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Early View

Review Article

A Protein from Nose-horned Viper Venom Holds Promise for the Development of an Innovative Drug for Venous Thromboembolism

Key words

snake venom;
haemostasis;
intrinsic tenase inhibitor; FIXa antagonist;
anticoagulant;
venous thrombosis

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Abstract: Investigating modes of action of natural toxins and venoms frequently inspire new therapies. Namely, many toxins precisely target specific receptors or pathways in organisms. For example, glucagon-like peptide-1-based drugs to treat diabetes and obesity, like Ozempic, were developed based on a peptide toxin from the venom of lizard *Gila monster*. In the venom of the nose-horned viper, we discovered and characterized the protein VaaSPH-1, which strongly inhibits blood coagulation via the intrinsic pathway. All current therapies for the prevention of venous thrombosis carry a high risk of severe bleeding as a side effect. Although numerous efforts have been made to improve treatment, no major breakthroughs have yet been achieved. The structure and mode of action of VaaSPH-1 are unique, making it a promising basis for the development of a safer anticoagulant drug for the treatment of venous thromboembolism that includes deep vein thrombosis and pulmonary embolism. Here, we describe our strategy for the development of innovative low molecular mass anticoagulant based on the structure of VaaSPH-1. The most promising candidate molecules are already in the process of patent protection.

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Blood coagulation process

Blood coagulation is an important physiological process that stops blood loss upon injury (1). The coagulation process can be divided into three pathways: (I) the extrinsic pathway, which is triggered by the extravascular tissue factor (TF) and the activated coagulation factor VII (FVIIa); (II) the intrinsic pathway, which involves only proteins present in the blood, such as FXIIa, FXIa, FIXa, and FVIIIa; and (III) the common coagulation pathway, which represents the identical final stages of both the extrinsic and intrinsic pathways and culminates in the formation of an insoluble fibrin network (Figure 1).

Coagulation is critical for survival and the prevention of blood loss following injury; however, when uncontrolled, it can pose severe threats, such as cardiovascular diseases (CVDs). Pulmonary thromboembolism is also associated with several underlying diseases in dogs and cats and antithrombotic therapy is often an important component of management (2, 3).

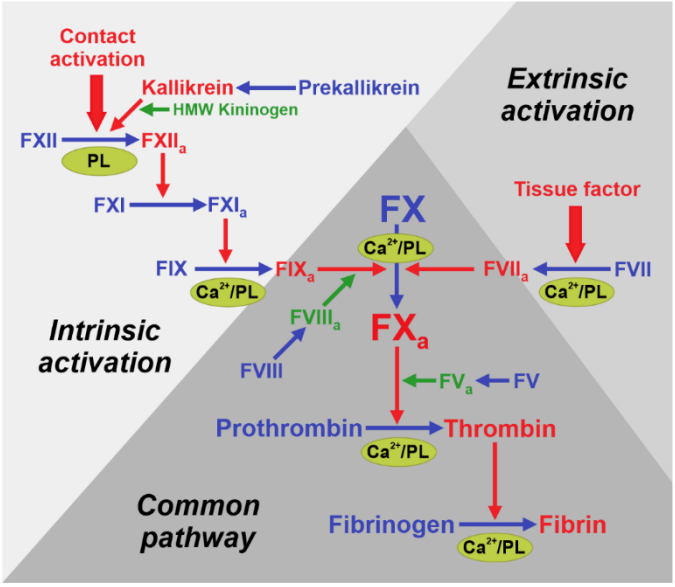

Figure 1: The blood coagulation cascade is divided into three pathways: extrinsic, intrinsic, and common. PL: phospholipids

Cardiovascular diseases

CVDs are the leading cause of death worldwide. According to the latest estimate by the World Health Organization (refer to the WHO webpage), CVDs accounted for 19.8 million deaths in 2022. This means that nearly one in three people globally die because of CVDs. European statistics is even more alarming: in Europe, CVDs cause more than 42.5% of all deaths, equating to roughly 10,000 deaths per day. In the European Union (EU), this proportion is slightly lower (37%) but still significantly above the global average (31%). The number of people living with CVD is much higher. As estimated by WHO, currently more than 120 million people in Europe and nearly 62 million people within the EU are suffering from some form of CVD. Despite notable advances in prevention, diagnosis, and therapy, CVDs continue to represent a major public health and socioeconomic challenge worldwide.

Venous thromboembolism

Our research specifically focuses on the pathological process known as venous thrombosis (VT) (4). VT is caused by increased activity of blood coagulation factors in the veins, resulting in excessive clot formation. The disruption of haemostasis, the balance of blood coagulation, may have genetic or acquired causes (5). VT ranks third among the leading causes of death related to CVDs, immediately after myocardial infarction and stroke. Up to three out of every thousand people die each year due to VT. This pathological process is the major cause of two serious CVDs, deep vein thrombosis (DVT) (6) and pulmonary embolism (PE) (7), which together are referred to as venous thromboembolism (VTE) (8). A blood clot, or thrombus, typically forms needlessly within the veins of the legs. The clot may obstruct blood flow in the vein, leading to deep vein thrombosis, or it may detach, travel through the bloodstream to the pulmonary arteries, block them, and cause a pulmonary embolism.

Current therapies for VTE are not optimal

Pathological blood clotting is treated with anticoagulants, which either directly reduce thrombin activity or inhibit its formation in the blood. Thrombin is the enzyme that converts soluble fibrinogen into an insoluble fibrin mesh-like network, the main structural component of a blood clot. For this purpose, various heparin formulations and vitamin K antagonists, such as warfarin, are used in current clinical practice. However, these drugs have numerous drawbacks, requiring continuous patient monitoring and precise dosage adjustment. For example, their effects are often unreliable due to potential interactions with food and other medications; they frequently require parenteral administration and, most importantly, carry a high risk of severe bleeding.

Currently available therapies for treating VTE act by inhibiting blood coagulation across multiple pathways (9, 10). Yet, the molecular causes of VTE lie in excessive blood clotting activity in veins due to dysregulation of the intrinsic pathway. Efficient blood coagulation via the extrinsic and common pathways is essential for survival, while the coagulation via the intrinsic pathway is not (11). Thus, existing VTE therapies often have a major adverse effect, difficult-to-control bleeding.

Searching for safer VTE therapy

Numerous efforts have been made to improve the treatment of VTE, but significant breakthroughs are still lacking. Oral anticoagulants with selective action on proteins in the coagulation cascade (NOACs: Non-vitamin K antagonist Oral AntiCoagulants) have been developed (12, 13). Dabigatran, apixaban, and rivaroxaban, three widely used NOACs, are more effective and predictable than traditional drugs. Nevertheless, the risk of bleeding remains, especially in cases of overdose, since effective antidotes are not available. There is, therefore, an obvious need for new anticoagulants for the treatment or prevention of VTE that would not pose a high risk of uncontrollable bleeding and whose effects could be safely reversed in the case of overdose.

In contrast to the inhibition of the extrinsic and common blood coagulation pathways, inhibition of the intrinsic blood coagulation pathway is not vital for survival. Therefore, the components of the intrinsic pathway represent therapeutic targets of choice in combating VTE. By specifically inhibiting coagulation through the intrinsic pathway, one could avoid the risk of uncontrolled bleeding during treatment, as the body would retain the ability to stop bleeding via the extrinsic and common pathways.

A key element of the intrinsic blood coagulation pathway is the intrinsic tenase complex (Figure 2). The complex consists of two blood proteins, two activated blood coagulation factors: FIXa, a serine protease (SP), and FVIIIa, a cofactor that enhances the specific enzymatic activity of FIXa (14, 15). The complex forms on the negatively charged surface of platelet membranes and proteolytically activates FX to FXa, ultimately leading to fibrin formation and blood clotting (Figure 1). Inhibition of the activity of FIXa and/or FVIIIa prevents the formation of the fibrin clot but does not block blood coagulation through the extrinsic or common pathways, thus avoiding the risk of life-threatening bleeding. Therefore, FIXa and FVIIIa are ideal therapeutic targets in the treatment of VTE.

Many compounds inhibiting FIXa have been evaluated for medical application with high expectations; however, their clinical development was discontinued at various stages for different reasons. The same fate befell all substances that targeted FVIIIa.

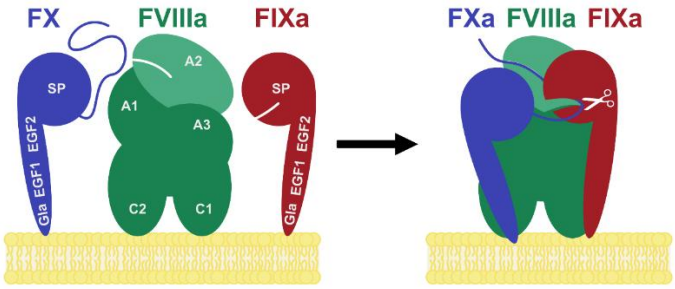

Figure 2: Schematic representation of the intrinsic tenase complex with its substrate – FX. The complex is formed by FIXa and its cofactor FVIIIa on the negatively charged surface of the platelet plasma membrane. FIXa catalyses the specific cleavage of FX, thereby activating it to FXa. A1, A2, A3, C1, and C2 are the protein domains in FVIIIa molecule while Gla (γ-carboxyglutamic acid-rich), EGF1 (epidermal growth factor-like domain 1), EGF2, and SP, are the domains in FIXa. The SP domain of FIXa is also termed the heavy chain of FIXa. The other three domains of FIXa—Gla, EGF1, and EGF2—together form the light chain. FIXa binds to the A2 domain of FVIIIa via its SP domain

Nose-horned viper venom prevents blood clotting

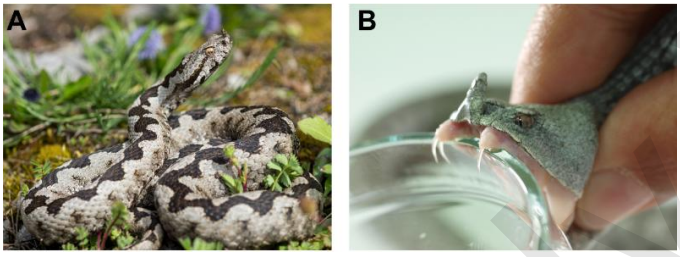

Figure 3: (A) The nose-horned viper (*Vipera ammodytes ammodytes*) is the most common viperid snake in the Balkans and the most dangerous of the European vipers. It is easily recognized by the characteristic horn-like scale on the tip of its snout. It is light brown, reddish-brown, or grey in colour. A zigzag pattern runs along its back. It can grow to a length of 65 to 90 cm. (B) Extraction or 'milking' of the venom, which is strongly anticoagulant

Animal venoms are a rich source of bioactive compounds and molecular tools for improving human and animal health. Some snake venoms have evolved over time into complex protein–peptide cocktails that strongly affect haemostasis, i.e., blood coagulation (16). Among these is the venom of the nose-horned viper (*Vipera ammodytes ammodytes*; Vaa) (17), the most venomous snake in Europe (Figure 3). A key feature of Vaa venom is its potent anticoagulant effect on human blood, making it a suitable starting point for our research.

Molecular basis of the anticoagulant activity of Vaa venom

We analysed the anticoagulant effect of the Vaa venom in detail at the molecular level and detected a protein without enzymatic activity that significantly prolonged the activated partial thromboplastin time (aPTT) in human plasma (18). This indicated that it preferentially inhibits the intrinsic pathway of blood coagulation. The protein was isolated from the venom in a homogeneous form.

Even at submicromolar concentrations, this protein rendered blood unable to clot in a clinically relevant sense, as thrombin formation in plasma dropped below the threshold required to trigger fibrin clot formation. Biochemical analysis demonstrated that the 35 kDa protein is glycosylated. Its amino acid sequence was determined and revealed that it structurally belongs to the SP family of proteins. The molecule, however, contains mutations on two out of three key amino acid residue positions forming the catalytic site in SPs, which explained its enzymatic inactivity. Therefore, the discovered anticoagulant protein is actually a serine pseudoprotease (19). This led to its naming as a homolog of SP from Vaa venom (VaaSPH-1).

VaaSPH-1 is unique, representing the first SP-structured protein that affects blood coagulation independently of enzymatic activity. The likelihood of directly using VaaSPH-1 as a drug is very low due to its large size and strong immunogenicity. However, it provides a natural model for developing smaller, structurally similar bioactive compounds more suitable for therapeutic applications.

Using molecular modelling and based on the crystal structure of a closely related SP (AHV_TL-I) from Siberian pit viper (*Gloydius halys*) venom, we built a three-dimensional (3D) model structure of the VaaSPH-1 (pink structure in Figure 4B). Simultaneously, we explored the molecular mechanism of VaaSPH-1's anticoagulant activity on human blood, demonstrating that it primarily results from VaaSPH-1 binding to the site on FVIIIa where FIXa normally binds (Figures 2 and 4). Competitive inhibition of FIXa binding to FVIIIa by VaaSPH-1 prevents the formation of the intrinsic tenase complex, thereby obstructing FXa formation and, consequently, fibrin clot formation.

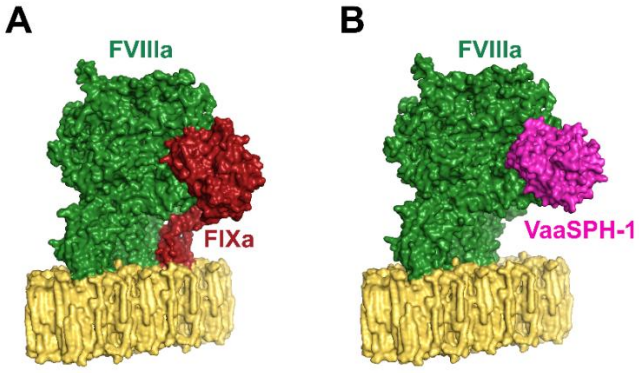

Figure 4: Docking of VaaSPH-1 into the intrinsic tenase complex. (A) Human intrinsic tenase consists of FVIIIa (green) and FIXa (red) on the negatively charged platelet plasma membrane (yellow). In the complex, all four FIXa domains (refer to Figure 2) make contacts with FVIIIa and the phospholipid membrane (yellow). (B) VaaSPH-1 is a homologue of the SP domain of FIXa. In the intrinsic tenase complex, the atomic coordinates of the FIXa SP domain were replaced with the atomic coordinates of VaaSPH-1 (pink). Then, molecular dynamics optimization and refinement was used to obtain thermodynamically most favourable structure

In a model of the intrinsic tenase complex on the phospholipid membrane (14), we replaced FIXa with either its heavy chain (only SP domain) or VaaSPH-1. Using computational protein docking, we identified the thermodynamically optimal binding state (Figure 4). In this state, we calculated the energies released during the formation of each complex: Van der Waals, electrostatic, and desolvation energies. Crucially, structure of the complex in which FIXa was replaced by VaaSPH-1 proved energetically as stable as the native intrinsic tenase complex (404 vs. 423 kcal/mol, respectively), supporting further studies.

The complex containing only the SP domain of FIXa (its heavy chain) and not the whole FIXa molecule was much less stable (225 kcal/mol). FIXa forms contacts with FVIIIa and the phospholipid membrane not only through its SP domain but also via its Gla and EGF domains (Figures 2 and 4). This means that the interaction between VaaSPH-1 and FVIIIa is stronger than the interaction between the SP domain of FIXa and FVIIIa, as analysed, particularly due to electrostatic energy. This result supports the development of innovative, therapeutically relevant inhibitors of intrinsic tenase that target FVIIIa, based on the structural features of the interaction surface of VaaSPH-1 with FVIIIa.

An additional incentive for continuing the project toward drug development was that an antidote to prevent potential overdose with an antagonist of FIXa binding to FVIIIa already exists. This is very important when evaluating the medical potential of a compound. In this case, either recombinant human FVIIIa or FIXa could serve as the antidote. Both preparations are approved for medical use in the treatment of haemophilia, A or B (20, 21).

The potential of VaaSPH-1 for developing a new generation of anticoagulants targeting the intrinsic blood coagulation pathway for the treatment of VTE is therefore significant. This molecule provides a highly promising structural model for designing a new type of low molecular mass selective inhibitors of the intrinsic tenase.

Development of innovative low molecular mass anticoagulants

As previously noted, VaaSPH-1 is unsuitable for direct therapeutic use due to its large molecular size and high immunogenicity. However, it represents a highly promising structural template for the design of smaller, low molecular mass FVIIIa-directed anticoagulants. The 3D model of the FVIIIa–VaaSPH-1 complex (18) is in the process of experimental validation by interaction site mapping using hydrogen–deuterium exchange mass spectrometry (HDX-MS) (22).

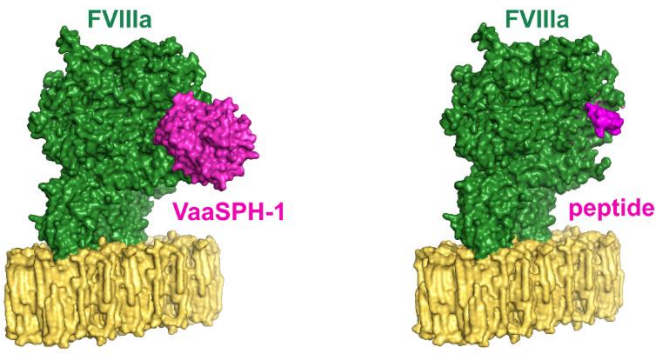

Figure 5: Short peptide is mimicking the interaction surface of VaaSPH-1 (pink) with the blood coagulation factor VIIIa (green). Binding of such peptide to FVIIIa would antagonize the binding of FIXa to the the same site, inhibiting the formation of the intrinsic tenase complex and consequently blood coagulation. Phospholipid membrane is yellow

Based on the increasingly refined picture of the interaction surface between VaaSPH-1 and FVIIIa, short peptides are being designed (Figure 5). Various *in silico* approaches—including homology modelling (MODELLER), protein–protein docking (HADDOCK), and molecular dynamics optimization (NAMD, VMD)—were employed to optimize their structures (18). The synthetic molecules are currently being evaluated *in vitro* for their ability to antagonize the binding of FIXa to FVIIIa, i.e., to inhibit formation of the intrinsic tenase complex, using surface plasmon resonance (SPR), activated partial thromboplastin time (aPTT) assay in human plasma, inhibition of FVIIIa–FIXa complex formation by native PAGE and intrinsic tenase interference assay.

Peptides demonstrating the strongest inhibition of FIXa–FVIIIa complex assembly are being tested for anticoagulant efficacy *in vivo* using a mouse carotid artery thrombosis model (23). The most promising peptide candidates for VTE treatment are currently undergoing patent protection.

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Conflict of interest: The authors declare that there is no conflict of interest.

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Protein iz strupa modrasa obeta razvoj inovativnega zdravila za zdravljenje venske trombembolije

I. Križaj, K. Požek

Izvleček: Preučevanje načinov delovanja naravnih toksinov in strupov pogosto navdahne razvoj novih načinov zdravljenja. Številni toksini namreč zelo natančno prizadenejo specifične receptorje ali signalne poti v organizmih. Na primer učinkovine homologne glukagonu podobnemu peptidu-1 za zdravljenje sladkorne bolezni in debelosti, kot je Ozempic, so bile razvite na osnovi peptidnega toksina iz strupa kuščarja gilske pošasti (*Gila monster*). V strupu modrasa smo odkrili in opisali protein VaaSPH-1, ki močno zavira strjevanje krvi po intrinzični poti. Vse trenutno dostopne terapije za preprečevanje venske tromboze so tvegane za pojav hudih krvavitev kot stranskega učinka. Kljub številnim prizadevanjem za izboljšanje zdravljenja te patologije večjega preboja še ni bilo. Struktura in način delovanja VaaSPH-1 sta edinstveni, zato ta molekula predstavlja obetavno osnovo za razvoj varnejše antikoagulantne učinkovine za zdravljenje venske trombembolije, ki vključuje trombozo globokih ven in pljučno embolijo. V članku je opisana strategija razvoja inovativnega nizkomolekularnega antikoagulanta na osnovi strukture VaaSPH-1. Najobetavnejše kandidatne molekule so že v postopku pridobivanja patentne zaščite.

Ključne besede: kačji strup; hemostaza; inhibitor intrinzične tenaze; antagonist FIXa; antikoagulant; venska tromboza