFPI* complex.

Progress curves of chromogenic substrate conversion can be fitted to the integrated rate equation (1, 2, 41) for slow-tight binding inhibition (Equation 2),

\[
A_t = A_0 + (v_1 \cdot t) + (v_0 - v_1) \cdot \left(1 - \exp(-k_{\text{obs}} \cdot t)\right)/k_{\text{obs}} \\
(\text{Eq. 2})
\]

Characteristics of the Interaction between Compound 3 and K1—The contact surface of compound 3, derived from atoms with an intermolecular distance of less than 4.5 Å, shows an overall hydrophobic character with some distinct positively and negatively charged hot spots that correspond to Arg-15AP, Lys-29 positioned near α1 of TFPI. Although the side chain of Phe-13AP interacts nonspecifically with α1, Gln-2AP forms a deeply buried contact in a pocket formed by the exit turn of α1 and strand β1.

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(\text{Eq. 2})
\]
in which $A_t$ is absorbance at 405 nm at time $t$; $A_0$ is initial absorbance at 405 nm; $v_0$ is final steady-state velocity; $v_0$ is initial velocity; $k_{obs}$ is apparent rate constant for the transition from $v_0$ to $v_f$ (FXa-TFPI to FXa-TFPI*). $v_0$ and $v_f$ values relative to rates of chromogenic substrate conversion by FXa in the absence of TFPI represent the extent of loose and tight FXa-TFPI complex formation.

The effectiveness by which TFPI inhibits FXa increases in the presence of phospholipids (PL) and is further enhanced when protein S is included in the reaction mixture (2) (Fig. 3, A–C). At all conditions, compound 3 impaired the inhibition of FXa by TFPI, but the antagonistic activity of the peptide decreased by the addition of PL and PL + protein S. Visual inspection of the progress curves suggests that the biphasic character of FXa inhibition is gradually lost at increasing compound 3 concentrations and that it is particularly the second phase of FXa inhibition, i.e. the slow conversion of the loose to the tight FXa-TFPI complex that is affected by compound 3.

That this is indeed the case is shown in Fig. 4A, which depicts the effect of compound 3 on the inhibition of FXa by a much higher TFPI concentration. This experiment was performed at a high TFPI concentration to get a reliable measurement of $v_0$ and $v_f$ (loose and tight complex formation) and the effect of compound 3 thereon. Fig. 4B shows that compound 3 indeed affects $v_f$ and has no effect on $v_0$, indicating that the peptide does not interfere with the formation of the loose FXa-TFPI complex, but prevents the isomerization step and the formation of the tight FXa-TFPI* complex.

To quantify the effect of compound 3, $v_f$ values obtained after fitting the progress curves using Equation 2 were plotted as a function of the compound 3 concentration and fitted to a hyperbola, which yielded an $E_{max}$ (extent of TFPI inhibition at saturating compound 3 concentration) and the $EC_{50}$ (the compound 3 concentration required to obtain 50% of the $E_{max}$). For these parameters the mean values ± S.D. and the number of experiments (n) are given.

Effect of Compound 3 on the Dissociation of Preformed FXa-TFPI Complexes—The data presented thus far indicate that compound 3 effectively blocks inhibition of FXa by TFPI. Fig. 5 shows that compound 3 is also able to reverse FXa-TFPI complex formation and dissociate preformed tight FXa-TFPI* complexes. In this experiment, FXa was incubated with TFPI during 45 min, which is sufficiently long to establish the inhibition equilibrium (Equation 1) and allow for full tight FXa-TFPI* complex formation (97% inhibition of FXa). Addition of varying
concentrations of compound 3 to the FXa-TFPI mixture after 45 min resulted in a concentration-dependent dissociation of FXa-TFPI* complexes and a recovery of active non-inhibited FXa. At compound 3 concentrations >10 nM, i.e. higher than the TFPI concentration, ~60% of the FXa activity was recovered.

Effect of Compound 3 on the Inhibition of TF-FVIIa-catalyzed FX Activation by TFPI—TFPI not only inhibits FXa, but also is a potent inhibitor of the TF-FVIIa complex and rapidly inhibits TF-FVIIa-catalyzed FX activation via formation of a quaternary TFPI-FXa-TF-FVIIa complex (3, 4). Compound 3 also blocks inhibition of TF-FVIIa-catalyzed FX activation by TFPI (Fig. 6). In the absence of compound 3, 0.36 nM TFPI almost completely inhibited FX activation within 2 min. Compound 3 reduced inhibition of FX activation by TFPI in a concentration-dependent manner. Although compound 3 concentrations in 10-fold excess of TFPI did not fully block TFPI, FXa generation was restored to 50% of that observed without TFPI at ~2 nM compound 3.

Effect of Compound 3 on TF-triggered Thrombin Generation in Plasma—The ability of compound 3 to block the anticoagulant activity of TFPI in plasma was tested by measuring its effect on TF-triggered thrombin generation in normal plasma (Fig. 7A), in normal plasma in which FVIII was inhibited with a polyclonal FVIII antibody (Fig. 7B), plasma from a hemophilia A (Fig. 7C) or hemophilia B patient (Fig. 7D), and in plasma from a hemophilia patient with a FVIII inhibitor (Fig. 7E). Compound 3 enhanced thrombin generation (thrombin peak height) in all plasmas tested to the level of normal plasma control or higher (hemophilia B patient plasma, Fig. 7D). The concentration of compound 3 that caused 50% enhancement of thrombin generation (EC$_{50}$) was similar in the five plasmas tested (Table 2). However, the extent by which compound 3 increased thrombin generation (thrombin peak height) was more pronounced in plasma without functional FVIII or FIX than in normal plasma (Table 2).

DISCUSSION

TFPI is a multi-Kunitz domain protease inhibitor that down-regulates the extrinsic pathway of the blood coagulation by inhibiting FXa and FVIIa. TFPI consists of a negatively charged N terminus, 3 Kunitz domains designated K1, K2, and K3, and a positively charged C terminus (5). With respect to the structure-function relationship of TFPI it has been reported that K1 binds to and inhibits FVIIa and K2 binds and inhibits FXa (4). However, there are several reports that indicate the involvement of TFPI domains other than K2 in the inhibition of FXa (8–11, 13). The physiological importance of TFPI as an anticoagulant protein is underscored by the observations that low levels of TFPI are associated with an increased risk of venous thrombosis (16, 17) but may protect FV-deficient patients from severe bleeding (18) and that inhibition of TFPI with antibodies, aptamers, and sulfated polysaccharides shortens the clotting time of plasma and restores coagulation in animal hemophilia models (19–23, 25, 26).

The objective of the current investigation was to develop peptide inhibitors of TFPI that enhance coagulation in hemophilia models and that might be used in the treatment of hemophilia patients. Using a library approach, we identified several peptides de novo that bind to TFPI and block FXa inhibition by TFPI. These peptides were analyzed and subsequently optimized by serial truncations and amino acid substitutions. This resulted in several peptides with an improved affinity for TFPI, one of which (compound 3) was further characterized in a structure-function study. Surface plasmon resonance (Biacore) experiments showed that compound 3 binds with high affinity to TFPI ($K_D$ 0.5 nM). The peptide also blocks inhibition of FXa by TFPI with an EC$_{50}$ of ~5 nM and TF-FVIIa-
catalyzed FX activation with an EC$_{50}$ of $\sim$2 nM. The fact that $K_D$ and EC$_{50}$ values are of the same order of magnitude shows that binding of compound 3 to TFPI is paralleled by blocking the ability of TFPI to inhibit FXa and FVIIa. Compound 3 not only blocked the inhibition of FXa and FVIIa by TFPI in model systems, but also exhibited TFPI antagonist activity in plasma (Fig. 7) and increased the thrombin peak height of low TF-triggered thrombin generation in normal plasma $\sim$3-fold with an EC$_{50}$ $\sim$16 nM (Table 2). Similar amounts of compound 3 were required to stimulate thrombin generation in plasmas without functional FVIII or FIX. However, compared with normal plasma, the extent of stimulation was substantially more pronounced (10–30-fold) in plasma from hemophilia patients and in plasma containing FVIII-inhibiting antibodies ($\sim$10-fold). The more pronounced increase of thrombin generation in plasmas without functional FVIII or FIX is likely explained by the fact that in these plasmas thrombin generation is fully dependent on TF-FVIIa, whereas in plasma with FVIII/FIX (normal plasma) there is a contribution to thrombin generation independent of TF-FVIIa, i.e. via FIXa-FVIIa, a pathway that is less sensitive to TFPI than the TF-FVIIa pathway. The large increase of thrombin generation in FVIII-deficient plasma may be caused by its high TFPI content of this particular plasma that resulted in very low thrombin generation in the absence of compound 3 (Fig. 7D) and upon inhibition with compound 3 will result in a high enhancement factor.

The crystal structure of K1 in complex with compound 3 was solved at 2.55-Å resolution. The complex structure highlights the relevance of both enthalpic and entropic contributions to the free energy of the protein-ligand interaction. Electrostatic interactions often determine the specificity of intermolecular recognition, as is also observed in the K1-compound 3 complex, e.g. Asp-32 interacting with Arg-15AP and Lys-19AP or Asp-10AP binding to Lys-29. However, the major gain in binding affinity was obtained by optimizing the entropy of binding. Following a dual strategy, we first minimized the internal degrees of freedom of TFPI antagonist compound 2 by stabilizing its structural modules. Examples include the introduction of an $\alpha$-methyl alanine at position 17AP acting as a helix-stabilizing element or D-proline at position 5AP acting as a turn-stabilizing element. These measures minimize the loss of entropy upon complex formation. Additionally, we aimed to gain entropy by

![Image](https://via.placeholder.com/150)

FIGURE 7. Effect of compound 3 on thrombin generation in different plasmas. Thrombin generation was determined with Calibrated Automated Thrombography as described under "Experimental Procedures" in: A, normal pooled plasma; B, normal plasma in which FVIII was inhibited with a polyclonal FVIII antibody (Z994); C, plasma from a hemophilia A, or D, hemophilia B patient, and E, plasma from a hemophilia A patient with a FVIII inhibitor at various compound 3 concentrations (1111, 370, 123, 41, 13.7, 4.6, 1.5, and 0.5 nM, dark to light gray). Thrombin generation curves of normal pooled plasma (upper dashed black) and hemophilia plasma (lower dashed black) are indicated in B–E.

| Plasma (donor-lot) | Mean EC$_{50}$ S.D. | Fold-increase of thrombin peak | TFPI–levels BU/ml |
|-------------------|---------------------|-------------------------------|------------------|
| Normal pooled plasma (2352) − anti-FVIII antibody | 16.3 2.2 | 2.8 x | Full length: 0.41 total: 1.29 |
| Normal pooled plasma (2352) + anti-FVIII antibody | 20.0 1.2 | 9.8 x | Full length: 0.41 total: 1.29 |
| FVIII-deficient plasma (GK995–2613) | 25.2 1.3 | 32.8 x | Full length: 0.58 total: 1.99 |
| FIX-deficient plasma (GK939–2702) | 16.8 1.9 | 11.4 x | Full length: 0.38 total: 1.67 |
| Patient plasma with FVIII inhibitor (GK1838–2624) | 20.4 0.7 | 8.9 x | Full length: 0.35 total: 1.55 |

BU, Bethesda unit.
Peptides Inhibiting TFPI

displacing surface-bound water molecules from the binding partners. The release of solvent molecules was optimized by designing highly complementary surface patches on the ligand with respect to the target protein, i.e. TFPI-K1.

TFPI is known to bind to FXa in a bivalent manner, where K2 as well as K1 including the acidic N terminus contribute to binding (8, 9). Although there exists a large body of evidence that indicates K2 binds to the active site via its RCL in a substrate-like manner, the binding mode of K1 and the acidic N-terminal segment to FXa are currently unknown. To investigate this question, we hypothesized that K1 will exploit one of the three major exosites that have been described for FXa and other related serine proteases. These exosites are located near the Ca\(^{2+}\) site (exosite I, located east to the active site); at the heparin binding site near the C-terminal helix (exosite II, located north to the active site); and at the cofactor Va binding site near the 170-helix (exosite III, located to the west of the active site). Among these possibilities exosite I can explain the observed calcium dependence of TFPI binding to FXa most easily (42).

The K1 surface patch that binds to the antagonistic peptide compound 3 largely overlaps with the putative FVIIa contact area, as deduced from docking K1 to the active site of FVIIa. The structure of the complex between compound 3 and K1 explains the antagonistic effect of compound 3 to the K1-FVIIa interactions. By contrast, the antagonist effect of compound 3 toward TFPI-FXa binding may be more complicated. Possibly, this effect involves conformational rearrangements that are induced upon compound 3 and FXa binding toward TFPI. Such reordering may be incompatible with and may lead to the interruption of the FXa-TFPI binding. This conclusion is consistent with the slow-tight binding kinetics of the FXa-TFPI complex.

It is interesting to note that inhibition of TFPI by compound 3 was not complete and that compound 3 only partially inhibited TFPI. The extent of inhibition of TFPI by compound 3 was most pronounced when FXa inhibition was investigated in the presence of Ca\(^{2+}\) ions only and gradually became less when phospholipids and protein S were included in the reaction mixture (Fig. 3, Table 1). This decrease of the TFPI antagonistic activity of compound 3 is likely explained by the fact that the effectivity of TFPI to inhibit FXa gradually increases in the order Ca\(^{2+}\), Ca\(^{2+}\)/phospholipid, and Ca\(^{2+}\)/phospholipid/protein S (1, 2). Furthermore, we observed that FXa inhibition by TFPI in the absence of compound 3 was biphasic and became monophasic at high concentrations of compound 3. The biphasic inhibition pattern in the absence of compound 3 is characteristic for a so-called slow-tight binding mechanism of inhibition (cf. Figs. 1, 3, and 4), which involves rapid formation of a low affinity TFPI-FXa complex followed by a slow transition to a tight FXa-TFPI\(^{\ast}\) complex (1, 2, 41). Our kinetic data indicate that binding of compound 3 to TFPI only affects the second slow phase of FXa inhibition, i.e. prevents the transition of the weak to the tight FXa-TFPI\(^{\ast}\) complex, but does not interfere with the rapid formation of the weak encounter complex (Figs. 4 and 5). This also explains why the antagonistic effect of compound 3 is less when FXa is inhibited by TFPI in the presence of phospholipids and protein S, which both have been reported to primarily decrease the \(K_i\) of TFPI and promote the formation of the encounter complex (1, 2). Because encounter complex formation is not affected by binding of compound 3 to TFPI, the extent of inhibition of TFPI by compound 3 is less in the presence of phospholipids and protein S.

The fact that compound 3 binds to the K1 domain of TFPI and acts as a TFPI antagonist indicates that the acidic N terminus and/or K1 domain of TFPI contribute to the inhibition of FXa by TFPI. Although the K2 domain of TFPI is considered to be responsible for the inhibition of FXa (4), there are several reports that indicate that other domains of TFPI also contribute to factor Xa inhibition because inhibition of FXa by full-length TFPI is \(\sim 1000\)-fold more effective than inhibition of FXa by the isolated K2 domain (9). Apparently, the K1 domain of TFPI significantly contributes to the differences in FXa inhibition by the K2 domain and TFPI by promoting transition of the weak encounter to the tight FXa-TFPI complex, which is in line with recent observations of Peraramelli et al. (12). Because the \(K_i\) of TFPI for the weak encounter complex formation is some 100-fold higher than the \(K_i\) for tight complex formation (1, 2), the N terminus and/or K1 domain of TFPI have an important contribution to the inhibition of FXa.

In summary, our data show that compound 3 is a potent TFPI antagonist that blocks inhibition of both FXa and the TF-FVIIa complex by TFPI and enhances thrombin generation in normal plasma and hemophilia plasma as well as in plasma from a hemophilia A patient with a circulating FVIII inhibitor. Because inhibition of TFPI with fucoidan, aptamers, and (monoclonal) antibodies shortens the clotting time of plasma and restores coagulation in animal hemophilia models (19–26), compound 3 can potentially be used to prevent bleeding in hemophilia patients. However, it should be emphasized that compound 3 differs from the above mentioned TFPI inhibitors in the sense that compound 3 selectively binds to the K1 domain of TFPI, whereas the aptamer BAX 499 binds to K1, K3, and the C-terminal domain (25) of TFPI and monoclonal antibody mAb2021 binds to the K2 domain (26). With respect to the binding site of fucoidan there are conflicting reports. Liu et al. (21) showed that fucoidan inhibits both full-length TFPI and a K1K2 construct that indicates that it binds to K1 and/or K2, whereas others (24, 43) reported that fucoidan requires the C terminus of TFPI to bind to and neutralize the anticoagulant activity of TFPI. It is not yet known which domain(s) of TFPI can be best targeted to obtain an optimal therapeutic effect of a TFPI inhibitor to provide a safe and efficient treatment of hemophilia patients.

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