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Target Specific Anticoagulant Peptides: A Review

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Abstract
Anticoagulant drugs are of crucial importance for the treatment and prophylaxis of thrombotic disorders. The use of traditional anticoagulants like heparin and warfarin is majorly associated with bleeding complications. In the quest for safer anticoagulation therapy, the interest for the isolation of novel anticoagulant compounds has shifted towards natural sources. Peptides can be considered as better alternative due to their therapeutic potential in the treatment of diseases. Peptides from hematophagous (blood-feeding) and venomous organisms have been recognized as potential anticoagulant agents. Of late, peptides derived from the hydrolysis of food proteins, including edible seaweeds, milk and seed proteins, have also shown to possess promising in vitro anticoagulant activity. To overcome the problems associated with regular anticoagulants, peptides targeting vital steps in the clotting cascade have been studied. This review focuses on anticoagulant peptides with known targets, inhibiting crucial factors in the coagulation cascade such as FXa, FXIa, FXIIa and FVIIa/TF complex, as well as peptides with unknown targets.

Keywords Anticoagulants · Peptides · Thrombosis · Coagulation

Introduction

Thrombosis illustrates a state of reduced blood flow inside a blood vessel as a result of clot formation (Mackman 2012). The process of clot development involving the complex interactions of vascular endothelium, platelets and clotting factors eventually manifests as venous thromboembolism [which encompasses deep vein thrombosis (DVT) and pulmonary embolism (PE)] or acute coronary syndrome (Mega et al. 2015). Thrombosis-associated deaths are still a concern. According to Raskob et al. (2014), thrombosis represents as a major contributor to the global burden of disease. Anticoagulation therapy is the mainstay for prevention and treatment of thrombosis (Alquwaizani et al. 2013); venous thromboembolism (Franchini and Mannucci 2016), acute ischemic stroke (Patterson et al. 2006) and atrial fibrillation (Katritsis et al. 2015).

Traditionally, unfractionated heparin (UFH) and warfarin have been used as anticoagulants in clinical settings (Hawkins 2004). Heparin is administered for patients requiring parenteral anticoagulation whereas warfarin for those requiring oral anticoagulation (Harter et al. 2015). Warfarin is a vitamin-K antagonist (VKA) that works by hindering the synthesis of vitamin-K dependent clotting factors. Unfractionated heparin is a heterogeneous mixture of polysaccharide polymers usually produced from intestinal mucosa of porcine. Heparin, with average molecular mass of 15 kDa, after cleavage produces heparin fractions of lesser molecular mass (~5 kDa) termed low-molecular-weight heparins (LMWH) (Hawkins 2004). It produces anticoagulation effect by binding and inhibiting clotting factor II activated (Thrombin) and factor X activated (FXa) with the aid of antithrombin (AT) protein (Hirsh et al. 2001). Though both the anticoagulants are highly efficacious at proper doses, they have a number of limiting factors that impede their clinical use. Bleeding is a serious issue related to warfarin therapy. Warfarin related bleeding is usually minor that requires no attention, but in the case of major bleeding the consequences are even worse (Snipelisky and Kusumoto 2013). The other limitation is the diet, wherein the intake of vitamin K rich foods may weaken or even eradicate the effect of warfarin (Hawkins 2004). In a recent report it was presented that the higher loading doses of warfarin induced deep vein thrombosis (DVT) in a patient who was being

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treated for atrial fibrillation (Binymin et al. 2014). On the other hand, the major limitation of heparin is that it causes the development of thrombocytopenia, termed heparin-induced thrombocytopenia (Ahmed et al. 2007).

Therefore, anticoagulants targeting crucial steps in the coagulation cascade are required for treatment and prophylaxis of thrombosis. In the quest of safer and effective anticoagulation therapy, anticoagulants isolated from alternative sources, such as food and natural sources, have increased (Amorim et al. 2011; Cao et al. 2017). Over the last two decades, peptides have attracted a huge amount of interest as therapeutics. Many natural and bioactive peptides possessing wide range of activities have been isolated and identified from natural sources (Yu et al. 2014; Shin et al. 2017). Peptides offer exciting opportunities as drug candidate as they combine the benefits of small molecules (cost, conformational restriction, membrane permeability, metabolic stability) and that of proteins (natural components, target specificity, high potency). Their high specificity and efficacy towards their target suggests their use in therapeutic applications (Craik et al. 2013).

This review focuses on anticoagulant peptides isolated from various sources targeting specific clotting factor(s) involved in the coagulation cascade as well as peptides for which specific targets are not known.

Overview of the Coagulation Cascade

The process of hemostasis functions by ceasing the blood flow at the site of injury without perturbing the normal blood circulation. Hemostasis consists of two main constituents termed primary and secondary hemostasis. The former refers to the aggregation of platelets and platelet plug formation. While the latter refers to the deposition of insoluble fibrin, the end-product of clotting cascade (Gale 2011). The coagulation cascade (Fig. 1) is a sequence of proteolytic reactions that occur through two distinct pathways termed intrinsic and extrinsic pathways. Though the cascade starts by two different mechanisms, it ends up converting soluble fibrinogen into insoluble fibrin. Both the pathways merge at the activation step of factor X in the cascade.

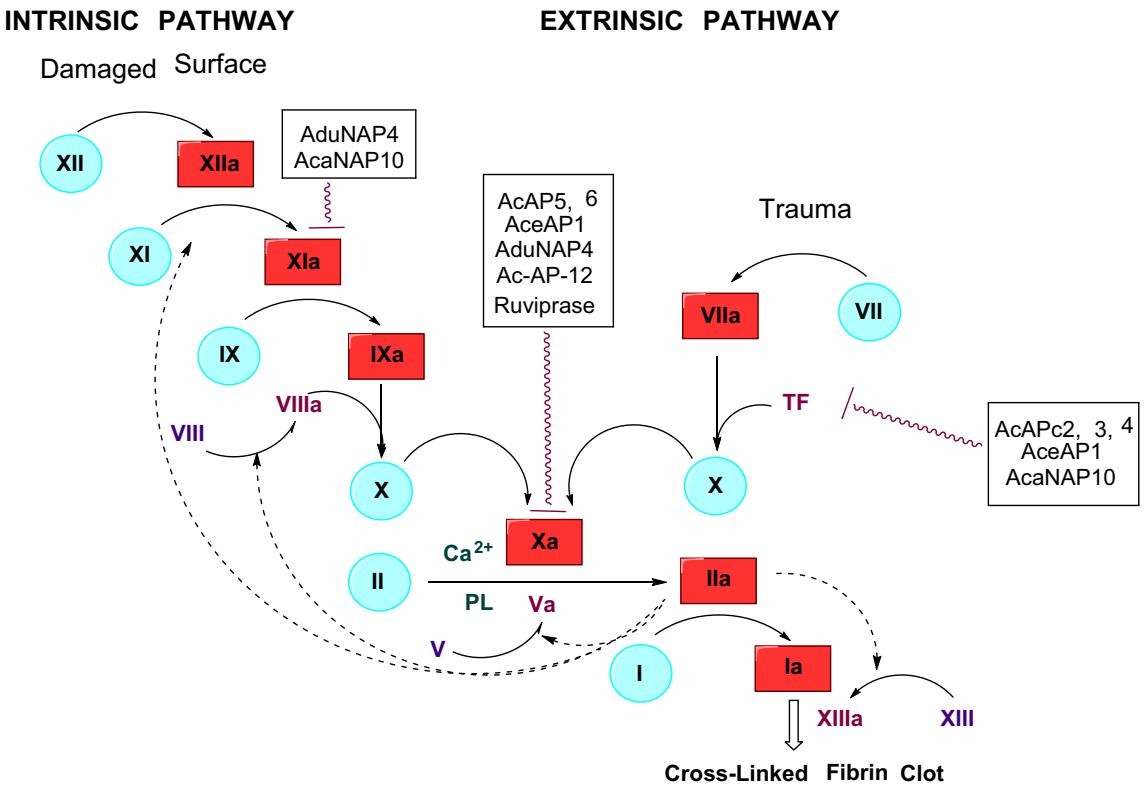

Fig. 1 Overview of the coagulation cascade involving intrinsic and extrinsic pathways depicted with potent peptide inhibitors. AcAP 5, 6—*Ancylostoma caninum* Anticoagulant Peptide-5, 6; AcAPc2, 3, 4—*Ancylostoma caninum* Anticoagulant Peptide-2, 3, 4; AcaNAP 10—*Ancylostoma caninum* Nematode Anticoagulant Peptide-10; Ac-AP-12—*Ancylostoma caninum* Anticoagulant Peptide-12; AceAP1—*Ancylostoma ceylanicum* Anticoagulant Peptide-12; AduNAP 4—*Ancylostoma duodenale* NematodeAnticoagulant Peptide-4; Factor I—Fibrinogen; Factor Ia—Fibrin; Factor II—Prothrombin; Factor IIa—Thrombin. Figure created using ChemDraw Professional version 15.0.0.106

The extrinsic pathway initiates upon endothelial barrier dysfunction and subsequent release of tissue factor (TF) (Mackman 2009). The TF binds to factor VIIa (FVIIa) on the phospholipid (PL) surface and forms a complex namely extrinsic tenase complex (FVIIa/TF/PL). This complex further mediates the activation of zymogens factor IX and X to FIXa (factor IX activated) and FXa (factor X activated) respectively (Kovalenko et al. 2017). In a similar fashion, the intrinsic or the contact pathway starts with FXII (factor XII), high molecular weight kininogen (HMWK), prekallekerin (PK) and FXI leading to the activation of factor XI to activated factor XI (FXIa) (Dahlback 2000). Consequently, factor IX gets activated by the action of activated factor XI (FXIa). On a phospholipid surface, FIXa along with its co-factor FVIIIa, forms the intrinsic tenase complex and mediate the activation of factor X (FX) (Palta et al. 2014).

Factor Xa then binds to the factor Va (FVa), it's co-factor, in the presence of Ca^{2+} on phospholipid membrane to form prothrombinase complex. This complex facilitates the conversion of Prothrombin (factor II) to Thrombin (factor IIa). Upon activation, thrombin catalyzes the activation of the factors V, VIII and XI. As a final act in the coagulation cascade, thrombin converts soluble fibrinogen (factor I) into insoluble fibrin (factor Ia) and forming a clot.

The thrombin thus generated further orchestrates the activation and aggregation of platelets, thereby complete cessation of the flow of blood occurs at the site of injury. As soon as the bleeding stops, fibrinolysis takes place to breakdown the blood clots. Plasmin, a serine protease, facilitates the destruction of clots. Plasminogen is a circulating plasma zymogen that gets converted to plasmin upon binding to plasminogen activators. Tissue-type plasminogen activator and urokinase-type activators are two main plasminogen activators involved in the activation of plasmin (Cesarman-maus and Hajjar 2005).

In spite of the presence of fibrinolytic mechanism, the hemostatic system sometimes fails to degrade the fibrin clots leading to obstruction of the blood flow and ultimately, thrombosis. Anticoagulants are a class of drugs that are generally used to treat blood clots. For over 50 years, conventional anticoagulants such as heparin and warfarin have been used that comes with several limitations. Now the search for anticoagulants with less adverse effects and better efficacy has shifted towards molecules isolated from natural sources. Molecules, such as proteins (Gou et al. 2017), sulfated polysaccharides (Zhang et al. 2010), and peptides (Mukherjee et al. 2014) have shown to possess anticoagulant activity. This review focuses on peptides exhibiting potential anticoagulant effect.

Peptides with Known Targets

Natural Anticoagulant Peptides

Many anticoagulant proteins and peptides have been isolated and have been commercialized. Hirudin is one such natural peptide isolated from hematophagous medicinal leech, *Hirudo medicinalis*. The 65 residue peptide is a potent inhibitor of thrombin (Greinacher and Warkentin 2008). Desirudin is a recombinant derivative of hirudin that has been clinically approved for use in the prophylaxis of DVT (Graetz et al. 2011). Bivalirudin is another analog of hirudin consisting of 20 amino acids. It is a direct thrombin inhibitor (DTI) sold under the trade name ANGIOMAX®. Bivalirudin has been rigorously evaluated under clinical trials as a DTI anticoagulant in patients undergoing percutaneous coronary intervention (PCI). Several studies have proved the usefulness of bivalirudin with reduced risk of bleeding. Secemsky et al. (2016) have studied the effectiveness of bivalirudin over unfractionated heparin during PCI for ST-segment elevation myocardial infarction (STEMI). The study found that there was an inclination towards bivalirudin as choice of anticoagulant for PCI during initial stage of study. It was also reported that bivalirudin is associated with reduced in-hospital bleeding compared with UFH-GPI (glycoprotein IIb/IIIa inhibitors) treatment. Bivalirudin has also been reported to be a safe anticoagulant in the prevention of thrombosis in children. The target specific nature of the bivalirudin peptide has been shown to resolve (partially and completely) thrombi in almost 60% of the children assessed in clinical trials (Young 2015).

Waxman et al. (1990) isolated an anticoagulant peptide termed Tick Anticoagulant Peptide (TAP) from another hematophagous organism, *Ornithodoros moubata*, a soft tick. The peptide exhibited a potent factor Xa inhibitory activity. The 3D structure of TAP has been solved by both NMR (Lim-Wilby et al. 1995) and XRD (St. Charles et al. 2000) techniques. Apart from natural peptides, few bioactive peptides possessing anticoagulant property have been isolated from proteolytic digestion of proteins. Bioactive peptides are inactive protein fragments that gets cleaved and activated upon treatment with digestive proteases (Mohanty et al. 2016). Apart from exhibiting anticoagulant activity (Indumathi and Mehta 2016), such peptides also exert different biological activities including antimicrobial (Lopez-Exposito et al. 2006), anti-oxidative (Li et al. 2017), anti-hypertensive (Lee et al. 2010), anti-cancer (Umayaparvathi et al. 2014), etc.

Marine species contain many important bioactive compounds including peptides exerting therapeutic benefits. Few marine derived bioactive anticoagulant peptides

have been isolated and been reviewed by Nasri and Nasri (2013). For their survival, the hematophagous (blood-feeding) organism feed on blood and therefore they contain anticoagulant proteins targeting blood clotting proteases. Over the years, natural compounds from hematophagous organisms have been exploited by researchers to identify novel anticoagulant molecules. Organisms such as leeches and hookworms have shown to possess lead molecules for anticoagulation therapy (Koh and Kini 2008).

FXa Inhibiting Peptides

FXa plays a pivotal role in blood coagulation cascade since it sits at the juncture of intrinsic and extrinsic coagulation pathways. It performs the enzymatic cleavage of prothrombin, its substrate, to generate thrombin (Leadley jr et al. 2001). The FXa inhibitors are divided into two groups: direct and indirect inhibitors. Direct FXa inhibitors may bind directly to free FXa or bound FXa from the prothrombinase complex, whereas the indirect FXa inhibitors, target free FXa but with the assistance of antithrombin (Rupprecht and Blank 2010).

Borensztajn and Spek (2011) emphasized that, FXa antagonists will end up being drugs of significant importance in antithrombotic therapy, owing to the crucial role of FXa in pathophysiology. Many naturally occurring anticoagulant bioactive peptides are specific inhibitors of FXa (Table 1).

Few natural peptides having anticoagulant and antithrombotic activities have been isolated from the venom of

Table 1 Peptides with known targets

Target	Peptide sequence/name	Size (kDa)	Effective concentration	Source	Reference
FXa	AcAP5	8.7	43 pM[a]	*Ancylostoma caninum*	Capello et al. (1995), Stanssens et al. (1996)
	AcAP6	8.7	996 pM[a]	*Ancylostoma caninum*	Capello et al. (1995), Stanssens et al. (1996)
	AceAP1	9.7	2 nM[a]	*Ancylostoma ceylanicum*	Harrison et al. (2002), Mieszczanek et al. (2004b)
	AduNAP4	9.44	7.34 nM[a]	*Ancylostoma duodenale*	Gan et al. (2009)
	Ac-AP-12	9.1	nr	*Ancylostoma caninum*	Jiang et al. (2011)
	TNGYT	0.55	41.14 mg/ml[b]	*Scolopendra subspinipes* Mutilans	Kong et al. (2013a)
	EVXWWWAQLS	4.42	0.16 µM[a]	*Daboia russelii russelii*	Thakur et al. (2014)
	LTFPRIVFVLG	1.26	9.02 µM[a]	*Agkistrodon acutus*	Chen et al. (2015)
FXIa	AduNAP4	9.44	42.45 nM[a]	*Ancylostoma duodenale*	Gan et al. (2009)
	AcaNAP10	17	17.96 nM[a]	*Ancylostoma caninum*	Deng et al. (2010)
FVIIa/TF	AcAPc2 AcAPc3 AcAPc4	nr	35 pM[a] nr nr	*Ancylostoma caninum*	Stanssens et al. (1996), Mieszczanek et al. (2004a)
	AceAP1	9.7	nr	*Ancylostoma ceylanicum*	Harrison et al. (2002), Mieszczanek et al. (2004b)
	AcaNAP10	17	nr	*Ancylostoma caninum*	Deng et al. (2010)
FXIIa	TDGSEDYGILEIDSR (partial)	12.01	1.5 µM[c]	*Limanda aspera*	Rajapakse et al. (2005)
FIXa	GELTPESGPDLFVH-FLDGNPSYSLYA-DAVPR	3.34	42.6 µg/ml[b]	*Urechis unicinctus*	Jo et al. (2008)
Intrinsic tenase, Extrinsic tenase, Prothrombinase complexes	nr	7.5	0.057 µg/ml[c] 0.114 µg/ml[c]	*Acanthaster planci*	Koyama et al. (1998) Karasudani et al. (1996)
Intrinsic tenase, Prothrombinase complexes	EADIDGDGQVNY-EEFVAMMTSK	2.47	13.6 µg/ml[b] 42.9 µg/ml[b]	*Mytilus edulis*	Jung and Kim (2009)

nr not reported

[a]K_i value

[b]IC_{50} value

[c]Inhibitory concentration

centipede *Scolopendra subspinipes* Mutilans. One such peptide possessing FXa inhibition activity was isolated from centipedes' venom by Kong et al. (2013a). The peptide with the sequence TNGYT was purified from the venom of *S. subspinipes* Mutilans. TNGYT was able to prolong the normal mice aPTT (activated partial thromboplastin time) clotting time as well as whole blood clotting time in mice at a concentration and dose of 8 mg/ml and 20 mg/kg, respectively. Authors assessed the FXa inhibition activity towards its chromogenic substrate and proved that the peptide could inhibit the activity in a dose-dependent manner with an IC_{50} of 41.14 mg/ml. To further support the binding of the peptide with FXa, the authors employed the use of molecular docking to study the peptide's interaction with its target. The docking analysis revealed that the peptide interacts with three residues and one residue in the S1 and S4 substrate binding pockets of the FXa, respectively.

Few anticoagulant peptides have shown to exhibit the property of inhibiting multiple clotting factors. Thakur et al. (2014) isolated a peptide inhibiting thrombin and FXa, non-enzymatically, from the venom of *Daboia russelii russelii*. The peptide (Ruviprase), with a mass of 4423.6 Da, did not share its sequence with any other existing thrombin and FXa inhibitors. Ruviprase prolonged the plasma recalcification time as well as PT (prothrombin time) in a dose-dependent manner and exhibited potency equivalent to that of heparin and warfarin. Authors reported that the Ruviprase hindered the amidolytic activity of thrombin towards its chromogenic substrate with inhibition constant (K_i) value of 0.42 µM. It was also determined that the peptide failed to inhibit the FXa's activity towards its chromogenic substrate, but inhibited FXa's action against its physiological substrate, prothrombin with K_i value of 0.16 µM. Moreover, the peptide efficiently lengthened the whole blood clotting time and was found non-toxic to mice at a dose of 2 mg/kg of the body weight. The structural characteristics and interactions of the peptide have been studied in detail. The secondary structure of the peptide has been determined to consist of 61.9% of α-helices and the rest as random coils, using circular dichroism (CD) spectroscopy. Interaction of the peptide with thrombin and FXa was further confirmed by the use of fluorescence spectrometer and surface plasmon resonance (SPR) analyses.

Further, Chen et al. (2015) reported a peptide with potent FXa inhibition activity from the venom of viper *Agkistrodon acutus*. The venom was hydrolyzed initially with four different enzymes, out of which neutrase appeared to be the best with highest degree of hydrolysis and FXa inhibitory activity. The neutrase hydrolysate was then further purified to obtain the anticoagulant peptide. The peptide (ACH-11), with sequence LTFPRIVFVLG and 1260.77 Da mass, directly inhibited FXa and platelet aggregation, as well. The influence of the peptide on the inhibition of amidolytic activity of FXa towards its chromogenic substrate was determined to be 9.02 µM. Ex vivo assay proved that ACH-11 could efficiently prolong the normal rat aPTT clotting time from 20.58 ± 0.33 to 158.05 ± 6.89 s, at a dose of 3 mg/kg without prolonging the bleeding time at the same concentration in rats.

Apart from venoms of snakes and centipedes, there are few naturally occurring potent anticoagulant peptides derived from hookworms. Hookworms are blood-feeding nematodes that dwell in the mammalian small intestine. Peptides, also termed Nematode anticoagulant peptides (NAPs), have been isolated from the genus *Ancylostoma*, majorly from *Ancylostoma caninum*, a dog parasite, and few others from *Ancylostoma ceylanicum* and *Ancylostoma duodenale*. *A.duodenale* is a major human pathogen, responsible for hookworm infection.

Capello et al. (1995) isolated a major anticoagulant peptide inhibiting FXa from *Ancylostoma caninum* termed *Ancylostoma caninum* anticoagulant peptide (AcAP). The peptide is the first reported anticoagulant from NAP anticoagulants family. Though the AcAP was screened for activity against different serine proteases, it specifically inhibited the proteolytic activity of FXa alone by 98%. AcAP also inhibited FXa in the prothrombinase complex (factor Xa, factor Va, phospholipids) thereby it hindered the catalysis of FXa's physiological substrate, prothrombin to thrombin with an IC_{50} value of 336 pM. The peptide's doubling time concentration for PT and aPTT was compared with that of recombinant Hirudin (rHIR) and Tick Anticoagulant Peptide (rTAP) and was found to be 35 nM for PT assay. This proved that AcAP is more effective than rHIR and rTAP, which has a doubling time concentration of 410 and 1256 nM respectively. While the required concentration for aPTT was 85 nM, it was less potent than that of rHIR's 32 nM and better than rTAP's 2365 nM.

The authors further employed molecular cloning strategy and developed AcAP's recombinant versions (Stanssens et al. 1996). Based on cDNA translational products, three recombinant forms of AcAP were obtained. Of which, two forms, designated AcAP5 (or) rNAP5 and AcAP6 (or) rNAP6, directly inhibited free FXa and bound FXa in the prothrombinase complex and the other form, named AcAPc2 (or rNAPc2), targeted FVIIa/TF complex. Of the three forms, AcAP5 turned out to be the best inhibitor of FXa that inhibited the amidolytic activity by 100% with a K_i of 43 ± 5 pM, whereas AcAP6 inhibited FXa by 87% with a K_i of 996 ± 65 pM. AcAP 5 and 6 also inhibited the thrombin generation by FXa in the prothrombinase complex with an inhibitory constant of 144 ± 15 pM (AcAP5) and 207 ± 40 pM (AcAP6).

The sequence data showed that the AcAP inhibitors contain 10 cysteine residues, analogous to those found in *Ascaris* inhibitor family. AcAP 5 and 6 have arginine and

phenylalanine as their reactive site residues that inhibit FXa by binding at the enzyme's catalytic centre.

Later, the antithrombotic efficacy of rNAP5 in canine models of arterial and venous thrombosis (Rebello et al. 1997) and coronary artery thrombolysis (Rebello et al. 2000) were studied which showed that the peptide is a potent antithrombotic agent. The structure of NAP5 has also been solved in its bound form with Factor Xa, by X-ray crystallography (Rios-steiner et al. 2007). The structure showed that the peptide can bind at the active site or exosite of activated FX and at exosite of zymogen factor X.

Though anticoagulant peptides have been isolated from *A. caninum*, which primarily is a dog parasite, Harrison et al. (2002) reported an 84-residue long anticoagulant peptide (AceAP1) from *Ancylostoma ceylanicum*, a hookworm species infecting humans. Initially, it was established that AceAP1 partially inhibited only factor Xa, later it was also found to inhibit FVIIa/TF complex in the presence of FXa (Mieszczanek et al. 2004b). The peptide was found to be less potent than AcAP5 in the aPTT assay. The doubling time concentration for AceAP1 and AcAP5 was estimated to be 73 nM and 560 pM, respectively, which indicated that AcAP5 exhibits a 100-fold inhibitory potential than AceAP1. The peptide was found to inhibit the amidolytic activity of FXa which was determined through single-stage chromogenic substrate assay.

Mieszczanek et al. (2004b) further deciphered the AceAP1's interaction. The reactive site residue of AceAP1 was predicted based on sequence alignment with the known serine protease inhibitors from *Ascaris* inhibitor family. The *Ascaris* inhibitors use P1 reactive site to bind with the catalytic site of the target protease and form a protease-inhibitor complex. The predicted P1 reactive site residue of AceAP1 was Arg40. Employing the site directed mutagenesis technique; a mutant was constructed with Ala replacing Arg40. The mutant did not inhibit the catalytic activity of the FXa in the chromogenic assay, which confirmed the role of predicted reactive site residue in the inhibition of protease activity.

Gan et al. (2009) cloned and expressed an anticoagulant peptide (AduNAP4) from adult human hookworm, *Ancylostoma duodenale*. The 81 residue peptide, having a theoretically predicted molecular mass of 9437.7 Da, showed dual inhibitory activity against human clotting factors Xa and XIa. AduNAP4's sequence shared = < 50% similarity with other FXa inhibitor peptides from the *Ancylostoma* inhibitor family. The catalytic activity of FXa was hampered efficiently by the peptide with K_i value of 7.34 ± 1.74 nM. The peptide effectively prolonged the normal aPTT clotting time of human plasma with a doubling concentration of 23.1 nM.

An anticoagulant peptide (Ac-AP-12) specifically inhibiting clotting factor Xa was isolated from the hookworm, *Ancylostoma caninum* (Jiang et al. 2011). As observed in other NAPs, Ac-AP-12 contains 10 conserved cysteine residues that form five intramolecular disulphide bonds. With a molecular weight of 9.1 kDa, the peptide significantly prolonged the normal human blood plasma aPTT and PT clotting time. The doubling concentration for aPTT and PT assay was found to be 60 and 75 nM, respectively. The inhibition in amidolytic activities of factors Xa and VIIa by the peptide was studied using their specific fluorogenic substrates. It was observed that Ac-AP-12 could inhibit only FXa's activity.

FXIa Inhibiting Peptides

The human coagulation factor XI is the zymogen of serine protease FXIa that acts in activation of factor IX in the coagulation cascade (Emsley et al. 2010). The zymogen factor XI becomes activated by FXIIa, thrombin and by autoactivation in the presence of negatively charged surfaces (Al-horani et al. 2016).

Though FXIa is vital for thrombin generation, results from animal models and humans suggest that the deficiency of the factor does not impair the hemostasis (Lowenberg et al. 2010). Bane Jr. and Gailani (2014) highlighted the importance of targeting factor XIa because of its role in thrombosis and less contribution in hemostasis.

Anticoagulant peptides targeting human coagulation factor XIa have rarely been isolated. In a first of its kind, a peptide (AduNAP4) from NAP family possessing factor XIa (and FXa) inhibition activity was cloned and expressed (Gan et al. 2009). The amidolytic activity of FXIa was inhibited with an inhibition constant of 42.45 ± 3.25 nM. AduNAP4 is the first peptide with FXIa inhibitory activity from *Ancylostoma* inhibitor family, albeit there have been many peptides reported against other clotting factors.

Another peptide (AcaNAP10) from NAP family with 80 residues with twin inhibition activity targeting FXIa and FVIIa/TF was isolated from hematophagous nematode *Ancylostoma caninum* (Deng et al. 2010). At concentrations 92.9 and 28.8 nM, AcaNAP10 was able to double the aPTT and PT clotting times of normal human plasma. Though the chromogenic substrate assay was performed on major coagulation proteases, the peptide inhibited only the amidolytic activity of FXIa and FVIIa (in the presence of EGR-FXa) up to 100%. The IC_{50} value of FXIa inhibition was 25.76 ± 1.06 nM.

FVIIa/TF Inhibiting Peptides

The AcAPc2 peptide, one of the cDNA clones from the AcAP inhibitors (Capello et al. 1995), is a potent FVIIa/TF complex inhibitor (Stanssens et al. 1996). AcAPc2 scantily inhibited FXa activity by 18% though it fully inhibited the thrombin generation by the bound FXa in the

prothrombinase complex with a K_i of 2385 ± 283 pM, which was lesser compared to the other inhibitors AcAP 5 and 6. AcAPc2 prolonged the PT time more than AcAP 5 and 6. Later, AcAPc2's potential against FVIIa/TF complex was investigated. The results proved that the AcAPc2 was able to inhibit the FVIIa/TF complex in the presence of fixed concentration of FXa with a K_i of 35 ± 5 pM. AcAPc2 was also able to inhibit FVIIa/TF complex even when the active site blocked FXa (EGR-FXa) was used. Contrasting to AcAP 5 and 6, AcAPc2 binds to FXa at exosite, i.e., at a site away from catalytic center.

The mechanism of inhibition of FVIIa/TF complex by rNAPc2 was then identified by Bergum et al. (2001). They proposed that the peptide initially binds to FXa or FX, after which the rNAPc2/FXa complex attaches to the FVIIa/TF complex in the presence of phospholipid membrane leading to the formation of a quaternary complex.

Later, clinical trials of rNAPc2 were initiated primarily focussing on thromoprophylaxis (Lee et al. 2001; Moons et al. 2003). In many phase-II clinical trials, the peptide has been proved to be efficient with promising results (Vlasuk and Rote 2002). The crystal structure of the rNAPc2-FXa complex has been determined and is available at RCSB PDB (Murakami et al. 2007).

Mieszczanek et al. (2004a) cloned and purified two anticoagulant peptides targeting FVIIa/TF complex from *Ancylostoma caninum*. The cDNA clones from the RNA extract of *A.caninum* were amplified using the AcAPc2's (Stanssens et al. 1996) primers. After initial screening, two cDNA clones were obtained. The translated sequences of the clones revealed homology to AcAPc2. The two homologues with 84 and 80 residues were termed AcAPc3 and AcAPc4, respectively. The potency of both the peptides was compared to that of AcAP5, the first NAP. The AcAPc3 and 4 were less potent in prolonging the aPTT than AcAP5, whereas for the PT assay, all the three peptides prolonged the clotting time. Using chromogenic substrate assay, it was proved that the peptides AcAPc 3 and 4 did not inhibit the amidolytic activity of the FXa. But the peptides successfully inhibited the FVIIa/TF complex in the presence of exogenous FXa, with AcAPc4 exhibiting higher activity than AcAPc3. These findings suggested that the AcAPc 3 and 4 inhibited their target in a fashion similar to that of AcAPc2.

The results from the in vitro studies of AceAP1 peptide proved that it also exhibits FVII/TF inhibitory activity (Mieszczanek et al. 2004b). The complete inhibition in the FVIIa/TF complex was possible in the presence of exogenous FXa. The AceAP1's activity against FVIIa/TF complex was assessed using both WT and mutant peptides. The WT peptide showed inhibitory activity against the FVIIa/TF complex whereas the Arg40 mutant did not. PT assay was performed for both the peptides, in which only the WT peptide prolonged the PT clotting time but not the mutant peptide. This further proved that AceAP1's mechanism of inhibiting FXa and FVIIa/TF complex were discrete.

The peptide AcaNAP10, as discussed above, is also a potent inhibitor of FVIIa/TF complex (Deng et al. 2010). As with other FVIIa/TF inhibitors, AcaNAP10 also required the use of FXa (EGR-FXa). AcaNAP10 efficiently hindered the amidolytic activity of FVIIa/TF complex with an IC_{50} of 123.9 ± 1.71 nM. The authors suggested that the mode of action of AcaNAP10 might be analogous to that of AcAPc2 since both the inhibitors utilize active site blocked FXa (EGR-FXa) for the inhibition of FVIIa/TF complex (Buddai et al. 2002).

Factor X and Prothrombin Activation Inhibiting Peptides

Marine species possess various bioactive compounds such as peptides that have high therapeutic importance (Cheung et al. 2015). Koyama et al. (1998) isolated one such anticoagulant peptide, inhibiting the activation of prothrombin and factor X, from starfish *Acanthaster planci*. At concentrations greater than 28.5 and 143 µg/ml, the peptide (Plancinin) significantly prolonged the aPTT and PT of normal human plasma. Plancinin did not inhibit the amidolytic activity of thrombin, plasma kallikrein and other proteases involved in the intrinsic coagulation cascade. But it effectively hampered the factor X activation by both intrinsic and extrinsic FXase complexes. On the other hand, the peptide also inhibited the factor II (prothrombin) activation by targeting the prothrombinase complex at a concentration exceeding 0.114 µg/ml. These findings suggested that the plancinin's site of action may be found at the activation steps of FX and prothrombin.

Another such marine peptide possessing factor IXa inhibitory activity has been isolated from *Urechis unicinctus*, a marine echiuroid worm (Jo et al. 2008). From the soluble extracts of the worm, the *U. unicinctus* anticoagulant peptide (UAP) (3.34 kDa) was isolated, which lengthened the clotting time only on aPTT, but not PT or TT. UAP was able to effectively prolong the normal aPTT clotting from a control time of 32.3 ± 0.9 to 192.8 ± 2.1 s, in a dose-dependent manner. Since the peptide prolonged the aPTT time, the authors studied its interaction with the clotting factors involved in the intrinsic pathway of coagulation (FIXa, FX, FXIa, FXIIa) using Surface Plasmon Resonance (SPR) spectrometer.

The SPR sensorgrams revealed that the peptide specifically interacted with FIXa. The sensorgrams also showed that the molecular interaction between FIXa and FX, a step required for the activation of FX, was also inhibited by the peptide. UAP lowered the activity of FIXa in normal plasma in a dose-dependent manner with an IC_{50} value of 42.6 µg/ml. The work concluded that UAP could be interacting with FIXa and thereby blocking the activation of FX by the

intrinsic FXase complex. In addition, the same group isolated a 2.5 kDa anticoagulant peptide from *Mytilus edulis* (blue mussel) (Jung and Kim 2009). The 22-mer *M. edulis* anticoagulant peptide (MEAP) was able to prolong the both aPTT and TT (thrombin time) clotting time but not PT. The aPTT extended from 35.3 ± 0.5 to 321 ± 2.1 s and TT from 11.6 ± 0.4 to 81.3 ± 0.8 s. The initial analysis of the MEAP revealed that it could retard the activity of factors IX, X and II. Further, the SPR sensorgrams confirmed that the peptide interacted with FIX, FX and FII with K_d value of 11.3 ± 0.69, 21.4 ± 1.28, and 65.6 ± 1.97 nM, respectively. The amidolytic assay showed that MEAP hindered the catalytic conversion of prothrombin to thrombin by the prothrombinase complex (FXa/FVa/Phospholipids) (IC_{50} 42.9 µg/ml) as well as FX activation by intrinsic tenase complex (FIXa/FVIIIa/Phospholipids) with an IC_{50} of 13.6 µg/ml.

FXIIa Inhibiting Peptide

The contact activation pathway comprises of plasma proteins factor XII (FXII), plasma kallikrein (PK) and high-molecular-weight kininogen (HMWK). Upon binding to negatively charged or uncharged surface, the zymogen FXII gets converted to serine protease FXIIa (factor XII activated) (Kenne and Renné 2014).

Renné et al. (2005) first described the FXII as a crucial player for thrombus formation using FXII-deficient mice model. The study also changed the long-standing concept that factor XII has no role in coagulation in vivo. Therefore, targeting FXII has low or no risk of uncontrolled bleeding, since the absence of the clotting factor does not affect the normal hemostasis in animals and humans (Kenne et al. 2015).

Rajapakse et al. (2005) isolated an anticoagulant from the hydrolysate of a fish protein. *Limanda aspera* (Yellowfin sole fish) protein was treated with seven different proteases, whose hydrolysates prolonged only the aPTT time. A single-chain monomeric protein termed YAP (Yellowfin sole anticoagulant protein), of 12.01 kDa mass, was isolated from the α-chymotrypsin hydrolysate, which displayed the highest aPTT clotting time. Using specific factor activity assay, YAP was evaluated for activity against the clotting proteins from intrinsic coagulation pathway, since it prolonged aPTT. YAP was found to be inhibiting FXIIa activity by 62.4% at 1.0 µM concentration. It was shown that at a concentration of 1.5 µM, YAP inhibited 100% activity of FXIIa. Further, native PAGE analysis confirmed that YAP is a specific inhibitor of FXIIa.

Peptides with Unknown Targets

Though a number of peptides possessing anticoagulant activity have been isolated, only a few (discussed above) have been studied for their interaction with specific clotting factor(s) of the coagulation cascade.

Few potent peptides having displayed promising activity in vitro have not been studied for their specific targets (Table 2). Indumathi and Mehta (2016) reported a peptide with potent anticoagulant activity from pepsin hydrolysate of *Porphyra yezoensis* (Nori). The peptide with 16 residues and 1.77 kDa molecular mass, prolonged the normal aPTT clotting time from 35 to 320 s in a dose-dependent manner.

Sabbione et al. (2015) have described the anticoagulant effect of albumins, globulins, p-globulins and glutelins from amaranth proteins. The proteins were treated first with alcalase followed by trypsin to generate low molecular weight species. The maximum inhibition of thrombus formation

Table 2 Peptides with unknown targets

Peptide sequence	Assay	Effective concentration	Source	Reference
NMEKGSSSVVSSRMKQ	aPTT	0.3 µM[a]	*Porphyra yezoensis*	Indumathi and Mehta (2016)
SQL	aPTT	1 mg/ml[b]	*Scolopendra subspinipes* Mutilans	Kong et al. (2013b)
HDFLNNKLEYE	aPTT, PT, TT	0.01 mg/ml[b]	*Whitmania pigra*	Ren et al. (2016)
nr	aPTT, TT Thrombin inhibitory assay	0.05 mg/ml[b] 0.74 mg/ml[b]	*Amaranthus mantegazzianus* (Pass cv Don Juan)	Sabbione et al. (2015)
YQEPVLGPVRGPFPIIV	Thrombin inhibitory assay	4.6%[c]	Casein	Rojas-Ronquillo et al. (2012)
VEPVTVNPHE	Thrombin inhibitory assay	0.012 mg/ml[a]	*Buthus martensii* Karsch	Ren at al. (2014)
SWAQL, GNHEAGE, CFNEYE	Thrombin inhibitory assay	0.2 mg/ml[b]	Peanut protein	Zhang (2016)

nr not reported

[a]IC_{50} value

[b]Inhibitory concentration

[c]Inhibition efficiency ratio

was exhibited by albumins in aPTT test whereas glutelins and its hydrolysate displayed maximum inhibition of thrombus formation in TT and thrombin inhibitory assay. Kong et al. (2013b) described an anticoagulant peptide from *Scolopendra subspinipes* Mutilans. The peptide (346 Da) with the sequence SQL prolonged normal aPTT from 40 to 71 s at a concentration of 1 mg/ml. Furthermore, Ren et al. (2016) isolated an anticoagulant peptide (molecular mass 1422 Da) from leech *Whitmania pigra*. The peptide effectively prolonged clotting time on aPTT, PT and TT at a concentration of 0.01 mg/ml. The investigators suggested that the peptide could interact with clotting factors involved in the intrinsic and extrinsic cascade since it prolonged the all the three clotting times.

Apart from performing laboratory-based clotting assays (aPTT, PT and TT) for determining the potency of anticoagulant peptides, few researchers have employed microplate reader based antithrombotic assay developed by Yang et al. (2007). The method incorporates the use of fibrinogen solution (0.1%), test sample and thrombin in a microplate reader at a wavelength of 405 nm.

Antithrombotic peptides from Bovine caseins were studied by Rojas-Ronquillo et al. (2012) using the method described above. Casein was hydrolysed, by *Lactobacillus casei* strain Shirota from commercial yoghurt. After purification and treatment with gastric enzymes pepsin and trypsin, a 17-mer peptide showing potent antithrombotic activity was isolated. The peptide YQEPVLGPVRGPFPIIV corresponded to the fragment 193–209 of beta-casein. The work concluded that the peptide can inhibit the action of thrombin on fibrinogen.

Ren et al. (2014) isolated an anticoagulant peptide from an edible scorpion, *Buthus martensii* Karsch. Powdered protein from the scorpion was enzymatically hydrolysed using Alcalase AF enzyme. The hydrolysate was further purified by ion-exchange, gel-filtration and RP-HPLC chromatography to yield a decamer peptide of 1119.8 Da molecular mass. The peptide, with sequence VEPVTVNPHE, inhibited thrombin activity with IC_{50} of 0.012 mg/ml. In a similar work, three peptides were isolated from Alcalase 2.4 L hydrolysate of peanut protein. The peptides were shorter with the sequences of SWAQL, GNHEAGE and CFNEYE. These peptides inhibited thrombin activity at a concentration of 0.4 mg/ml (Zhang 2016).

Owing to the fact that most of the above discussed peptides are derived from protein hydrolysates of food sources, they may not have the side effects that synthetic drugs impart. Another advantage is the shorter size that supports the candidature of peptides as drugs. Despite these facts and given their strong in vitro anticoagulant activity, their specificity of inhibition is unknown. As the sites of action are unexplored, the further evaluation of the peptide drug candidates for clinical trials might be hindered. Further studies are therefore required to establish the inhibition mechanism of peptides towards the clotting factors involved in the coagulation cascade.

The specific factor inhibitory activity of peptides can be determined using chromogenic substrate assay. This is the most commonly used technique adopted in many studies (discussed in section 'Peptides with known Targets') to identify a peptide's specificity of inhibition. Chromogenic substrates are short synthetic peptides that contain residues analogous to that of the enzyme's natural substrate along with para-nitroaniline (p-NA). This assay is a colorimetric analysis requiring the use of purified peptide (ligand), clotting factor (protein), chromogenic substrate and a microplate reader. Upon cleavage by its specific clotting factor, the p-NA will release a yellow color compound with molar absorption coefficient at 405 nm. The intensity of the color produced will be directly proportional to the activity of the clotting factor (Budzynski 2001). Therefore, there will be a reduction in the intensity of the yellow if the peptide binds to the clotting factor under study. Thus the selectivity of the peptide for different clotting factor can be identified.

Further, the molecular interaction between the clotting factor and peptide can be deciphered using sensitive analytical technique such as surface plasmon resonance (SPR) (Thakur et al. 2014; Jo et al. 2008). The method involves pH scouting for the concentration of the ligand protein. Then clotting protein (ligand), to be analyzed, is immobilized on a carboxymethylated dextran sensor chip. Once the immobilization is complete, the peptide (analyte) is made to flow with the running buffer, through the protein-immobilized chip and the binding assay is performed. The output is obtained as a sensorgram plot containing response units (RU) against time with which the interaction kinetics can be analysed.

As the peptides with unknown targets possess promising anticoagulant activity, they deserve to be investigated further.

Conclusion

Better therapeutic agents with less adverse effects are the need of the hour for treating thrombotic disorders. The main drawback associated with conventional anticoagulants is the risk of bleeding. Peptides can be considered as an ideal choice for safer anticoagulation therapy given their target specific nature. Thus far, there is an increase in number of studies reporting anticoagulant peptides from various sources. Apart from hematophagous organisms, anticoagulant peptides have also been isolated from protein hydrolysates of food sources. Though there are several reports of food protein hydrolysate-derived anticoagulant peptides, the data on their specificity of inhibition are sparse and needs to be studied further.

The clotting factors responsible for fibrin development are inhibited by regular anticoagulants and this result in increased bleeding risk. Therefore, focus should be directed towards targeting factors XI and XII for safer anticoagulation therapy as enough evidences have suggested their substantial role in thrombosis and modest role in hemostasis. Novel peptides inhibiting these coagulation factors should be studied in detail and be given due importance. Even though many peptides have been extensively studied for their anticoagulant activity, to the best of our knowledge, the NAPc2 is the only peptide therapeutic which has been evaluated in the clinical trials. Hence there is a requirement for further detailed clinical studies of anticoagulant peptides.

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Compliance with Ethical Standards

Conflict of interest Azeemullah A. Syed and Alka Mehta declare that they have no conflict of interest.

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