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Tick anti-hemostatics: targets for future vaccines and therapeutics

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For ticks, a significant obstacle in obtaining a blood meal is counteracting the hemostatic system of the host. To this end, ticks have developed a broad array of anti-hemostatics, which is reflected in the presence of structurally related tick proteins with different functions. Disruption of blood flow which blocks successful tick feeding makes anti-hemostatics attractive targets for anti-tick vaccines. Moreover, the limited number of drugs currently available for a range of important cardio-vascular diseases makes ticks a potential source of novel therapeutics. This review aims to summarize the key features of tick anti-hemostatics, their structures, mode of action and possible future application as vaccines and novel therapeutic agents.

Tick anti-hemostatics

Hemostasis occurs following vascular injury and comprises three distinct events: vascular constriction, platelet aggregation and blood coagulation. Following hemostasis, clot dissolution occurs, enabling the resumption of blood flow after tissue repair (Figure 1). Ticks have developed a diverse array of anti-hemostatic agents that are considered to be essential for successful feeding and tick survival (Box 1). These anti-hemostatics have been found in salivary glands, saliva, eggs and hemolymph, appearing not only to prevent blood clot formation in the host, as well as the ingested blood meal, but also to regulate hemolymph coagulation in the tick itself.

In recent years, alternative ways to control ticks have been developed, including the employment of anti-tick vaccines with either concealed or exposed antigens [1]. Tick anti-hemostatics are predominantly exposed antigens because they are secreted from tick salivary glands by regulated exocytosis [2,3]. The importance of these inhibitors to successful tick feeding is exemplified by the finding of many such compounds in the tick salivary gland transcriptomes of *Ixodes pacificus* [4], *Ixodes scapularis* [5,6] and *Haemaphysalis longicornis* [7]. Although each tick species has its own feeding preference and most have probably developed a repertoire of anti-hemostatics applicable for its own requirements [8], it is evident that thrombin, factor X (FX) and platelet aggregation and adhesion are shared obstacles that every tick species has to overcome.

To date, many anti-hemostatic activities have been described in ticks but relatively few have been characterized fully. This review summarizes the current information available on well described tick molecules that target blood coagulation, platelet adhesion and aggregation and fibrin(o-gen)lysis. Finally, the possibilities of tick anti-hemostatics as anti-tick vaccines and novel therapeutics are discussed.

Inhibitors of blood coagulation

Thrombin inhibitors

The serine proteinase α-thrombin is the key enzyme in hemostasis (Figure 1). Thrombin interacts with its array of substrates, cofactors and receptors through its catalytic or primary site and secondary recognition sites, anion-binding exosite 1 and 2 (Figure 2).

A total of 17 tick thrombin inhibitors have been identified [apart from sequences available in expressed sequence tag (EST) libraries and published data wherein activity has not been established], of which the sequence information for seven is known (Table 1). Sequence alignments of ornithodorin, amblin, boophilin, bovine pancreatic trypsin inhibitor (BPTI) [9] and savignin [10] indicate conserved cysteine residues, protease-recognition loops and kunitz domains. Studies on the mode of action of savignin and ornithodorin (89% sequence similarity) propose a mechanism by which these inhibitors insert their N-terminal residues into the active site of thrombin, while the linker region is buried inside thrombin and the C-terminal domain helix binds to the basic fibrinogen recognition exosite 1 (Figure 2), which explains their slow-binding kinetics [10,11]. Although it is possible that amblin and boophilin share a similar mechanism of action, this remains to be proven. The high sequence and possibly even secondary and tertiary structure similarity make them valuable in studying possible cross-reactivity among species and so yielding potentially valuable insight for vaccine design.

By contrast, madanin 1 and 2 demonstrate no sequence similarities to any known thrombin inhibitors from

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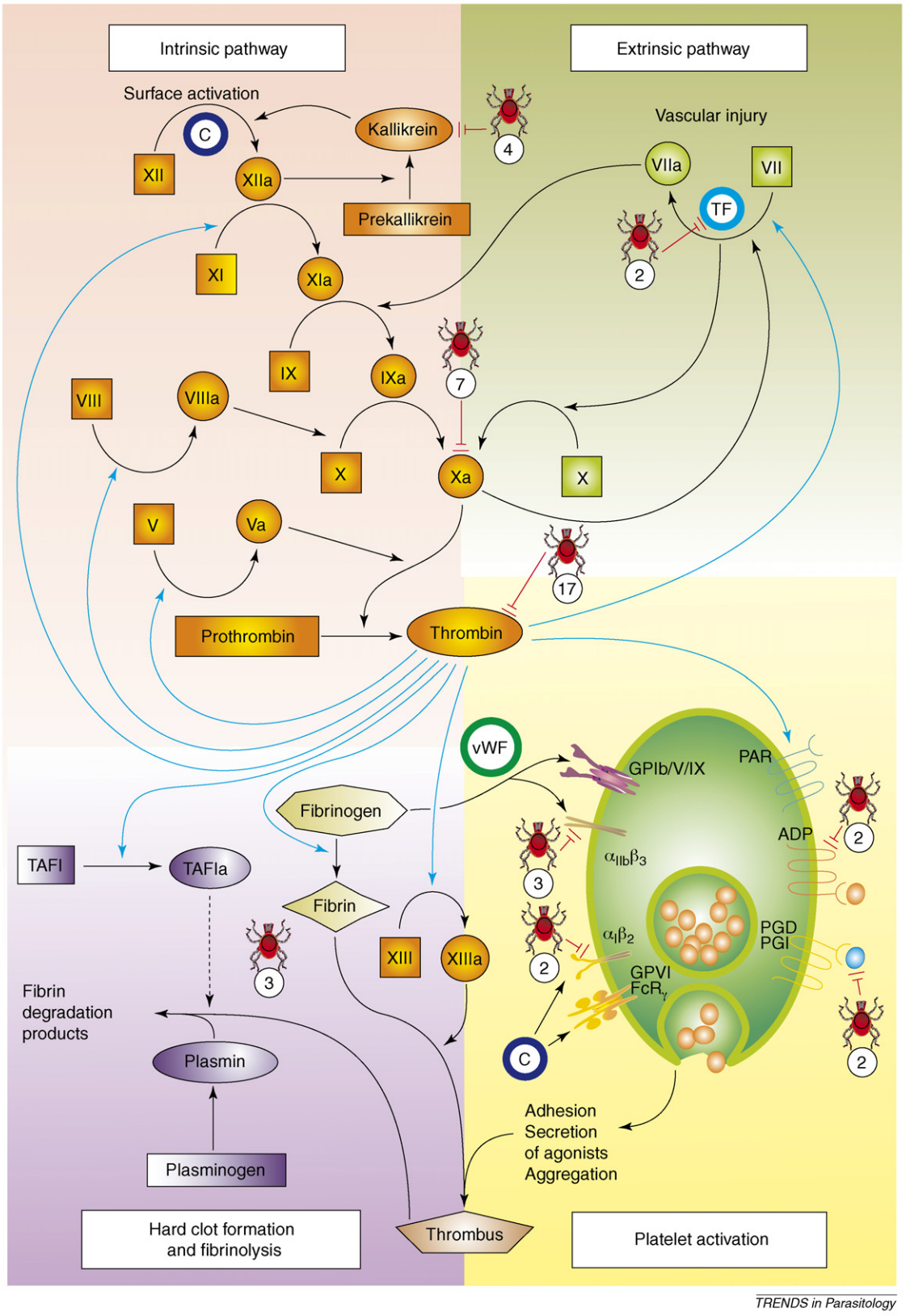


Figure 1. Sites of inhibition by tick anticoagulants. Hemostasis can be divided into basic components for the formation of a hemostatic plug. The first component is the coagulation cascade consisting of intrinsic (orange) and extrinsic pathways (green). They are activated by exposed C, and TF released by endothelial cells, respectively. The cascade consists of a series of proteolytically activated serine proteases and leads to prothrombin activation to thrombin. Thrombin activates several clotting factors and cleaves fibrinogen to form fibrin and also activates circulating blood platelets through PARs. The fibrin monomers are cross-linked by thrombin-activated factor XIII. Platelet activation (yellow) is initiated by adhesion of platelet membrane receptors to vWF and collagen. This leads to the binding of plasma fibrinogen through GPIIb/IIIa. Platelet activation results in secretion of platelet agonists, such as ADP, which cause platelet aggregation. In fibrinolysis, the TAFI increases clot dissolution time (broken arrow). In the figure, tick images indicate the sites of inhibition by various tick anti-hemostatics. The numbers below the tick images indicate the number of described tick inhibitors for these specific sites. Note the multiple functions of thrombin (blue arrows) and the central role of FXa in both the extrinsic and intrinsic pathways, rendering inhibitors of these two factors strong candidates as anti-tick-feeding vaccines and/or therapeutics. Abbreviations: $\alpha_{IIb}\beta_3$ and $\alpha_I\beta_2$, integrin receptors; ADP, adenosine diphosphate

Box 1. How do ticks obtain a blood meal successfully?

To acquire a blood meal, ticks penetrate the host's skin, damaging capillary and small blood vessels. This leads to the activation of the hemostatic system (blood coagulation and platelet aggregation) and, subsequently, the immune system of the host.

These host defense mechanisms need to be circumvented by ticks for successful feeding. Ticks do this successfully through the injection in the saliva of a large array of pharmacologically active molecules into the feeding lesion. These bioactive substances include immunomodulators, vasodilators, anticoagulants, inhibitors of platelet adhesion and aggregation and fibrin(ogen)olytic agents.

Following vascular damage, platelets are activated by a variety of agonists (such as ADP, thrombin and collagen) that bind to specific platelet membrane receptors. Once activated, the platelet $\alpha_{IIb}\beta_3$ fibrinogen receptor undergoes a conformational change and binds fibrinogen, leading to the cross-linking of activated platelets and subsequent aggregation, which forms a plug that seals the damaged blood vessel. The platelet activation and aggregation cascade can be targeted by ticks at several stages. First, ticks inhibit the activation of platelets by producing substances that either remove agonists and/or compete with the agonist for binding to its receptor. Second, aggregation is inhibited by tick molecules that block the binding of fibrinogen to $\alpha_{IIb}\beta_3$ receptors on activated platelets. Ticks even produce substances that are able to displace fibrinogen from its receptor, which results in the separation of aggregated platelets.

Host blood coagulation is a cascade of the consecutive activation of different serine proteases. Both the intrinsic (collagen-activated) and extrinsic (tissue factor-activated) pathways are activated by tick feeding. They converge at the activation of factor X with conversion of prothrombin to thrombin. Activated thrombin converts the plasma protein fibrinogen to fibrin, which forms a network that is the main constituent, together with platelets and erythrocytes, of the blood clot. Tick anticoagulants are serine protease inhibitors. To date, most identified tick anticoagulants are inhibitors of factor X (or FXa) and thrombin or of both.

In addition, ticks have developed proteases and protease inhibitors that affect fibrinolysis. This is essential for dissolving a clot that might have formed during feeding as well as preventing clotting of the ingested blood meal in the tick gut. Fibrinolysis is a natural process by which the host solubilizes fibrin clots. This is mediated by a serine protease, plasmin, which is derived from the proteolytic activation of plasminogen. Several proteases can activate plasminogen, the most important being tissue-type plasminogen activator (t-PA). Fibrinolysis is inhibited by thrombin-activatable fibrinolysis inhibitor (TAFI). Ticks are capable of accelerating fibrinolysis by producing an inhibitor that blocks the action of TAFI. Ticks also secrete metalloproteases in their saliva, which can degrade fibrin clots (fibrinolytic activity) as well as fibrinogen (fibrinogenolytic activity).

blood-feeding organisms. They contain clusters of acidic residues in the central region similar to those of hirudin, tsetse thrombin inhibitor anophelin and thrombostatin. These acidic residues are proposed to bind to exosite 1 [12].

H. longicornis serpin-2 (HLS-2) was identified in the hemolymph of *H. longicornis* and belongs to the serpin family. Although it exhibits anti-thrombin activity, it is probably involved in regulating hemolymph coagulation. Vaccination of rabbits with this concealed anti-hemostatic resulted in 44% mortality in nymphal and adult ticks [13]. HLS-1, another serpin-like antigen expressed in the midgut, inhibits the intrinsic pathway by an unknown mechanism. Vaccination of rabbits with recombinant HLS-1 resulted in 44% and 11% mortality of nymphs and adults, respectively [14]. These are the first examples of concealed tick anti-hemostatics as possible vaccine candidates.

Several human diseases are associated with the onset of thrombosis. Current anti-thrombotic agents have limitations and the development of new drugs continues to be researched actively [15]. The various tick thrombin inhibitors that interact with either the active site and/or exosite 1 are thus valuable as research tools in the rational design of small-molecule [16] and peptide [17,18] anti-thrombotics. Currently, only the synthetic derivative of hirudin, from the leech *Hirudo medicinalis*, has been approved for clinical use [15,19].

Inhibitors of tissue factor (TF), FX and activated FX (FXa)

Both the intrinsic and extrinsic pathways converge at FX, making it an attractive target for therapeutic and vaccine development. The best-characterized tick FXa inhibitor is tick anticoagulant peptide (TAP) from *Ornithodoros moubata*. To date, it has been produced [20–22], subjected to structure determination [23–25] and shown to be an effective anti-thrombotic in numerous animal models [26]. NMR [23] and crystal structures [24,25], in combination with the model suggested for the ornithodorin–thrombin complex [11], have been used to explain the mode of inhibition and binding to FXa. Similar to ornithodorin, the three N-terminal residues of TAP bind to the active site and two separate segments of TAP bind to an exosite of FXa [25] (Figure 2). The two-step binding mechanism of TAP is explained by an initial slow-binding to the exosite, which induces the N-terminal of TAP to rearrange and lock into the active site. A similar mechanism is proposed for the FXa inhibitor from *Ornithodoros savignyi* [27].

Sequence alignments of TAP and the FXa inhibitor from *O. savignyi* show strict conservation of cysteine residues and various areas of conservative amino acid substitutions. Their N-terminal amino acids, which are involved in forming the enzyme-inhibitor complex, are identical, whereas only eight out of the 15 residues involved in exosite binding are identical. These inhibitors do differ, however, regarding residues 20–28, which are proposed by homology modeling to stabilize the structure through side-chain interactions with the hydrophobic C-terminal helix [27].

TF pathway inhibitors (TFPIs) are examples of FXa-dependent inhibition of the coagulation pathway through inhibition of the factor VIIa (FVIIa)–TF complex. By means of cDNA library sequencing and BLAST (basic logical alignment search tool) analysis, TFPI homologues, named ixolaris and penthalaris, were identified in *I. scapularis*. These are distinct both functionally and structurally from its endogenous counterpart human TFPI. Ixolaris is a two kunitz domain protein, which inhibits prothrombinase assembly *in vitro* by binding to the FX heparin-binding site. It is useful in studying FX exosites as well as the various interactions among factor Va (FVa), FVIIa, TF, FX, FXa and prothrombin [28,29]. Most importantly, ixolaris binds to FX, γ-carboxyglutamic acid domainless FXa (des–Gla–FXa) as well as active site-blocked FXa [29,30].

receptor; C, collagen; GPIb/VI/X and GPVI/FcRγ, glycoprotein-receptor complexes; PAR, protease-activated receptor; PGD/PGI, prostacyclin receptors; TAFI, thrombin-activatable fibrinolysis inhibitor; TF, tissue factor; vWF, von Willebrand factor.

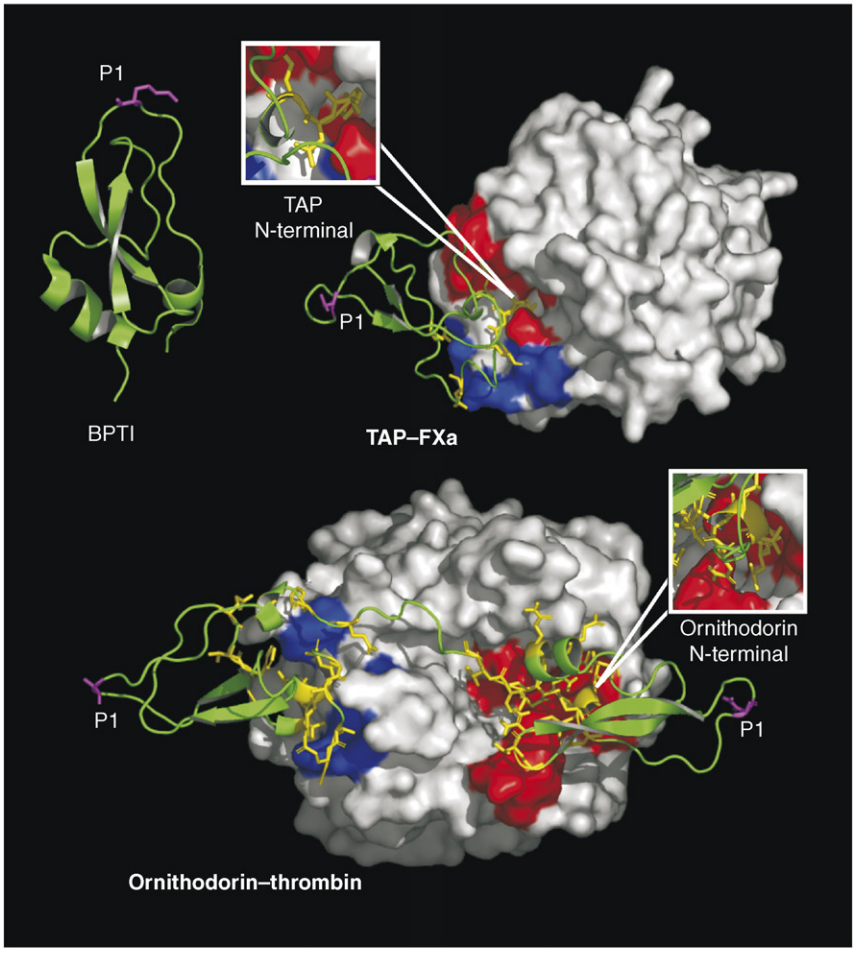


Figure 2. Structures of BPTI, TAP–FXa and ornithodorin–thrombin complexes. Crystal structure data for only two tick anti-hemostatics, TAP and ornithodorin, are known. These tick inhibitors (green) are shown docked into their respective target enzymes. The exosites (blue) and active sites (red) of both thrombin and FX are shown. Inhibitor residues interacting with these sites are indicated in yellow. Note that, unlike the canonical mechanism, it is not the P1 residues (purple) of TAP and ornithodorin but their N-terminals (yellow) that are inserted into their respective target enzymes. In both inhibitors, the conserved BPTI-fold (upper left corner) is visible. Structures were obtained from the RCSB protein databank (PDB IDs 1bpi, 1KIG and 1toc) and modeled using PyMol™ (v.097).

Recent studies identified ixolaris-related sequences in *I. scapularis* [5] and *I. pacificus* [4], which remain to be characterized. Ixolaris tested *in vivo* using a rat thrombus model displayed a potent anti-thrombotic effect, a long plasma half-life (34–40 h) and minor bleeding [30]. Further studies in higher vertebrate models might confirm the efficacy of ixolaris as a therapeutic agent.

Penthalaris 1, 2 and 3 identified in *I. scapularis* each contains five kunitz-domains. Penthalaris 1 binds FX and FXa tightly as scaffolds for its inhibition of the FVIIa–TF complex [31]. Structurally, penthalaris resembles kunitz-type inhibitors in that it contains positive residues at the P1 positions of kunitz domains 1, 2 and 5 but it differs in the residue found at P1 of kunitz domain 3 and the number of disulphide bridges. The penthalaris group of proteins has been expanded recently to include a further eight sequences in *I. scapularis*, which are assumed to be derived from six different genes [5]. Moreover, penthalaris homologues were also identified in *I. pacificus* [4]. All of these proteins remain to be characterized further.

The presence of another novel family of FX inhibitors exemplifies the importance of tick anti-hemostatics in successful feeding. The FXa inhibitor salp14, together with some 30 paralogues, has been identified in *I. scapularis*. Salp9 putative anticoagulant (Salp9Pac), which shares 70% identity with salp14, has no anticoagulant activity and is weakly immunogenic. Salp14 is, however, immunodominant [32]. Immunoprecipitation of tick saliva with anti-salp14 antibodies eliminated 87% of the anticoagulant activity [32]. Silencing of salp14 and its paralogues resulted in a 60–80% reduction of FXa activity and a 50–70% decline in engorgement weights of adult female *I. sacpularis*, thus emphasizing its role during adult tick feeding [33]. By contrast, salp14 silencing in naïve nymphs had no effect on the engorgement weights, suggesting that nymphs and adults feed by different mechanisms [34]. The presence of orthologues for ixolaris, penthalaris and salp14 make them an attractive target for anti-tick vaccines. Immunization with a single protein might, however, result in cross-reactivity with structurally similar but functionally different tick antigens.

Kallikrein–kinin system inhibitors

Plasma kallikrein is a serine protease that participates in the intrinsic blood coagulation pathway and production of kinins from kininogens, thus regulating various biological

Table 1. Properties of tick anti-hemostatics[a,b]

Species and name	Accession numbers	Source	Target	Mass (kDa)	Recombinant expression	Kinetics	Structure
Thrombin inhibitors							
Amblyomma americanum Americanin	n/d	SG	Thrombin	12	No	Ki: 0.073 ± 0.0054 nM Slow, tight binding, competitive	n/d
A. hebraeum Amblin	AY437082, AAR97367	HL, Synganglion	Thrombin	17.4	Yes	Ki: 0.02 ± 0.005 μM	Two kunitz-like domains
Amblyomma variegatum	n/d	SG	Thrombin	n/d	No	n/d	n/d
Boophilus calcaratus Calcaratin	n/d	SG	Thrombin	14.5	No	n/d	n/d
B. microplus Boophilin (G2 and H2)	AJ30447, AJ304446 CAC82582, CAC82583	Unknown	Thrombin	G2: 14 H2: 14	No	Ki: 1.8 nM	Two kunitz-like domains
B. microplus BmAP	n/d	Saliva	Thrombin active site	55–60	No	Ki: 100 nM	n/d
B. microplus Microphilin	n/d	Saliva	Thrombin exosite 1	1.8	No	IC50: 5, 5 μM	n/d
Hyalomma dromedarii	n/d	Egg, embryos	Thrombin	n/d	No	n/d	n/d
H. dromedarii NTI-1	n/d	Nymphs	Thrombin and FXa	3.2	No	Thrombin Ki: 11, 7 μM Noncompetitive	n/d
H. dromedarii NTI-2	n/d	Nymphs	Thrombin and FXa	15	No	Thrombin Ki: 211 nM Competitive	n/d
H. longicornis Madanin 1	AY245439, AAP04349	Saliva	Thrombin exosite 1	6.8	Yes	Kd(app): 25 nM Competitive	n/d
H. longicornis Madanin 2	AY145440, AAP04350	Saliva	Thrombin exosite 1	7.1	Yes	Kd: 2.96 μM Kd(app): 34.5 nM Competitive	n/d
H. longicornis Chimadanin/ HLSG-g21	AB218911, BAE00177	SG	Thrombin (catalytic- and exo-site?)	7.5	Yes	n/d	n/d
H. longicornis HLS2	AB162827, BAD11156	HL	n/d	44	Yes	n/d	Serpin-like
I. ricinus Ixin	n/d	WT extract	n/d	7	No	n/d	n/d
O. moubata Ornithodorin	P56409	SG	Thrombin active site and exosite 1	12.6	No	Ki: 10 pM Slow, tight binding, competitive	Two kunitz-like domains Distorted BPTI-like fold
O. savignyi Savignin	AF321524, AAL37210	SG	Thrombin active site and exosite 1	12.4	No	Ki: 4.9 ± 1.4 pM Slow, tight binding, competitive	Two kunitz-like domains Single chain linker
FX, FXa and tissue factor pathway inhibitors (TFPI)							
H. dromedarii	n/d	Nymphs	FXa	15	No	Ki: 134 nM Uncompetitive	n/d
Hyalomma truncatum	n/d	SG	FXa	17	No	Ki: 0.69 nM Uncompetitive	n/d
I ricinus	n/d	WT	FXa	n/d	No	n/d	n/d
I. scapularis Ixolaris	AF286029, AAK83022	SG	FX (heparin-binding exosite)	15.7	Yes	Kd (FXa): 520 ± 18 pM	Two kunitz-like domains BPTI-like fold
I. scapularis Penthalaris	AF483716, AAM93638	SG	Unknown (TFPI)	35	Yes	IC_{50}: ~100 pM	Five tandem kunitz domains
I. scapularis Salp14	AF209921, AAK97824	Saliva	FXa	14	Yes	IC_{50}: ~140 nM	n/d
Rhipicephalus appendiculatus	n/d	SG	FXa, not the active site	65	No	n/d	n/d
O. moubata TAP	GI84694, GI1421459	SG	FXa	6	Yes	Ki: 0.18 nM Slow, tight binding, competitive	BPTI-kunitz

Table 1 (*Continued*)

Species and name	Accession numbers	Source	Target	Mass (kDa)	Recombinant expression	Kinetics	Structure
O. savignyi FXI	GI25991384, AF452887	SG	FXa	7	Yes	Ki: 0.83 ± 0.1 nM Slow, tight binding, competitive	Homology models indicate structure similar to TAP
Uncharacterized intrinsic (INPI) and extrinsic (EXPI) pathway inhibitors							
B. microplus	n/d	Eggs, larvae	INPI, EXPI	n/d	No	n/d	n/d
Dermacentor andersoni	n/d	SG	INPI, EXPI	n/d	No	n/d	n/d
H. longicornis HLS1	Full-length sequence published	Gut	INPI	41	Yes	n/d	Serpin-like
Rhipicephalus evertsi evertsi	n/d	SG	EXPI	n/d	No	n/d	n/d
O. savignyi BSAP1	n/d	SG	EXPI	9.3	No	n/d	n/d
O. savignyi BSAP2	n/d	SG	EXPI	9.7	No	n/d	n/d
Kallikrein-Kinin inhibitors							
B. microplus BmTI-A	P83609_1, P83609_2	Larvae	Unknown	18	Yes (chimera, synthetic gene)	Ki (Elastase): 1.4 nM Ki (Plasma kallikrein): 120 nM	BPTI-kunitz like
B. microplus BmTI-D	P83607 Partial N-terminal	Larvae	Unknown	8	No	Ki (Plasma kallikrein): 12 nM	BPTI-kunitz like
R. sanguineus RsTIQ2	Partial N-terminal	Larvae	Unknown	12	No	Ki (Elastase): 1.3 nM Ki (Plasma kallikrein): 22 nM	BPTI-kunitz like
H. longicornis Haemaphysalin	Full-length sequence published	SG	FXII, FXIIa and HK	16	Yes	Kd (FXIIa): 2.49 ± 0.73 nM	Two kunitz-like domains
Platelet aggregation/adhesion inhibitors							
D. variabilis Variabilin	Full-length sequence by Edman degradation	SG	$\alpha_{IIb}\beta_3$	5	No	IC_{50}: 157 nM	Related to snake neurotoxin family
O. moubata Apyrase	n/d	Saliva	ADP	n/d	No	n/d	n/d
I. scapularis Apyrase	n/d	Saliva	ADP	n/d	No	n/d	n/d
O. moubata Moubatin	L04129, AAA29432	WT	Unknown (collagen-dependent platelet activation)	17	Yes	IC_{50}: 100 nM	Lipocalin-like
O. moubata TAI	n/d	SG	$\alpha_1\beta_2$	15	No	IC_{50}: 8 nM	n/d
O. moubata Disagregin	P36235	SG	$\alpha_{IIb}\beta_3$	6	No	Kd: 39–42 nM IC_{50}: 104 ± 17 nM	BPTI-kunitz like
O. savignyi Apyrase	n/d	SG	ATP, ADP	67	No	Km: 0.93 mM Kcat: $3.11e^{-6}$/s	n/d
O. savignyi Savignygrin	AAM54047, AF452885 AAM54048, AF452886	SG	$\alpha_{IIb}\beta_3$	7	No	IC_{50}: 50–130 nM Kd: 50–70 nM	BPTI-kunitz like
Fibrin(ogen)olytic agents							
R. bursa TCI	AY794405, AAW72225	WT extract	Plasma carboxy-peptidase B	7.8	Yes	Ki (human TAFI): 1.2 nM Tight binding	C-terminal resembles metallo-carboxypeptidase inhibitors
I. scapularis MP1	AY264367, JC7969	SG	Fibrin or fibrinogen	36.9	No	n/d	n/d
I. ricinus Iris	AJ269658, CAB55818	Saliva	Elastase	44	Yes	Ka (HLE): 4.7e6	Serpin

[a]Abbreviations: HK, high molecular weight kininogen; HL, hemolymph; HLE, human leukocyte elastase; n/d, not determined; SG, salivary gland; TAFI, thrombin-activatable fibrinolysis inhibitor; TAP, tick anticoagulant peptide; TCI, tick carboxy peptidase inhibitor; WT, whole tick.
[b]A version of this table including full references can be found in the supplementary material online.

activities [35]. Although the *in vivo* significance of this pathway in blood coagulation is questionable, it is included because many different tick inhibitors (Table 1) affecting this pathway exhibit anti-hemostatic activity.

Boophilus microplus trypsin inhibitor A (BmTI-A), a BPTI–kunitz serine protease inhibitor from larvae, affects the intrinsic pathway and inhibits trypsin, neutrophil elastase and human plasma kallikrein [35]. A more potent BPTI–kunitz family kallikrein inhibitor, BmTI-D, which does not affect elastase, was also identified. Immunization trials with BmTIs showed 72.8% efficacy to interfere with engorgement of *B. microplus* ticks feeding on cattle [36]. Characterization of the response showed that BmTI-A was the most antigenic and that BmTI-D was only weakly antigenic [37]. Expression of a BmTI-A–carrapatin synthetic chimera resulted in a potent kallikrein-specific inhibitor, which also exhibits cysteine-proteinase inhibition [38]. Although this construct was unable to elicit an immune response in cattle, it is postulated to be a valuable tool in the development of specific plasma kallikrein inhibitors [38]. Because BmTIs have not been found in tick salivary glands or saliva but are concentrated in eggs and larvae, they might be an example of a concealed antigen that might induce a stage-specific immune response. The identification of *Rhipicephalus sanguineus* trypsin inhibitor Q2 (RsTIQ2), a homologous BPTI–kunitz kallikrein and elastase inhibitor, provides the opportunity to exploit this group of inhibitors (BmTIs and RsTIs) as possible cross-protective vaccines. A preliminary study indicated that BMTI-antisera cross-reacts with RsTIs, with the strongest response against RsTIQ7 [39].

Hemaphysalin (from *H. longicornis*) is another kunitz-type inhibitor that inhibits the reciprocal activation between FXIIa and kallikrein [40]. The C-terminal domain mediates inhibition by binding to high molecular-weight kininogen and FXII in a Zn^{2+}-dependent manner, preventing them from binding to the activated cell surface [41]. Although it did not inhibit the amidolytic activities of FXIIa, FXIa, FIXa, FXa or thrombin, it did prolong plasma clotting time, probably by inhibiting the generation of FXIa, FXIIa, FXa and kallikrein [40].

Inhibitors of platelet aggregation and adhesion

Vascular injury exposes agonists, such as collagen and von Willebrand factor (vWF), facilitating the adhesion of platelets to the site of injury (Figure 1). Ticks are able to circumvent platelet responses by means of apyrases and inhibitors of collagen- and fibrinogen-dependent platelet aggregation (Figure 1, Box 1).

Apyrases facilitate the hydrolysis of ATP and ADP to monophosphate derivatives and are capable of dissolving aggregated platelets [42]. To date, only partial sequences for putative apyrases have been described for *I. scapularis* [6], *Argas monolakensis* (GenBank ABI52806) and *O. savignyi* (C. Stutzer, MSc thesis, University of Pretoria, 2007), indicating that they are members of the 5′-nucleotidase or CD73 family [6]. A 5′-nucleotidase enzyme from *B. microplus* hydrolyzes both ATP and ADP [43], although its effect on platelet aggregation and adhesion remains to be confirmed. Expression library immunization (ELI) with the putative nucleotidase from *I. scapularis* showed a 50% reduction in tick infestation [44]. Antiserum raised against the *O. savignyi* apyrase indicated it to be antigenic [45]. Cross-protection and adverse effects have not been investigated on the host.

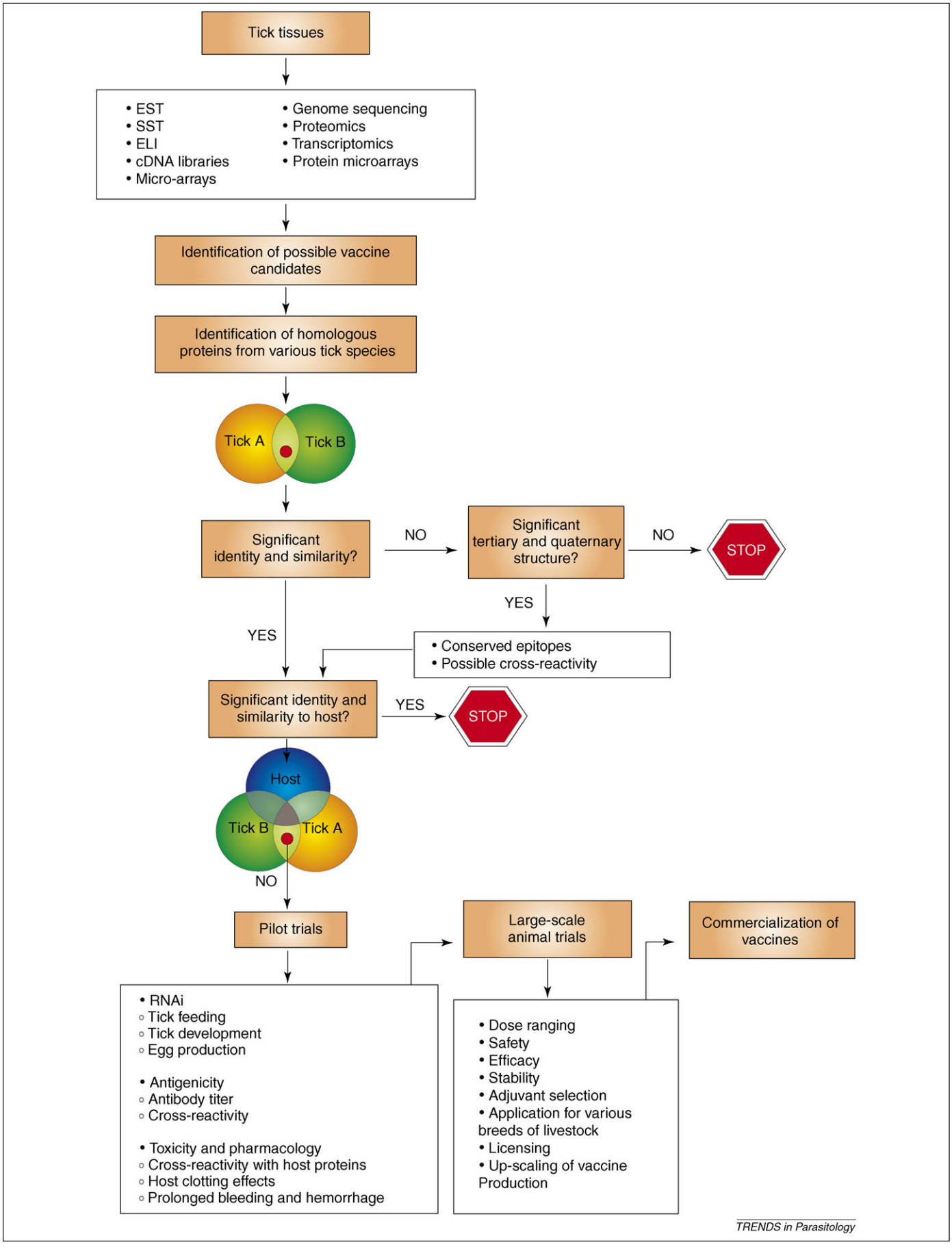
Tick-adhesion inhibitor (TAI) and moubatin inhibit collagen-dependent platelet aggregation [46,47]. TAI inhibits adhesion of the $\alpha_2\beta_1$ integrin (GPIa/IIa) to collagen but does not affect aggregation [46]. By contrast, moubatin (a lipocalin) does not affect platelet adhesion to collagen but inhibits collagen-induced platelet activation by an unknown mechanism, possibly by pathway inactivation or association with a glycoprotein–receptor complex [46,48].

Fibrinogen-dependent platelet activation relies on the interaction of glycoprotein complex Ib/IX/V (GPIb/IX/V) and the integrin $\alpha_{IIb}\beta_3$ with fibrinogen or vWF to adhere platelets to the growing thrombus [49]. The recognition of a ligand by the integrin $\alpha_{IIb}\beta_3$ is achieved through a recognition motif Arg–Gly–Asp (RGD) or a dodecapeptide (HHLGGAKQAGDV). Several tick proteins have been characterized that inhibit the activation of platelets through the integrin $\alpha_{IIb}\beta_3$ [50–52], such as savignygrin and disagregin (Table 1). Savignygrin contains a RGD motif that is proposed to bind to the fibrinogen-binding site [51]. Disagregin lacks these motifs and is therefore unique in both its sequence and mechanism [50]. High sequence similarity between savignygrin and disagregin (~60%) might infer possible cross-protection within argasid families [51]. Ixodid antagonists related to snake neurotoxins have been identified for *Dermacentor variabilis* (variabilin) [52] and from transcriptome analysis for *I. pacificus* and *I. scapularis* (ixodegrins), respectively [4]. Although both ixodegrins and variabilin exhibit their function through a RGD motif, variabilin lacks the cysteine-stabilized loop that is involved in presentation of the functional motif [52]. To date, only the antigenicity of savignygrin has been demonstrated [45]. The efficacy of platelet aggregation and adhesion inhibitors as vaccine candidates remains unaddressed. To date, only potato apyrase has been tested as a possible therapeutic using an *in vivo* rabbit thrombosis model [53].

Fibrin(ogen)olytic agents

Tick carboxypeptidase inhibitor (TCI) from *Rhipicephalus bursa* accelerates fibrinolysis *in vitro* [54]. TCI shows no amino acid sequence homology to any other proteins, other than its C-terminus, which resembles other metallo-carboxypeptidase inhibitors. The structures of recombinant TCI (rTCI) bound to bovine carboxypeptidase A and human carboxypeptidase B indicated that TCI consists of two structurally similar domains, each consisting of an α-helix followed by an antiparallel β-sheet. TCI binds to mammalian carboxypeptidases in a double-headed manner, whereby the C-terminus binds to the active site and the N-terminal domain binds to an exosite [55].

An *Ixodes ricinus* immunosuppressor, iris, another serpin, was shown recently to affect fibrinolysis *in vitro* by binding to its natural substrate, leukocyte elastase. Apart from its high affinity for elastases, it also inhibited serine proteases (tissue-plasminogen activator, thrombin and FXa) and increased platelet adhesion. The inhibition of elastase is probably more important in preventing tick rejection than being a true anti-hemostatic. This is the


Tick tissues
• EST
• SST
• ELI
• cDNA libraries
• Micro-arrays
• Genome sequencing
• Proteomics
• Transcriptomics
• Protein microarrays
Identification of possible vaccine candidates
Identification of homologous proteins from various tick species
Tick A
Tick B
Significant identity and similarity?
NO
Significant tertiary and quaternary structure?
NO
STOP
YES
YES
• Conserved epitopes
• Possible cross-reactivity
Significant identity and similarity to host?
YES
STOP
Host
Tick B
Tick A
NO
Pilot trials
Large-scale animal trials
Commercialization of vaccines
• RNAi
◦ Tick feeding
◦ Tick development
◦ Egg production
• Antigenicity
◦ Antibody titer
◦ Cross-reactivity
• Toxicity and pharmacology
◦ Cross-reactivity with host proteins
◦ Host clotting effects
◦ Prolonged bleeding and hemorrhage
• Dose ranging
• Safety
• Efficacy
• Stability
• Adjuvant selection
• Application for various breeds of livestock
• Licensing
• Up-scaling of vaccine Production
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first description of an ectoparasite serpin affecting both host hemostasis and immunity [56].

Finally, metalloproteases with α-fibrinogenase and fibrinolytic activities have been reported from *I. scapularis* [57] and *O. savignyi* (M. Mahlaku, MSc Thesis, University of Pretoria, 2002). The metalloprotease from *O. savignyi* also disaggregates platelets, probably by hydrolyzing the fibrinogen cross-linked platelets.

Tick anti-hemostatics as possible vaccine candidates

A large number of tick anti-hemostatics have been identified, isolated and characterized to date, however, sequence, kinetic and structural data are lacking for the vast majority (Table 1). The application of high-throughput techniques (Figure 3) in combination with bioinformatic and high-throughput structural analysis and the rapid expansion of sequence and structural databases should prove valuable in the identification of new tick anti-hemostatics.

These new sequences will contribute to the identification of more orthologous families, which are highly attractive as vaccine candidates owing to the high probability of cross-reactivity. To date, five such orthologous families have been identified: the BPTI–kunitz thrombin inhibitors, ixolaris, penthalaris, salp14 and BmTIs/RsTIs. During vaccination trials, targeting a family, such as salp14 and BmTIs, resulted in 50–70% and 72.8% efficacy to interfere with tick feeding and development, respectively. To date, targeting exposed antigens seems to be more successful in that vaccination with the concealed antigens HLS-1 and HLS-2 resulted in only 11–44% mortality. Vaccination or even silencing of genes encoding a tick anti-hemostatic simultaneously with a cocktail of other possible tick antigens or genes has not yet been investigated.

Apart from ixolaris, which has been shown to be functionally and structurally distinct from its human counterpart TFPI, little effort has been made to ensure that tick anti-hemostatics differ from the counterparts in their hosts to limit any possible side effects. The nature of the immune response elicited by these inhibitors in vaccination trials is still unclear, as is the possible systemic responses that might result from the introduction of recombinant inhibitors. Thus, the interaction of antibodies raised by tested targets has to be evaluated in terms of what they recognize and what negative effects might be incurred in the vaccinated host.

Occurrence of gene duplication in both ixodid and argasid tick anti-hemostatics has been described. This can be advantageous for universal vaccine design if conserved epitopes and amino acids are maintained and recognized by the host immune response. Identification of cross-reactive homologues might be aided by bioinformatic and phylogenetic analysis.

Increased use of RNA interference in tick research will probably result in the validation of more tick anti-hemostatics as suitable vaccines. However, it must be noted that, during gene silencing, homologous genes that possibly encode functionally different proteins might be silenced and this could affect processes other than anticoagulation. This will complicate the validation of a single anti-hemostatic. Aptamers, which are capable of discriminating between enzyme isotypes [58], might be valuable in contributing to RNAi studies. Combining RNAi studies with proteomic and gene expression analysis will not only enhance our knowledge regarding tick responses during gene silencing but could also expand our repertoire of possible vaccine candidates.

Tick anti-hemostatics as novel therapeutics

Despite the impressive amount of inhibitors identified to date, there is only a limited number in clinical use. This can be ascribed to the vast number of criteria these compounds must meet before being allowed for use clinically, such as high selectivity, metabolic stability, low serum protein binding, low toxicity and slow elimination from the blood stream, among others [16]. To date, only preliminary validation of TAP and ixolaris as possible therapeutics has been performed *in vivo* using animal models. TAP, which has been studied since the 1990s, has never been tested in humans for various reasons, including a slow onset of action and because it is antigenic [15,59]. Using TAP as a scaffold for the rational design of therapeutics or even for the identification of smaller, less antigenic functional domains has not been investigated. Only a single study in rats has been performed for ixolaris and this awaits future validation.

Because tick anticoagulants bind to their respective target molecules in a highly specific manner, they are attractive molecular tools. Examples include ornithodorin, which has been used successfully to map thrombin exosites [11], and ixolaris, which has added to our understanding of prothrombinase complex formation [29]. Therefore, tick anti-hemostatics could enhance our understanding of host coagulation as well as tick physiology. Even if tick-derived anticoagulants are never approved for clinical use, any structural and functional information obtained from investigating these inhibitors will aid in the design of either synthetic peptides or peptidomimetics that might be developed further as novel therapeutics.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pt.2007.07.005.

Figure 3. Flowchart for the possible identification and verification of anti-tick vaccine candidates. Various techniques are suitable for the initial identification of vaccine candidates. Suitable candidates should share regions of identity or similarity between tick species to ensure cross-protection. They should, however, not share identity with host proteins, which will exclude unwanted side effects. Verification is done by means of pilot vaccine trials and subsequent large-scale field trials before commercialization. Abbreviations: ELI, expression library immunization; EST, expressed sequence tag; SST, signal sequence trapping.

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