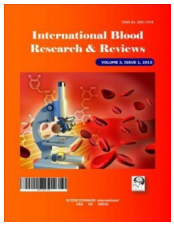

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Thrombin Structural and Functional Determinants as Therapeutic Targets

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Thrombin is the final serine protease generated during blood coagulation. This enzyme possesses a complex and fascinating structure composed not only by an active site but also by two positively charged patches, called anion binding exosites o simply exosites 1 and 2, as well as several surface loops and a Na+ binding region. These complex structural determinants make of thrombin a highly versatile enzyme with multiple and opposed roles within haemostasis by cleaving different substrates, and interacting with diverse cofactors and inhibitors. However, it is well known that thrombin actions are not limited to haemostasis. Thrombin has also multiple functions or pleiotropic effects, interconnecting coagulation to other systems, including the immune and the nervous system. This review focus on thrombin as therapeutic target of direct thrombin inhibitors, and highlights the pharmacology of anticoagulants in clinical use; including unfractionated heparin, low-molecular-weight heparins as well as lepirudin, desirudin, bivalirudin, argatroban and dabigatran etexilate. Adverse effects, antidotes and monitoring of these anticoagulants are discussed in detail. Finally, we also review recent advances on the development of aptamers as thrombin inhibitors.

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Keywords: Thrombin; structure; function; direct thrombin inhibitors; heparins; hirudin; argatroban; dabigatran; aptamer.

ABBREVIATIONS

aPTT : Activated partial thromboplastin time
AT : Antithrombin
DVT : Deep vein thrombosis
DTI : Direct thrombin inhibitor
HIT : Heparin-induced thrombocytopenia
IV : Intravenous
LMWHs: Low molecular weight heparins
PARs : Protease-activated receptors
PF4 : Platelet factor 4
PCI : Percutaneous coronary intervention

1. INTRODUCTION

Thrombosis is the formation of a blood clot that obstructs vessel flow of arteries and veins [1]. The mechanisms underlying both events are different. In general, arterial thrombosis is triggered by the rupture of an atherosclerotic plaque; thrombi are rich in platelets and are referred to as white-clots. In opposition, venous thrombosis or thromboembolism occurs due to changes of the composition of the blood and/or changes in the vessel walls (endothelium), which favor clotting, and is referred to as red clots (rich in fibrin with trapped red blood cells). The arterial and venous thrombosis are prevented and treated differently. The drugs used to treat arterial thrombosis are mainly directed to inhibit platelet activation and aggregation, and those for venous thrombosis are designed to reduce the activity of certain coagulation proteases. The antiplatelet therapy includes ADP-receptor antagonists and inhibitors of cyclooxygenase, protease-activated-receptor type-1 (PAR1) and $\alpha_{IIb}\beta_3$ integrin. On the other hand, vitamin K antagonists, heparins and direct inhibitors of factor Xa are used for anticoagulation. Thrombin inhibitors are applied to both arterial and venous thrombosis, since in both cases thrombin formation is the required step to trigger clot formation [1].

We review thrombin functions, structure and regulation by diverse inhibitors, and deal with more depth the pharmacology of heparins and direct thrombin inhibitors.

2. THROMBIN FUNCTIONS

Blood clotting involves a physical change from a liquid into a semi-solid state. This transition occurs due to the generation of thrombin. Thrombin is a serine protease that is formed once coagulation is triggered either by a vascular lesion or other stimuli that promotes tissue factor (TF) exposure.

Thrombin has multiple and opposed roles in haemostasis: procoagulant [2], anticoagulant [3], profibrinolytic and antifibrinolytic [4,5]. The procoagulant function includes the activation of different zymogens, cofactors, platelets receptors, and fibrinogen. Thrombin catalyzes its own generation through activation of factor V, VIII, and XI. Thrombin is the only physiologic enzyme that modifies fibrinogen leading to fibrin formation. Once formed, fibrin monomers polymerize producing the tridimensional scaffold of the clot, which is stabilized by activated factor XIII that is also activated by thrombin.

Thrombin downregulates its own generation after its binding to thrombomodulin through the activation of Protein C (PC) that inactivates factors VIIIa and Va exhibiting anticoagulant properties. Thrombin also shows profibrinolytic activity in cultured human umbilical vein endothelial cells by stimulating both tissue plasminogen activator (tPA) release and p11 synthesis [6,7]. On the surface of endothelial cells, the annexin A_2-p11 complex acts as receptor for plasminogen and tPA, increasing plasmin formation [8].

Thrombin regulates negatively fibrinolysis by activating thrombin activatable fibrinolysis inhibitor (TAFI) that slows fibrin degradation by removing the C-terminal Lys and Arg residues from fibrin [9] and by stimulating PAI 1 secretion [10]. The multiple and opposite roles of thrombin in haemostasis are summarized in Table 1.

The action of thrombin is not limited to haemostasis. Thrombin has multiple functions or pleiotropic effects, most of them have been described in *in vitro* systems [11]. Thrombin induces the proliferation of different cells type [12-14]. Thrombin can induce the release of vasoactive cytokines, and chemoattractants [15-17]. It is itself a chemoattractant [13]. Thrombin increases the permeability of vascular endothelium [18], promotes adhesion to endothelial cells [19], and induce contraction of smooth muscle cells [20]. Thrombin has been implicated in angiogenesis [21], growth and metastasis of tumors [22], the initiation of bone

Table 1. Summary of thrombin functions in haemostasis

Function	Action
Procoagulant	• Activation of coagulation factors V, VIII, XI and XIII. • Activation of fibrinogen (fibrinopeptide A and B release). • Platelet activation
Anticoagulant	• Activation of protein C
Profibrinolytic	• Stimulation of tPA secretion • Stimulation of p11 synthesis
Antifibrinolytic	• Activation of TAFI • Stimulation of PAI 1 secretion

absorption [23], regulation of neurite outgrowth [24], and muscle development [25]. More recently, thrombin has been implicated in inflammation [26] and inflammatory pain [27,28], in synaptic transmission and plasticity [29]. These thrombin cellular functions are mediated at least in part by a small family of G protein-coupled protease-activated receptors (PARs). These receptors are activated by irreversible proteolytic cleavage, leading to the exposure of a new amino-terminus, which serves as a tethered ligand unique for each receptor. Three of the four members of this receptor family, PAR1, PAR3 and PAR4 are cleaved and activated by thrombin and therefore are considered as functional thrombin receptors [2]. Other serine proteases can also activate PAR1 and PAR4. However, PAR3 is activated only by thrombin and PAR2 is not cleaved by thrombin.

The model of thrombin allostery, which explained how the enzyme activity was regulated by Na+ [30,31] is not longer sustained. Different technical approaches have shown that thrombin exists as an ensemble of conformations of zymogen-like forms (apo-thrombin) that upon the binding of a ligand switches toward allosterically stabilized conformations of protease-like forms [32,33]. This knowledge has provided new insights of how thrombins´ multiple functions are regulated [34].

3. THROMBIN GENERATION

Prothrombin is a vitamin K-dependent zymogen that must be proteolytically activated to express enzymatic activity. Like other vitamin K-dependent factors (including factors VII, X and IX), prothrombin bind to phospholipids surface via its γ-carboxyl glutamic acid residues or Gla domains to form the prothrombinase complex. This complex also includes the serine protease factor Xa, the cofactor Va, and calcium ions, which are essentials for the binding of Gla domains to negatively charged phosphate groups on phospholipids [35]. The prothrombin activation requires the cleavage of two peptide bonds by factor Xa. Dependent on the order of cleavage, prethrombin 2 or meizothrombin could be formed as intermediate [36]. The cleavage at Arg_{271}-Thr_{272} bond yields fragment 1+2 and prethrombin 2. Then the cleavage of Arg_{320}-Ile_{321} bond in prethrombin 2 yields full active thrombin. Cleavage of prothrombin at Arg_{320}-Ile_{321} bond generates meizothrombin that has enzymatic activity to small synthetic substrates but not to macromolecules as fibrinogen [37]. Meizothrombin is autocatalytically cleaved at Arg_{156} releasing fragment 1; generating meizothrombin des-fragment 1, which by subsequent autocatalytic cleavage at Arg_{283} is rapidly converted into thrombin. Thrombin is the only vitamin K-dependent protease generated in blood coagulation that lost its membrane anchoring Gla domain and can freely diffuse to encounter its multiple substrates in several environments.

The coagulation extrinsic pathway forms only tiny amount of thrombin in the area where tissue factor is exposed (approximately 1 nM), due to the fact that tissue factor-factor VIIa-factor X (Xa) complex is rapidly inhibited by tissue factor pathway inhibitor (TFPI). These trace amounts of thrombin are enough to activate platelets and other coagulation factors that generate in situ a burst of thrombin higher than 500 nM [38]. Typically low thrombin concentrations (<10 nM) are associated with platelet activation and thick, loosely organized fibrin strands that are more susceptible to fibrinolysis, while high concentrations produce a stable clot characterized by tightly-packed fibrin strands [39]. It is plausible that fibrin formation during thrombin generation includes a complex spatial component. The physical progression of procoagulant activity from the tissue factor-bearing cell (low levels of thrombin generation) to the activated platelet surface (rapid burst of thrombin generation) it is likely to induce in space a thrombin gradient. Given the direct effects of thrombin concentration on fibrin formation, this gradient may therefore cause the formation of a

range of fiber thicknesses across a region of growing thrombus [38].

4. THROMBIN STRUCTURE

Thrombin is a serine protease composed by a light chain (A chain) of 49 residues, and a heavy chain (B chain, the catalytic subunit) of 259 residues (for recent reviews see references [32-34]). Thrombin removes autocatalytically the first 13 amino acids from the A chain to form α-thrombin [40]. The A and the B chains are linked together by a disulphide bridge. The catalytic triad (active site) is composed by His_{57}, Asp_{102}, and Ser_{195} [41] (Fig. 1A).

The primary cause of the narrow specificity of α-thrombin is given by the presence of the 60 and the 148 insertion loops at the border of the canyon-like active site cleft, which block the access of many macromolecular substrates or inhibitors [42]. The extended Na^+-binding loop is composed of residues 215–225 (Fig. 1B), and these and adjacent residues on the 180 loop (184–194) form the primary specificity (S1) pocket, where is accommodated the side chain of Arg residue (P1) present in most of thrombins´ substrates and inhibitors.

The external surface of thrombin has two positively charged patches that are responsible of substrate/inhibitors thrombin interactions named anion binding exosites, in near opposition on the thrombin surface [43]. The exosite 1 is composed of both insertion loops 30-40 and 70-80 [41,44]. Due to its interaction with fibrin/ogen, this patch is also designated as fibrinogen recognition site [45,46]. The second positively charged surface patch, the exosite 2, has an even stronger positive electrostatic field [44] that extends from the intermediate helix towards the C-terminus of thrombin. Due to its interaction with heparin this positively charged patch has been also named as heparin binding site. The overall structure of thrombin is depicted in Fig. 2.

Thrombin exosites bind substrates, cofactors, and some inhibitors. The exosite 1 recognizes fibrin/ogen, coagulation factors XIII and XI, PAR1, PAR4, heparin cofactor II and hirudin [47-49], while exosite 2 binds prothrombin fragment 2, heparin, GPIbα, fibrinogen γ´ [50], and polyphosphates [51]. Some substrates use both exosites, as thrombomodulin, factors V and VIII [43].

There is an increasing body of evidence supporting that exosite 1 and 2 are communicated [50,52-54]. The occupancy of exosite 1 by a ligand decreased the affinity of thrombin toward ligands of exosite 2, and *vice versa.*

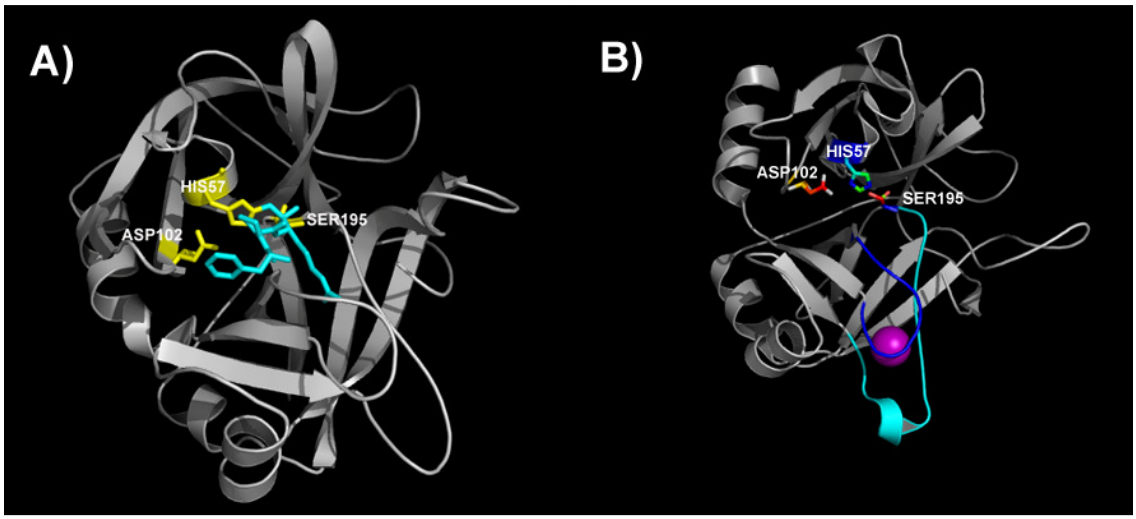

Fig. 1. Representation of the thrombin catalytic triad and Na^+-binding loop

A) Ribbon diagram of thrombin B chain was performed from 1PPB. The amino acids of the catalytic triad: His_{57}, Asp_{102} and Ser_{195} are highlighted in yellow. The thrombin molecule was co-crystallized with PPACK (cyan). B) Ribbon diagram of thrombin B chain from 1JOU. Since in the 1JOU the Ser_{195} is mutated to Ala, in order to represent the active site, the Ala_{195} was replaced by Ser. The amino acids of the catalytic triad are highlighted in rainbow. The sphere in purple represents the Na^+ ion surrounded by loops 184-194 in cyan and 215-225 in blue. The molecules were generated using PYMOL 1.7.4.1

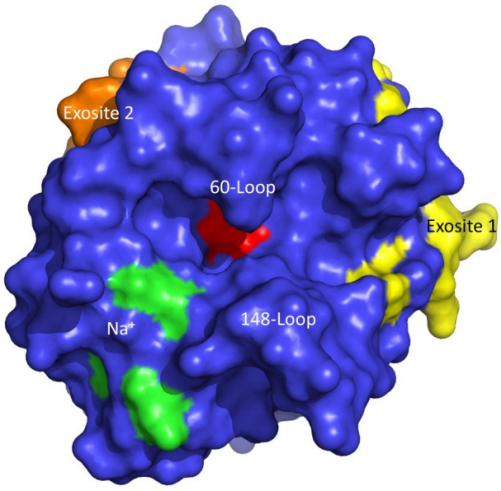

Fig. 2. Thrombin overall structure

The surface of thrombin is represented. The exosite 1 (east, yellow) and 2 (west, orange) are located in opposite direction. The residues that comprise the active center are highlighted in red, and Na+ binding loop in green. This model was generated with PDB file 1PPB [44] using PYMOL 1.7.4.1

5. PHYSIOLOGICAL THROMBIN INHIBITORS

The regulation of thrombin generation is vital to the maintenance of the hemostatic balance. One of the major mechanisms by which the amount of active thrombin is kept under control is through irreversible inhibition of thrombin by non specific protease inhibitors, such as α1-proteinase and α2-macroglobulin as well as by serine protease inhibitors (also called serpins), specifically antithrombin and heparin cofactor II. The rate of thrombin inactivation by antithrombin and heparin cofactor II is greatly accelerated in the presence of heparin and certain glycosaminoglycans [55,56].

Recently, it has been reported that the cartilage oligomeric matrix protein (COMP), a matricellular protein also known as thrombospondin-5, also inhibits thrombin by interaction with both exosites. COMP is also expressed in and secreted by platelet [57].

5.1 Antithrombin

The physiological inhibitor of thrombin is antithrombin (AT), ATIII in the old nomenclature. AT is a single chain glycoprotein of 432 amino acids, with three intramolecular disulfide bonds and four glycosylation sites at Asn_{96}, Asn_{135}, Asn_{155}, and Asn_{192} [58]. The predominant form of the plasmatic human AT (AT α) is fully glycosylated. However, a minor fraction (AT β) lacks the carbohydrate side chain at Asn_{135}, and shows a slightly higher affinity for heparin [59,60]. AT β appears to be an effective inhibitor of the thrombin activity after vascular injury.

Thrombin inactivation is the result of the formation of an equimolar tight complex with AT, where the active site of thrombin is inaccessible to substrates [61]. The inactivation by AT alone is rather slow and occurs via a two step mechanism, which involves the formation and subsequent stabilization of the AT-thrombin complex [62].

The slow inhibition of thrombin by AT is primarily due to the low affinity between the two proteins in the initial complex. The assembly of the AT-thrombin complex involves the interaction between the active site of thrombin and the reactive Arg_{393}-Ser_{394} bond in the carboxy terminal region of AT [63]. The attack of the enzyme on this bond induces a conformational change that arrests the cleavage of the reactive bond and traps the enzyme in a stable complex. However, a fraction of the enzyme escapes this trapping by completing the cleavage of the bond before the conformational change occurs, resulting in release of free enzyme and cleaved inhibitor [62].

The rate of this reaction is greatly accelerated by heparin, a strong negatively charged, highly sulfated glycosaminoglycan. The predominant pathway for the heparin-accelerated inhibition of thrombin by AT appears to be a reaction between the binary AT-heparin complex and free thrombin. This ternary complex is formed first by the binding of thrombin to heparin, and then diffusing along the heparin chain to encounter the bound inhibitor. The binding of thrombin and inhibitor to the same heparin molecule (in a ternary complex) is essential for a full rate enhancement (Fig. 3A) [62,64].

The AT also inactivates other proteases of the intrinsic coagulation system, primarily factor IXa and factor Xa, and non-coagulation serine proteases such as plasmin, C1s and trypsin [65].

5.2 Heparin Cofactor II

Although the activity of thrombin generated during blood coagulation is regulated primarily by AT, the extravascular thrombin activity,

associated with inflammation and wound healing, is thought to be inhibited by heparin cofactor II (HCII) [66]. This inhibitor has several features that make it unique among heparin-binding serpins. HCII accelerates thrombin inhibition by binding dermatan sulfate [67]. HCII is also unique because it has Leu_{444} at the P1 position, whereas most serpins, and thrombin substrates contain an Arg [44,68]. The presence of a P1-Leu enables HCII chymotrypsin inhibition faster than thrombin without glycosaminoglycans [69]. In addition to a glycosaminoglycan-binding site, HCII has a highly acidic N-terminal that interacts with thrombin exosite 1. It seems that this acidic tail occupies HCII's own heparin-binding site that is displaced in the presence of heparin, and becomes available for binding to thrombin exosite 1. The exosite interaction allows the binding of an unfavorable P1 residue to the active center of thrombin [70]. Therefore, thrombin inhibition by HCII in presence of glycosaminoglycans occur by binding of the acidic region of HCII to exosite 1, and the glycosaminoglycans moiety to exosite 2 [66].

6. THROMBIN AS THERAPEUTIC TARGET

6.1 Heparins

The increased rate of thrombin inhibition by AT or HCII in presence of glycosaminoglycans makes unfractionated heparin (UFH) and its depolymerized products, low molecular weight heparins (LMWHs), the most widely used parenteral anticoagulants. They have been recommended for prevention and treatment of venous thrombosis in surgical and acutely ill medical patients [71].

UFH is a natural anticoagulant synthesized and secreted primarily by mast cells. As a therapeutic product, it is derived from porcine intestinal mucosa, or bovine lung. UFH is a heterogeneous mixture of branched sulfated glycosaminoglycans with molecular weights ranging from 5 to 40 kDa, with a mean molecular weight of 12 to 15 kDa [72]. UFH is heterogeneous not only respect to molecular size, but also to anticoagulant activity and pharmacokinetic properties.

UFH produces its major anticoagulant effect by inactivating thrombin and FXa through an AT-dependent mechanism. UFH has a pentasaccharide motif that binds with high-affinity to lysine residues on AT. Heparin interacts with thrombin exosite 2 and the formation of the ternary complex leads to thrombin inhibition. Heparin then dissociates from the ternary complex and can be reused. The UFH-AT complex is 100 to 1,000 times more potent than AT alone [73,74]. Moreover, the action of UFH-AT complex on thrombin, not only prevents fibrin formation but also thrombin-induced activation of factor V, VIII, XI and IX, as well as platelets. Thereby, UFH prevents further growth and propagation of a formed thrombus, but the UFH–AT complex is unable to inactivate the thrombin within or bound to the clot due to its relatively large size and the obligate co-occupation of AT and thrombin on the same heparin chain [56,75-77]. Additionally, the UFH–AT complex inhibits the activity of other clotting factors, including factors IXa and XIIa. However, only about one third of UFH molecules contains the pentasaccharide sequence that binds to AT. This fraction is responsible for most of its anticoagulant activity.

Moreover, UFH activates HCII and thereby inactivates thrombin through a second mechanism. This interaction is charge-dependent, but it is pentasaccharide-independent and requires a higher concentration of UFH than that required for thrombin's´ AT-mediated inactivation. The HCII-mediated anticoagulant effect of UFH could operate in cases of severe AT deficiency.

Other anticoagulant effect of UFH results from an AT- and HCII-independent modulation of factor Xa generation. It is charge-dependent, is mediated by heparin binding to factor IXa, and requires very high doses of UFH to produce an anticoagulant effect [78].

Furthermore, the biologic activity and bioavailability of UFH is also limited by its high binding capability to plasma proteins and cells, which reduces its anticoagulant activity and contributes to the variability of the anticoagulant response to heparin among patients, and to heparin resistance [79].

UFH is given parenterally because it is not absorbed in the gastrointestinal tract due to its large molecular size and anionic nature. After subcutaneous (SC) injection, the anticoagulation effect of UFH is usually around one to two hours. When rapid anticoagulation is needed, UFH may be given intravenously [79,80]. The bioavailability of subcutaneous UFH is dose-dependent. The bioavailability ranges from 30% at lower doses to as much as 70% at higher doses. The plasma

half-life of UFH is approximately 30 to 90 minutes in healthy adults, and rises with increasing UFH doses.

UFH is cleared through the combination of two dose-dependent mechanisms. Low doses of UFH are cleared mostly by heparinases and desulfatases that enzymatically inactivate heparin molecules that are bound to endothelial cells and macrophages. UFH is also eliminated by the kidneys. Renal clearance is a first-order process that is slower and nonsaturable, and predominantly occurs at very high doses. Therapeutic doses of UFH are eliminated by a combination of these two mechanisms. Renal and hepatic dysfunction reduce the rate of UFH clearance [80].

New agents with more predictable pharmacokinetic profiles such as the low molecular weight heparins (LMWHs) have proven to be just as, or more effective for the same indications than UFH.

LMWHs are produced by treating UFH chemically or enzymatically to yield products with lower molecular weight of approximately 5 kDa. For example, enoxaparin (Lovenox®, Aventis) is prepared by treating UFH by chemical benzylation followed by alkaline depolymerization, tinzaparin (Innohep®, Leo and Pharmion) by enzymatic depolymerization of UFH with heparinase, and dalteparin (Fragmin®, Pharmacia & Upjohn) by controlled nitrous acid depolymerization [81]. These LMWHs have been approved by the federal drug administration (FDA), and are commercially available for use in USA. Other LMWHs that are used in Europe and in other countries include reviparin, nadroparin, bemiparin and certoparin.

LMWHs also contain the pentasaccharide motif that bind to AT, although they are not long enough to bind thrombin. For this reason, LMWHs lose some of their effect against thrombin, and its main effect is the inhibition of factor Xa activity. LMWHs have distinct biochemical and pharmacological properties, e.g. they differ in their average size from 4.3 to 5.8 kDa, and also in the ratio of inhibitory activity toward thrombin and factor Xa [78,81].

Compared to UFH, LMWHs have higher bioavailability (85-99%) after SC administration. The elimination half-life of anti-Xa activity is 2.4 h for dalteparin, 3.0 h for tinzaparin and 4.3 h for enoxaparin [82]. LMWHs are predominantly eliminated by renal filtration, so their half-lives are extended in renal failure. Moreover, the anti-Xa activity persists longer than the anti-thrombin effect due to the faster clearance of the longer heparin molecules.

6.2 Direct Thrombin Inhibitors

In the last 20 years a number of proteins and peptides with different molecule mass and well established antithrombin activity have been isolated from the salivary glands of several bloodsucking animals. Most of these inhibitors have undergone genetic engineering and thus, they have been available in adequate amounts for experimental and clinical pharmacological studies. Hirudin is the most widely studied direct thrombin inhibitor (DTI) which was isolated from the european leech Hirudo medicinalis [87]. It neutralizes only thrombin, and not other clotting serine proteases. At present, approximately 20 natural variants have been described [88,89]. The major form of natural hirudin is a polypeptide of 65 amino acids. All hirudins display a disulfide-linked N-terminal core and a highly acidic C-terminal tail. They exhibit the same cysteine spacing, a conserved N-terminal sequence that is either Val-Val or Ile-Thr and a sulfated tyrosine (Tyr_{63}) in the C-terminal part of the peptide. Hirudin reacts rapidly with thrombin, forming a tight equimolar complex with a Ki of 21 fM [90].

All hirudins bind tightly to thrombin by interacting with the active site and exosite 1 (Fig. 3B). The structure of hirudin has been determined both in solution [91,92] and in crystal complexes with thrombin [93,94]. In solution, the N-terminal domain forms a compact structure from Tyr_3 to Lys_{47}, but the first two residues, and the C-terminal tail are disordered. However, in the thrombin-hirudin complex, the amino terminal two residues and the C-terminal tail become ordered, and interact with the enzyme. The interaction with the catalytic site involves a unique hydrogen bond between the hirudin amino terminus and Ser_{195} of the catalytic triad. Furthermore, by sitting over the active site, the N-terminal core blocks the access of substrates to thrombin's active site [47,93,94], and simultaneously the C-terminal tail occupies the exosite 1 (Fig. 3B).

The salivary glands of other bloodsucking animals like tics, assassin bugs and mosquitoes also contain a large number of DTIs of different structures. Some examples of these natural thrombin inhibitors are rhodniin and dipetalogastin, isolated from the assassin bugs

Rhodnius prolixus and *Dipetalogaster maximus*, respectively [83,84]; anophelin purified from the salivary gland of the mosquito *Anopheles albimanus* [85], or savignin isolated from the salivary glands of the tick *Ornithodoros savignyi* [86], among others. It is common in these inhibitors a bivalent binding mode. They interact with thrombin at two different sites, generally the active site and exosite 1. Moreover, they share common mechanisms for thrombin inhibition, characterized by strong affinities for thrombin with Ki ranging from picomolar to femtomolar values and generally, their kinetic pattern correspond to slow tight binding inhibitors.

Genetic engineering techniques also allowed the modification of natural occurring inhibitors, as in the case of dipetarudin, designed replacing the bulky C-terminal structure of dipetalogastin II by the C-terminal tail of hirudin. Biochemical analysis revealed that dipetarudin has molecular mass of 7560 Da and inhibits thrombin with a Ki value of 0.45 pM [87].

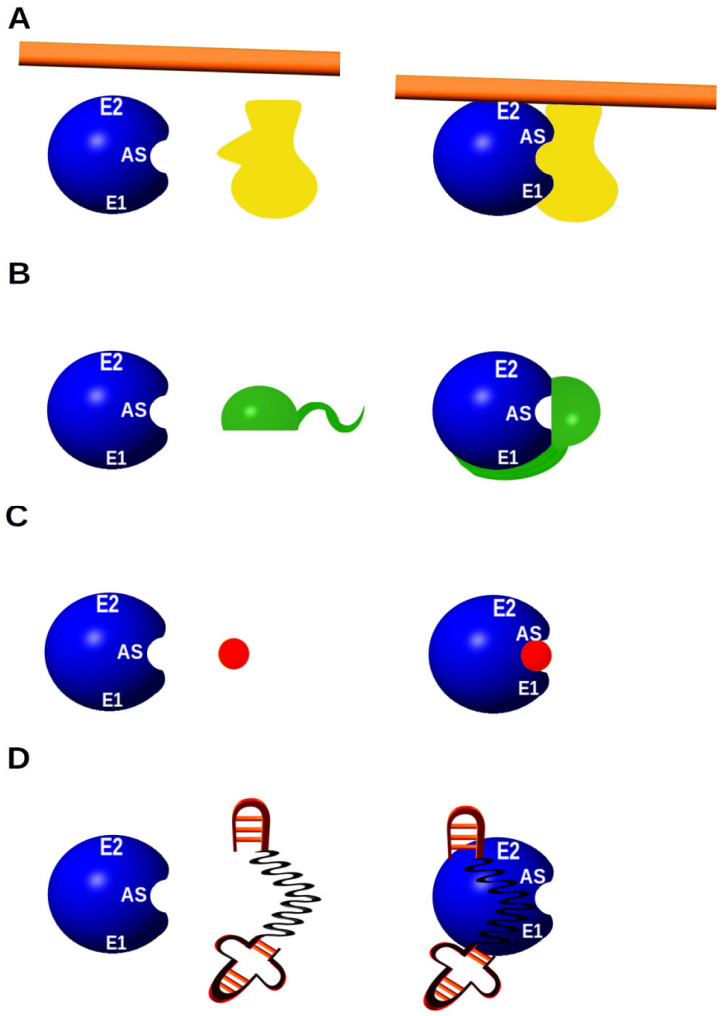

Fig. 3. Thrombin inhibitors

The enzymatic activity of thrombin can be downregulated by different strategies: A) Physiological Inhibitors or serine protease inhibitors present in blood that bind irreversibly to thrombin such as AT. Thrombin inactivation results by the formation of an equimolar ternary complex between thrombin (blue), AT (yellow) and heparin (orange), in which the active site (AS) of thrombin is inaccessible to substrates. B) Bivalent inhibitors block thrombin activity at two different sites, the AS and exosite 1 (E1), as desirudin (green) and bivalirudin. C) Univalent inhibitors interact with thrombin only at the active site (AS), as dabigatran and argatroban (red). D) Nucleic acid-derived thrombin inhibitors or aptamers are single-stranded RNA or DNA molecules that can bind to either exosite 1 (E1) or exosite 2 (E2) (univalent) or both (bivalent), as represented in this figure

The efforts for developing DTIs have been focused on the synthesis of peptide mimetic with antithrombin activity. Several laboratories have synthesized large numbers of thrombin inhibitors. A common principle for designing inhibitors is the modification of their substrates. The most useful skeleton in designing active-site directed thrombin inhibitors was the sequence Phe-Pro-Arg-H, especially if phenylalanine is in the D-configuration [88]. Furthermore, another of the building blocks used to develop synthetic inhibitors is arginine with optimal C and/or N-terminal modifications. In general, these thrombin inhibitors are univalent by interacting only with thrombin active site (Fig. 3C). They have weaker affinities for thrombin (Ki in the nanomolar range) than natural occurring DTIs and with diverse types of inhibition that could be irreversible, reversible competitive or reversible non-competitive.

The DTIs have been extensively used in order to prevent and treat thrombosis. Up today, four parenteral: desirudin (Iprivask and Revasc, Canyon Pharmaceuticals), lepirudin (Refludan, Bayer HealthCare Pharmaceuticals), bivalirudin (Angiomax, the Medicines Company), and argatroban (Argatra, GlaxoSmithKline), and one oral DTI, dabigatran etexilate (Pradaxa, Boehringer Ingelheim), have been approved as anticoagulants for clinical use. The first three inhibitors are hirudin-derived analogs, while argatroban and dabigatran are non-hirudin like synthetic thrombin inhibitors.

DTIs are classified according to their interaction with thrombin, as mentioned previously. The bivalent DTIs desirudin, lepirudin, and bivalirudin block thrombin at two different sites—the active site and exosite 1, while the univalent DTIs like argatroban and dabigatran interact with thrombin only at the active site [89].

DTIs do not depend on cofactors to inhibit thrombin. They can inhibit free thrombin as well as thrombin bound to fibrin [75,90]. DTIs are also able to inhibit other thrombin-catalyzed reactions, such as PARs activation on platelets and other cells [91,92].

Each DTI has unique properties and are prescribed for different indications. They differ in potency, pharmacokinetics, dosage regimen and routes of administration (Table 2).

6.2.1 Lepirudin

Hirudin was first obtained in recombinant form in yeast cells as lepirudin (Refludan®). Lepirudin was the first DTI to be approved for clinical use for the treatment of heparin-induced thrombocytopenia (HIT) complicated by thrombosis. Lepirudin is identical to natural hirudin except for a leucine at the N-terminal end of the molecule, and the absence of a sulfate group on Tyr_{63} that reduces tenfold its affinity for thrombin (0.22 pM) [93,94].

The recommended lepirudin dose was 0.4 mg/kg as a single intravenous (IV) injection, followed by a continuous infusion of 0.15 mg/kg per h for 2 to 10 days, or longer. Lepirudin is currently not available in the market; it was withdrawn by the manufacturer in 2012. This was a business decision and not due to safety concerns (http://www.drugs.com/drug-shortages/lepirudin-injection-924).

6.2.2 Desirudin

Desirudin (Iprivask®), another recombinant hirudin produced in *Saccharomyces cerevisiae* was approved in USA for thrombosis prophylaxis after major orthopedic surgery. Desirudin is used to decrease the risk of deep vein thrombosis (DVT) and pulmonary embolism (PE) in patients undergoing hip replacement surgery. It was also approved for DVT prophylaxis in Europe under the trade name Revasc®.

Desirudin is nearly identical to hirudin with the exception that it lacks a sulfate group on Tyr_{63}. As expected, the affinity constant is comparable to that of lepirudin.

Desirudin is the only DTI that is administered subcutaneously (SC) at a fixed dose, and it does not need activated partial thromboplastin time (aPTT) monitoring. The usual dose is 15 mg immediately before surgery and every 12 h in patients with normal renal function. It is rapidly absorbed after SC administration, reaches maximum drug plasma concentration after 1 to 3 h at doses ranging from 0.1 to 0.75 mg/kg, with nearly 100% bioavailability [95]. Desirudin has an elimination half-life of approximately 3 h following SC administration [96].

Desirudin is primarily metabolized and eliminated by the kidneys [95]. Approximately 50% of the administered dose is eliminated unchanged in the urine [97], the rest is eliminated as metabolites lacking of 1 or 2 carboxy-terminal amino acids with lower inhibitory activity, degraded by kidney's carboxypeptidases [98]. In patients with renal impairment, overdose might occur even with standard dosage regimen.

Table 2. Direct thrombin inhibitors in clinical use

Name	Molecular mass (Da)	Ki (nM)	Binding site on thrombin	Trade name	Indication	Half-life (min)	Dose	Route of elimination	Monitoring
Desirudin	6985.5	0.00026	Active site and Exosite 1	Iprivask® Revasc®	Thrombosis prophylaxis after major orthopedic surgery	180	SC bolus of 15 mg before surgery and every 12 hours	Renal	Only necessary in patients with renal dysfunction
Bivalirudin	2180.2	2.3	Active site and Exosite 1	Hirulog®, Angiomax®	Anticoagulation by invasive intravascular surgery	25	IV bolus of 0.75 mg/kg followed by an infusion of 1.75 mg/kg*h	Proteolytic cleavage (80%) and renal mechanisms (20%)	aPTT 1.5-2.5 times the baseline values
Argatroban	526.6	39	Active site	Argatra®	Thrombosis treatment and prophylaxis in patients with HIT and during PCI with or at risk for HIT	39 to 51	IV bolus followed by an infusion of 2 µg/kg*min	Hepatic metabolism	aPTT 1.5–3 times the baseline values
Dabigatran	627.7	4.5	Active site	Pradaxa®	Prevention of stroke and systemic embolism in non-valvular atrial fibrillation	480	300 mg by oral route as one 150 mg capsule twice daily	Renal	Only necessary in patients with renal dysfunction

HIT: Heparin-induced thrombocytopenia; PCI: Percutaneous coronary intervention; SC: Subcutaneous; IV: Intravenous; aPTT: Activated partial thromboplastin time

Therefore, dosage adjustment and aPTT monitoring are recommended for these patients [99].

6.2.3 Bivalirudin

Bivalirudin (Hirulog®, Angiomax®) was approved for parenteral use during invasive intravascular surgery such as percutaneous coronary intervention (PCI), including percutaneous transluminal coronary angioplasty (PTCA), balloon angioplasty and PTCA with stenting. The use of bivalirudin for heparin-induced thrombocytopenia (HIT) has been reported. Bivalirudin has been effective in trials of angioplasty in patients with HIT [100] but it is not approved for treatment of HIT [101,102].

Bivalirudin is a synthetic analog of hirudin. It is a 20-amino acid peptide with a molecular weight of 2180.19 Da. Bivalirudin contains 2 structural domains: the N-terminal sequence (D-Phe_1-Pro_2-Arg_3-Pro_4) that binds to the thrombin´s active site, and the C-terminal sequence that binds to exosite 1. Both domains are connected by a tetraglycyl spacer. Bivalirudin has a binding affinity for thrombin of 2.3 nM that is approximately 10,000 times less than those of recombinant analogs. During its interaction with thrombin, bivalirudin is cleaved at Arg_3-Pro_4 bond, losing its inhibitory activity.

Bivalirudin is administered by continuous IV infusion. The recommended dose is an IV bolus of 0.75 mg/kg, immediately followed by an infusion of 1.75 mg/kg*h for the duration of the surgical intervention or up to 4 h post-PCI if required. The dose adjustment is recommended to maintain aPTT between 1.5-2.5 times the baseline values. Bivalirudin is cleared from plasma by a combination of proteolytic cleavage (80%) and renal mechanisms (20%), therefore, dose needs only to be adjusted in patients with moderate or severe renal impairment. Its elimination half-life is 25 min in patients with normal renal function [103,104].

6.2.4 Argatroban

Argatroban (Argatra®) was approved for thrombosis treatment and prophylaxis in patients with heparin-induced thrombocytopenia (HIT). Argatroban is also indicated as anticoagulant in adult patients undergoing PCI with or at risk for HIT [105].

Argatroban is a derivate from the amino acid L-arginine [106]. It has a molecular mass of 526.6 Da and interacts reversibly with the catalytic site of thrombin with a Ki of 39 nM [107,108]. Argatroban is 54% bound to human serum proteins, with binding of 20% to albumin and 34% to α1-acid glycoprotein. It is metabolized mainly by the liver and has a short elimination half-life ranging between 39 and 51 min [109-111].

Because of its short half-life, argatroban is usually administered as an IV bolus followed by an infusion of 2 μg/kg*min, titrated to achieve an aPTT 1.5–3 times the baseline value [109,112]. The anticoagulant effects are immediately achieved, and steady-state levels are maintained until the infusion is discontinued or the dose adjusted. Little or no argatroban is found in plasma after 4 h infusion cessation [107].

Caution should be taken when argatroban is administered to patients with hepatic dysfunction. Lower dose should be administered, and carefully titrated until the desired level of anticoagulation is achieved. No dosage adjustment is necessary in patients with renal dysfunction.

6.2.5 Dabigatran

Dabigatran Etexilate (Pradaxa®) is the only oral DTI. Dabigatran etexilate is licensed in more than 75 countries worldwide for the prevention of venous thromboembolism (VTE) after elective hip or knee arthroplasty. It is also indicated for the prevention of stroke and systemic embolism in non-valvular atrial fibrillation (AF), with at least one risk factor for stroke. Recently, dabigatran has been licensed for the treatment of DVT and PE and the prevention of recurrent DVT and PE in adults [113,114]. In all indications, fixed-dose regimens of dabigatran etexilate have provided effective anticoagulation. It is given at a dose of 220 mg once daily in patients undergoing elective total hip or knee replacement, and 150 mg twice daily for secondary prophylaxis for the treatment of VTE or stroke prevention in AF [115].

Dabigatran etexilate is a low molecular weight prodrug without pharmacological activity. After oral administration is rapidly absorbed and quickly hydrolyzed to dabigatran by nonspecific ubiquitous esterases in blood, gut, and liver, in a mechanism that is independent of the CYP enzymes and other oxidoreductases. The bioavailability after oral administration of dabigatran is only 6.5%. Moreover,

approximately 35% binds to plasma proteins, therefore relatively high doses must be given to ensure therapeutic plasma concentrations.

Dabigatran plasma concentrations and anticoagulant effects are dose dependent and predictable. It peaks 1.5 h after oral administration [116] or within 4 h with food. Due to its predictable pharmacokinetics and pharmacodynamics, dabigatran does not require routine monitoring [116].

The elimination half-life of dabigatran after oral administration is 8 h after a single dose, and ranges from 12 to 14 h after multiple doses in patients with normal renal function, which permits once- or twice-daily intake, and a fast offset of action [114,117]. There is no unexpected accumulation of dabigatran after multiple dosing. Dabigatran clearance is predominantly renal, with 80% excreted unchanged in the urine, and needs a dose adjustment when administered to subjects with renal dysfunction [118]. Age-related differences in dabigatran exposure are largely related to renal function, although there is a small additional effect due to advanced age. Studies on the pharmacokinetics and pharmacodynamics of dabigatran in healthy elderly subjects indicate that, compared with young healthy subjects, dabigatran bioavailability increases 1.7 to 2-fold in elderly subjects [119].

The pharmacokinetic profile of dabigatran can be affected by concomitant administration of several drugs. Dabigatran etexilate is a substrate for p-glycoprotein; thus drugs that inhibit or induce p-glycoprotein could potentiate or attenuate the anticoagulant effect of dabigatran [120].

6.3 Other Aspects of the Anticoagulant Therapy

6.3.1 Side effects

Hemorrhagic complications are the most common adverse effects of anticoagulant therapy. However, in addition to bleeding complications, heparin has limitations based on its ability to induce the formation of antibodies that activate platelets, leading to heparin-induced thrombocytopenia (HIT). The principal antigen is the complex between heparin and platelet factor 4 (PF4), a small positively charged molecule of uncertain biological function, normally found in the α-granules. Heparin's affinity for PF4 depends upon its molecular weight, chain length and degree of sulfation, which explains the differences in the incidence of HIT observed with different heparins [121]. HIT occurs in 3 to 5% of patients who receive intravenous UFH but less than 1% in patients receiving LMWHs. Paradoxically, despite thrombocytopenia bleeding is rare. Rather, HIT is strongly associated with thromboembolic complications involving both the arterial and venous systems. The heparin–PF4–IgG multimolecular immune complex activates platelets via their FcγIIa receptors, causing the release of prothrombotic platelet-derived microparticles. These microparticles in turn promote excessive thrombin generation, frequently resulting in thrombosis [122]. Moreover, a long-term UFH therapy can also affect bone metabolism, leading to osteoporosis. All of the non-hemorrhagic limitations of heparin are caused by the binding properties of UFH to proteins. LMWHs have a more favorable benefit/risk ratio than UFH. Indeed, LMWHs are associated with less major bleeding and non-hemorrhagic side effects than UFH [123,124].

The most common side effect of direct thrombin inhibitors is bleeding. DTIs can cause also hypersensitivity reactions, including anaphylaxis. Antibodies formation following the administration of recombinant hirudins have been reported, and hypersensitivity reactions are particularly common in persons who have been treated repeatedly with recombinant hirudins. Bivalirudin is a relatively small polypeptide and may therefore have minimal antigenicity in comparison with lepirudin and desirudin. However, cross-reactivity with bivalirudin has also been observed in 40% of patients with hirudin-induced antibodies. Fatal anaphylactic reactions following administration of hirudins are rare [125].

Several authors have reported some side effects for dabigatran including higher incidence of gastrointestinal bleeding – especially in older patients and those with low body weight [126]. Moreover, there was a trend towards a higher incidence of myocardial infarction in patients treated with dabigatran. Experts worldwide recommend caution with the use of dabigatran in clinical practice due to a number of unresolved issues, including lack of effective laboratory monitoring as well as safety concerns about its use in elderly and in patients with renal insufficiency [127].

6.3.2 Antidotes

In spite of heparin’s contraindications and side-effects, the clinical use of heparin remains high

because its anticoagulant activity can be controlled by an antidote, the polypeptide protamine.

UFH associated hemorrhages are treated by discontinuing the drug. Additionally, for severe hemorrhage, heparin may be neutralized by protamine sulfate at a dose of 1 mg for every 100 units. Protamine should be administered immediately after stopping heparin infusion. Intravenously infused protamine binds to heparin and hereby neutralizes its anticoagulant effects [128]. Treatment with fresh frozen plasma or platelet infusions is ineffective for controlling UFH-induced hemorrhages.

The treatment of major hemorrhage associated with LMWH is similar to UFH. However, the elimination half-life of LMWHs is longer. LMWH can partly be neutralized by protamine (30 – 40%), which binds to the fraction of longer heparin molecules in the LMWHs and neutralizes their antithrombin activity. For the smaller molecules in the LMWH with anti-factor Xa activity no antidote is available [128]. As with UFH, fresh frozen plasma or platelet transfusions are ineffective for LMWH-associated hemorrhages.

On the other hand, specific antidotes to DTIs are still under clinical development. Some different approaches have been used to antagonize dabigatran; one is based on an active site-mutated Ser_{195}Ala thrombin (Ser_{195}Ala-IIa), and its trypsinized derivative (T-Ser_{195}Ala-IIa). At the same dose in in vitro clotting tests, T-Ser_{195}Ala-IIa was more effective than Ser_{195}Ala-IIa in antagonizing dabigatran effects, both in human plasma and in a mouse model [129]. A second approach is a Fab-fragment of a humanized monoclonal antibody (idarucizumab; Boehringer Ingelheim). It acts by competitively displacing dabigatran from thrombin to reverse anticoagulation. Dabigatran has an affinity for idarucizumab that is 350 times greater than that for thrombin [130]. Also a small molecule (aripazine; PER977; Perosphere Inc.) has been reported to antagonize the effects of LMWH, dabigatran and some factor Xa inhibitors [131].

The anticoagulant effect of DTIs can be also antagonized by the administration of one or a cocktail of coagulation factors, or via removal of the active form of the drug. Indeed, beneficial effects has been reported on recombinant activated human factor VII (rhFVIIa) administration in dabigatran associated hemorrhage, although there is no clear evidence of its effectiveness [132]. rhFVIIa is an activated form of coagulation factor VII that bypasses the need for factor VIIIa and factor IXa. The use of rhFVIIa in non-hemophilic patients may increase the risk of thromboembolism and should be used with caution. Furthermore, an activated prothrombin complex concentrate (APCC) containing factor VIIa overcame the effects of hirudin, as measured by bleeding and whole blood coagulation times in rabbits [133].

Another study comparing four-factor prothrombin complex concentrate (4FPCC), fresh frozen plasma (FFP), and rFVIIa showed that 4FPCC was most efficacious in promoting survival, preventing intracerebral hematoma expansion, and reducing tail vein bleeding in mice anticoagulated with dabigatran, while FFP and rFVIIa showed little benefit [134]. Unfortunately, there is no complete agreement regarding the efficacy of APCC and 4FPCC for hirudin and dabigatran reversal [135].

For argatroban and bivalirudin, there is not specific antidotes available, although this may be less needed considering their relatively short half-lives [128]. Discontinuation leads to a fast reduction in the anticoagulant effects due to metabolism and elimination of the drug. Moreover, a case of overdose of argatroban has been managed with fresh frozen plasma and tolerated without significant complications [136].

Dialysis or haemofiltration can be also used to remove DTIs with the use of special dialysis membranes. Indeed, dabigatran can be dialyzed because of its relatively low plasma protein binding. Nevertheless, dialysis can reduce the plasma concentration of bivalirudin by only 25% and neither hirudin nor argatroban are eliminated by dialysis. It is important to highlight that these procedures take time and are difficult to perform in acute bleeding patients [131].

6.3.3 Monitoring

All parenteral DTIs (with exception of desirudin) require routine laboratory monitoring [135]. For desirudin and dabigatran, this monitoring is only necessary in patients with renal insufficiency (Table 2).

The activated partial thromboplastin time (aPTT) remains the most widely used monitoring test. Although the increase in aPTT is initially linear, as the dose of a DTI increases, it has a

curvilinear dose-response curve at higher levels of inhibitor [115]. Therefore, aPTT does not always correlate with the drug levels. This makes aPTT not suitable for precise quantification of DTIs´ anticoagulant effects. Even with this limitation, aPTT is widely used for DTI monitoring. Although thrombin time (TT) is highly sensitive, it cannot be used to monitor DTI activity because it exceeds its maximum measurable value, even for sub-therapeutic DTI levels [115]. Prothrombin time (PT) is relatively insensitive to plasma DTI levels and therefore it cannot be used [115,137].

Ecarin clotting time (ECT), a meizothrombin generation test performed with plasma or with whole blood, is more reliable for DTI monitoring because it shows a linear correlation to the DTIs levels over a wide range of concentration [138], including very high doses such as those used during cardiopulmonary bypass (CPB) [125]. Another newer meizothrombin assay, the ecarin chromogenic assays (ECA), provides a linear dose-response curve for all DTIs independently of the patient's prothrombin and fibrinogen levels [139]. These tests are relatively easy to perform, but unfortunately they are not widely available yet.

Since few years ago, a diluted thrombin time (Hemoclot) is commercially available and presents a linear response to dabigatran plasma levels but its availability is an issue [140].

6.4 Aptamers

During the last decade, it has been explored the synthesis of thrombin inhibitors from nucleic acids. Aptamers (or decoys) are single-stranded nucleic acid molecules (RNA/DNA) that can directly inhibit protein function by binding to their targets with high affinity and specificity, selected by a process known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [141,142].

The first thrombin binding aptamer (TBA), called HD1 or ARC 183, inhibits fibrinogen conversion to fibrin and thrombin-induced platelet aggregation [143,144]. HD1 has a 15-nucleotide G-quadruplex structure [d(5'GGTTGGTGTGGTTGG3')], which binds to prothrombin´s pre-exosite 1 and thrombin´s exosite 1 (Ki of 86 and 34 nM, respectively) [145]. HD1 is a better anticoagulant than Hir_{54-63}, it prolongs PT and aPTT 2-fold more than Hir_{54-63} [146]. This aptamer had no effect on thrombin inhibition by AT, with or without glycosaminoglycans. However, it affects 2-fold thrombin inhibition by HCII alone, and 200 and 30-fold in the presence of heparin and dermatan sulfate, respectively [147]. HD1 inhibits thrombin bound to fibrin, and has in vivo a short elimination half-life of approximately 2 min, which allows for rapid reversal of its effects. This aptamer was evaluated in Phase 1 clinical trial (Archemix and Nuvelo) during bypass surgery; however, it failed to show its efficacy [148]. A modified HD1 aptamer (mTBA): 3'GGT5'-5'TGGTGTGGTTGG3' has been synthesized with a 10-fold higher thrombin affinity [149], as an anticoagulant during coronay artery bypass graft (CABG) (http://www.hcp.com/content5788.html). However, it seems that it is no longer in clinical development.

Later a more potent second generation DNA aptamer (NU172) was developed. NU172 is composed by 26 nucleotides and has a Ki of 0.1 nM. It was under phase 1 and 2 clinical trial in healthy volunteers [150], and in patients undergoing off-pump CABG surgery (NCT00808964, http://clinicaltrials.gov). Although at present this study has not been updated. More recently, two new RNA aptamers have been synthesized (R9D-14 and R9D-14T). They bind thrombin exosite 1, hereby they inhibit thrombin functions dependent on exosite 1 binding. R9D-14T significantly prolongs aPTT and PT, and it is stronger than HD1 [151].

Two specific aptamers that bind to exosite 2 have been also synthesized: HD22 (141) and Tog25 (also called Toggle-25) [152]. HD22 has a Ki of 0.5 nM, and competitively inhibits binding of thrombin to platelet GpIα [153]. In addition, it limits the glycosaminoglycan-catalyzed inhibition of thrombin by serpins [154]. Tog25 is a 29-fluoropyrimidine modified RNA aptamer with a Ki for thrombin of 2 nM. It has a nominal effect on clotting times and on thrombin-mediated platelet activation [152,155].

Another aptamer, HD1-22, was synthesized by connecting HD1 and HD22 through a poly dA linker (15 nt). HD1-22 binds both exosites with a Ki of 0.65 nM, without blocking the active center of the enzyme (Fig. 3D) [156]. A series of clotting experiments showed that anticoagulant activity of HD1-22 was as effective as bivalirrudin and more effective than argatroban. HD1-22 also displayed slightly stronger inhibition on thrombin-induced platelet aggregation than bivalirudin.

In spite of the extensive research performed on these compounds and their benefits including the

lack of toxicity or immunogenicity [157] and the advantage of having antidotes, up to present they have not yet been approved for clinical use.

7. CONCLUSIONS

Recent investigations have demonstrated that thrombin exists in multiple conformations from an inactive state (apothrombin) to an active state with enzymatic activity. The interchange between these conformations is regulated by the binding of molecules to the exosite 1 and/or 2, which are also allosterically interconnected. The allosteric regulation of thrombin by Na+ is not longer sustained, although Na+ still remains as an important functional regulator of thrombin activity. This complex structure make of thrombin a highly versatile enzyme with multiple roles within and outside of haemostasis. The advances in the knowledge of thrombin structure and functions have allowed the design of specific thrombin inhibitors.

The qualities of an ideal thrombin inhibitor should be an easy route of administration, high efficacy and safety (with minimal contraindications or adverse effects), rapid onset, a therapeutic half-life, minimal or no monitoring, reversible action by an antidote and low cost of production. All these qualities have not yet been achieved in a single drug. Each of the DTIs available today has been able to incorporate some, but not all of these features. This encourages the search for designing new thrombin inhibitors that incorporate as much as possible the characteristics mentioned above. Thrombin binding aptamers have interesting anticoagulant and pharmacological properties, but whether they will be available for clinical use as anticoagulants should still be addressed.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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