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Platelet aggregation Inhibitors from Hemetophagous Animals

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Abstract

Salivary glands from blood-sucking animals (*e.g.*, mosquitoes, bugs, sandflies, fleas, ticks, leeches, hookworms, bats) are a rich source of bioactive molecules that counteract hemostasis in a redundant and synergistic manner. This review discusses recent progress in the identification of salivary inhibitors of platelet aggregation, their molecular characterization, and detailed mechanism of action. Diversity of inhibitors is remarkable, with distinct families of proteins characterized as apyrases that enzymatically degrade ADP or as collagen-binding proteins that prevent its interaction with vWF, or platelet integrin $\alpha 2\beta 1$ or GPVI. Molecules that bind ADP, TXA₂, epinephrine, or serotonin with high affinity have also been cloned, expressed, and their structure determined. In addition, a repertoire of antithrombins and an increasingly number of RGD and non-RGD disintegrins targeting platelet $\alpha \text{IIb}\beta 3$ have been reported. Moreover, metalloproteases with fibrinogen(olytic) activity and PAF phosphorylcholine hydrolase are enzymes that have been recruited to the salivary gland to block platelet aggregation. Platelet inhibitory prostaglandins, lysophosphatidylcholine, adenosine, and nitric oxide (NO)-carrying proteins are other notable examples of molecules from hematophagous salivary secretions (herein named sialogenins) with antihemostatic properties. Sialogenins have been employed as tools in biochemistry and cell biology and also display potential therapeutic applications.

Keywords

Aegyptin; Ixolaris; D7-short; RPAI-1; Nitrophorin; Anophelin; Lipocalin; Tick; Mosquito; Sand fly; Ixodegrin; Ornatin; Metalloproteases; Sialogenin

1. Hematophagy and platelet activation

Hematophagous animals have evolved various strategies to counteract their host hemostatic system and to successfully feed on blood. In evolution, it has been suggested that insects may have been initially nest parasites or predators on other insects. They may also have fed on secretions from vertebrate eyes or other mucosal membranes where access to a meal is easier and does not require piercing of the skin. Perhaps this was the first adaptation toward acquiring the ability to penetrate the skin in search of blood (Francischetti, et al., 2009, Ribeiro and Francischetti, 2003). Actually, blood-feeding may occur through two different pathways: from hemorrhagic pools that accumulate in tissues following skin lacerations, or through direct cannulation of venules or arterioles. For example, mosquitoes and bugs

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cannulate the arterioles and venules deep in the skin, sometimes reaching several millimeters. In contrast, sand fly mouthparts penetrate up to 0.5 mm into their host skin and therefore can feed only from superficial hematomas caused by laceration of capillaries. Tabanids, which have cutting scissors, can also lacerate several vessels simultaneously, potentially forming a large hematoma. Further, ticks introduce their mouthparts into the host and generate a feeding cavity from which they feed on blood (Francischetti, et al., 2009, Ribeiro and Francischetti, 2003). These animals express molecules from hematophagous salivary secretions, herein named sialogenins (from the Greek *sialo*, saliva; *gen*, origin, source; and *ins* for proteins). Sialogenins target the host response to injury, modulate immune response, prevent pain, and other mechanisms associated with blood feeding. While several functions of sialogenins have been reviewed before (Francischetti, et al., 2009, Koh and Kini, 2009, Maritz-Olivier, et al., 2007, Ribeiro and Francischetti, 2003, Steen, et al., 2006), this review focuses on those that target platelet aggregation.

Platelets have a central role in hemostasis. The first step in the hemostatic cascade is platelet interaction with the exposed extracellular matrix (ECM), which contains a large number of adhesive macromolecules such as collagens and fibronectin. Under conditions of high shear, such as in small arteries and arterioles, the initial tethering of platelets to the ECM is mediated by interaction between the platelet receptor glycoprotein (GP)Ib and vWF bound to collagen (Furie and Furie, 2005, Jackson and Schoenwaelder, 2003, Ruggeri, 2002, Watson, et al., 2005). GPIb to vWF binding dissociates rapidly and is insufficient to mediate stable adhesion but rather maintains the platelet in close contact with the exposed surface. This interaction allows the collagen receptor GPVI (Jandrot-Perrus, et al., 2000) to bind to collagen. This triggers the conformational change of integrins (*e.g.*, $\alpha_2\beta_1$) to a high-affinity state, thereby enabling them to mediate firm adhesion to collagen and also promotes the release of TXA₂ and ADP (Furie and Furie, 2005, Jackson and Schoenwaelder, 2003, Ruggeri, 2002, Watson, et al., 2005).

ADP binds to the G_q-protein-linked P2Y₁ receptor on platelets, which causes a change in cell shape, mobilization of calcium, and initiation of reversible aggregation via stimulation of PLC. It also binds the G_i-linked P2Y₁₂ receptor to amplify aggregation via inhibition of adenylyl-cyclase-mediated cyclic AMP production. The resulting platelet activation leads to PI-3-kinase activation, granule secretion, and “inside-out” activation of integrin $\alpha_{IIb}\beta_3$, which increases its affinity for fibrinogen and vWF. These ligands then bind to the receptors to form bridges between adjacent platelets, which results in “outside-in” signalling and aggregation (Kahner, et al., 2006, Varga-Szabo, et al., 2008). Accordingly, sustained ADP-induced platelet aggregation requires activation of both P2Y₁ and P2Y₁₂ receptors. TXA₂ activates platelets through the TP receptors that are coupled to G_q and G_{12/13}. While it promotes shape change, activation of this pathway is unable to induce sustained platelet aggregation in the absence of a G_i-coupled receptor agonist such as ADP, explaining the sensitivity of TXA₂ mimetic (U46619)-induced platelet aggregation to apyrases (Kahner, et al., 2006, Varga-Szabo, et al., 2008).

Granule secretion also releases the weak agonists serotonin (5-HT) and epinephrine. While serotonin is not a pro-aggregatory molecule, it binds to G_q-coupled 5-HT_{2A} receptor and activates PLC, leading to shape change. On the other hand, epinephrine inhibits adenylyl cyclase via a G_i-coupled α_{2A} adrenergic receptor without noticeable effects on platelet aggregation; however, when added together, both weak agonists induce platelet aggregation comparable to ADP. Further, epinephrine and serotonin potentiate platelet aggregation by a number of platelet inducers tested at low concentrations, decreasing their threshold for aggregation; this property appears to be particularly relevant *in vivo*, where aggregation occurs as a result of several different agonists acting synergetically and in a redundant manner (Kahner, et al., 2006, Varga-Szabo, et al., 2008).

Activated platelets also promote the generation of thrombin—the most potent of all physiological platelet agonists. Thrombin acts predominantly via protease-activated receptors 1 and 4 (PAR1 and PAR4) expressed on platelets. Thrombin cleaves a portion of the N-terminus of these receptors, unmasking the sequence that serves as its ligand; this modification activates the receptor and triggers multiple signal transduction pathways that modulate thrombosis, coagulation, and inflammation (Coughlin, 2005). In addition, activated platelets secrete a number of proinflammatory molecules and may contribute to local inflammatory processes by a number of mechanisms (Gawaz, et al., 2005). Finally, physiologic inhibitors of platelet aggregation have been described including endothelium CD39 apyrase, which degrades ADP; nitric oxide (NO), which inhibits platelet aggregation through cGMP; and PGI₂, which shuts down different pathways of platelet activation by increasing intracellular cAMP through PGI₂ receptors (Ruggeri, 2002, Varga-Szabo, et al., 2008). Figure 1 displays the basic mechanisms of platelet aggregation and inhibition.

2. Sialogenins that inhibit platelet aggregation

2.1. Apyrases (ATP-diphosphohydrolase EC 3.6.1.5)

The saliva of many hematophagous animals including mosquitoes, bugs, sand flies, fleas, triatomines, and ticks contains large amounts of apyrase activity (ATP-diphosphohydrolase EC 3.6.1.5), which hydrolyses ATP and ADP into AMP and P_i , thus inhibiting platelet aggregation (Ribeiro and Francischetti, 2003). Apyrases are ubiquitous, conceivably because ADP is an important platelet aggregation inducer released by damaged cells at the site of injury and from activated platelets (Watson, et al., 2005).

Three classes of apyrases have been characterized at the molecular level in different blood-sucking arthropods. One is the 5'-nucleotidase family, which have been cloned from the salivary gland of mosquitoes *Anopheles gambiae* (Lombardo, et al., 2000) and *Aedes aegypti* (Champagne, et al., 1995), bugs such as *Triatoma infestans* (Faudry, et al., 2004), soft tick *Ornithodoros savignyi* (Stutzer, et al., 2009) and from the hard tick *Rhipicephalus (Boophilus) microplus* (Liyoun, et al., 1999). The platelet aggregation inhibitor chrysopsin has also been cloned from the salivary gland of the tabanid *Chrysops* sp. It has been suggested that it inhibits platelet aggregation as a non-RGD antagonist of integrin $\alpha IIb\beta 3$ (Reddy, et al., 2000); however, its sequence clearly indicates it is a member of the 5'-nucleotidase family that clades with *An. gambiae* apyrases (Stutzer, et al., 2009). Although it has negligible apyrase activity, it remains possible that inhibition of integrin function developed secondarily to degradation by ADP, as it activates integrin $\alpha IIb\beta 3$ (Reddy, et al., 2000). Another family of apyrases has been initially identified in the bed bug *Cimex lectularius* and confirmed to be enzymatically active (Valenzuela, et al., 1998); members of this family were later discovered in sand fly *Phlebotomus papatasi* (Valenzuela, et al., 2001) and *Lutzomyia longipalpis* salivary glands (Charlab, et al., 1999). Of note, *Cimex*-type apyrase (Valenzuela, et al., 1998) has been found in eukaryotes and to be fully active as a nucleotidase (Dai, et al., 2004). Therefore, this discovery validates and exemplifies how the study of salivary proteins as sources of (known) biologic activities may provide leads for the identification of the (unknown) function of human counterparts based on sequence similarity. Finally, the flea *Xenopsylla cheopis* salivary gland expresses a third type of apyrase—not yet obtained in recombinant form—whose sequence resembles that of the CD39 family of nucleotidases (Andersen, et al., 2007) found in the endothelium. Therefore, degradation of ADP through recruitment of different families of apyrases has been a consistent strategy employed by blood-sucking animals to inhibit hemostasis in general and platelet activation in particular.

2.2. ADP-binding proteins

2.2.1. RPAI-1—*Rhodnius prolixus* aggregation inhibitor 1 (RPAI-1) is a 19-kDa lipocalin isolated from the salivary gland of *R. prolixus* (Francischetti, et al., 2000). RPAI-1 was the first sialogenin whose function was identified as ADP-binding protein ($K_D \sim 50$ nM). RPAI-1 also displays high-affinity binding to ATP, AMP, adenosine (Ado), AP4A, and α, β Met ADP but does not bind to inosine, guanosine, uridine, or cytidine, implying that Ado structure is necessary for binding (Francischetti, et al., 2002). Despite its high affinity, it only inhibits platelet aggregation triggered by low doses of ADP (< 0.5 μ M) (Figure 2A) or appropriate concentrations of collagen (Figure 2B), arachidonic acid, U46619, TRAP, PAF, and A23187 (Francischetti, et al., 2002, Francischetti, et al., 2000), which is ADP dependent (Kahner, et al., 2006, Watson, et al., 2005). In fact, high doses of ADP or other pro-aggregatory substances will consistently not (or only marginally) be affected by this family of inhibitors, as the amount of ADP released rapidly saturates the mopping up ability of the inhibitor that occurs at a 1:1 stoichiometry (Francischetti, et al., 2000). This is important to keep in mind to avoid a given inhibitor being erroneously classified as a specific inhibitor of collagen, an agonist that—at the usual *in vitro* concentration of ~ 1 μ g/ml—is particularly sensitive to ADP scavengers. RPAI-1 also inhibits platelet aggregation under high shear observed in the microcirculation as demonstrated by platelet function analyzer (PFA-100) or clot signature analyzer (Francischetti, et al., 2002).

Two questions are often raised about the true biological function of lipocalins such as RPAI-1 *in vivo*. The first is whether the concentration of the inhibitor found in saliva is compatible with the concentrations of pro-aggregatory components produced upon injury at the microcapillary level. The second is whether a high-affinity ADP-binding protein is needed in the saliva, because apyrases effectively inhibit platelet aggregation. Accordingly, computer simulation experiments were performed where the rate of scavenging or hydrolysis of ADP by RPAI-1 or *R. prolixus* (RP) apyrase (a 5/-nucleotidase)—which have distinct K_m and K_{cat} for ADP—were compared. The simulation results indicate that RPAI-1 (high affinity for ADP, no hydrolysis) is functionally more effective at low ADP concentrations (< 1 μ M). In contrast, effective degradation of ADP by the RP apyrase (low affinity for ADP and enzymatic degradation) occurs when the ADP concentration is at least 1000- to 5000-fold higher (50 μ M) than that readily removed by RPAI-1. Of note, ADP physiological plasma concentration is ~ 0.1 μ M, and it can reach 1–2 μ M at the site of injury provoked by a Simplate device; 0.2–0.4 μ M ADP is known to be biologically active (Francischetti, et al., 2002, Francischetti, et al., 2000). Accordingly, RPAI-1 and RP apyrase coexisting in the same secretion have precise and complementary biologic functions in preventing platelet aggregation *in vivo*.

2.3. TXA2-binding proteins

2.3.1. Moubatin and TSGP3—Moubatin is a sialogenin cloned from the salivary gland of the soft tick *Ornithodoros moubata* salivary gland (Keller, et al., 1993, Waxman and Connolly, 1993). Recombinant moubatin inhibited collagen-stimulated aggregation of washed human platelets with an IC_{50} of about 100 nM, while aggregation induced by other ligands was not affected. Moubatin did not inhibit platelet adhesion to collagen, supporting the view that it uniquely inhibited a step involved in platelet activation; however, at higher concentrations (2–6 μ M), moubatin diminished the second phase of aggregation induced by ADP, inhibited aggregation in response to submaximal concentrations of the TXA2 mimetic U46619, and competed for the binding of a TXA2 receptor antagonist to platelet membranes. (Keller, et al., 1993). Therefore, moubatin behaved similarly to RPAI-1, because at high concentrations it affects ADP and U46619-induced platelet aggregation (Francischetti, et al., 2000). It has been suggested that moubatin interferes with a pathway

associated with TXA₂ receptor (Keller, et al., 1993), but the molecular target of moubatin remained elusive.

More recently, moubatin and another lipocalin from *O. savignyi* (TSGP3) were expressed in *Escherichia coli* and shown to inhibit collagen-induced platelet aggregation (Mans and Ribeiro, 2008). As described before (Keller, et al., 1993), inhibition of platelet aggregation was partial, and shape change was still present as depicted in Figure 2C (Mans and Ribeiro, 2008). Of note, isothermal titration calorimetry (ITC) demonstrated that Moubatin and TSGP3 bind cTXA₂ with high affinity (24 and 5 nM, respectively), but not ADP (Mans and Ribeiro, 2008). The implications of scavenging TXA₂ are many, taking into account that this prostanoid is a potent platelet aggregation agonist as well as a vasoconstrictor. In fact, moubatin (and TSGP3) relaxes rat aorta pre-constricted by U46619 and inhibits contraction induced by U46619 in a concentration-dependent manner. It is likely that the concentrations of moubatin and TSGP3 in saliva is enough to counteract TXA₂ produced *in vivo*, inhibiting vasoconstriction and platelet aggregation at the site of feeding (Mans and Ribeiro, 2008). Accordingly, it was previously shown to exist as ~5% of the total protein present in salivary gland extracts of *O. savignyi*. It is reasonable to suggest that other lipocalins including moubatin are expressed at a similar level. If it is assumed that only 50% of the salivary gland protein is secreted during feeding and that the feeding site is between 10–50 µl in volume, then moubatin would be present at ~3–18 µM. This is well above the concentrations needed by TXA₂ to induce vasoconstriction or platelet aggregation and given the high affinities for TXA₂ (Mans and Ribeiro, 2008).

2.3.2. Putative ADP or TXA₂-binding proteins

2.3.2.1. Pallidipin: This is a 19-kDa sialogenin from the salivary gland of *Triatoma pallidipennis*. Pallidipin reportedly blocks collagen-mediated aggregation of platelets (Noeske-Jungblut, et al., 1994). While it does not attenuate platelet aggregation stimulated by other agonists (including ADP, thrombin, U46619, PMA), it is unclear whether it inhibits low doses of ADP-induced platelet aggregation. As described above for RPAI-1, the concentration of platelet agonist tested *in vitro* is critical when one is investigating the function of a potential ADP-binding lipocalin (Francischetti, et al., 2000). It is thus possible that it operates as an ADP-binding protein or a TXA₂ scavenger. This contention is based on high sequence similarity to RPAI-1, the fact that it does not block collagen-induced shape change (a GPVI-mediated event), and the finding that relatively high concentrations of pallidipin are needed to inhibit platelet aggregation (Noeske-Jungblut, et al., 1994). Isothermal titration calorimetry experiments using recombinant pallidipin may clarify this issue. As reported for moubatin and RPAI-1, pallidipin had no effect on platelet adhesion to immobilized soluble collagen, indicating that it does not affect integrin α₂β₁ (Noeske-Jungblut, et al., 1994).

2.3.2.2. Triplatin: Triplatin has been cloned from the salivary gland of *T. infestans* and shown to inhibit platelet aggregation induced by collagen but not ADP, AA, U46619, or thrombin. Triplatin inhibits Fc receptor γ-chain phosphorylation induced by collagen, which is mediated by GPVI. Triplatin also inhibits platelet aggregation induced by collagen-related peptide (CRP), a GPVI agonist. While it has been suggested that triplatin targets GPVI, a direct demonstration has not been provided (Morita, et al., 2006). In fact, CRP-induced platelet aggregation is inhibitable by ADP receptor antagonists and cyclooxygenase blockers (Watson, et al., 2005). It is thus unclear whether the results observed were due to another activity such as ADP- or TXA₂-binding properties. Additionally, triplatin displays high sequence similarity to pallidipin-1, works only at high concentrations, and at > 1 µM does not eliminate completely CRP- or collagen-induced shape change (Morita, et al., 2006). It is prudent to say that triplatin has not been confirmed as a GPVI antagonist.

2.3.2.3. Longicornin: Longicornin is a 16-kDa sialogenin from the hard tick *Haemaphysalis longicornis*. Although the N-terminus of longicornin has been identified, it has not been cloned. It inhibits collagen-induced platelet aggregation without affecting other inducers or interfering with platelet adhesion (Cheng, et al., 1999); it is not known, however, whether low doses of other agonists are inhibitable by longicornin. It cannot be excluded that longicornin binds either ADP or TXA₂.

Finally, a number of lipocalins reportedly bind to AA and to leukotrienes. For example, moubatin, TSGP2, and TSGP3 bind to AA with K_D 34–69 nM, suggesting that it may under certain circumstances mop up AA (Mans and Ribeiro, 2008); however, these three proteins also interact with LTB₄ with higher affinity (~ 20 nM), while moubatin and TSGP3 are TXA₂ ligands. While the relative inhibitory contribution of moubatin and related lipocalins to inhibition of AA, TXA₂, or LTB₄ *in vivo* is not known, it is plausible that the prostanoid and not AA is the most relevant ligand for this family of lipocalins, due to the higher affinity and intense pharmacologic activity at sites of vascular injury and inflammation.

2.4. Epinephrine and serotonin-binding proteins

2.4.1. Amine-binding protein (ABP)—A biogenic ABP that belongs to the nitrophorin group of lipocalins has been discovered in *R. prolixus* salivary gland. Using isothermal titration calorimetry and the Hummel-Dreyer method of equilibrium gel filtration, K_D values of 102, 24, and 345 nM were found for serotonin, norepinephrine, and epinephrine, respectively (Andersen, et al., 2003). While ABP does not block platelet aggregation by moderate concentrations of collagen or ADP, it inhibits platelet aggregation induced by a combination of low concentrations of ADP and either serotonin or epinephrine. Potentiation of aggregation induced by low concentrations of collagen along with serotonin (or epinephrine) is also inhibited as shown in Figure 2D (Andersen, et al., 2003). This occurs because serotonin activates the phospholipase C pathway via the 5-HT_{2A} receptor but not the G_i-dependent pathway and consequently induces only shape change when administered alone. Epinephrine binds to G_i-coupled adrenergic receptors, inhibiting adenylyl cyclase and eliciting no detectable aggregation response by itself (Andersen, et al., 2003).

The presence of ABP in the circulation would therefore cause a localized increase in the agonist threshold concentration for platelet aggregation. In *R. prolixus* salivary gland, this protein would act in concert with RPAI-1, NP7, and salivary apyrase, reducing the concentration of weak agonists in the vicinity of the feeding site and thereby attenuating the overall stimulus for platelet aggregation (Andersen, et al., 2003).

2.4.1. D7—The D7 protein family is one of the most abundantly expressed sialogenins of mosquitoes and one of the first to be cloned from the salivary glands of insects. Recombinant short D7 of *An. gambiae* were shown to bind serotonin and norepinephrine with high affinity, and it also binds histamine and epinephrine with lower affinity. D7 members from *Aedes* sp. have been identified as high-affinity serotonin-binding proteins with specificity similar to *An. gambiae* short D7 (Calvo, et al., 2006). Elucidation of the crystal structure and their binding pockets revealed that the amino domain of the long D7 protein of *Aedes* sp. could bind a hydrophobic compound, which was identified as cysteinyl leukotrienes (Calvo, et al., 2009). While the function of short D7 is reportedly to modulate tonus of vessels, it is plausible that scavenging serotonin may block potentiation of platelet activation by serotonin, as described above for ABP from *R. prolixus*.

2.4.2. Monotonin, TSGP-1 and SHBP—Monotonin has been isolated from the salivary gland of the soft tick *Argas monolakensis* and shown to bind to serotonin with high affinity ($K_D < 2$ nM) (Mans, et al., 2008); it may also block platelet aggregation as reported for ABP

from *R. prolixus* (Andersen, et al., 2003). It has been calculated that the concentration of monotonin in the saliva is compatible with antihemostatic activity at the site of injection (Mans, et al., 2008). The crystal structure of monotonin has been determined and indicates that the protein has a single binding site for serotonin. From the conserved features of these proteins, a tick lipocalin biogenic amine-binding motif could be derived that was used to predict biogenic amine-binding (BAB motif) function in other tick lipocalins. Another serotonin-binding protein, TSGP1, has been characterized from *O. savignyi*, and its sequence also contains the BAB motif. It binds 5-HT ($K_D \sim 6$ nM) with a stoichiometry of ~ 1 (Mans, et al., 2008). Finally, SHBP from *Dermacentor reticularis* is another lipocalin that binds serotonin (and histamine) with high affinity and may share antiplatelet properties with monotonin and TSGP1 (Sangamnatdej, et al., 2002).

2.5. Collagen-binding proteins

2.5.1. Aegyptin and Anopheline antiplatelet protein (AAPP)—Aegyptin is a 30-kDa salivary sialogenin from *Ae. aegypti* that displays a unique sequence characterized by glycine, glutamic acid, and aspartic acid repeats. It specifically blocks collagen-induced human platelet aggregation and granule secretion ($IC_{50} \sim 50$ nM) without affecting aggregation induced by other agonists. Figure 2E shows tracings of collagen-induced platelet aggregation where, in the presence of 60 nM aegyptin, the onset time for shape change is increased, while at higher doses (120 nM), shape change was abolished. Surface plasmon resonance (SPR) experiments demonstrate that aegyptin binds to collagen ($K_D \sim 1$ nM) but does not interact with vitronectin, fibronectin, laminin, fibrinogen, or vWF (Calvo, et al., 2007). Aegyptin inhibits collagen-induced platelet aggregation (IC_{50} 100 nM) and blocks vWf interaction with collagen type III under static conditions and platelet adhesion to collagen under flow conditions at high shear rates with similar IC_{50} (~ 300 nM) (Calvo, et al., 2007). More recently, the sequence RGQOGVMGFO, which mediates collagen interaction with vWF, has been identified as a high-affinity binding site for aegyptin. Because it also interacts with the linear peptide RGQPGVMGFP and heat-denatured collagen, triple-helix and hydroxyproline residues are not a prerequisite for binding. Aegyptin also recognizes (GPO)₁₀ and GFOGER peptides with low-affinity, which represent GPVI and integrin $\alpha 2\beta 1$ binding sites in collagen, respectively (Calvo et al., unpublished observations). Consistent with these results, aegyptin interferes with platelet interaction with soluble collagen and prevents collagen binding to recombinant GPVI. *In vivo* experiments show that aegyptin prevents laser-induced carotid thrombus formation in the presence of Rose Bengal, a model where collagen reportedly plays an important role. Inhibition of thrombus formation is observable at 50 μ g/kg, while occlusion of the carotid took more than 80 min at doses of 100 μ g/kg. Rats treated at antithrombotic concentrations did not bleed significantly, suggesting that aegyptin is a suitable molecule to inhibit platelet-collagen interactions *in vivo* (Calvo et al., unpublished observations).

Anopheline antiplatelet protein (AAPP) from *Anopheles stephensi* saliva is a 30-kDa protein that displays strong sequence similarity to aegyptin. Recombinant AAPP directly binds to immobilized collagen and specifically inhibits collagen-induced platelet aggregation ($IC_{50} \sim 25$ nM) and intracellular Ca^{2+} increase. It blocks both platelet integrin $\alpha 2\beta 1$ -dependent and GPVI-expressing Jurkat cell-mediated adhesion to collagen. Intravenous injection of AAPP in rats inhibited collagen-induced platelet aggregation *ex vivo* (Yoshida, et al., 2008).

2.5.2. Leech antiplatelet protein (LAPP)—LAPP was identified in the salivary glands of the leech *Haementeria officinalis*. Recombinant LAPP inhibits collagen-mediated platelet aggregation under test-tube stirring conditions ($IC_{50} \sim 60$ –100 nM) without affecting other agonists. It also blocks platelet adhesion to soluble collagen under static conditions ($IC_{50} \sim 80$ nM), a step mediated by integrin $\alpha 2\beta 1$ (Connolly, et al., 1992, Keller, et al., 1992).

Consistent with these results, rLAPP prevents integrin α -I domain binding to collagen with $IC_{50} \sim 2 \mu\text{g/ml}$ (125 nM) (Depraetere, et al., 1999). rLAPP also inhibits platelet adhesion to collagen type I (IC_{50} 70 nM) at high shear rate (1600 s^{-1}) and prevents binding of vWF to collagen type III (van Zanten, et al., 1995). While rLAPP inhibits platelet deposition to cross sections of human atherosclerotic coronary arteries (van Zanten, et al., 1995) it did not block collagen graft thrombosis in baboons, suggesting that inhibition of collagen alone is not enough to prevent thrombosis (Schaffer, et al., 1993), possibly because tissue factor exposure plays an important role in graft models. The structure of LAPP has been determined and consists of a compactly folded C-terminal domain and an N-terminal region that is disordered in the crystal. The C-terminal domain folds similarly to the N-domain of hepatocyte growth factor and has been classified as the so-called PAN domain (Huizinga, et al., 2001). A pattern of four conserved cysteines forming two disulfide bonds and of five residues with a conserved hydrophobic character may be characteristic of this type of fold.

2.5.3. Saratin—Saratin, isolated from the saliva of the leech *Hirudo medicinalis*, binds to collagen with high affinity ($K_D \sim 50 \text{ nM}$; SPR experiments). It is a potent inhibitor of vWF binding to collagen ($IC_{50} \sim 1 \mu\text{g/ml}$; $\sim 100 \text{ nM}$) according to adhesion experiments performed at high shear rates (Barnes, et al., 2001). Platelet adhesion to collagen at 300 s^{-1} is not inhibited at $10 \mu\text{g/ml}$ ($1 \mu\text{M}$) saratin, and only very high concentrations of saratin ($200 \mu\text{g/ml}$; $\sim 20 \mu\text{M}$) inhibit platelet aggregation by collagen. Therefore, it has been suggested that saratin, in contrast to aegyptin, LAPP, and calin, is specific for the vWF-collagen interaction. More recently, however, saratin was shown to inhibit binding of the $\alpha 2$ integrin subunit I domain to collagen and prevents platelet adhesion in the presence of ADP and TXA2 inhibitors, suggesting that it interferes with integrin $\alpha 2\beta 1$ binding to collagen in addition to inhibiting vWF-collagen binding (White, et al., 2007).

Structural determination of saratin-collagen complex in solution has been revealed by NMR spectroscopy. Saratin has high structural homology to LAPP, which is a distant member of the PAN domain superfamily. In fact, when comparing the secondary structure elements of the solution structure of saratin with the structure of LAPP, both proteins show a very similar arrangement. The structural homology in core regions of the two proteins suggests that both bind to collagen in a similar way (Gronwald, et al., 2008).

Because of its therapeutic potential, saratin has been tested *in vivo* and found to significantly decrease platelet adhesion, intimal hyperplasia, luminal stenosis, and thrombosis after carotid endarterectomy in rats without increasing bleeding time. It also decreases venous anastomotic intimal hyperplasia in a canine dialysis model. Further, under stenotic shear conditions of 800 s^{-1} , saratin reduced (40% to 60%) platelet deposition triggered by human denuded vessel walls, fatty streaks, severely damaged vessels, and atherosclerotic plaque (Cruz, et al., 2001, Vilahur, et al., 2004). These results support the view that collagen-binding proteins may be beneficial as antithrombotic agents under certain pathologic conditions.

2.5.4. Calin—Calin is a semipurified substance ($\sim 65 \text{ kDa}$.) from *H. medicinalis* that has not yet been molecularly characterized. It inhibits platelet aggregation by collagen ($IC_{50} \sim 6.5$ to $13 \mu\text{g/ml}$; 100 – 200 nM) and adhesion to immobilized collagen ($IC_{50} \sim 22 \mu\text{g/ml}$; 400 nM) (Deckmyn, et al., 1995). Notably, it prevents binding of vWF to coated collagen under static conditions (IC_{50} $10 \mu\text{g/ml}$) and high shear rates (1300 s^{-1} ; $\sim 80 \mu\text{g/ml}$) (Depraetere, et al., 1999, Harsfalvi, et al., 1995). Therefore, calin inhibits direct platelet-collagen interactions and vWF-binding to collagen. This dual effect may contribute to prevention of thrombus formation observed under flow (Harsfalvi, et al., 1995). *In vivo* activity of calin has been tested in a thrombosis model in hamsters based on vessel clamp damage to the femoral vein. Results show that intravenous calin inhibited thrombus formation in this

model with an ED₅₀ of 0.07 mg/kg and complete inhibition with 0.2 mg/kg. No effects were seen on coagulation tests or bleeding times, whereas *ex vivo* aggregation induced by collagen was inhibited dose dependently (Deckmyn, et al., 1995). Because the molecular identity of calin is not known, the precise mechanism of action remains unclear.

2.6. Integrin $\alpha 2\beta 1$ antagonists

TAI is a 15-Kda sialogenin purified from the *O. moubata* salivary gland that has not been molecularly cloned. It inhibits platelet adhesion to soluble collagen under static conditions (IC₅₀ 8 nM) without affecting the onset or maximum aggregation triggered by collagen or other platelet agonists. TAI also affects endothelial cell adhesion to collagen and has partial inhibitory activity for fibronectin-mediated platelet adhesion. Further, it outcompetes anti- $\alpha 2\beta 1$ monoclonal antibody Gi9 binding to platelets, suggesting it is an integrin $\alpha 2\beta 1$ antagonist (Karczewski, et al., 1995).

HPI has been purified from *Ancylostoma caninum* hookworms and shown to inhibit platelet aggregation by epinephrine, thrombin, and ADP. It also blocks platelet adhesion to fibrinogen and collagen, suggesting that it targets integrin $\alpha 2\beta 1$ and integrin $\alpha IIb\beta 3$; however, HPI expressed in *E. coli* is devoid of platelet inhibitory properties. It remains to be proven that is HPI an integrin $\alpha 2\beta 1$ antagonist (Del Valle, et al., 2003).

2.7. Delivery of NO

2.7.1. Nitrophorins—In *R. prolixus*, NO is stored in the lumen of the salivary glands as a stable nitrophorin(NP)-NO complex. Binding is tight at the pH of the salivary gland (~ 5), but affinity is lower at the pH of the host (~ 7.5), a property that facilitates release of NO upon saliva injection (Montfort, et al., 2000). Of interest, the ability to transport NO has evolved independently in the bedbug *C. lectularius*, and is again heme-based but apparently does not involve the lipocalin fold (Valenzuela, et al., 1995). The structure of the NP4-NO complex has been determined. Binding of NO induces a conformational change in the spacious distal pocket, and the NO moiety becomes completely buried and surrounded by hydrophobic groups. By releasing NO at the site of an arthropod bite, NP conceivably inhibits platelet aggregation. This assumption has been confirmed *in vitro* in experiments where one NP family member (NP7) loaded with NO reportedly inhibits ADP-induced platelet aggregation and promotes disaggregation of platelets (Figure 2F) in addition to being an anticoagulant through binding to phosphatidylserine (Andersen, et al., 2004).

2.8. Increase in intraplatelet cAMP

R. prolixus salivary glands accumulate phospholipids, mainly phosphatidylcholine and lysoPC (75 μ g/ml in the lumen), and in the saliva (Golodne, et al., 2003). Of note, Lyso-PC (25–150 μ g/ml) inhibits platelet aggregation and induces a progressive increase in the cytosolic concentration of cAMP. In addition, salivary lysoPC incubated with porcine arterial endothelial cells for 24 h increases NO production. Accordingly, salivary lysoPC may affect platelets through an increase of intraplatelet cAMP and endothelium-derived NO. The calculated concentration of lysoPC in the feeding site is ~ 15 μ g/ml, which is close to the range capable of full inhibition of platelet aggregation (Golodne, et al., 2003), particularly when other salivary inhibitors are delivered simultaneously.

Prostaglandin I₂ (prostacyclin) has been discovered in the saliva of *Ixodes scapularis* at high concentrations (~ 1.5 μ M) (Ribeiro, et al., 1988). As an agonist of PGI₂-receptors, it induces cAMP increase—a potent intracellular platelet aggregation inhibitor. PGF₂ α has also been identified in *Amblyomma americanum* saliva (Aljamali, et al., 2002) and at appropriate concentrations may contribute to inhibition of platelet aggregation through a small increase in intraplatelet cAMP or TXA₂ receptor antagonism (Jones, et al., 2009). Finally, adenosine

was identified in sand flies *P. papatasi* and *Phlebotomus perniciosus* at concentrations that may reach ~ 0.8 mM, at the site of feeding, a concentration above the adenosine IC₅₀ (~ 1 μM) for inhibition of ADP-induced platelet aggregation, via an adenosine receptor and cAMP-dependent pathway (Ribeiro, et al., 1999).

2.9. Thrombin inhibitors

A number of sialogenins target thrombin and have been expressed or synthesized in active form. These inhibitors are generally low-molecular-mass molecules, sometimes without cysteines, which allows easy chemical synthesis. They have distinct mechanisms of action targeting exosite I, the catalytic site, or both. Inhibition is either competitive or noncompetitive. The reader is referred to other reviews that discuss in detail the molecular and functional aspects of anticlotting sialogenins (Francischetti, et al., 2009, Koh and Kini, 2009, Maritz-Olivier, et al., 2007, Ribeiro and Francischetti, 2003, Steen, et al., 2006).

2.10. Integrin αIIbβ3 antagonists (disintegrins)

In the presence of RGD peptides, platelets do not aggregate to any agonist, because fibrinogen cannot crosslink them. Several tick proteins have been characterized that inhibit the activation of platelets through the integrin αIIbβ3. Variabilin (from *Dermacentor variabilis*) is a 4-cysteine, 5 Da RGD-containing disintegrin that blocks ADP-induced platelet aggregation (IC₅₀ ~ 150 nM), and prevents integrin αIIbβ3 binding to immobilized fibrinogen (IC₅₀ 9 nM). It also attenuates osteosarcoma cell adhesion to vitronectin (IC₅₀ ~ 100 nM), suggesting that it targets αvβ3 (Wang, et al., 1996). Of interest, ixodegrins found in *Ixodes pacificus* and *I. scapularis* transcriptomes (Francischetti, et al., 2005) display sequence similarity to variabilin, including RGD position, but have two additional cysteines as shown by the Clustal alignment depicted in Figure 3A. Ixodegrin remains to be produced in recombinant form to confirm its disintegrin activity.

Another tick disintegrin, named disagregin (from *O. moubatta*) lacks RGD motifs and the cysteine stabilized loop that is critical to present RGD motif to integrins. It potently blocks ADP-induced platelet aggregation (IC₅₀ 150 nM) and platelet adhesion to fibrinogen. Disagregin binds integrin αIIbβ3 in activated platelets with K_D ~ 40 nM but does not affect endothelial cell adhesion to fibrinogen or vitronectin, confirming its specificity (Karczewski, et al., 1994). Likewise, savignygrin (from *O. savignyi*) contains a RGD motif and inhibits platelet aggregation by a number of agonists including ADP (IC₅₀ 130 nM). It blocks binding of α-CD41 to platelets as well as binding of purified αIIbβ3 to fibrinogen (Mans, et al., 2002). Notably, cysteine arrangement of savignygrin is similar to that of the bovine pancreatic trypsin inhibitor (BPTI) family of serine protease inhibitors, suggesting that it presents the RGD motif using the Kunitz-BPTI protein fold (Mans, et al., 2002). Savignygrin-like molecules have also been cloned from the soft tick *O. coriaceus*. (Francischetti, et al., 2008). More recently, monogrin was purified from the salivary gland of soft tick *A. monolakensis*. It is a RGD-containing 10-kDa protein with sequence homology to savignygrin (and disagregin); it also presents the RGD integrin-recognition motif on the BPTI loop. Both recombinant and purified monogrins block ADP-induced platelet aggregation (IC₅₀ ~ 150 nM) but not initiation of shape change (Figure 2G) and were shown by surface plasmon resonance to interact with integrin αIIbβ3. It has been calculated that monogrin concentration in the feeding cavity is between 0.8–4 μM, which is well within the range of its inhibitory properties (Mans, et al., 2008). Figure 3B shows the Clustal alignment and the putative cysteine pairs for savignygrin, monogrin and disagregin. Figure 3C shows the predicted secondary structure for savignygrin which is based on Kunitz protein fold.

In insects, disintegrins have been identified solely in tabanids, where members of the tabinhibitin family acquired such a domain. This 25-kDa RGD-containing protein blocks

platelet aggregation by different agonists with concentrations < 40 nM (Ma, et al., 2009). RGD disintegrins have not been found in fleas, sand flies, or bugs, although recently one was discovered by transcriptome analysis of anopheline mosquito (*Anopheles darlingi*) and found to belong to the aegyptin-like family.

Leeches are another source of disintegrins. Decorsin from *Macrobdella decora* has been obtained in recombinant and synthetic active forms. It contains an RGD sequence and reportedly inhibits fibrinogen binding to immobilized integrin $\alpha\text{IIb}\beta 3$ (IC_{50} 1.5 nM); however, it attenuates platelet aggregation at considerable higher concentrations (IC_{50} 500 nM) (McLane, et al., 1995, Seymour, et al., 1990). At low doses (1.5–3 $\mu\text{g}/\text{mouse}$), it protects mice against death from pulmonary thromboembolism (McLane, et al., 1995). The decorsin structure has been determined by NMR and shown to be similar to hirudin despite their distinct primary sequences (Krezel, et al., 1994). Ornatin is the name for a family of six isoforms of RGD-containing disintegrins (~ 5 kDa) purified from whole leech (*Placobdella ornata*) homogenate. It potently inhibits fibrinogen binding to integrin $\alpha\text{IIb}\beta 3$ ($\text{IC}_{50} \sim 5$ nM) but, like decorsin, inhibits platelet aggregation at higher concentrations ($\text{IC}_{50} \sim 300$ nM). A recombinant form of ornatin has been expressed in *E. coli* with a 60-fold decrease in activity, indicating that appropriate folding is critical (Mazur, et al., 1991).

2.11. Fibrinolytic metalloproteases and PAF-acether hydrolase

Besides apyrases, two other sialogenins with enzymatic activity potentially inhibit platelet aggregation. Metalloprotease activity targeting the A α chain of fibrinogen and fibrin only has been found in *I. scapularis* saliva, and a number of cDNAs coding for metalloproteases have been sequenced (Francischetti, et al., 2003, Francischetti, et al., 2009). Another family of enzymes (tablysin) targeting both A α and B β chains of fibrin(ogen) has been cloned from *Tabanus yao Macquart* (Tabanidae) salivary glands (Ma, et al., 2009). These enzymes belong to the repolysin family of metalloproteases commonly found in Viperidae venom (Francischetti, et al., 2003). Hementin and hementerin from leeches *Haementeria ghilianii* and *Haementeria depressa*, respectively, degrade α , β , and γ chains of fibrinogen and fibrin (Budzynski, 1991, Chudzinski-Tavassi, et al., 1998). Through cleavage of fibrinogen, these enzymes may inhibit platelet aggregation and clot formation.

A PAF phosphorylcholine hydrolase was found in the salivary glands and saliva of the human-feeding mosquito *Culex quinquefasciatus* and shown to inhibit PAF-induced platelet aggregation. While this enzyme has not yet been cloned, it may prevent platelet activation through neutrophil-derived PAF (Ribeiro and Francischetti, 2001).

3. Concluding remarks

Molecules involved in platelet activation are important targets of sialogenins from different hematophagous animals. Notably, only inhibitors targeting molecules directly involved with activation of hemostasis have been discovered. Accordingly, agonists that are only produced upon platelet activation or endothelial denudation—such as thrombin, collagen, ADP, TXA₂, and serotonin—are obvious targets for sialogenins with antiplatelet activity. The same is true for disintegrins targeting $\alpha\text{IIb}\beta 3$, which only becomes functionally available to bind fibrinogen after platelet activation. In fact, no salivary antagonists have been described so far targeting constitutively expressed receptors such as GPIb, GPVI, PARs or inhibitors of plasma proteins that occurs at high concentrations such as vWF, prothrombin, cofactors V and VIII, etc. despite their critical role in hemostasis. It appears that these molecules are likely not ideal targets for sialogenins because they are not easily neutralized due to high plasma concentration, or high receptor density in non-activated cells. They may actually remove sialogenins (usually present in minute amounts) from the site where they have been injected, decreasing their effective concentration. This is very much in contrast to snake

venom toxins (usually found in large amounts), which have been reported to bind to GPVI, vWF, prothrombin, and cofactors (Lu, et al., 2005). Of interest, some sialogenins have structural features (e.g., positively charged residues) that direct them to the membrane of activated platelets through interaction with phosphatidylserine (Andersen, et al., 2004). Conceivably, this targets the sialogenins to activated cells, increasing the effective concentration of the inhibitor at sites of inflammation; this may also avoid diffusion into flowing blood. In conclusion, antiplatelet sialogenins from salivary glands are remarkably functionally diverse. They likely work in a synergistic and redundant manner to keep hemostatic tonus as low as possible so as to facilitate blood-feeding, as depicted in Figure 4. Finally, antiplatelet sialogenins may be useful tools in cell biology and may also have potential for therapeutic applications.

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Fig. 1. Platelet activation. Pro-aggregatory molecules ADP, thrombin, and collagen activate platelets through specific receptors, leading to PLA2 and PLC activation, granule release, production of TXA2 and activation of integrin $\alpha_{IIb}\beta_3$. Inhibitory signals include endothelial apyrase (not shown), prostacyclin, and NO (for details see text).

Fig. 2.

Platelet inhibition by sialogenins. (A) ADP-binding proteins, *e.g.*, RPAI-1. Low concentrations of ADP (< 0.5 μ M)-induced platelet aggregation including shape change is dose-dependently inhibited by RPAI-1 which has only marginal effects at higher concentrations of ADP (> 1 μ M) (Francischetti, et al., 2000). (B) Collagen (1 μ g/ml)-induced platelet aggregation is attenuated by RPAI-1 without abolishing shape change (Francischetti, et al., 2002). (C) TXA₂-binding protein, *e.g.*, moubatin. Collagen-induced platelet aggregation is attenuated by moubatin without abolishing shape change (Mans and Ribeiro, 2008). (D) Serotonin-binding protein, *e.g.*, ABP. Serotonin (0.5 μ M)-induced potentiation of aggregation by low doses of collagen (0.75 μ g/ml) is blocked by ABP (0.8 μ M) (Andersen, et al., 2003). (E) Collagen-binding protein, *e.g.*, aegyptin. Aggregation induced by collagen (2 μ g/ml) is accompanied by delay in onset time for shape change at 60 nM aegyptin; no shape change or aggregation is observable at higher concentrations of the inhibitor (Calvo, et al., 2007). (F) NO-delivery, *e.g.*, nitrophorins. ADP (3 μ M)-induced

platelet aggregation and shape change is attenuated by NP2 (0.4 μM) when added before stimulus. NP2 also promotes disaggregation when added (arrow) after addition of ADP (Andersen, et al., 2004). (G) Disintegrin targeting $\alpha\text{IIb}\beta 3$; *e.g.*, monogrins. ADP (20 μM)-induced platelet aggregation is prevented by monogrins (0.3 μM), while initiation of shape change is not affected (Mans, et al., 2008). Concentrations of inhibitors are given next to each tracing of platelet aggregation. ΔT , transmittance.

Fig. 3. Disintegrin from blood-sucking arthropods. (A) Clustal alignment for variabilin (Wang, et al., 1996) and ixodegrin (gi 51011476) (Francischetti, et al., 2005, Francischetti, et al., 2009) (B) Clustal alignment and putative disulfide pattern for savignygrin (gi 21435983) (Mans, et al., 2002), disagregin (gi 545738) (Karczewski, et al., 1994), and monogrin (gi 114152966) (Mans, et al., 2008). (C) The predicted secondary folding for savignygrin is shown. The RGD sequence is indicated in gray.

Fig. 4.

Hematophagy and inhibition of platelet aggregation. Platelets adhere to collagen at sites of vascular injury through vWF, GPIb and specific receptors collagen receptors GPVI and integrin $\alpha 2\beta 1$. This event is accompanied by release of secondary mediators (e.g., ADP, epinephrine and serotonin) and generation of TXA2. During this process, thrombin may be produced through exposure of tissue factor. These agonists activate the signaling pathway, culminating with activation of integrin $\alpha IIb\beta 3$, which binds fibrinogen (Gross and Weitz, 2009, Watson, et al., 2005). The target for each family of inhibitor is indicated by a number. The target for PAF phosphorylcholine hydrolase is not shown. The name of each inhibitor is shown beside each picture exemplifying a different hematophagous animal found in different genus and/or species. Epi, epinephrine; 5HT, serotonin.

Table1

Sialogenins that inhibit platelet aggregation.

Targets and Inhibitors	Species	Molecular mass (mature protein)	Target	R/S/P	Comments and References
ADP (hydrolysis)					
Apyrase	<i>A. gambiae</i>	61	ADP	N/N/N	5'-nucleotidase family (Lombardo, et al., 2000).
Apyrase	<i>A. aegypti</i>	60	ADP	N/N/N	5'-nucleotidase family (Champagne, et al., 1995).
Apyrase	<i>T. infestans</i>	60	ADP	Y/N/Y	5'-nucleotidase family (Faudry, et al., 2004).
Apyrase	<i>O. savignyi</i>	63	ADP	Y/N/N	5'-nucleotidase family (Stutzer, et al., 2009).
Apyrase	<i>Chrysops sp.</i>	59	ADP	Y/N/Y	5'-nucleotidase family (Reddy, et al., 2000). Chrysopsin.
Apyrase	<i>B. microplus</i>	62	ADP	Y/N/N	5'-nucleotidase family (Liyou, et al., 1999).
Apyrase	<i>C. lectularius</i>	38	ADP	Y/N/N	Cimex family (Valenzuela, et al., 1998).
Apyrase	<i>P. papatasi</i>	36	ADP	N/N/N	Cimex family (Valenzuela, et al., 2001).
Apyrase	<i>L. longipalpis</i>	35	ADP	N/N/N	Cimex family (Charlab, et al., 1999).
Apyrase	<i>X. cheopis</i>	47	ADP	N/N/N	CD39 family (Andersen, et al., 2007).
ADP (binding)					
RPAL-1	<i>R. prolixus</i>	19	ADP, 50 nM	Y/N/Y	Binds to other adenine nucleotides (Francischetti, et al., 2002, Francischetti, et al., 2000). No apyrase activity.
TXA (binding)					
Moubatin	<i>O. moubata</i>	17	cTXA2, 24 nM	Y/N/Y	Also binds AA, LTB4 (Keller, et al., 1993, Mans and Ribeiro, 2008).
TSGP3	<i>O. savignyi</i>	16	cTXA2, 5 nM	Y/N/Y	Also binds AA, LTB4, and C5 (Mans and Ribeiro, 2008).
Putative ADP or TXA2 (binding)					
Pallidipin	<i>T. pallidipennis</i>	18	Specificity ADP?; TXA2?	Y/N/Y	Possible ADP or TXA2 binder (Noeske-Jungblut, et al., 1994).
Triplatin	<i>T. infestans</i>	19	ADP?; TXA2?	Y/N/Y	Possible ADP or TXA2 binder. GPVI antagonist unclear. (Moria, et al., 2006).
Longicornin	<i>H. longicornis</i>	16	ADP?, TXA2?	N/N/Y	Possible ADP or TXA2 binder (Cheng, et al., 1999).
EPI or 5-HT (binding)					
ABP	<i>R. prolixus</i>	19	Specificity/affinity 5-HT, 102 nM EPI, 345 nM	Y/N/Y	Blocks potentiation of platelet aggregation induced by serotonin and EPI (Andersen, et al., 2003). Binds NE (KD, 24 nM).
D7r1-4 (short)	<i>A. gambiae</i>	15	5-HT, 1 nM	Y/Y/N	Displays low affinity for histamine and EPI. D7r2,-3 binds NE (KD 3nM) (Calvo, et al., 2006).

Targets and Inhibitors	Species	Molecular mass (mature protein)	Target	R/S/P	Comments and References
AeD7L (long)	<i>A. Aegypti</i>	33	5-HT, 0.5 nM	Y/Y/N	High affinity for cysteinyl leukotrienes. Low affinity for histamine and EPI. Binds NE (KD 0.1 nM) (Calvo, et al., 2009).
Monotonin	<i>A. monolakensis</i>	20	5-HT, < 2 nM	Y/Y/N	Presents a BAB motif (Mans, et al., 2008).
SHBP	<i>D. reticularis</i>	22	5-HT, 0.6 nM	Y/N/N	Binds histamine with high affinity (Sangamnatdej, et al., 2002).
Collagen					
Affinity for collagen/IC ₅₀ for platelet aggregation					
Aegyptin	<i>A. aegypti</i>	30	1 nM, 50 nM	Y/N/Y	Blocks aggregation and adhesion to collagen at static condition or at high shear rates (Calvo, et al., 2007). Antithrombotic <i>in vivo</i> .
AAPP	<i>A. stephensi</i>	30	?, 25 nM	Y/N/Y	Blocks aggregation and adhesion to collagen at static condition. Antiplatelet <i>ex vivo</i> (Yoshida, et al., 2008).
Calin	<i>H. medicinalis</i>	65	?, 100 nM	N/N/Y	Molecular identity(ies) unknown. Blocks aggregation and adhesion to collagen at static condition or at high shear rates (Harsfalvi, et al., 1995). Antithrombotic <i>in vivo</i> (Deckmyn, et al., 1995).
LAPP	<i>H. officialis</i>	13	?, 100 nM	Y/Y/Y	Blocks aggregation and adhesion to collagen at static condition or at high shear rates. Antithrombotic <i>in vivo</i> (Keller, et al., 1992).
Saratin	<i>H. medicinalis</i>	12	50 nM, 20 μM	Y/Y/Y	Preferentially blocks platelet adhesion to collagen at high shear rates (vWF-dependent) (Barnes, et al., 2001). Antithrombotic <i>in vivo</i> (Cruz, et al., 2001).
Integrin α2β1					
IC ₅₀ for platelet aggregation					
Tick adhesion inhibitor (TAI)	<i>O. moubatta</i>	5	8 nM	N/N/Y	Inhibits platelet adhesion to soluble collagen but does not affect platelet aggregation. (Karczewski, et al., 1995).
Hookworm platelet inhibitor (HPI)	<i>A. caninum</i>	20	NA	Y/N/Y	Inhibits α2β1 and α1bβ3. Recombinant form not active (Del Valle, et al., 2003).
Nitric oxide (NO)					
IC ₅₀ for platelet aggregation					
Nitrophorin (NP7)	<i>R. prolixus</i>	19	~ 10 nM	Y/N/Y	Inhibits ADP-induced platelet aggregation and causes disaggregation (Andersen, et al., 2004).
Cimex nitrophorin	<i>C. lectularius</i>	32	NA	N/N/N	Putative inhibitor of platelet aggregation (Valenzuela, et al., 1995).
Platelet cAMP					
IC ₅₀ for platelet aggregation					
PGI2	<i>I. scapularis</i>	352	~ 1 nM	na/na/N	Increases intraplatelet cAMP (Ribeiro, et al., 1988).
LysoPC	<i>R. prolixus</i>	518	~ 10 μg/ml	na/na/Y	Increases intraplatelet cAMP (Golodne, et al., 2003).
Adenosine	<i>P. papatasi</i>	267	~ 1 μM	na/na/N	Increases intraplatelet cAMP (Ribeiro, et al., 1999).
Thrombin					
Affinity and mechanism					

Targets and Inhibitors	Species	Molecular mass (mature protein)	Target	R/S/P	Comments and References
More than 20 inhibitors	See (*)	See(*)	See(*)	See(*)	(*) Usually low mol mass proteins, with or without cysteines. Usually bind to exosite, catalytic site or both. See (Koh and Kini, 2009, Maritz-Olivier, et al., 2007, Ribeiro and Francischetti, 2003, Steen, et al., 2006).
Integrin αIIbβ3					
IC₅₀ for platelet aggregation					
Disagregin	<i>O. moubata</i>	6	104 nM	N/N/Y	Without RGD. Kunitz-containing protein (Karczewski, et al., 1994).
Decorsin	<i>M. decora</i>	4	500 nM	Y/Y/Y	With RGD. Synthetic form active. Protect mice against death from pulmonary thromboembolism (McLane, et al., 1995).
Ornatin	<i>P. ornata</i>	5	130 nM	N/N/Y	With RGD (Mazur, et al., 1991).
Savignygrin	<i>O. savignyi</i>	7	130 nM	N/N/Y	With RGD. Kunitz-containing protein (Mans, et al., 2002).
Integrin αIIbβ3 (continued)					
IC₅₀ for platelet aggregation					
Variabilin	<i>D. variabilis</i>	5	157 nM	N/N/Y	With RGD (Wang, et al., 1996).
Tabinhibitin	<i>T. yao</i>	25	<40 nM	N/N/Y	With RGD (Ma, et al., 2009).
Ixodegrin	<i>Ixodes</i> sp	7	NA	N/N/N	With RGD (Francischetti, et al., 2005).
Savignygrin-like	<i>O. coriaceus</i>	7	NA	N/N/N	With RGD. Kunitz-containing protein (Francischetti, et al., 2008).
Monogrin	<i>A. monolakensis</i>	10	150 nM	Y/N/Y	With RGD. Kunitz-containing protein (Mans, et al., 2008).
Fibrinolytic enzymes					
Specificity/affinity					
Metalloprotease	<i>I. scapularis</i>	37	Fibrin(ogen), ?	N/N/N	Degrades A α -chain of fibrinogen and fibrin. Degrades fibronectin but not collagen (Francischetti, et al., 2003).
Metalloprotease (Tablysin)	<i>T. yao</i>	35	Fibrin(ogen), ?	N/N/N	Degrades A α or B β -chains of fibrinogen (Ma, et al., 2009).
Metalloprotease (Hementin)	<i>H. ghilianii</i>	~80	Fibrin(ogen)/K m 1 μ M	N/N/N	Degrades A α , B β , and γ chains of fibrinogen and fibrin (Budzynski, 1991).
Metalloprotease (Hementerin)	<i>H. depressa</i>	80	Fibrin(ogen)/?	N/N/N	Degrades A α , B β , and γ chains of fibrinogen and fibrin (Chudzinski-Tavassi, et al., 1998).

R, obtained in recombinant form.

S, structure available.

P, inhibition of platelet aggregation tested with recombinant or purified protein.

na, not applicable.

NA, not available

5-HT, serotonin; EPI, epinephrine; NE, norepinephrine.