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CLINICAL & BASIC RESEARCH

In Silico Design of Novel Anticoagulant Peptides targeting Blood Coagulation Factor VIIa

Manal S Q Al-Amri,[1] Khalid Alrasadi,[2] Riad Bayoumi,[1] *Yajnavalka Banerjee[1]

تصميم ببيتايد مانعات التخثر بنموذج (In Silico) ضد عامل التخثر VIIa

منال العامري، خالد الرصادي، رياض بيومي، ياجنافالكا بانيرجي

الخلاصة: **الهدف:** يبدأ التخثر المتعاقب عند حدوث إصابة في الأوعية الدموية لمنع النزف، غير أن حصول التخثر غير المرغوب فيه يكون مضرا ويستوجب استخدام مانعات التخثر للوقاية والعلاج. تفضل مانعات التخثر التي تعمل على تعطيل خطوة محددة أو إنزيم معين في عملية التخثر أكثر من غيرها لأنها تقلل حدوث الأعراض الجانبية. الخطوة الرئيسية لاكتشاف مانع التخثر الجديد يشمل تصميما حسب طريقة (In Silico) . هذه الدراسة تبين أهمية ببيتايد مانعات التخثر حسب طريقة (In Silico) ضد عامل التخثر (VIIa). **الطريقة:** باستخدام قاعدة (Proline bracket) وأيضا توظيف المعلومات الحيوية مثل أداة بحث الرصف الأساسي العائد للمركز القومي للمعلومات الحيوية التقنية ونموذج (T–coffee) المقدم من قبل مختبر علم الأحياء الجزيئية الأوربي – معهد المعلومات الحيوية الأوربي ونماذج أخرى عديدة، قمنا بتصميم خمسة مانعات للتخثر ثنائية التكافؤ تستهدف عامل التخثر (VIIa)، باستخدام مثبطات ضد العامل (VIIa) وهي هيماكسيتين (أ) من سم الكوبرا الأفريقية وببيتايد مثبط للعامل (VIIa) كقوالب. تم اشتقاق ستة ببيتايدات من هيماكسيتين (أ) التي ربطت مع ببيتايد مثبط للعامل (VIIa) المتداخلة مع مساحات للبولي ألانين، وحللت بواسطة برنامج النموذج السويسري المطور في المعهد السويسري للمعلومات الحيوية وبرنامج عرض المختبر الشبكي للتأكد من الاستقرار الهيكلي. **النتائج:** حصلنا على اثني عشر ببيتايد، خمسة منها أظهرت الاستقرار الهيكلي.الخلاصة: إن الببيتايدات الخمسة التي تم الحصول عليها في هذه الدراسة كمانعات للتخثر ربما تؤدي إلى تخثر الدم، ولا بد من إجراء المزيد من الدراسات ألمختبرية والقياسات في الجسم الحي. أعطت هذه الدراسة تصورا عن كيفية استخدام النماذج البسيطة على الشبكة في التصميم المنطقي للأدلة المحتملة لاستهداف الجزئيات الفسيولوجية المحددة.

مفتاح الكلمات: مانع التخثر، عامل VIIa، تصميم الدواء بطريقة In Silico

abstract: ***Objectives:*** The coagulation cascade initiated during vascular injury prevents bleeding. Unwanted clot formation is however detrimental and requires the use of anticoagulants for prophylaxis and treatment. Anticoagulants targeting a specific step or an enzyme in the clotting process are most preferred as they minimise disadvantageous side-effects. A principal step in the discovery of novel anticoagulants encompasses the in silico design of potential leads. This study depicts the in silico design of peptide anticoagulants targeting coagulation factor VIIa. ***Methods:*** Applying the proline bracket rule and using various bioinformatics tools: the basic alignment search tool (BLAST) of National Center for Biotechnology Information; the T-coffee module provided by European Molecular Biology Laboratory-European Bioinformatics Institute, and several modules available on the ExPASy server, we designed five bivalent chimeric anticoagulants targeting factor VIIa, using factor VIIa inhibitors – hemextin A from *Hemachatus haemachatus* (African Ringhals cobra) venom and factor VIIa exosite-inhibitor peptide as templates. Six peptides were derived from hemextin A, which were concomitantly fused with factor VIIa exosite-inhibitor peptide intermediated by a polyalanine spacer, and analysed for structural stability using the SWISS-MODEL software developed at the Swiss Institute of Bioinformatics and WebLab ViewerPro (Version 4.2). ***Results:*** Twelve chimeric peptides were obtained; only five exhibited stable structures in silico. ***Conclusion:*** The five peptides obtained are probable anticoagulant leads that should be further evaluated using suitable *in vitro* and *in vivo* assays. Further, this study shows how simple web-based modules can be used for the rational design of probable leads targeting specific physiological molecular targets.

Keywords: Anticoagulant; Factor VIIa; In silico drug design

Advances in Knowledge

1. *Five potential bivalent chimeric peptide inhibitors of coagulation factor VIIa have been designed, which should be assessed using in vitro and in vivo assays.*

[1]*Department of Biochemistry, College of Medicine and Health Sciences, Sultan Qaboos University, Muscat, Oman;* [2]*Department of Biochemistry, Sultan Qaboos University Hospital, Muscat, Oman.*

**Corresponding Author email: yaj@squ.edu.om*

2. Depiction of a scheme to design rationally peptide based drugs using simple web-based software tools

Application to Patient Care

1. *The designed peptides, if viable in clinical trials, will provide the clinical community with an arsenal of anticoagulants targeting clot initiation thereby helping in the treatment and prophylaxis of thromboembolic and associated disorders.*
2. *The study has translational significance since it provides a simple and viable strategy to design peptide based drugs targeting different diseases/disorders.*

Blood coagulation is a physiological response to vascular injury in which zymogens of serine proteases present in the plasma milieu are sequentially activated culminating in the formation of the fibrin clot.[1-3] Coagulation factor VIIa (FVIIa), along with cofactor tissue-factor (TF), act as the prima ballerinas to initiate the coagulation cascade.[4-6] The extrinsic pathway is responsible for initiating the clotting process whereas the intrinsic pathway propagates the process of coagulation. A comprehensive description of both these pathways is summarised in Figure 1.

Although coagulation reactions are pivotal for appropriate homeostasis, unwanted clot formation can mediate debilitating affects leading to thrombosis and associated disorders. Anti-coagulants are pivotal for the prevention and treatment of thromboembolic disorders, and ~0.7% of the Western population receives oral anticoagulant treatment.[7] Coumarins and heparin are the most well known clinically used anticoagulants. Coumarins inhibit the activity of all vitamin K-dependent proteins, including procoagulants (thrombin, FXa, FIXa, and FVIIa) and anticoagulants (activated protein C and protein S). Heparin mediates its anticoagulant activity by enhancing the inhibition of thrombin and FXa by antithrombin III.[8,9] The non-specific mode of action of these anticoagulants accounts for their therapeutic limitations in maintaining a balance between thrombosis and haemostasis. These limitations have provided the impetus for the development of new anticoagulants that target specific coagulation enzymes or a particular step in the clotting process.[10,11] Because of its relatively low concentrations in blood (10 nM) and its pivotal role in the initiation of the coagulation reactions,[12] FVII/FVIIa is an attractive drug target for the development of novel and specific anticoagulant agents.

So far, only two proteins that specifically inhibit the TF-FVIIa complex have been evaluated in clinical trials, viz. tissue factor pathway inhibitor (TFPI) and nematode anticoagulant peptide c2 (NAPc2). TFPI is an endogenous inhibitor of this complex, whereas NAPc2 is an exogenous inhibitor isolated from canine hookworm (*Ancylostoma caninum*).[13-15] Of these, only NAPc2 has future viability as a probable anticoagulant lead. In a phase II study, NAPc2 showed promise in preventing venous thromboembolism after elective knee replacement surgery.[16,17] Because of the lack of natural inhibitors that specifically interfere with FVIIa activity, a number of artificial inhibitors have been designed and developed. They include proteins that block the association of TF and FVIIa, such as antibodies against TF and FVIIa, TFAA (a TF mutant with reduced cofactor function for FX), FFR-VIIa (inactivated form of FVIIa with 5-fold higher affinity for TF compared with native FVIIa), and peptides derived from TF and FVIIa.[18-22] In addition, two series of peptide exosite inhibitors were selected from phage display libraries for their ability to bind to the TF-FVIIa complex.[18,19] They bind to two distinct exosites on the serine protease domain of FVIIa and exhibit steric and allosteric inhibition.[21]Although both peptide classes are potent and selective inhibitors of the TF-FVIIa complex, they fail to inhibit 100% activity even at saturating concentrations. This is overcome either by the fusion of the two peptides,[22] or by using a protease switch with substrate phage.[20] A number of synthetic compounds have also been designed as active-site inhibitors of FVIIa as well as the TF-FVIIa complex.[23,24] A number of naphthylamidines have recently been reported to have FVIIa inhibitory activity. They were synthesised by the coupling of amidinobenzaldehyde analogs to a polystyrene resin. However, apart from inhibiting FVIIa activity, these synthetic compounds nonspecifically inhibit the activity of other blood coagulation serine proteases.[25]

In the past, we reported the identification and

Figure 1: The current model of the blood coagulation cascade. There are two blood coagulation pathways, the contact activation or "intrinsic" pathway and the primary or "extrinsic" pathway. These multicomponent processes are illustrated as enzymes, inhibitors, zymogens, or complexes. The contact activation pathway has no known bleeding cause associated with it; thus, this pathway is considered accessory to haemostasis. On injury to the vessel wall, tissue factor, the cofactor for the extrinsic tenase complex, is exposed to circulating FVIIa and forms the extrinsic tenase. FIX and FX are converted to their serine proteases FIXa and FXa, which then form the intrinsic tenase and the prothrombinase complexes with their cofactors, respectively. The combined actions of the intrinsic and extrinsic tenase and the prothrombinase complexes lead to an explosive burst of the key enzyme thrombin (IIa). In addition to its multiple procoagulant roles, thrombin also can act in an anticoagulant capacity when combined with the cofactor thrombomodulin in the protein Case complex. The product of the protein Case reaction, activated protein C (APC), inactivates the cofactors FVa (cofactor for FXa) and FVIIIa (cofactor for FIXa). The cleaved species, FVai and FVIIIai, no longer support the respective procoagulant activities. Once thrombin is generated through procoagulant mechanisms, thrombin cleaves fibrinogen (releasing fibrinopeptides A and B [FPA and FPB]) and activates FXIII to form a cross-linked fibrin clot. Thrombin–thrombomodulin also activates a thrombin activate-able fibrinolysis inhibitor that slows fibrin degradation by plasmin. The procoagulant response is downregulated at various stages by the stoichiometric inhibitors, tissue factor pathway inhibitor (TFPI) and antithrombin III (AT-III). TFPI serves to attenuate the activity of the extrinsic tenase trigger of coagulation. AT-III directly inhibits thrombin, FIXa, and FXa. The accessory pathway provides an alternate route for the generation of FIXa. Thrombin has also been shown to activate FXI. The fibrin clot is eventually degraded by plasmin yielding soluble fibrin peptides.

in-depth characterisation of a novel FVIIa inhibitor, christened hemextin AB complex, from the venom of the African Ringhals cobra.[26-29] This synergistic anticoagulant complex consists of two proteins, hemextin A (HA) and hemextin B (HB), both of which belong to the three-finger family of snake venom proteins [Figure 2]. Of the two subunits, HA individually possesses anticoagulant activity whereas HB is inactive. Unlike NAPc2, HA of hemextin AB complex does not use coagulation factor Xa as a scaffold for FVIIa inhibitory activity positing a very unusual mechanism of anticoagulation.[29] Thus HA provides us with a novel and intriguing anticoagulant lead that can be further developed into an anticoagulant.

In this study, we report the design of a short chimeric anticoagulant mimetic (with probable FVIIa inhibitory function) using peptides derived from HA (by applying the proline bracket rule) and fusing them with an already characterised peptide FVIIa-exosite inhibitor,[20] using simple web-based bioinformatics. Only five of the 12 designed peptides exhibited structural stability and warrant assessment with regards to anticoagulant activity. This study therefore not only provided a foundation in the quest for the rational design of anticoagulants

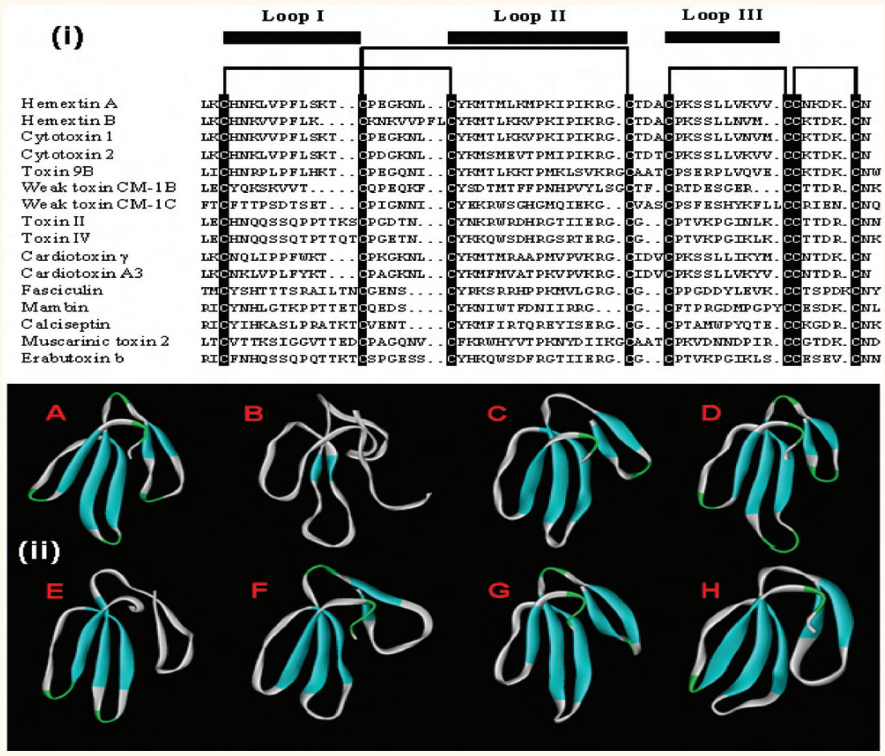

Figure 2: Analysis of primary structure of HA. **1)** Comparison of amino acid sequence of hemextin A with other sequences of the three-finger toxin family. The conserved cysteine residues are indicated in black. The four conserved disulfide linkages and the segments contributing to the three loops are outlined. Comparison were made with the following sequences: cytotoxin 1, cytotoxin 2, toxin 9B, weak toxin CM-1B, weak toxin CM-1C, toxin II, toxin IV are from *Hemachatus haemachatus* (African Ringhals Cobra); carditoxin γ from *Naja nigricollis* (Black spitting cobra); carditoxin A3 from *Naja atra* (Taiwan cobra); fasciculin from *Dendroaspis angusticeps*; mambin from *Dendroaspis jamesoni*; calciseptin from *Dendrosaspis polylepsis*; muscarinic toxin 2 from *D. angusticeps*; erabutoxin b from *Laticauda semifasciata*. **2)** Three-dimensional structural similarity among three-finger toxins from snake venoms **A)** Erabutoxin a (1QKD); **B)** α-bungarotoxin (2ABX); **C)** cardiotoxin V4 (1CDT); **D)** κ-bungarotoxin (1KBA); **E)** candoxin (1JGK); **F)** fasciculin 2 (1FAS); **G)** muscarinic toxin MT-2 (1FF4; R Menez et al., pers. comm., 2002); (H) FS2 toxin (1TFS). Note all these 'sibling' toxins share a similar structural fold; three β-sheeted 'fingers' start from the core. These β-sheeted loops are numbered right to left as loop I, II and III, respectively. However, these toxins differ from each other in their biological activities.

specifically targeting FVIIa, but also depicted a scheme that can be utilised in the rational design of drug leads using web-based bioinformatics.

Methods

The complete sequence of HA protein had been determined earlier using Edman degradation protein chemistry, and is depicted below (using the single letter amino acid code): LKCHNKLVPFLSKTCPEGKNLCYKMTMLKMPKIPIKRGCTDACPKSSLLVKVVCCNKDKCN. The sequence of the 26-mer FVIIa-exosite inhibitor peptide was obtained from the literature,[22] and is as follows: EEWEVLCWTWETCERGEGVEELWEWR.

The various software employed in this study are freely available on the Internet and do not require special access to any paid database or server. Most of these modules are Windows based, however some operate at an optimum on a LINUX platform.

Sequence alignment involving HA involved a detailed search of homologous sequences from the National Center for Biotechnology Information protein database,[30,31] following which the basic alignment search tool (BLAST)[32-34] was employed. The sequence alignment of HA was carried out by looking at the position of the cys residues using the online T-coffee module provided by the European Molecular Biology Laboratory-European Bioinformatics Institute.[35] In order to look at the various specifics of the amino acid sequence, appropriate modules available on the ExPASy server were used.[36] In order to model

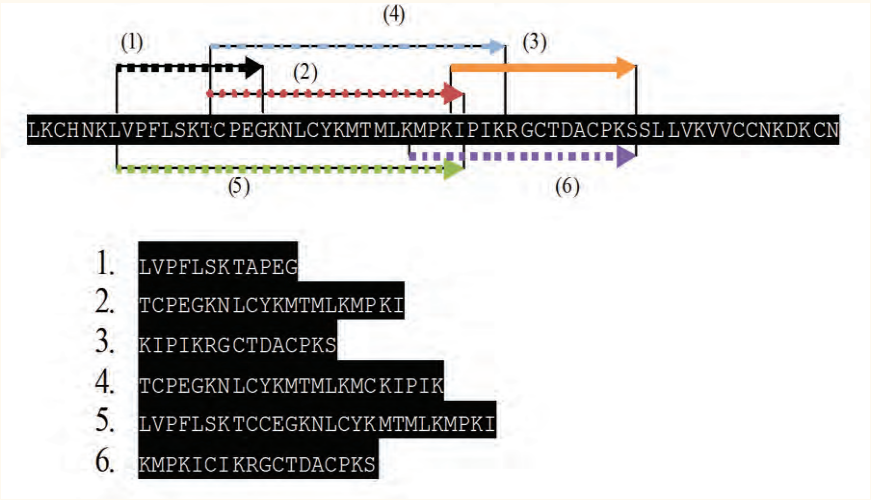

Figure 3: Peptides derived from HA. Six short peptides were derived from HA using the proline bracket rule. We kept two amino acids on the flanking region of each proline residue in order to provide structural stability to the peptide.

the three-dimensional structures of the HA and chimeric peptides, the sequences of the proteins were submitted to the SWISS-MODEL developed by the Protein Structure Bioinformatics group at the Swiss Institute of Bioinformatics (SIB) and the Biozentrum at the University of Basel.[37]

The raw data obtained were then analysed using the WebLab ViewerPro (Version 4.2) (Accelrys, Inc. San Diego, CA, USA). The surface charge distribution of the modeled as well solved structures obtained from the protein data bank were analysed using the Molecular Graphics Laboratory Tools (MGLT) package obtained from The Scripps Research Institute (TSRI) molecular modeling server (TSRI, La Jolla, CA, USA) The Python Molecular Viewer (PMV) of the MGLT package was used predominantly in this study.[38,39]

Results

Our finding on the alignment of HA, was that HA belongs to the three-finger family of snake venom proteins [Figure 2A] and not to the family of snake venom serine protease inhibitors which are Kunitz type inhibitors with a very different cys bonding pattern. Proteins belonging to this group exhibit a characteristic β-sheet structure [Figure 2B]. In order to design short peptides rationally from HA, we used the proline bracket rule.[40,41] In this rule, unique physicochemical properties of proline, the most commonly found imino acid in proteins, are used for the prediction of active sites. The distinct properties of proline residues are due to the bulky pyrrolidine ring and the lack of protons important for hydrogen bond formation in the α-helix and β sheet. Proline residues are the common structural elements found in the flanking segments of protein-protein interaction sites and they enhance the protein-protein interactions. The proline residues most likely reduce the number of possible conformations due to their ability to constrain the conformation of the neighbouring residues. The short segments of 3-7 residues flanked by proline residues have been identified as the potential protein-protein interaction sites directly from the amino acid sequence of the protein. A case in point is that incorporation of proline residues on either or both sides of the interaction site of an antiplatelet peptide (peptide that prevents platelet aggregation), IARGDMNA, enhances the antiplatelet activity to approximately the same extent (1.5- to 2.5-fold). The incorporation of proline residues on both sides enhances the activity by 7- to 13-fold. This enhancement of the biological activity of the peptide is probably due to a reduction in the number of possible conformations of the peptide, without introducing the rigidity that would accompany cyclization.[40] Therefore we hypothesised that proline residues will be present on the flanking regions of the probable interaction/active sites. Based on this premise, we designed the six short peptides from HA [Figure 3]. We also kept two amino residues beside each proline residue on each side to provide structural stability to the

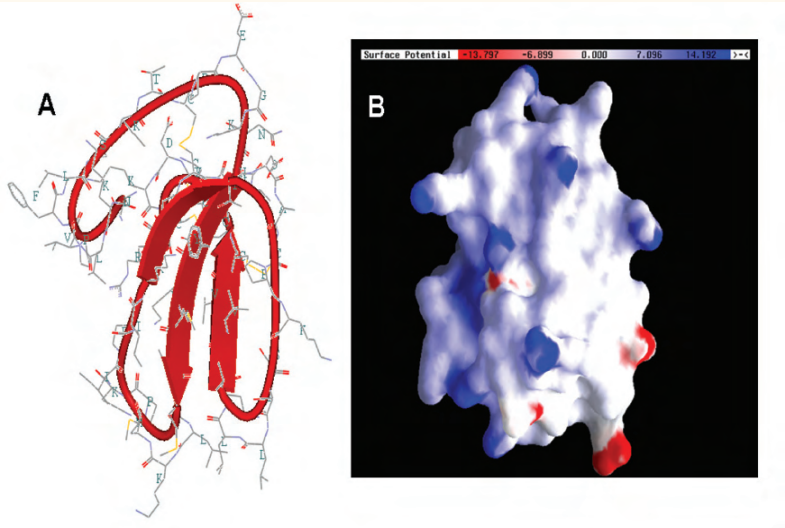

Figure 4: Modeled three dimensional structure of HA. **A)** The ribbon representation of the three-dimensional structure of HA (Red). The amino acid residues are represented in line model with their corresponding one letter code. Note: HA exhibits a characteristic three-finger structure and is predominantly β-sheeted like other members of the three-finger family (Figure 2B). **B)** Surface potential calculation of HA using PyMOL molecular viewer. HA is predominantly basic as revealed by the scale on top of the figure (blue represents positively charged patches where as red represents negatively charged patches).

designed peptide.

For the modelling of the HA 3D-structure, the sequence alignment showed that HA has conserved cys residues with other members of the three-finger toxin (3-FTX) family, which indicated that HA is a 3-FTX. Earlier studies involving circular dichroism spectroscopy also have supported this finding.[26] In order to confirm further that HA has a similar architecture to three-finger proteins, we modelled the HA. As observed in Figure 4 A, it has a characteristic fold of a three-finger toxin, with anti-parallel β-sheets. Further, we mapped the surface charge distribution of HA using PyMOL molecular viewer (PMV) [Figure 4B]. HA shows a predominance of blue or positively charged patches, from which it can be concluded that HA is a basic protein and therefore will have a higher propensity of binding to negatively charged regions on its corresponding target molecule. This observation was further confirmed by the calculating the theoretical PI for HA, which was found to be 9.34, confirming that the protein is of basic nature.

In order to predict the binding sites of HA on FVIIa, the structure of FVIIa was modelled using the X-ray crystallographic structure of TF-FVIIa as a template (PDB ID 1DAN).[42] As shown in Figure 5, FVIIa consists of two chains, the heavy and the light chains. The heavy chain consists of the protease domain of the enzyme, whereas the light chain consists of two epidermal growth-factor-like domains and a γ-carboxyglutamic acid domain. HA does not bind to the light chain of FVIIa (data not shown) and therefore its binding site is located on the heavy chain. The heavy chain of FVIIa consists of a patch of negatively charged residues (highlighted in yellow in Figure 5) and HA, being predominantly basic in nature, will have its binding/interaction site localised to this acidic patch. Therefore, peptides derived from HA are supposedly going to bind to this acidic or negatively charged patch on FVIIa.

As to the design of chimeric peptides, since peptides derived from HA will bind to the acidic patch on the FVIIa molecule, we fused these HA derived peptides with another peptide that binds to the basic patch on the heavy chain. This peptide has already been characterised with regards to the structure-function details of the peptide.[20,21] The binding site of the peptide on the FVIIa heavy chain is shown in Figure 6 A and was determined using X-ray crystallography (PDB ID 1JBU). The peptide is inherently highly acidic in nature and binds to a predominantly positively charged region on the FVIIa heavy chain, as shown in the surface potential plot in Figure 6 B. HA derived peptides were fused with this FVIIa exosite-inhibitory peptide. A spacer consisting of polyalanine residues was introduced between the HA derived and FVIIa exosite-inhibitory peptides in order to maintain

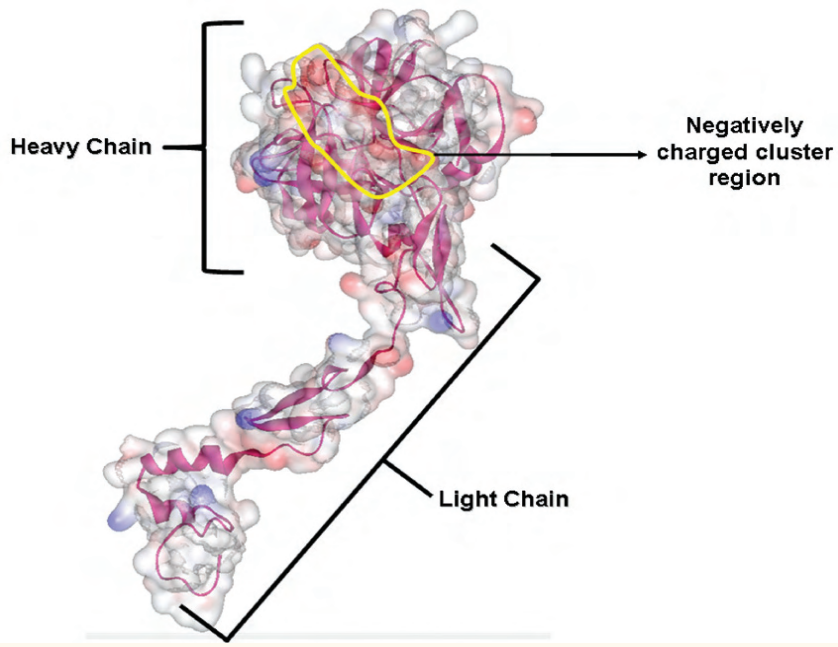

Figure 5: Modeled three dimensional structure of FVIIa. FVIIa consists of heavy and light chains. The model was obtained from the solved structure of the TF-FVIIa complex (PDB ID 1DAN). The negatively charged cluster on the FVIIa molecule localized on the heavy chain is highlighted in yellow.

the distance between the acidic and the basic patches. The designed chimeric peptides are shown in Table 1.

The structural stability of the chimeras was assessed by modelling their structure in a way similar to that used for HA. Only five of the 12 chimeric proteins were found to be structurally stable [Figure 7A-E], whereas the 3D-structure modelling of the other peptides failed, thus indicating their structural instability.

Discussion

In the present study, we designed bivalent anticoagulant peptides targeting coagulation FVIIa, the clotting factor responsible for the initiation of the clotting reactions, employing simple web-based bioinformatics. For our study, we used two different proteins as templates: HA, a FVIIa inhibitor isolated and characterised from the venom of *Hemachatus haemachatus* (African Ringhals cobra), and factor VIIa exosite-inhibitor peptide which is a synthetic peptide identified in phage-display libraries.

The complete sequence alignment of HA revealed that it belongs to the three-finger family of snake venom proteins. This family of proteins is found only in the venoms of elapids (cobras, kraits and mambas) and hydrophids (sea snakes) and not those of vipers and crotalids (rattlesnakes). They contain four or five disulphide bridges, of which four are conserved in all members. Accordingly, all proteins of this family show a similar pattern of protein folding: three β-stranded loops extending from a central core containing the four conserved disulphide bridges [Figure 2]. Despite the overall similarity in structure, these polypeptides differ from each other in their biological activities. However, because of their well folded and compact structure, proteins belonging to this family are attractive as drug leads (if the parent protein is pharmacologically active) or are used as molecular scaffolds in the peptide based drug design.[43] For our study one of the principal reasons for choosing HA was its well-folded and compact structure.

The idea of designing a bivalent inhibitor for a coagulation serine protease was first successfully implemented in case of thrombin, where two peptides acetyl-(d)F-P-R-P-Q-S-H-N-D-G-D-F-E-E-I-P-E-E-Y-L-Q (binding to the catalytic active site) and (d)F-P-R-P-G-G-G-G-N-G-D-F-E-E-I-P-E-E-Y-L (binding to the exosite of thrombin) were covalently linked, and the resultant peptide mimicked the activity of hirudin and had higher inhibitory potency than the individual

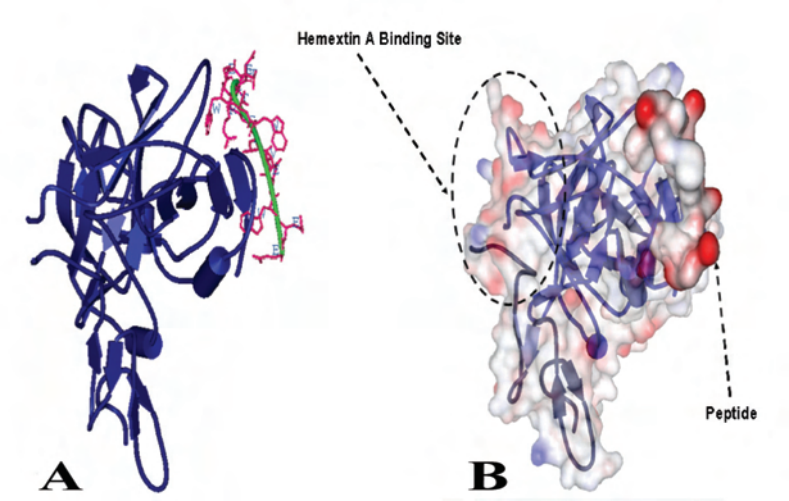

Figure 6: Binding site of the FVIIa-inhibitory peptide on FVIIa molecule. **A)** The ribbon representation of the FVIIa heavy chain molecule (blue) in complex with the FVIIa-inhibitory peptide (green) **B)** Surface charge distribution of the heavy chain and the inhibitory peptide. Note that the inhibitory peptide is negatively charged binding to a positively charged region on the FVIIa molecule keeping the HA binding site on FVIIa molecule free.

peptides.[44] Some bivalent peptides, although having weak binding properties, have been derived from the activation sequences of thrombin-activated receptors, also referred to as protease-activated receptors (PARs). These peptides carry the LDPR sequence and binding motifs targeting the fibrinogen-recognition exosite of thrombin.[45,46] The essence of designing such inhibitors is to use peptides which are structurally stable and do not have overlapping binding sites.

In our study, we targeted coagulation FVIIa, since this serine protease is responsible for the initiation of the clotting cascade. Therefore, inhibiting the activity of this protein will inhibit the cascade in its initial stages. Also the levels of this protein in blood is low in comparison to some of the other coagulation serine proteases, necessitating the use of low levels of the designed anticoagulant, a key factor involved in the success of any drug.

Based on the surface charge calculations [Figure 4 B], it is pertinent that HA is a basic protein and therefore peptides derived from it will bind to predominantly negatively charged regions on its target molecule. Also, previous studies have shown that HA is a specific inhibitor of FVIIa.[29] Based on this premise we identified the regions of FVIIa which have a high density of negatively charged clusters. Such clusters exist in both the heavy and light chains of the FVIIa molecule. However, these clusters in the light chain are not accessible to HA since they are involved in co-factor (TF binding) as revealed in the crystallographic structure solved by Banner et al.[42] and later confirmed by us (data not shown). Therefore, we localised the negatively charged cluster on the heavy chain of the protein using the solved structure of TF--FVIIa (PDB ID 1DAN) as the model [Figure 5, highlighted in yellow].

In order to derive peptides from HA we used the proline bracket rule [Figure 3] (see above with regards to the details). In our next step, we needed to select an FVIIa-inhibitory peptide whose binding site will not overlap with HA derived peptides. After a literature search we found a peptide (EEWEVLCWTWETCERGEGVEELWEWR) which is predominantly negatively charged (PI = 3.98) as the ideal candidate to be used for the design of the chimera. Firstly, because of its inherent acidic nature, it will be involved in binding to a patch of residues which are positively charged and secondly [Figure 6 A and B], the presence of a disulfide bond in the peptide guarantees its having a well defined fold. Since HA derived peptides are positively charged and might interact with the FVIIa-inhibitory peptide, we introduced a spacer of nine alanine residues in between them. The designed chimeras were then assessed for structural stability using the appropriate tools mentioned in the Methods section. Only five of the designed 12 chimeras were found to be structurally viable

Table: 1. Designed Chimeric Peptides and Result of Structural modeling

Hemextin A (peptide)	Terminal link	Chimera (HA p + Lazarus p)	Structure Analysis
1.			
A- LVPFLSKTAPEG	COOH-terminal	LVPFLSKTAPEG + EEWEVLCWTWETCERGEGVEEELWEWR	No recognisable fold was obtained
B- LVPFLSKTAPEG	NH2-terminal	EEWEVLCWTWETCERGEGVEEELWEWR + LVPFLSKTAPEG	No recognisable fold was obtained
2.			
A- TCPEGKNLCYKMTMLKMPKI	COOH-terminal	TCPEGKNLCYKMTMLKMPKI + EEWEVLCWTWETCERGEGVEEELWEWR	No recognisable fold was obtained
B- TCPEGKNLCYKMTMLKMPKI	NH2-terminal	EEWEVLCWTWETCERGEGVEEELWEWR + TCPEGKNLCYKMTMLKMPKI	No recognisable fold was obtained
3.			
A- KIPIKRGCTDACPKS	COOH-terminal	KIPIKRGCTDACPKS + EEWEVLCWTWETCERGEGVEEELWEWR	No recognisable fold was obtained
B- KIPIKRGCTDACPKS	NH2-terminal	EEWEVLCWTWETCERGEGVEEELWEWR + KIPIKRGCTDACPKS	Distinct fold was observe wafter modeling
4.			
A- TCPEGKNLCYKMTMLKMCKIPIK	COOH-terminal	TCPEGKNLCYKMTMLKMCKIPIK + EEWEVLCWTWETCERGEGVEEELWEWR	Distinct fold was observe wafter modeling
B- TCPEGKNLCYKMTMLKMCKIPIK	NH2-terminal	EEWEVLCWTWETCERGEGVEEELWEWR + TCPEGKNLCYKMTMLKMCKIPIK	Distinct fold was observe wafter modeling
5.			
A- LVPFLSKTCCEGKNLCYKMTMLKMPKI	COOH-terminal	LVPFLSKTCCEGKNLCYKMTMLKMPKI + EEWEVLCWTWE TCERGEGVEEELWEWR	Distinct fold was observe wafter modeling
B- LVPFLSKTCCEGKNLCYKMTMLKMPKI	NH2-terminal	EEWEVLCWTWETCERGEGVEEELWEWR + LVPFLSKTCCEGKNLCYKMTMLKMPKI	Distinct fold was observe wafter modeling
6.			
A- KMPKICIKRGCTDACPKS	COOH-terminal	KMPKICIKRGCTDACPKS + EEWEVLCWTWETCERGEGVEEELWEWR	No recognisable fold was obtained
B- KMPKICIKRGCTDACPKS	NH2-terminal	EEWEVLCWTWETCERGEGVEEELWEWR + KMPKICIKRGCTDACPKS	No recognisable fold was obtained

Notes: + denotes the nine alanine spacer; HA p denotes the peptide fragment derived from hemextin A; Lazarus p denotes the peptide designed at Genentech CA, USA by Dr Bob Lazarus and his colleagues.

[Figure 7 A-E]. One of the intriguing highlights of the study was that the designed structurally viable peptides had the positively and negatively charged residues separated as in observed in Figure 7.

Also, we designed various combination of chimeras where the HA derived peptides were placed at the amino-terminal as well as the carboxy-terminal ends. Switching of ends was found to affect the stability of the designed chimera. As shown in Table 1, for combination 3, the presence of the FVIIa-inhibitory peptide on the carboxy-terminal end leads to structural instability of the designed chimera, whereas its presence on the amino-terminal does not affect its structural viability.

These putative bivalent anticoagulants still need to be evaluated in various *in vitro* and *in vivo* assays before they can be assessed in clinical trials. One of the advantages of the current strategy is that it can be successfully applied without the involvement of large financial commitment by researchers with limited knowledge of bioinformatics. However, the designed chimeric anticoagulant, although structurally viable, might lose its activity since the fold it attains might not favour its binding to its specific interaction sites.

Conclusion

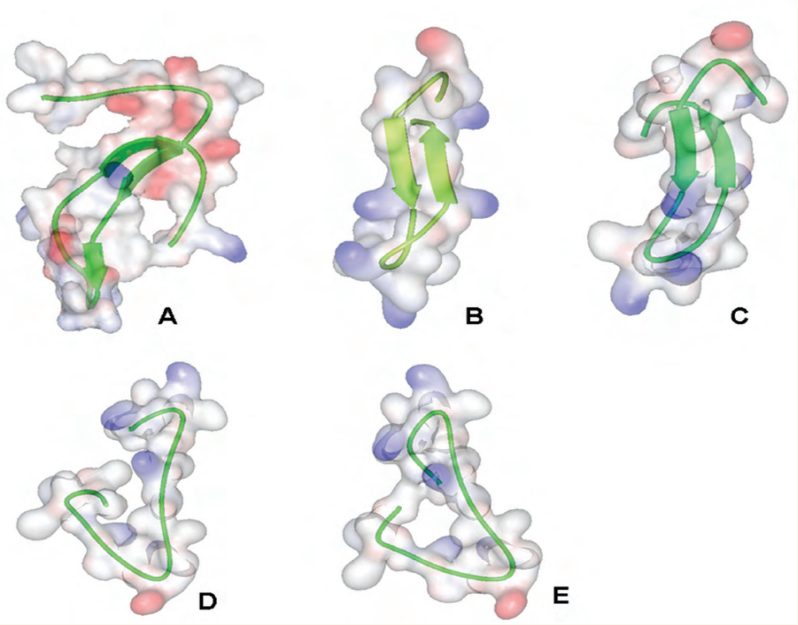

Figure 7: Structure of the five viable chimeric peptides. **A-E)** The ribbon representation of the three-dimensional structure of five designed viable chimera along with their surface charge distribution. **A)** Peptide 3 in Table 1; **B)** and **C)** are peptide 4 in Table 1; **D)** and **E)** are peptide 5 in Table 1. Blue Represent positively charged regions and red negatively charged regions respectively..

In summary, we designed bivalent inhibitors for FVIIa using simple bioinformatics. The five structurally viable inhibitors will be assessed for functional potency using appropriate *in vitro* and *in vivo* assays/experiments in the near future. Although a great deal of effort was involved in the analysis of data and literature search during the study, it was relatively inexpensive since all the tools used were freely available on the Internet. Further, a similar strategy can be used for designing peptide based drugs for other physiological target molecules. Thus this study introduces a novel paradigm in strategy design for drug-leads.

CONFLICT OF INTEREST

The authors reported no conflict of interest.

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