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Recent Advances on Plasmin Inhibitors for the Treatment of Fibrinolysis-Related Disorders

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Abstract

Growing evidence suggests that plasmin is involved in a number of physiological processes in addition to its key role in fibrin cleavage. Plasmin inhibition is critical in preventing adverse consequences arising from plasmin overactivity, e.g., blood loss that may follow cardiac surgery. Aprotinin was widely used as an antifibrinolytic drug before its discontinuation in 2008. Tranexamic acid and ε-aminocaproic acid, two small molecule plasmin inhibitors, are currently used in the clinic. Several molecules have been designed utilizing covalent, but reversible, chemistry relying on reactive cyclohexanones, nitrile warheads, and reactive aldehyde peptidomimetics. Other major classes of plasmin inhibitors include the cyclic peptidomimetics and polypeptides of the Kunitz and Kazal-type. Allosteric inhibitors of plasmin have also been designed including small molecule lysine analogs that bind to plasmin's kringle domain(s) and sulfated glycosaminoglycan mimetics that bind to plasmin's catalytic domain. Plasmin inhibitors have also been explored for resolving other disease states including cell metastasis, cell proliferation, angiogenesis, and embryo implantation. This review highlights functional and structural aspects of plasmin inhibitors with the goal of advancing their design.

Keywords

plasmin(ogen); serine proteases antifibrinolytics; tranexamic acid; aprotinin; cyclic peptidomimetics; glycosaminoglycan mimetics; allosteric inhibition

1. INTRODUCTION

Given the key role of plasmin in fibrinolysis, plasmin inhibitors are used in the clinic to treat hyperfibrinolysis-associated bleeding events and adverse consequences. Hyperfibrinolysis-associated bleeding occurs in many major surgeries that require blood transfusion.[1] Approximately 55% of cardiac surgery patients receive blood transfusions.[2] Such transfusions are associated with longer hospital stay, multi-organ dysfunction and increased mortality.[3] The estimated cost of transfusing one unit of blood is $700–$1200 in United States,[4] which justifies the use of relatively inexpensive antifibrinolytic agents.

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CONFLICT OF INTEREST

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Nearly 70% of cardiac surgeries performed in United States utilize antifibrinolytics to reduce the economical burden.[5] Antifibrinolytics can also be used as adjunct therapy to control hemorrhage in some cases of disseminated intravascular coagulation (DIC), which is a hyperfibrinolysis-associated secondary disorder.[6, 7] DIC, in particular, occurs in 40% of patients with sepsis leading to a mortality rate of 50–75%.[7] DIC patients with a primary hyperfibrinolytic state and having severe bleeding can be treated with lysine analogs, a group of interesting plasmin inhibitors.[6] In addition, hemophilia,[8] menorrhagia,[9] von Willebrand syndrome,[10] and thrombolytics-induced bleeding can be fully or partially managed by the antifibrinolytic activity of plasmin inhibitors.[11]

Hemostasis concludes with thrombin cleavage of fibrinogen to generate fibrin monomers that are rapidly crosslinked by factor XIIIa (Fig. 1).[12] Under normal physiologic conditions, the fibrin-rich clot is dissolved by plasmin, which is produced following activation of plasminogen. This activation process takes place on the surface of fibrin. The *C*-terminal region of fibrin monomers that are rich in lysine residues facilitate binding to the lysine-binding sites (LBSs) on both plasminogen and tPA[13-16] resulting in the formation of fibrin-plasminogen-tPA ternary complex, which initiates the plasminogen activation process. The plasmin so formed remains bound to fibrin. Plasmin that disengages from fibrin is rapidly neutralized by α_2-antiplasmin, which is present in plasma at high concentrations. This helps localize plasmin's proteolytic activity.[17, 18] Fibrinolysis can also be regulated by thrombin-activatable fibrinolysis inhibitor,[19] plasminogen activator inhibitor-1 and -2,[20, 21] or α_2-macroglobulin.[22] In a clinical setting, fibrinolysis can be enhanced by using thrombolytics such as the fibrin-cleaving preparations of streptokinase, urokinase, or tissue plasminogen activator (tPA).[23]

Plasmin is a serine protease that exhibits trypsin-like broad specificity, particularly when plasminogen is activated by urokinase plasminogen activator (uPA) in a cell surface receptor-mediated process.[24, 25] Plasmin generated at the cell surface plays a critical role in degradation of extracellular matrix (ECM) resulting in modulation of several processes including tissue remodeling,[26, 27] cell invasion and metastasis,[28, 29] chemotaxis,[29, 30] wound healing and tissue repair,[31-33] neuritogenesis,[34, 35] ovulation and embryo implantation,[36, 37] and others.[38] Plasmin itself can degrade components in the ECM such as laminin,[39] fibronectin,[40] and collagens.[41] Plasmin also contributes indirectly to ECM degradation by its activation of matrix metalloproteinases (MMPs), which degrade other ECM components.[38, 42-44] In fact, potential benefits of plasmin inhibition have been projected for angioedema,[45, 46] chronic inflammatory responses,[47, 48] embryogenesis,[37, 49] and lymphoid malignancies.[50, 51] Thus, plasmin inhibitors may also be of value as anticancer and antiinflammatory agents in addition to their well-known therapeutic use as antifibrinolytic agents.[45-51]

In this review, the plasminogen-plasmin system and its central physiological role in the fibrinolytic pathway will be discussed in an attempt to understand fundamental rationale for plasmin inhibitors design and use. Recent advances made on the design, discovery, and development of plasmin inhibitors as antifibrinolytic agents will be more elaborately discussed. Current plasmin inhibitors in use or development belong to either the orthosteric (competitive) or the allosteric (noncompetitive) class of inhibitors. Structurally,

the inhibitors belong to one of three chemical classes including peptidomimetics, natural and engineered polypeptides, and polymeric sulfated glycosaminoglycan (GAG) mimetics. The broad categories of plasmin inhibitors reported in the literature imply considerably diverse structures and mechanisms. Finally, studies on the role of plasmin in other pathophysiological events and the potential benefits of plasmin inhibitors under these conditions will also be outlined.

2. THE PLASMINOGEN-PLASMIN SYSTEM

Human plasmin is known to be the key player in the fibrinolytic system (Fig. 1). It catalyzes the proteolytic cleavage of blood clots. Plasmin is derived from its zymogen, plasminogen, which is a single-chain polypeptide biosynthesized as 810 amino acid residues. The mature form of this zymogen is a glycoprotein comprising 791 residues arising from the cleavage of a 19-residue leader peptide upon secretion.[27, 52-54] Although plasminogen is primarily biosynthesized in liver, its messenger RNA has been detected in other tissues.[55] Plasminogen is physiologically activated by the action of either tPA or uPA, of which the former appears to be the primary activator for intravascular events, whereas the latter is mainly important for extravascular cell surface events. The plasminogen/plasmin structural architecture is relatively complex as depicted in Figure 2.[27, 52-54]

Structurally, plasmin has two polypeptide chains linked by two disulfide linkages. The *N*-terminal heavy chain (Glu1-Arg561) comprises five sequential kringle (K1–K5) domains each of which is a triple disulfide-linked polypeptide comprising approximately 80 residues.[27, 53] Each kringle domain contains an LBS characterized by anionic and cationic centers interrupted by a hydrophobic channel. For example, the K1 domain contains anionic and cationic centers made up of two Asp and two Arg residues, respectively, and also a hydrophobic groove that is lined with three Tyr residues.[56, 57] The LBSs facilitate binding of small and large molecules, such as Lys-like ligands,[58-62] inorganic chloride,[63] fibrin(ogen),[64, 65] bacterial proteins,[66, 67] mammalian cell surfaces,[68, 69] and the main physiological inhibitor α_2-antiplasmin.[17, 18] Interestingly, despite similarity among the five LBSs, structural differences do exist among them leading to differential recognition of a ligand by the five domains. For example, the affinity of antifibrinolytic drug 6-aminohexanoic acid increases in the order K1 > K3 > K2 > K5 > K4.[27] Likewise, although the K3 domain possesses a dysfunctional LBS, a single Lys311Asp mutation results in enhanced function.[70] The other structural feature of plasmin is the *C*-terminal light chain (Val562-Asn791), which is the trypsin-like catalytic domain having the triad of His603, Asp646, and Ser741 residues (His57, Asp102, and Ser195 in chymotrypsinogen numbering).[71] The catalytic domain appears to bind to α_2-macroglobulin, a physiological inhibitor of plasmin.[22, 72]

A very important component of the plasminogen-plasmin system is the 77-residue activation domain, named as plasmin-apple-nematode domain.[73-75] Physiologic activators cleave the scissile bond Arg561-Val562 in full-length plasminogen (Glu1-plasminogen) between the heavy chain (K5) and the light chain (catalytic domain) to produce the active plasmin (Glu1-plasmin or full-length plasmin).[76] Another scissile bond that has been identified is Lys77-Lys78, cleavage of which appears to be self-catalyzed leading to the formation of a shorter

length plasminogen (Lys78-plasminogen) or a shorter length plasmin (Lys78-plasmin). A fundamental difference between the two forms is the conformation of the corresponding protein. For example, Glu1-protein exists in more tight conformation that is favored by the chloride ion, whereas Lys78-protein exists in a more open conformation that appears to be favored by Lys-like ligands.[53] Crystal structures shedding light on the structural aspects of plasminogen have been published recently.[74, 75]

Although plasmin is a trypsin-like protease with significant similarity to other enzymes in the superfamily, structural differences between these enzymes do exist and these differences have aided the design of selective active site inhibitors. Of particular interest is the absence of segment 95–100 in plasmin(ogen), which led to the discovery of the most selective plasmin inhibitor belonging to the cyclic peptidomimetics class.[11] Furthermore, several approaches including positional-scanning synthetic combinatorial library screening[77-79] and noncombinatorial sparse matrix peptide library screening[80] have been performed to deduce the plasmin active site specificity. Although a general consensus on plasmin specificity has not been derived as of yet, some understanding has been deduced.[81] Using macromolecule protein substrates, it was suggested that the P1 specificity of plasmin includes basic amino acids Lys and Arg with a slight preference for Arg; P2 residues can be Leu or Ser, and to a lesser extent Pro, Ala, or Phe; P3 preference is for Arg, followed by Ser, Gln, or Gly; and P4 specificity is for Pro and Ala, and to a lesser extent Arg. On the C-terminal side of the scissile bond, Ser and Ala are overwhelmingly favored for the P1’ position, Arg, Ser, or Val are preferred at the P2’ position, Ser and Pro are favored at the P3’ position, and Gly, Pro, or Leu are favored at the P4’ position. In studies with smaller substrates having P4–P1 domains, plasmin was found to prefer Lys at the P1 position (to a lesser extent Arg), and Trp, Phe, and Tyr at the P2 position. However, there was significant uncertainty with respect to P3 and P4 positions suggesting that most amino acids may be tolerated in the two subsites.[77-81]

3. PLASMIN INHIBITORS AS ANTIFIBRINOLYTICS

A. Peptidomimetic Inhibitors

1. Lysine Analogs—*ε*-Aminocaproic acid (EACA or 6-aminohexanoic acid, **1**) and tranexamic acid (TXA, **2**) are synthetic derivatives of the amino acid lysine (Fig. 3) and are antifibrinolytic agents used in the clinic today.[4, 6, 82-85] EACA and TXA were discovered by Okamoto et al. in the 1950s.[86-88] The two agents do not bind in the active site of plasmin, rather, they bind to the LBSs of the kringle domains on plasmin(ogen) and prevent tPA/uPA-induced activation of plasminogen activation to plasmin.[4, 6, 82-85] TXA is approximately tenfold more potent per unit dose than EACA.[89, 90] TXA produces higher and sustained antifibrinolytic activity than EACA. Structurally, TXA is the *trans*-stereoisomer of 4-aminomethyl-cyclohexane carboxylic acid. Initial studies to assess its antifibrinolytic activity were performed using a mixture of *cis*- and *trans*-isomers. However, Okamoto et al. concluded later that the *trans*-isomer is the only active species in the mixture.[87, 89, 90] The two analogs demonstrate different pharmacokinetic profiles. EACA is orally well absorbed and reaches a maximal plasma level of 30 mg/L but shows a shorter half life (60 min). Nearly 80% of EACA is excreted in urine in the unmetabolized form. In contrast, TXA

shows a half life of 80 min, is recovered in the urine in an unchanged form, and requires a therapeutic concentration of 5–10 mg/L. TXA shows a higher volume of distribution and crosses the blood–brain barrier.[90] Complications associated with the use of either derivative include renal failure (more with EACA),[91] seizures (more with TXA),[91, 92] and rhabdomylosis.[5]

Several studies have implicated the use of lysine analogs to reduce perioperative and postoperative blood loss in cardiac surgeries,[1, 2, 5] orthotopic liver transplantation,[82, 84] menorrhea,[9, 93] DIC,[6, 7] von Willebrand syndrome,[10, 85] and some forms of hemophilia.[8] For example, TXA saves an average of 300 mL of blood per patient during cardiac surgery and reduces blood transfusion by 32%.[1] It reduces blood loss by 40%, thereby reducing number of transfusions in liver transplantation.[87] Both analogs have been exhaustively studied to establish their safety profiles and have shown a superior safety margin, particularly in cardiac procedures, with less than 1% of cases associated with severe complications. Both analogs effect significant cost-savings relative to other antifibrinolytics.[5]

Several biochemical studies have shed light on the mechanism of TXA/EACA. The two lysine analogs bind to kringle domains of plasmin(ogen).[72] Kringle domain 1 (K1) appears to be the primary target of TXA (1.1 μM) and EACA (9.0 μM), while their affinities are intermediate for K4 and low for K2, K3, and K5.[72] Both TXA and EACA do not affect the amidolytic activity of plasmin, miniplasmin, and streptokinase-plasmin complex but inhibit plasmin and miniplasmin-mediated fibrinolysis. At the molecular level, miniplasmin contains only the K5 domain (instead of the five kringles present in plasmin) and the catalytic domain, which implies that the two lysine derivatives inhibit fibrinolysis by binding to K5.[72] However, this does not imply that other kringles are not involved. TXA and EACA reduce the rate of inhibition of plasmin by α_2-antiplasmin, which utilizes K1–K3.[72] The two lysine derivatives do not affect activated partial thromboplastin time (APTT) or prothrombin time (PT) but prolong euglobulin clot lysis time at 10 mM concentration.[72] These studies suggest that TXA and EACA are allosteric inhibitors.

Structure-activity relationship (SAR) studies have been performed as to enhance the moderate efficacy of TXA and EACA and to minimize their side effects. A lysine ester, N^{α}-acetyl-L-Lys-methyl ester (**3**, Fig. 3), was found to inhibit the amidolytic activity of plasmin variants, in contrast to TXA and EACA, suggesting competitive inhibition. Inhibitor **3** did not inhibit plasmin-mediated fibrinolysis and also did not reduce plasmin inhibition by α_2-antiplasmin at 100 μM. Inhibitor **3** did not affect APTT and PT as well as euglobulin clot lysis time, which was in contrast to TXA and EACA.[72] This suggested that allosteric inhibition through the kringle domains required carboxylic acid and a free amine on a Lys analog. Other SAR studies also confirmed the role of free amine and carboxylic acid groups for antifibrinolytic activity.[89] Studies on homologous aminocarboxylic acids showed that the antifibrinolytic activity depended critically on the distance between the free amine and carboxylic acid groups (~7 Å).[89] Yet interestingly, replacing the C_2-C_5 segment with a benzene ring (presumably only 4.5 Å in length), as in **4** (Fig. 3), was found to increase potency by fivefold. Saturating the benzene ring to produce *cis*- and *trans*-isomers led to the discovery of *trans*-TXA (**2**), which is tenfold more potent than EACA and is most often used nowadays. Several derivatives of *trans*-TXA were synthesized including

4-aminomethyl-bicyclo-2,2,2-octane carboxylic acid (**5**, Fig. 3), however none displayed superior potency.

The crystal structure of human plasminogen K1 domain with TXA and EACA shows that the amine of the two ligands recognizes K1 Asp54 and Asp56 residues, while the carboxyl moiety binds to Arg70 and Arg34. A hydrogen bond between the carboxyl group and the phenolic group of Tyr63 appears to contribute to binding affinity. The methylene groups of the ligands are stabilized by van der Waals contacts with the side-chain atoms of Trp61 and Tyr71.[57]

To optimize EACA's pharmacokinetic/dynamic profiles, several short peptides based on the EACA moiety were synthesized and evaluated.[94-100] Analogs containing residues such as L-Lys, L-Leu, L-Nle, or L-Cys demonstrated variable antifibrinolytic activity relative to the parent EACA. Of note was the L-Lys derivative that exhibited tenfold better antifibrinolytic potency (IC_{50} < 200 μM).[94]

More recently, Boström et al. reported the use of a computational technique coupled with a low-throughput screening to find **6** (Fig. 3), which displays an electrostatic potential similar to TXA.[101] Inhibitor **6** was found to be about four times as potent as TXA with an IC_{50} for plasma clot lysis of 0.8 μM. Inhibitor **6** is predicted to exhibit GABA-A activity, which leads to convulsions, similar to that associated with TXA. Yet, **6** has been proposed as a good lead for further development.[101]

Finally, the low bioavailability of TXA (~34%)[93] was sought to be resolved through its maleamic acid[102] and acyloxyalkyl carbamate derivatives.[103] In November 2009, Lysteda™, a novel TXA formulation, was approved by the FDA to treat heavy menstrual bleeding.[93] This oral formulation was designed to minimize gastrointestinal adverse effects. The formulation requires less frequent dosing because of its higher per-tablet dosage relative to the immediate-release preparation.[93]

2. Trans-4-aminomethylcyclohexanecarbonyl Conjugated Inhibitors—Okada et al. and others have reported that plasmin's chromogenic substrate D-Ile-Phe-Lys-*p*NA (7, Fig. 4) has a low K_m (20 μM) as well as mild inhibitory action (IC_{50} 30 μM).[104] Hence, this substrate was used as a template to help design more potent active site inhibitors. Replacing the *p*-nitroanilide group with more hydrophobic moieties such as *p*-benzoylanilide (BZA, **8**), *p*-acetylanilide or 3-(*p*-dimethylaminobenzoyl) anilide increased the IC_{50} some 6–9-fold as measured by plasmin fibrinolysis.[104] Yet, the analogs were better inhibitors than **7** as these molecules were minimally hydrolyzed by plasmin.[104] Same investigators attempted to replace the P3-P2 domain of substrate **7**. Introducing a tosyl group, instead of the D-Ile-Phe motif as in Tos-Lys-*p*NA, displayed an IC_{50} of 700 μM (against S-2251) and 780 μM (against fibrin).[104] The above two efforts were combined to design Tos-Lys-BZA (**9**, Fig. 4), which inhibited plasmin better (IC_{50} 140–150 μM).[104]

These initial studies were exploited to develop irreversible inhibitors containing the chloromethyl ketone moiety. Replacing P1′ (BZA) inhibitor **8** (Fig. 4) with chloromethyl ketone moiety led to an inhibitor with an excellent K_d of 1.75 μM and a catalytic efficiency

(k_2/K_i) of 77,000 $M^{-1}s^{-1}$. But the inhibitor lacked selectivity as it also inhibited thrombin, plasma kallikrein, factor Xa, and trypsin.[105] An analog, D-Ile-Phe-Arg-CH_2Cl, synthesized on the basis of inhibitor **8**, was actually found to be a more potent inhibitor of plasma kallikrein.[105]

Teno et al. have reported Tra-Lys-BZA (**10**, Fig. 4) as a moderately potent, active site-directed plasmin inhibitor displaying IC_{50} of 15 and 6.1 μM against chromogenic substrate S-2251 and fibrin, respectively.[106] This inhibitor was intuitively designed from *trans*-TXA, which replaced the D-Ile-Phe domain of inhibitor **8**.[106] Interestingly, **10** inhibited fibrinogenolysis too with an IC_{50} of 13 μM.[106]

Dissecting the inhibitor Tra-Lys-BZA (**10**) into three structural domains gave some interesting structure-function insights. It was found that L-Lys is an optimal central part and replacing it with ornithine or D-Lys abolished inhibition of plasmin. Substituting the terminal Tra domain with racemic 4-aminocyclohexanecarbonyl or 3-aminocyclohexanecarbonyl groups resulted in essentially inactive analogs. Likewise, the *cis*-isomer of Tra diminished the activity toward chromogenic substrate and fibrin nearly 27- and 43-fold, respectively. Likewise, replacing the Tra group with the tosyl group led to 10–25-fold decrease in inhibitory effect on plasmin amidolysis, fibrinolysis, and fibrinogenolysis.[106] However, introducing 5-aminopentoyl or 6-aminohexanoyl (EACA) group in the place of Tra group maintained inhibition potency against amidolysis (IC_{50} 12–16 μM), fibrinolysis (IC_{50} 10–17 μM), and fibrinogenolysis (IC_{50} 36 μM). Replacement of the third domain, the BZA group, with substituted piperidineamide, *p*-nitroanilide, 5,6,7,8-tetrahydro-2-aminoronaphthyl amide, or *p*-methoxycarbonylanilide resulted in compounds that did not inhibit plasmin amidolytic or fibrinolytic activity at concentrations as high as 500 μM. But substituting only the 4-benzoyl group of BZA with aceyl group resulted in moderately potent fibrinolysis inhibitor **11** (IC_{50} 9.3 μM).[106] Last, replacing the central amide linker with an ester also reduced the overall inhibitor potency.[106] Thus, **10** was arguably the best inhibitor in the series.

Mechanistically, **10** is a competitive inhibitor that bound in the active site of plasmin. Plasmin's S1 subsite interacts with the basic side chain of Lys or Arg residues. Using this information, two derivatives were synthesized to gain insights on **10**'s mode of binding. The free amino groups of Lys or Tra moieties in **10** were individually protected by the benzyloxycarbonyl (Cbz) group. While the former derivative retained inhibitory activity, the latter derivative was significantly less active.[106] This implied that the free amine of Tra, and not that of Lys, interacted with the S1 Asp of plasmin.

The observation that the Tra group when conjugated to appropriate amino acids induces potent plasmin inhibition led to the design of new peptidomimetics with three domains (P1-P1′-P2′) in which P1 is the Tra group.[107-109] The most potent plasmin inhibitor in the new set had 2-bromobenzyloxycarbonyltyrosine as P1′ group and *p*-acetylanilide as P2′ group. This compound **12** inhibited plasmin amidolysis with an IC_{50} of 0.23 μM. But **12** also inhibited plasma kallikrein, urokinase, and thrombin with IC_{50} of 0.37, 43, and 63 μM, respectively.[107] An organic chemistry-driven approach was developed to improve the three domain peptidomimetic. The P2′ domain was modified with branched and unbranched

alkylamides, *p*-alkylanilides, pyridineanilides, pyridinealkylanilides, or a Tra group.[108] Each of these displayed plasmin inhibition potency comparable to the parent inhibitor **12**. Their specificity over thrombin and urokinase was significantly improved but not against kallikrein or trypsin. This suggested that the S2′ subsites in both plasmin and kallikrein tolerate bulky hydrophobic groups as opposed to thrombin and urokinase. Thus, inhibitor **13** was designed with a P2′ octylamide and contained P1 and P1′ groups of **12**.[108] Compound **13** inhibited plasmin with an IC_{50} of 0.8 μM (amidolysis) or 0.23 μM (fibrinolysis) as well as plasma kallikrein (IC_{50} 16 μM) and trypsin (IC_{50} 1.6 μM) but not urokinase or thrombin (both $IC_{50} > 50$ μM).[108] Further replacement of the protecting group on P1′ Tyr from 2-bromobenzyloxycarbonyl to 4-pyrdinylmethyl group produced inhibitor **14** (Fig. 4), which displayed better plasmin inhibition (IC_{50} 0.53 μM) and 60-fold higher selectivity over plasma kallikrein (IC_{50} 30 μM). However, **14** lost specificity against urokinase (K_i 5.3 μM).[109] To restore selectivity against urokinase, the P2′ octylamide was modified to contain a terminal amine as in **15**, which improved the selectivity index eightfold against plasma kallikrein, urokinase, and thrombin.[109, 110] To capitalize on these gains, a set of four domain peptidomimetics (P2-P1-P1′-P2′) were synthesized based on **12**. The Tra group at P1 was modified to 4-aminocyclohexylalanine, P1′ and P2′ groups were retained as in **12** and a tosyl group was replaced for P2. These changes neither improved potency nor enhanced specificity of inhibition.[107]

3. Cyclohexanone-Based Inhibitors—Seto and colleagues were the first to report that 4-heterocyclohexanone moiety gives reversible, covalent, active site inhibitors of several serine and cysteine proteases.[111] The inhibitor's ketone moiety is attacked by nucleophilic active site Ser or Cys of the protease to form a reversible hemiketal adduct. Steric relationship between the heteroatom and the carbonyl group was proposed as key to enhance the reactivity of the molecule.[112, 113] Compound **16** (Fig. 5) was the first to be designed for plasmin.[111] It relied on three interactions including those of the S1 and S3 subsites with the two hexylamine chains. Inhibitor **16** displayed a K_i of 400 μM against plasmin with approximately threefold selectivity over trypsin and more than 25-fold selectivity over thrombin and kallikrein.[111] To increase its potency, one hexylamine was replaced with D-Ile-L-Phe, a favorable P3–P2 plasmin motif derived earlier, to give **17** (Fig. 5). One diastereomer of **17** displayed threefold better inhibition of plasmin (K_i 50 μM) than the other diastereomer and good selectivity over trypsin, thrombin, and kallikrein (34-, 15-, and 13-fold, respectively). Lineweaver-Burk analysis confirmed that inhibitor **17** was a reversible competitive inhibitor of plasmin. The importance of **17**'s hexylamine chain was realized when replacing it with a hydrogen eliminated plasmin inhibition potential (**18**, K_i 9–16 mM, Fig. 5). Likewise, replacement of the heteroatom with a hydrogen, as in **19**, also lost potency by approximately threefold.[111] Finally, **20** was found to be about 12- and fivefold less potent than inhibitors **17** and **19**, respectively suggesting the importance of covalent hemiketal adduct formation in inhibition.[111]

Abato et al. exploited another cyclohexanone core structure to design the next generation of bidirectional plasmin inhibitors.[114] A combinatorial chemistry approach was developed to synthesize some 400 potential inhibitors, each of which contained two variations at positions 2 and 6 of the cyclohexanone core that fit into the S2 and S2′ subsites (Fig.

6). Based on an initial screening, four inhibitors (**21**–**24**, Fig. 6) were chosen for further analysis. Inhibitor **21** having two Trp residues demonstrated the highest potency in the group with a K_i of 5–10 μM and at least 38-fold selectivity over the cysteine protease papain. Phe-containing inhibitors were less potent (K_i > 100 μM) and less selective.[114] To improve upon the potency, a hexylamine chain was added so as to engage the S1 subsite of plasmin. However, the resulting diastereomeric mixture (**25**, Fig. 6) did not show much improvement.[114, 115] A follow-up study developed an extended bidirectional cyclohexanone inhibitor comprising five domains corresponding to P3-P2-cyclohexanone-P1′-P2′-P3′.[116] A combinatorial chemistry approach synthesized 400 compounds, of which **26** displayed an IC_{50} of 2.7 μM against plasmin and a selectivity index of > 150-fold over kallikrein, thrombin, and trypsin. This suggested a preference for Trp at the P3, P2, and P2′ sites. Likewise, Tyr was found to be favored at the P3′ site as other residues displayed slightly weaker inhibition.[116]

The success of the ketone–hemiacetal trapping strategy led Seto and co-workers to further optimize the type and size of the cyclic ketone core structure, the residue at the P2 domain, the amine cation at the P1 domain, and the *N*-terminus of a four-domain inhibitor P4-P3-P2-P1.[117] Varying the P2 domain to either Phe (as in inhibitor **27**) or Trp did not affect inhibition potency (~22 μM). In contrast, Cbz removal from the *N*-terminus (as in inhibitor **28**) diminished the activity ~17-fold suggesting its significance in binding to the S4 subsite of plasmin. Replacing the nuclear oxygen of the heterocyclic ring of **27** with a methylene or a protected amine reduced the potency by 45- or 16-fold, respectively,[117] suggesting its probable role in enhancing electrophilicity of the ketone. Supporting this hypothesis was the observation that a sulfone moiety in the place of the nuclear oxygen, as in inhibitor **29**, enhanced the potency by approximately twofold. Likewise, expansion to a six-membered ring essentially eliminated inhibitory potential (IC_{50} 1000 μM). With regard to the P1 domain, butylamine, pentylamine, heptylamine, or 4-aminocyclohexylmethyl containing molecules were less active than inhibitor **27** (Fig. 7), except for the hexylamine containing derivative **30** that displayed twofold improved IC_{50}. Combining these results with that of the sulfone derivative **29** led to inhibitor **31**, which showed an IC_{50} of 5.7 μM, the most potent inhibitor in this series.[117] No selectivity data have been published for this set of plasmin inhibitors.

Overall, the designed reversible, covalent, hemiketal-based competitive inhibitors of plasmin demonstrate moderate inhibitory potency (low μM) with the **26** being the best in this class. Unfortunately, further biochemical or crystallographic studies have not been reported on this inhibitor which has stymied development of new directions. Also, no in vivo studies have been published. It is possible that the size and the peptidic nature of this inhibitor is a detriment for such a translation. Nevertheless, it is important to recognize that this chemical class has led to three important observations on plasmin specificity and cooperativity. Plasmin strongly prefers Lys or Arg at the P1 position and extended hydrophobic and aromatic residues, particularly Trp, at the P2 position. Plasmin also favors hydrophobic residues (Ile, Phe, and Tyr) at P2′. Furthermore, the inhibition studies show that cooperative binding in the S1 and S2 subsites of plasmin is important for enhancing specificity. A positively charged P1 group that binds in the S1 subsite indiscriminately positions any P2

aromatic group (Trp, Tyr, or Phe) to bind in the S2 pocket. In contrast, inhibitors that do not bind in the S1 subsite prefer Trp at P2, perhaps due to favorable van der Waals or hydrogen-bonding interactions with the S2 subsite.[115] Thus, the S2 substrate specificity can be altered depending on whether S1 is occupied or not. S3 and S3′ subsites prefer to bind hydrophobic and/or aromatic residues such as Trp, Phe, Tyr, or Leu. Less hydrophobic residues, such as Ala, Ile, or Val, show good affinity for plasmin, whereas polar amino acids, such as Orn and His, and conformationally constrained residues, such as Pro and hydroxyPro, are detrimental for binding to the S3′ subsite.[115] For this class of inhibitors, it was found that Trp is the preferred residue at P2, P3, and P2′, whereas Tyr is preferred at the P3′ site. These results are consistent with previous reports on the specificity of plasmin substrates and inhibitors.[77, 78, 118]

4. Cyclic Peptidomimetic Inhibitors—Cyclization of known peptide inhibitors to form macrocylic peptidomimetics has recently emerged as a viable drug design strategy.[119, 120] An important feature that has helped inspire this protocol is that substrate-like inhibitors are likely to bind in the form of an antiparallel *β*-strand to the active site of trypsin-like serine proteases. The conformational constraint introduced by cyclization should have two special advantages over the corresponding linear peptides. First, cyclization is expected to reduce the entropic penalty for binding by decreasing flexibility. Second, cyclization reduces proteolytic processing by physiologic peptidases, which can improve bioavailability.[121-123]

To put this concept to work for plasmin, Xue and Seto synthesized cyclic analogs by treating the free *N*-terminus of a Tyr-containing analog of inhibitor **28** with appropriate bromoalkanoyl chloride followed by NaI/acetone-mediated cyclization.[117] Inhibitors **32**, **33**, and **34**, containing macrocyles with varying atoms (Fig. 8) showed reasonable improvement in the inhibition potency relative to that of parent molecule **28**. The best analog **32** contained a 19-atom macrocycle and showed ~15-fold improvement over **28**.[117] However, **33** and **34** did not exhibit more than 20% inhibition at a concentration as high as 250 *μ*M.[124]

These results do not appear to demonstrate much potential arising from the cyclization strategy. One reason could be the absence of optimal elements for plasmin inhibition in the acyclic precursors. This led Saupe and Steinmetzer to prepare a series of macrocyclic peptidomimetics by simple amidation, metathesis, or click chemistry so as to link the P2 and P3 chains of inhibitor **35**.[125] The designed molecule **36** (Fig. 8) was a potent plasmin inhibitor with a K_i of 0.77 nM. This inhibitor exhibited a selectivity index of 12324-, 268-, and 3896-fold over thrombin, factor Xa, and activated protein C, respectively.[125] However, **36** inhibited plasma kallikrein with high potency too (K_i 2.4 nM).

The success of the Saupe and Steinmetzer strategy appears to reside in the starting acyclic lead **35** (Fig. 8), which inhibited plasmin (K_i 0.81 nM), plasma kallikrein (K_i 0.075 nM), thrombin (K_i 667 nM), factor Xa (K_i 42 nM), and activated protein C (K_i 0.61 nM). The nonselective inhibitor **35** contained 4-amidinobenzylamide as an Arg mimetic at the P1 position, 3-guanidinomethylphenylalanine at the P2 position, D-phenylpropylglycine at the P3 position, and benzylsulfonyl moiety at the P4 position. A comparison of the crystal structures of several serine proteases shows that plasmin's active site is devoid of the 95–100

segment, the so-called 94-shunt. This allows continuity of proximal (S1 and S2 subsites) and distal (S3 and S4 subsites) binding pockets rendering the active site wide and open. This accounts for the relative nonspecificity of plasmin, which can cleave many different substrates in a fashion similar to trypsin. Saupe and Steinmetzer exploited this feature as to devise cyclic analogs having bulky group in the vicinity of the 99 residue. The cyclic loop of the designed inhibitors was expected to fit well into plasmin active site but generate steric clash with other serine proteases that contain this segment, as is the case of thrombin, factor Xa, factor IXa, factor XIa, factor VIIa, activated protein C and uPA. They used the '*i* to *i*+1' cyclization strategy as opposed to the '*i* to *i*+2' strategy utilized by Xue and Seto.[124] The shorter cyclization strategy increased bulkiness and rigidity in the macrocycles and enhanced their selectivity over other homologous proteases.

The same study explored the SAR further through 11 additional cyclic inhibitors containing 1,3- or 1,4-disubstituted benzene, 1,4-disubstituted piperazine, 1,3-disubstituted pyridine or *N*-oxide pyridine, *n*-butyl, or butylene linkers. Owing to rigidity, inhibitors with smaller rings (17-membered) generally exhibited better potency (K_i < 3 nM) and selectivity relative to inhibitors with larger rings (24 to 26-membered rings).[125] A plausible mode of binding and selectivity was explained through molecular modeling, which suggested that the benzamidine group of inhibitor **36** fits into the S1 subsite, as expected, and hydrogen bonds to both the carbonyl of Gly219 as well as the side chain oxygen of Ser190. Also the benzylsulfonyl group of **36** seems to fit into the S4 subsite and the sulfonyl group hydrogen bonds to the side chain of Gln192, which is also involved in interacting with the P2 carbonyl group. The two triazole rings with the 1,3-substituted aromatic linker were hypothesized to be directed toward the large open binding pocket. Superimposition of thrombin, factor Xa and activated protein C structures onto the docked plasmin-**36** complex showed that the triazole-containing linker would sterically clash with the 95–100 segment of the related proteases.[125] At the same time, steric clash is greatly reduced for plasma kallikrein because of its small and flexible S2 subsite residue (Gly99), which explained the observed comparable potency. Overall, the modeling results corroborated with the observed selectivity profile of inhibitor **36**.

To enhance the selectivity of inhibitor **36**, especially against kallikrein, and to improve its aqueous solubility, additional cyclic peptidomimetics were synthesized.[11] Inhibitor **37**, containing a 26-membered, piperazine linker-based macrocycle, was found to display better selectivity profile in comparison to inhibitor **38**, which had a 24-atom macrocycle (Fig. 8).[125] This suggested that the 1,4-disubstituted piperazine linker was a better lead for further optimization. In a new set, the $-CH_2-$of 4-aminomethylene-Phe present in **37** was eliminated to yield **39**, which inhibited plasmin with 13-fold (K_i 0.68 nM) better potency than **37**. Inhibitor **39** was 100-fold more selective over trypsin, 470-fold over kallikrein, and > 15,000-fold selective over thrombin, factor Xa, and activated protein C.[11] Replacing the 1,4-diacetylpiperazine linker with the 1,4-dipropionylpiperazine linker to give a 26-membered macrocycle **40** (Fig. 8) further enhanced the potency (K_i plasmin 0.2 nM) as well as selectivity over kallikrein, thrombin, factor Xa, and activated protein C. But its selectivity over trypsin was reduced (**40** 38.3 nM).[11] Additional modifications of piperazine ring had marginal impact on plasmin inhibition.[11] Inhibitors **39** and **40** also

did not affect many other proteases including the enzymes of the coagulation cascade (Table I) and possessed excellent solubility in 0.9% saline. Both inhibitors efficiently inhibit the tPA-induced fibrinolysis in human plasma. Clot lysis time was doubled at 280 nM for inhibitor **39** and 180 nM for inhibitor **40**. Both inhibitors showed no anticoagulant activity in plasma as demonstrated by APTT, PT, and thrombin time (TT) assays as well as insignificant binding to hERG, natrium, and L-type calcium ion channels at 10 μM concentration. Incubation with human liver microsomes revealed not much metabolism for both inhibitors as the IC_{50} for the inhibition of various Cyp-P450 enzymes was found to be > 10 μM.[11]

To get atomistic insight on the interactions of **39** and **40** within the active site of human plasmin, molecular modeling was employed. Computational docking revealed that the amidino group of the P1 domain fits well into S1 subsite and hydrogen bonds to the carbonyl group of Gly219 as well as the side chain oxygen of Ser190.[11] The amide NH of the P1 residue binds to the carbonyl oxygen of Ser214. A hydrogen bond was predicted between the P2 carbonyl oxygen of the two inhibitors and the side chain amide of Gln192. Sulfonyl oxygens were involved in hydrogen bonding to the NH of Gly219. A comparison of the two inhibitor conformations suggested better fit of **40** in plasmin's active site because of stronger van der Waals interactions. Interestingly, no hydrogen bond between the linker segment and plasmin was identified. Finally, a steric clash between the 95–100 loop of kallikrein, thrombin, factor Xa, activated protein C and trypsin and the piperazine linker of inhibitors **39** and **40** was predicted by overlaying the models, thus corroborating the observed high selectivity profile.[11]

Overall, the cyclization strategy was highly successful in designing potent and selective substrate-like inhibitors of plasmin. It puts forward two inhibitors **39** and **40** as clinically relevant candidates, although the molecules appear to have been not tested in animal models.

5. Miscellaneous Agents

Nitrile warheaded inhibitors: An attractive strategy in the design of covalent enzyme inhibitors is that of transforming a peptidic substrate into a nitrile warhead-based inhibitor. The strategy has been reported for several serine proteases with reasonable success.[126, 127] Teno et al. utilized tripeptide D-Ile-Phe-Lys-*pNA* (**7**, Fig. 4) to design a new class of covalent inhibitors of plasmin utilizing the nitrile warhead concept. In this strategy, the scissile bond of **7** was replaced by a nitrile warhead,[128] which was found to selectively inhibit plasmin with a nearly fourfold improved IC_{50} (78 μM). This initial success inspired design of new nonpeptidic, nitrile warhead-containing inhibitors (Fig. 9). Molecular modeling studies predicted optimal positioning of the nitrile group of a lead molecule **41** with regard to the active site Ser. In addition, the lysyl side chain would fit into the S1 subsite, the *meta*-substituent would occupy the S2 subsite, and the *para*-picolyl group would bind to the S2 subsite. Thus, seven analogs of **41** containing various aromatic substituents at the P3 domain were synthesized. Of these, **42** (Fig. 9), in which the P3 domain is *N*-(4-fluorobenzyl)-3-indole propionamide, was found to be most potent (IC_{50} 140 μM) and selective over plasma kallikrein and urokinase (IC_{50} > 1000 μM). It is worth mentioning that

replacing the picolyl moiety of **42** with a methyl group abolished plasmin inhibition, but retained plasma kallikrein inhibition.[128]

In a parallel study, five different tripeptidic nitrile warheads of D-Ile-Phe-Lys-CN type were studied containing variations at the Phe-containing P2 domain.[129] The lead tripeptide showed a plasmin IC_{50} of 78 μM and 13-fold selectivity over plasma kallikrein and urokinase. Introducing *O*-benzyl, *O*-picolyl or *O*-picolyloxybenzyl Tyr (**43**, Fig. 9) in the place of Phe enhanced the potency against plasmin by —sevenfold to tenfold. However, this decreased the selectivity against plasma kallikrein (IC_{50} 210–320 μM). P1 domain modifications to imidazole or triazole ethylene, or butyl side chains rendered the compounds inactive. Likewise, substitution of the P3 D-Ile with Ile, Gly, acetyl, or pivaloyl significantly diminished plasmin inhibition suggesting that the S3 subsite of plasmin prefers a branched hydrophobic residue, which was consistent with previous reports.[129]

Aldehyde-based peptidomimetic inhibitors: In the manner of the nitrile group as a warhead, the aldehyde group can also be used to replace the scissile bond and develop a warhead that covalently nullifies the activity of active site Ser of enzymes. Swedberg and Harris identified two tetrapeptide aldehydes from a library as potent active site inhibitors of plasmin.[80] Tetrapeptides Ac-Lys-Met(sulfone)-Tyr-Arg-H and Ac-Arg-Met(sulfone)-Tyr-Arg-H inhibited plasmin with K_i of 3.1 and 9.9 nM, respectively. The former also inhibited trypsin and plasma kallikrein with IC_{50} of 95 and 366 nM, respectively. In addition, the molecules inhibited fibrinogenolysis by plasmin at 250 nM. Molecular modeling studies of the tetrapeptide binding to the catalytic plasmin unit showed that the aldehyde group is likely to be well accommodated in S1 subsite and multiple hydrogen bonds between the two interacting partners are likely to form. In addition, Tyr of the tetrapeptide was likely to interact with His603 (His57 in chymotrypsinogen numbering) of the active site through *pi–pi* interactions.[80] It is important to mention that this study's results are at variance with previous residue level specificity conclusions.[77-79] For example, substrates with Arg, rather than Lys, were cleaved more efficiently by plasmin. There was a slight preference for Tyr at P2 in contrast to previous studies showing preference for Trp or Phe. There was no clear evidence that plasmin prefers a basic residue at P4, rather preference for Val or Phe was observed. Finally, the high in vitro potency and selectivity of the tetrapeptide aldehyde inhibitors has not been translated to in vivo evaluation as yet.

CU-2010: CU-2010 (renamed MDCO2010 (**44**), Fig. 9) is a peptidomimetic inhibitor.[130, 131] The inhibitor has a benzamidinomethylamine moiety at the *C*-terminus mimicking a P1 motif. Inhibitor **44** displays a K_i of 2.2 nM against plasmin, but also good potency against other proteases including factors Xa (K_i 45 nM), XIa (K_i 18 nM), XIIa (K_i 5200 nM), and IIa (K_i 1700 nM) (Table I). It is especially more potent against plasma kallikrein (K_i 0.019 nM).[130] Human whole blood clot lysis was suppressed by inhibitor **44** at 150 nM, which is 2- and 18-fold better than that for aprotinin and TXA, respectively.[130] CU-2010 had a significant effect on APTT and a weak effect on PT, while aprotinin prolonged only the APTT at comparable concentration.

Considering its interesting inhibition profile, CU-2010 half-life was studied and found to be 20 min in dogs and rats following intravenous administration, which increases to 45 min for

its pegylated analog CU-2020.[132] But pegylation had variable effect on inhibition potencies of factor XIIa (4.5-fold increase), plasmin (fourfold loss), kallikrein (fourfold decrease), and factor XIa (31-fold decrease), while retaining factor Xa and thrombin inhibition potential.[132] Both CU-2010 and CU-2020 reduced blood loss by approximately threefold relative to the vehicle (149 ± 24 mL) after cardiopulmonary bypass surgery in a canine model, which was similar to that observed for aprotinin.[132] Despite the controversial lack of specificity, CU-2010 not only dose-dependently reduced postoperative blood loss, similar to aprotinin, but also improved the postischemia recovery of myocardial and endothelial functions in a canine model of cardiac surgery. This probably arises from its kallikrein inhibition that results in an antiinflammatory effect.[133]

Surprisingly, Phase 2b study of CU-2010 was stopped because of some evidence of enhanced risk.[134] This could be because of CU-2010's prolongation of activated clotting time arising from inhibition of coagulation factors Xa, Xia, and activated protein C. Activated clotting time is widely used for monitoring heparin anticoagulation in patients during cardiac surgery and its prolongation by CU-2010 may interfere with the evaluation of a patient's anticoagulation state leading to underdosing of heparin.[135]

ONO-3307, nafamostat, and gabexate: ONO-3307 (**45**), nafamostat (**46**), and gabexate (**47**) (Fig. 9) are guanidine/amidine-based, highly nonselective serine proteases inhibitors. The first two molecules inhibit plasmin with a K_I of 0.31 μM, whereas **47** inhibits with sixfold lower potency.[136] They also inhibit trypsin, thrombin, and kallikrein competitively ($K_I < 5$ μM) and their effects can be reversed by dialysis.[136] ONO-3307 and gabexate were shown to completely inhibit the deposition of labeled fibrinogen in kidney, lung, and liver in experimental animal model of t-AMCHA-induced thrombosis in rats at 10 mg/kg/hr. In addition, 100 μM ONO-3307 was shown to inhibit elastase release from stimulated human leukocytes. Likewise, the three inhibitors inhibited thromboplastin release from stimulated leukocytes at ~10 μM dosage.[136] Due to the combined effect on thrombin (coagulation) and plasmin (fibrinolysis), ONO-3307 was tested as a treatment for DIC[137] and pancreatitis.[138-140] Yoshikawa et al. found a protective effect against endotoxin-induced DIC in rats with 10 or 100 μg/kg/h of ONO-3307 as measured by fibrin degradation products, number of renal glomeruli with fibrin deposition, and other effects.[137] Likewise, Hirano and others observed beneficial effects of ONO-3307 in rat acute pancreatitis.[138-140]

Both nafamostat and gabexate have been approved for clinical use in Japan and Italy. Reports suggest that prophylactic intravenous nafamostat mesilate reduces the frequency of postendoscopic retrograde cholangiopancreatography pancreatitis.[141] Nafamostat is also useful as an anticoagulant during continuous venous hemodialysis[142] and it attenuates postreperfusion syndrome during liver transplantation.[143] Likewise, gabexate appears to be pharmacologically useful in pancreatitis,[144] liver transplantation,[145] and hemodialysis anticoagulation.[146] Both drugs are also potentially beneficial in the treatment of DIC.[147, 148]

B. Polypeptide Inhibitors of Plasmin–Natural and Engineered

Plasmin can be functionally regulated by several polypeptide inhibitors of the Kunitz or Kazal type. Despite their structural similarity to plasmin substrates, the polypeptides act

as extremely potent inhibitors. The polypeptides bind to the active site of plasmin in a substrate-like fashion utilizing the enzyme's catalytic triad,[149, 150] but the products of hydrolysis remain associated due to extremely slow hydrolysis, which favors resynthesis of the peptide bond.[149-152] This inhibition mechanism is widely known as the Finkenstadt and Laskowski mechanism.[151, 152] Several reasons have been proposed to explain such an inhibitory mechanism including (1) the extreme rigidity of the protease–polypeptide complex stemming from the internal network of stabilizing hydrogen and disulfide bonds in the binding loops of the protease inhibitors, (2) the poor orientation of the reactive groups resulting in a nonproductive complex, or (3) the orientation of the leaving group in the acyl-enzyme complex, which favors the reverse reaction to regenerate the Michaelis complex.[149, 150]

A brief description of the loop sequences for each natural polypeptide inhibitors of plasmin along with their K_i's is provided in Table II. The Schechter and Berger nomenclature is used to describe the inhibitory peptide sequence.[153] Each polypeptide is a competitive inhibitor, which blocks the active site without inducing any major conformational change and forms an antiparallel β sheet in the active site of proteases. The inhibitors typically display nanomolar affinity but suffer from poor selectivity. Nearly all sequences are natural, except DX-1000[154] and KD1-L17R,[155] which have been engineered. Of these, bovine pancreatic trypsin inhibitor (BPTI) has been therapeutically used as antifibrinolytic agent.[156]

1. Natural Kunitz-Type Inhibitors

Bovine pancreatic trypsin inhibitor (BPTI, also called aprotinin): BPTI is a Kunitz-type serine protease inhibitor of bovine pancreatic trypsin that was introduced independently by Kraut et al.[157] and Kunitz and Northrop in the 1930s.[158] Structurally, it is a single polypeptide chain that is crosslinked by three disulfide bridges. It can be obtained from the pancreas, parotid gland, and the lung of cows, however, only the lung form is clinically used. It is a 58 residue basic protein containing six Arg residues at positions 1, 17, 20, 39, 42, and 53 as well as four Lys residues at positions 15, 26, 41, and 46.[156] To identify the P1 residue, chemical labeling with *N*-carboxy-*DL*-alanine anhydride in the presence and absence of trypsin was employed. Lys15 was the only basic residue that remained unmodified in the presence of trypsin suggesting its binding in the S1 anionic pocket. This was further confirmed through sequencing of the tryptic digest that identified Lys15-Ala16 as the scissile bond.[159]

Aprotinin targets several serine proteases (Tables I and II).[156] The broad specificity of wild-type aprotinin can be altered through site-specific mutagenesis.[160-165] For example, Lys15Arg mutation did not impact plasmin inhibition, but increased antifactor Xa (~484-fold) and antithrombin activity (~17-fold).[164] Likewise, Lys15Val aprotinin exhibited higher potency against leukocyte elastase.[160]

Aprotinin was widely used in antifibrinolytic therapy for reducing perioperative bleeding and the need for blood transfusions during organ transplantation, and orthopedic, cardiac, and thoracic surgeries.[1, 2, 5, 82-84, 90, 91] Aprotinin reduced the high risk of allergic reactions and transmission of infections associated with multiple blood transfusions.[3, 4, 83, 166, 167] In addition, the polypeptide has other beneficial effects due to its activity against

serine proteases of the coagulation and inflammatory systems that help reduce some adverse consequences during cardiopulmonary bypass.[168-170] Likewise, the coagulation and inflammatory complications arising from contact with the artificial surfaces of heart and lung devices also tend to be lower because of aprotinin.[52] Evaluation of aprotinin in different in vitro and in vivo models of fibrinolysis, coagulation, and thrombus formation has been reported.[171] Fibrinolysis was inhibited by aprotinin with an IC_{50} of 0.16 μM. Aprotinin also inhibited in vivo thrombus formation and reduced rat tail bleeding time in a dose-dependent manner. It appears that aprotinin reduced blood loss and the need of blood transfusion by an average of 45–60%.[1, 172]

Aprotinin is monomeric in solution, but exists as a decamer at high-salt concentrations. Inorganic sulfate neutralizes its basic amino acids and stabilizes the decameric form. A clinical counterpart of such sulfate–aprotinin interaction is the heparin–aprotinin interaction, for which a model has been proposed.[173] Heparin is a mixture of highly sulfated polysaccharide chains that is biosynthesized in the mast cells along with aprotinin.[174] This implies that bovine heparin may carry aprotinin, which may impact the heparin anticoagulant therapy.[175] Aprotinin is known to interfere with heparin binding to platelets and appears to reduce peri- and postoperative blood loss resulting from heparin usage.[176, 177]

Despite the many advantageous effects of aprotinin, results from the BART clinical trial led to suspension of aprotinin's clinical use in May 2008.[178, 179] This large multicenter study found that even though the risk of bleeding was lowest in the aprotinin group for patients undergoing high-risk cardiac surgery in comparison to TXA and EACA groups, mortality was significantly higher, which led to early termination of the trial. A recent Cochrane review also concluded that the risk of death was higher with aprotinin.[180] Aprotinin has been also reported to increase the incidences of renal failure,[178, 181, 182] myocardial infarction,[178] vein graft hypercoagulation,[183] anaphylactic shock,[184] and mortality.[179, 181, 182] However, several subsequent studies have shown the BART results to be controversial.[185, 186] Health Canada published a safety review of aprotinin in September 2011, which suggested that the benefit of using aprotinin in cardiac surgery might offset the risk.[83, 187] Accordingly, aprotinin was made available again in Canada for restricted use in isolated coronary bypass graft surgery. The European Medicines Agency also recommended lifting the suspension of aprotinin in February 2012 after a review of the risks and benefits of antifibrinolytic drugs.[83]

2. Other Natural Kunitz-Type Inhibitors

Alzheimer amyloid precursor protein and its homolog: The Kunitz domain of Alzheimer amyloid precursor protein homolog (KD APPH) was found to inhibit plasmin with a K_i of 81 nM. KD APPH inhibited several other proteases too including trypsin (K_i 0.02 nM), plasma kallikrein (K_i 86 nM), glandular kallikrein (K_i 8.8 nM), chymotrypsin (K_i 78 nM), and factor XIa (K_i 14 nM).[188] Likewise, the Kunitz domain of amyloid precursor protein (KD APP), also reported in the literature as Kunitz protease inhibitor of protease nexin-2 (KPI PN2), potently inhibits trypsin, chymotrypsin, and factor XIa with K_i of 0.02, 6.0, and 0.7 nM, respectively, and moderately inhibits plasmin, plasma kallikrein, and glandular kallikrein (K_i 42–82 nM).[188] Mutagenesis at specific sites in the recognition

sequence of KPI PN2, especially P1 Arg→Lys and P2′ Met→Lys/Arg (Table II), fine tuned activity against plasmin (K_i 8 nM) at the cost of activity against factor XIa and kallikrein.[189] Although wild-type KPI PN2 was potently antithrombotic, the double mutants were antifibrinolytic without displaying antithrombotic complications.[189]

Tissue factor pathway inhibitor-1 (TFPI-1) and TFPI-2: The first Kunitz domain of human TFPI-2 (KD TFPI-2) inhibited plasmin (K_i 10 nM), while also inhibiting trypsin (K_i 7 nM).[190] In contrast, recombinant domain 1 of hTFPI-1 inhibited several enzymes including factor VIIa/tissue factor (K_i 250 nM), cathepsin G (K_i 200 nM), and plasmin (K_i 26 nM). Its recombinant domain 2 inhibited factor Xa (K_i 90 nM), trypsin (K_i 0.1 nM), and chymotrypsin (K_i 0.75 nM), while Kunitz domain 3 appeared to have no inhibitory function.[191]

Bikunin: Placental bikunin is a 170-amino acid human serine protease inhibitor containing two Kunitz-type inhibitory domains, the *N*-terminal$_{(7–64)}$ Kunitz domain, and the *C*-terminal$_{(102–159)}$ Kunitz domain.[192, 193] The two domains can function independently and can also direct ternary complexation with selected serine proteases.[192, 193] Both domains inhibited several enzymes of the intrinsic coagulation and fibrinolytic pathways including plasmin (K_i 0.5–1.0 nM), trypsin (K_i ~0.03 nM), chymotrypsin (K_i ~2 nM), plasma kallikrein (K_i ~0.7 nM), and pancreatic kallikrein (K_i ~0.5 nM). But there were some interesting differences too. Tissue kallikrein was more potently inhibited by *C*-terminal$_{(102–159)}$ Kunitz domain (K_i 0.13 nM). However, the high potency could not be easily exploited because of considerable cross reactivity.

L1T1: A 174-residue polypeptide L1TI was isolated from *Leucaena leucocephala* seeds and found to inhibit human plasmin with a K_i of 0.32 nM.[194] Other enzymes (plasma kallikrein, trypsin, and chymotrypsin) were inhibited with at least 20-fold lower potency. L1TI prolonged clotting time in the APTT assay, but not in PT or TT assays, suggesting its primary effect on the contact activation pathway. L1TI also inhibited kinin release from high molecular weight kininogen, decreased carrageenin-induced edema, and lowered bradykinin suggesting an antiinflammatory effect.[194]

Tick-derived protease inhibitor: Another natural plasmin inhibitor (K_i 55 nM) is tick-derived protease inhibitor (TdPI), which is a 97-amino acid polypeptide isolated from *Rhipicephalus appendiculatus*. The polypeptide also inhibited human tryptase (K_i ~1.5 nM) and trypsin (K_i 5.6 nM),[195] but it does not affect urokinase, thrombin, factor Xa, factor XIIa, elastases, kallikreins, cathespsin G, granzyme B, chymase, and chymotrypsin.[195]

Textilinin-1 and -2: Textilinin-1 and -2 are Kunitz-type serine protease inhibitors isolated from the Australian snake *Pseudonaja textilis* having 59 residues and ~45% and 43% identity to aprotinin, respectively.[196] Both inhibitors contain six conserved cysteines common to all Kunitz-type inhibitors and bind tightly to plasmin with K_i of ~0.11–3.5 nM.[197] Both inhibitors reduced blood loss by a substantial 60% in a murine tail vein bleeding model.[196] Although textilinin-1 appears to be a more specific plasmin inhibitor than aprotinin, the latter was able to inhibit clot lysis better.[198] Textilinin-1 was several-fold less potent than aprotinin in inhibiting kallikrein (plasma and tissue), trypsin and plasmin

(Tables I and II). But it was more specific than aprotinin with respect to direct inhibition of tPA, urokinase, activated protein C, and elastase.[199] The crystal structure of free textilinin-1 has just been reported and found to be similar to that of aprotinin.[200] Also, the crystal structure of textilinin-1–microplasmin complex has been solved.[201] The narrower specificity of textilinin-1 most probably arises from its bulkier P1′ Val in comparison to the P1' Ala present in aprotinin.[201] Textilinin-1 appears to be worth investigating further as an antifibrinolytic agent.[202]

AvKT1, DrKln-II, and Bt-KTI: A plasmin inhibitor was isolated from *Araneus ventricosus* spider and found to be a Kunitz-type protease inhibitor (AvKTI).[203] Recombinant AvKTI having a 57-amino acid Kunitz domain inhibited plasmin (K_i 4.9 nM), neutrophil elastase (K_i 169 nM), trypsin (K_i 7.3 nM), and chymotrypsin (K_i 37.8 nM).[203] Likewise, recently a slow and tight-binding inhibitor of plasmin (K_i 0.2 nM) was isolated from Russell's viper (*Daboia russelii*) venom and named as Kunitz-type protease inhibitor (DrKIn-II).[204] It appeared to inhibit plasmin more than most other proteases screened. It prolonged APTT but not PT. DrKIn-II demonstrated antifibrinolytic activity in fibrin plate assay and prolonged the clot lysis time. Finally, DrKIn-II inhibited formation of fibrin/fibrinogen degradation product in a coagulation-stimulated mice model and diminished murine tail bleeding time.[204] The third Kunitz-type polypetide Bt-KTI is a 58-residue inhibitor from bumblebee *Bombus terrestris* venom.[205, 206] Bt-KTI is antifibrinolytic owing to its potent plasmin inhibition activity.

Tryptogalinin: Tryptogalinin is a tick-derived Kunitz-type serine protease inhibitor isolated from *Ixodes scapularis*.[207] Besides its potent inhibition of β-tryptase (K_i 0.01 nM), tryptogalinin potently inhibited plasmin, trypsin, and chymotrypsin with K_i values of 5.8, 0.5, and 0.4 nM, respectively. It also moderately inhibited matriptase and elastase (K_i ~14 and 19 nM, respectively). This inhibitor did not inhibit coagulation enzymes (thrombin, factors Xa, XIa, or Xlla), chymase, kallikren, or plasminogen activators indicating its special specificity features.

3. Natural Kazal-Type Inhibitors—Kazal-type serine protease inhibitors are similar to the Kunitz-type inhibitors in terms of their size (40–60 residues) and the number of the conserved disulfide bridges. Yet, the difference arises from the combination of the six conserved cysteines in forming the disulfide bridges. Although cysteines 1 and 5, 2 and 4, and 3 and 6[208] combine for Kazal-type inhibitors, it is 1 with 6, 2 with 4 and 3 with 5 for Kunitz-type polypeptides.[209] This results in their characteristic three-dimensional structures that are different. Five natural Kazal-type plasmin inhibitors are described below.

Infestins: Infestins are Kazal-type serine protease inhibitors found in the midgut of the Chagas' disease vector, *Triatoma infestans*.[210] Infestins are composed of seven Kazal domains. Although native infestin did not show plasmin inhibition, recombinant infestin 1–4 (fourth to seventh Kazal domains), infestin 3–4, and infestin 4 inhibited plasmin potently with K_i of 1.1, 0.4 and 2.1 nM, respectively.[210-214] These Kazal domains also inhibited other serine proteases including thrombin, trypsin, factor XIIIa, and factor Xa, although not all were inhibited by each polypeptide. Structural characterization of the seven Kazal domains

revealed that domains second to fifth share high-sequence homology and thus, inhibitory properties.[211] However, the data appears to indicate that infestin's antiplasmin activity stems from the seventh Kazal domain (infestin-4). The plasmin inhibitory activity of infestins has not been exploited for antifibrinolytic purposes. Instead, infestins are being studied in molecular imaging.[214]

Bdellin-KL: Bdellin-KL is a trypsin (K_i 3.6 nM) and plasmin inhibitor (K_i 8.6 nM) from the leech *Hirudo nipponia* and comprises a 48-amino acid Kazal-type protease inhibitor domain at the *N*-terminal (fragment 19–66) sequence.[215] The intact protein (fragment 19–155) displayed similar inhibitory profile. Interestingly, its plasmin inhibitory activity remained intact up to 90°C at pH 1 and up to 50°C at pH 2 or 12.

clTI-1: Recently, Kubiak et al. identified a Kazal-type trypsin inhibitor, named as clTI-1 from the liver of *Mleagris gallopavo* chicken.[216] This inhibitor avidly binds to human plasmin (K_d 83 nM) and different forms of trypsin (K_d 0.08–2.2 nM). Interestingly, the P1–P1' sequence was found to be Arg^{P1}–$Asp^{P1'}$.[216, 217] Not much additional information is available on this molecule.

4. Other Kazal-Type Inhibitors—Other inhibitors belonging to the Kazal-type inhibitor family include the bikazin salivary inhibitor isolated from *Canis familiaris* dog submandibular glands and a trypsin inhibitor from *Aedes aegypti* (AaTI). The former has two structural domains, of which domain I inhibits trypsin and plasmin (K_i 2 μM) and domain II inhibits chymotrypsin, elastase, and subtilisin.[217] The recombinant form of the latter inhibitor inhibited human plasmin, trypsin, and thrombin with K_i of 3.8, 0.15, and 320 nM, respectively.[218, 219]

5. Natural non-Kunitz-Type or Non-Kazal-Type Inhibitors—Plasminostreptin has been reported as a plasmin inhibitor isolated from *Streptomyces antifibrinolyticus* microbe.[220] It does not belong to either Kunitz-type or Kazal-type inhibitor class. Plasminostreptin inhibited human plasmin (ID_{50} 8 μg), bovine trypsin (ID_{50} 1 μg) and several microbial alkaline proteases (e.g., subtilisin (ID_{50} 2.5 μg)), but not thrombin, elastase or kallikrein.[221] Plasminostreptin inhibited plasmin by forming a stoichiometric complex of 1:1 and doubled the clot lysis time at 8 μg dose.

6. Engineered Kunitz-Type Protease Inhibitors—Kunitz domains appear to be attractive platforms to design novel therapeutic proteins.[154, 155, 222-226] Several reasons contribute to this characteristic. Structurally, Kunitz domains are relatively small peptides of about 60 amino acids that exhibit high stability because of the presence of three disulfide linkages.[227] They can be engineered to possess high stability to inactivation by oxidants, high temperatures, and extreme acidity or basicity. Their expression in yeast occurs with high efficiency and yields.[154, 155] Finally, literature reports their relatively safe use in humans, although concerns about their immunogenicity, bioavailability, and plasma half-life have to be kept in mind. We describe here two promising engineered Kunitz domains that have been developed for potential use as antifibrinolytics to substitute the recently withdrawn aprotinin.

DX-1000: Markland et al. reported the iterative use of phage display to generate small libraries of Kunitz domains having nearly human sequences and possessing high affinity and specificity for human plasmin.[154] A series of libraries having variants of the first Kunitz domain of hTFPI-1 were iteratively generated through variations at 13 positions in the P1 region (residues 10–21) and in the second loop (residues 31–39). These two regions comprise the end of the Kunitz domain, which interacts with the serine protease target. One particular engineered protein (EPI-P302 or DX-1000) was identified to have interesting antifibrinolytic profile comparable to aprotinin.

DX-1000 binds tightly to human plasmin (K_i 0.087 nM), which reflects about 300-fold increase in the affinity relative to the parent domain KD hTFPI-1 (Table I). It was also several thousand-fold selective over plasma kallikrein (K_i ~250 nM), factor Xa (K_i 1.3 μM), human chymotrypsin (K_i 10 μM), urokinase (K_i > 100 μM), and human neutrophil elastase (K_i ~800 nM). Interestingly, DX-1000 did not inhibit human thrombin at the highest concentration tested (K_i >> 100 μM).[154] Comparison of DX-1000 sequence with its parent KD hTFPI-1 revealed seven differences including position 10 (*E* vs. D), 11 (*T* vs. D), 15 (*R* vs. K), 17 (*R* vs. I), 18 (*F* vs. M), 19 (D vs. K), and 21 (W vs. F).[154] Interestingly, the plasmin inhibitory activity of DX-1000 remained essentially unaffected by temperatures as high as 85°C, or by prolonged incubation at pH 10, or in the presence of chloramine T, a very strong oxidant.[154] DX-1000 was recently evaluated in hemostasis and coagulation studies.[226] In these studies, DX-1000 inhibited plasmin with a high affinity (K_i 0.1 nM) and did not inhibit human tPA or uPA (K_i > 10 μM). Despite the good antiplasmin activity, DX-1000 was evaluated as an antineoplastic agent[226] rather than as an antifibrinolytic agent.

KD1-L17R: An interesting example of an engineered Kunitz-type plasmin inhibitor is KD1-L17R. It is the first Kunitz domain of hTFPI-2 in which the P2′ residue was mutated to Arg.[155] Human TFPI-2 contains three Kunitz domains, of which the first domain was found to inhibit several enzymes of the digestive system, coagulation pathways, and the fibrinolytic pathway. The second Kunitz domain was found to inhibit factor Xa and the third domain has no known inhibitory function.[191, 228, 229] Taking these selectivities in consideration, a rational structure-based approach was developed that led to the design of KD1-L17R, which selectively inhibited fibrinolysis but not coagulation.

The reason why a single mutation was sufficient to engineer high selectivity is that digestive and coagulation proteases prefer hydrophobic residues at the P2′ position in their substrates and inhibitors, whereas plasmin favors a basic residue. The cationic P2’ in the engineered inhibitor was expected to interact with the strongly electronegative Glu73 and Glu143 present in the S2’ subsite of plasmin.[230] In contrast, the S2′ subsite in plasma kallikrein, factor XIa and factor VIIa comprises a hydrophobic pocket that prefers nonpolar residues. Biochemical studies indicated that KD1-L17R had a K_i of 0.9 nM against plasmin and a K_i of at least 3000 nM against other serine proteases (Table I). This was in contrast to KD1-WT, which was less potent against plasmin (K_i 6 nM).[155]

KD1-WT prolonged the clotting time in APTT and PT assays. In contrast, KD1-L17R did not prolong the clotting times as expected of its higher selectivity. In the fibrinolysis assay, KD1-L17R inhibited tPA-induced plasma clot fibrinolysis with an IC_{50} of 0.36 μM,

which was at least fivefold better than KD1-WT and tranexamic acid. Mouse liver laceration animal model was also exploited to assess the effect of KD1-L17R on the amount of blood loss. It was found that the amount of blood loss was reduced by ~84% by KD1-L17R, ~70% by aprotinin, ~52% by TXA, and ~10% by KD1-WT. These results suggest that KD1-L17R is very effective in inhibiting plasma clot fibrinolysis and in reducing blood loss in animal models in a manner comparable to clinically used aprotinin.[155]

Based on the fact that KD1-L17R and aprotinin share similar size, and that the dose at which each satisfies the required therapeutic outcome is also similar, it was proposed that they both share similar pharmacokinetics. KD1-L17R did not induce renal toxicity in mice when used at doses higher than that of aprotinin and resulted in no detectable histopathologic changes in major organs including heart, lungs, brain, and liver.[155] KD1-L17R also did not cause any seizures as observed for TXA.[231] These results suggested that KD1-L17R was likely to have a better safety profile than existing antifibrinolytic agents. At this point, the potential of this engineered protein is being further studied.

It is important to mention here that several reports indicate the value of direct inhibition of plasmin in reducing blood loss during on-pump cardiothoracic surgery.[225, 232, 233] For example, DX 88 is a mutated version of the Kunitz domain from human lipoprotein-associated coagulation inhibitor domain 1. It was studied for two major indications; hereditary angioedema, for which it was approved in 2009,[232] and reduction of blood loss during on-pump cardiothoracic surgery, in which it failed relative to TXA.[225, 233] Likewise, DXX 88 is a potent kallikrein inhibitor (K_i 25 pM). But it also inhibits plasmin (K_i 29 nM). The inhibitor has good safety and tolerability profiles.[217, 232, 233] Hence, the failure for the second indication does not necessarily implicate any dangerous risk, yet it does emphasize the importance of direct inhibition of plasmin to reduce blood loss over kallikrein inhibition.

C. Sulfated Polymers as Plasmin Inhibitors

1. Heparin and Heparan Sulfate—Heparin is a variably sulfated, highly heterogeneous and polydisperse mixture of linear sulfated GAGs. Structurally, heparin is composed of repeating disaccharide units of glucosamine and uronic acid (Fig. 11A).[234] Heparin has long been clinically used as an anticoagulant to prevent and treat thromboembolic diseases. Heparin's anticoagulant activity stems from its binding to antithrombin (AT), which enhances inactivation of coagulation enzymes, particularly thrombin and factor Xa.[12]

In addition to its effect on the coagulation system, heparin was also found to affect the fibrinolytic system. Yet, how heparin interacts with different components of the fibrinolytic pathway remains controversial.[235] The reason for this is that many of the experimental conditions used were not physiological. Nevertheless, the heparin–plasmin interaction has received significant attention, particularly to explore a route to novel antifibrinolytic agents. Even here, it is important to recognize that heparin's impact on plasmin activity, direct or indirect, seems to be limited at NaCl concentrations higher than 25 mM.

Plasmin inhibition can be brought about by other plasma inhibitors including α_2-antiplasmin, α_1-antitrypsin, C1 esterase inhibitor, α_2-macroglobulin, and AT. Highsmith et

al. studied the interaction of AT and plasmin.[236] AT was found to be a time-dependent inhibitor of plasmin's proteolytic activities. Sodium dodecylsulfate gel electrophoresis indicated that AT forms a stoichiometric covalent complex, which is stable in the presence of denaturing or reducing agents. The presence of heparin accelerated plasmin inhibition by AT by about 60-fold. However, increasing the plasmin concentration relative to heparin reduced the rate.[237]

Heparin bound directly to plasmin with a K_D of 10 nM[238-240] and induced a conformational change in its active site.[241] Interestingly, heparin enhanced hydrolysis of several chromogenic substrates through an increase in k_{CAT} (K_M unchanged). The polysaccharide also enhanced the rate of enzyme inactivation by *N*-α-tosyl-lysine-chloromethylketone, an active site histidine targeting agent, but not by phenyl-methylsulfonyl fluoride, an active site serine probe. Likewise, heparin increased the heat sensitivity (denaturation) of plasmin when a P1-Lys chromogenic substrate was used, but did not affect enzyme activity when P1-Arg substrates were used. These results suggest that heparin induced microscopic environmental changes in the active site of plasmin by binding to an allosteric site.[241] In fact, kinetic studies using chromogenic substrate S-2251 demonstrated that heparin is a noncompetitive inhibitor of plasmin with a K_i of 4.9 μM.[240]

Recently, Chander et al. reported plasmin inhibition under physiological conditions by a covalent complex of AT and heparin.[235] Results revealed that the increase in plasmin inactivation is brought about by inducing a conformational change in the enzyme. The study also found that plasmin inhibition was decreased in the presence of fibrin, fibrinogen, and EACA.[235]

Earlier studies have shown that oleic acid exhibits a contradictory effect on the amidolytic and fibrinolytic activity of plasmin. Although oleic acid stimulated plasmin amidolysis of S-2251, it inhibited cleavage of the physiological substrate fibrin.[242, 243] Competitive studies with lysine analogs indicated that oleic acid bound to the LBSs of plasmin and induced a conformational change to bring about such contradictory effects. The effect of oleic acid on plasmin was later exploited to enhance the inhibitory action of heparin. Combining oleic acid and heparin in a single molecule, as in *N*-oleoyl heparin, yielded a more potent inhibitor of plasmin (IC_{50} 16 nM).[244] *N*-oleoyl heparin was also devoid of antifactor Xa activity, which means that it may carry only antifibrinolytic activity.[244] Other GAGs including hyaluronic acid, chondroitin sulfate, dermatan sulfate, and heparan sulfate did not inhibit the peptidolytic activity of plasmin. Yet, oversulfation of chondroitin sulfate generated an antiplasmin molecule (IC_{50} 60 nM), especially when four sulfates were present per disaccharide unit.[244] Plasmin inhibition by *N*-oleoyl heparin was highly dependent on salt and detergent concentration suggesting a role for both ionic and hydrophobic interactions. Lineweaver-Burk analysis indicated that *N*-oleoyl heparin is a noncompetitive inhibitor with a K_i of 8 nM.[244]

Plasmin's heparin-binding site(s) is(are) yet to be determined. The site(s) is(are) most probably located in the catalytic domain, but could also be in the kringle domains or both. Several researchers have exploited these putative heparin-binding site(s) to introduce heparin

mimetics that are capable of potently inhibiting plasmin.[245-247] Three classes of heparin mimetics functioning as plasmin inhibitors are described in the following sections.

2. Sulfated Low Molecular Weight Lignins (LMWLs)—Sulfated LMWLs have been developed as polymeric, nonsaccharide mimetics of heparin that utilize dual ionic and hydrophobic interactions in binding to proteins.[248] Three sulfated LMWLs, $CDSO_3$, $FDSO_3$, and $SDSO_3$ (Fig. 11B), were synthesized and found to be potent inhibitors of human plasmin as well as thrombin.[245, 249] The polymers were synthesized in two steps involving a chemo-enzymatic process in which horseradish peroxidase-catalyzed oxidative coupling of 4-hydroxycinnamic acid monomers was followed by sulfation using triethylamine-sulfur trioxide complex. The enzymatic process relies on a radical coupling reaction, which results in polymers containing multiple inter-residue linkages, such as β-O-4 and β-5, and heterogeneity and polydispersity resembling low molecular weight heparins. The three sulfated LMWLs displayed molecular weights in the range of 3000–4000 Da, chain lengths in the range of 5–15 units and 0.3–0.4 sulfate groups per unit.

Chromogenic substrate hydrolysis assays indicated that $CDSO_3$ potently inhibited the amidolytic activity of human plasmin with an IC_{50} value of 0.24 μM and efficacy of nearly 100%. $FDSO_3$ and $SDSO_3$ were threefold and fivefold less potent. Indirect AT-mediated inhibition of plasmin was also evaluated and found to have limited contribution to the overall inhibition process.[245] Among the three polymers, $FDSO_3$ was tested for the inhibition of fibrin cleavage using an in vitro transmittance assay, which revealed good reduction in fibrinolysis over 0–6.4 μM concentration. Mechanistically, sulfated LMWLs were found to disrupt the plasmin's catalytic apparatus as revealed by Michaelis–Menten kinetics in presence of $CDSO_3$. Binding of $CDSO_3$ to plasmin affected both K_M and V_{MAX} of the hydrolysis suggesting a mixed inhibition mechanism. Competitive binding studies indicated that $FDSO_3$ competed well with 30 nM and 300 nM full-length heparin, which implied binding to the heparin-binding site on plasmin.[245]

Although sulfated LMWLs are good inhibitors of plasmin, they inhibit several other serine proteases,[250, 251] including thrombin, factor Xa, factor IXa, factor XIa, leuckocyte elastase, and cathepsin G (Table I).[250, 251] Especially for thrombin, extensive biochemical studies and site-directed mutagenesis studies have revealed that sulfated LMWLs bind in exosite 2, which is the heparin-binding site in thrombin.[252] Thus, superimposition of the thrombin and plasmin structures helped identify the corresponding residues in plasmin that may recognize sulfated LMWLs.[245, 252] The authors hypothesized that Arg644, Lys645, Arg637, Arg776, and Arg779 of plasmin may be important (Fig. 12).

Overall, sulfated LMWLs can be considered as nonspecific inhibitors that may be useful in preventing and/or treating complex pathologies. Clinically, several disordered conditions are known in which a cluster of serine proteases are active including pancreatitis, DIC, and cancer.[253] Thrombin and plasmin are the two critical enzymes in DIC, in which coagulation and fibrinolysis processes are aberrant. Extensive clotting due to thrombin action in DIC is followed by bleeding due to plasmin action.[6, 7, 137, 148] Hence, the dual inhibitory function of LMWLs may lead to a novel therapy for DIC. Also, inhibiting thrombin and plasmin could offer an efficient strategy to treat thrombotic cancers.[26, 27, 42, 48, 243] Plasmin also

activates MMPs, which play important roles in tissue remodeling and have been recently indicated to contribute to emphysema.[254] Thus, direct inhibition of neutrophil elastase and plasmin-mediated indirect inhibition of MMPs could represent a novel therapy for chronic obstructive pulmonary diseases. This was recently demonstrated by Saluja et al. in an in vitro antiemphysema study of sulfated LMWLs.[255]

3. Chemically Modified Dextran Sulfate Derivatives—Considering plasmin's biological roles in tissue remodeling (in addition to fibrinolysis), Ledoux reported synthetic dextran sulfates (DS) that mimic heparin's antiplasmin activity.[246] Four chemically modified DSs were synthesized from dextran T40. This included DSs that were carboxymethylated (RG1100), *O*-sulfonated (RG1003), carboxymethylated and *O*-sulfonated (RG1503), and carboxymethylated, amidated, and *O*-sulfonated (RG1192). The resulting polymers of 40–140 kDa size had variable levels of hydrophobic/hydrophilic substitution (Fig. 11D).[246]

S-2251 hydrolysis assay indicated that RG1100 and RG1003 were inactive at pH 7.4 and 37°C, but the dual carboxylated and *O*-sulfonated derivative, RG1503, inhibited human plasmin with an IC_{50} of 20 nM and a moderate efficacy of 30%. The most potent activity was derived upon introducing aromatic hydrophobic elements (benzyl groups) through amidation on the carboxylated polymer. The resulting triple-modified dextran derivative RG1192 was found to potently inhibit plasmin with an IC_{50} of 2 nM and efficacy of 80%. Interestingly, RG1192 inhibited tPA also (IC_{50} 34 nM, efficacy 70%) but did not affect trypsin, chymotrypsin, and uPA.[246]

Mechanistically, both RG1503 and RG1192 were found to be reversible tight binding, noncompetitive inhibitors of human plasmin and displayed a stoichiometry of 1:6 and 1:20, respectively. The derivatives relied significantly on their sulfate groups for inhibiting plasmin. Using physiological salt concentration, only RG1192 retained an inhibitory activity toward plasmin (efficacy 70%, K_i 0.31 μM). Competitive studies in presence of EACA, kringle 1–3, kringle 4, or kringle 5 indicated that RG1192 preferred to bind to the LBSs on kringles 1, 4, and 5 with variable affinites but not to the active site. RG1192 bound to plasminogen also with high affinity (K_d 0.03 μM). SDS-PAGE analysis revealed that RG1192 renders the Arg561-Val562 scissile bond of plasminogen inaccessible and/or unrecognizable by uPA. This implies that RG1192 could contribute to the regulation of plasmin activity by three plausible mechanisms; (1) inhibition of plasmin generation due to plasminogen binding; (2) inhibition of tPA so as to prevent plasmin generation; and (3) direct and noncompetitive plasmin inhibition. Analysis by SDS-PAGE further revealed that plasmin-catalyzed degradation of fibronectin and laminin was inhibited by RG1192 (1–2.5 μM).[246] In addition, the DSs protected various heparin-binding growth factors against proteolytic degradation in vitro and thus enhanced their bioavailability.[256, 257] The derivatives also inhibited the enzymatic activity of neutrophil elastase.[258] The inhibitory profile of DSs partially accounted for their roles in in vivo models of tissue repair and wound healing.[256-262] Despite this success, reports on their antifibrinolytic activity to reduce blood loss have not been published as yet.

4. Sulfated Polyvinylalcohol-Acrylate Copolymers—Four sulfated polyvinylalcohol-acrylic acid (PVA) copolymers were prepared as heparin mimetics having

varying number of negative charges (40.5–73.5%) and molecular weights (5600–8800 Da, Fig. 11C).[247] The four copolymers were named PVA30, PVA38, PVA47, and PVA64 according to their sulfate content. Each copolymer, except for PVA30, inhibited small molecule hydrolysis by plasmin (IC_{50} 80–110 nM) in pH 7.4 buffer containing 150 mM NaCl. Miniplasmin (kringles 1–4 absent) and microplasmin (kringles 1–5 absent) were similarly inhibited (IC_{50} 28–31 nM and 120–140 nM, respectively).[247] These results suggested that the binding site(s) for sulfated PVA polymers is(are) likely to be in the catalytic domain of plasmin. Under similar conditions, heparin had no effect on plasmin activity at concentrations as high as 2.2 μM, although it did inhibit plasmin at low ionic strengths of buffer.[247] A change in the intrinsic fluorescence of plasmin was observed for all four copolymers indicating a possible change in conformation upon binding. PVA38, PVA47, and PVA64 also inhibited the fibrinolytic activity of plasmin with an IC_{50} of 1.6–2.2 μM, whereas heparin was not effective under similar conditions.[247] PVA47 decreased the fibrin dissolution when co-added with plasminogen and tPA but had no effect on fibrinolysis when co-added with fibrin-bound plasminogen and tPA. This probably implied that the polymers are not effective when plasmin was generated from plasminogen that is attached to the fibrin but inhibit plasmin in its free, solution form.[247] Earlier, these copolymers had been reported to inhibit thrombin with potencies similar to that against plasmin.[263]

4. PLASMIN INHIBITORS FOR OTHER DISEASES

The above discussion was focused on structural and mechanistic aspects of plasmin inhibitors with respect to their antifibrinolytic activity so as to treat hyperfibrinolysis-associated bleeding consequences. However, plasmin inhibitors can potentially serve several other roles. We highlight other applications of plasmin inhibitors, especially as antimetastatic, antiproliferative, antiangiogenesis agents as well as in embryo implantation.

A. PASI-535

Wanaka et al. reported that PASI-535 (**48**, Fig. 10) potently inhibited plasmin-mediated fibrinolysis (IC_{50} 2.9 μM) and fibrinogenolysis (IC_{50} 4.5 μM). Interestingly, PASI-535 suppressed ascites retention and growth of tumor cells in sarcoma-180 bearing mice following its subcutaneous injection for 5 days at 30–50 mg/kg/day.[264] This activity was nearly 40 times better than that for TXA, which reduced both ascites retention and tumor growth at ~2 g/kg/day.[264]

B. YO-2

Another interesting discovery was that of a selective plasmin inhibitor (**14**, Fig. 4). This agent not only inhibited plasmin-mediated fibrinolysis (IC_{50} 0.36 μM), but also induced apoptosis of both M1 melanoma and HT29 colon carcinoma cell lines within 24 hr.[50] Mechanistic studies revealed that inhibitor **14** activated caspase-3 at low doses (10–20 μg/mL).[50] The pro-apoptotic activity rose from a combination of intranucleosomal DNA fragmentation and enhancement in capsase-3, capsase-8, and capsase-9-like activities.[265, 266] Further, inhibition of tumor growth by **14** was examined using HT29 human colon carcinoma, HT18 human melanoma, and HT58 human B-cell lymphoma inoculated as xenografts into immuno-deprived mice.[267] Its antimetastatic activity was also investigated

in the B16 mouse melanoma muscle-lung model. Results indicated that **14** inhibited the growth of all xenografts by 40–50%, when administered at a dose of 0.4–2.0 mg/kg. It also effectively decreased the number of lung metastasis found in mice inoculated with B16 melanoma at 4 mg/kg.[267] Recently, **14** was shown to reduce T-cell lymphoid tumor growth by suppressing MMP-9-dependent $CD11b^+$ $F4/80^+$ myeloid cell recruitment.[51]

C. Pegylated DX-1000

Recently, engineered polypeptide DX-1000 was modified using methoxy-PEG-succinimidyl propionate (mPEG-SPA, M_r 5000 Da) at its three lysines (positions 10, 54, and 55) and the amino terminus to produce pegylated DX-1000 (4PEG DX-1000).[226] Pegylation increased the K_i for plasmin by 2.5-fold to 0.23 nM. Both DX-1000 and 4PEGDX-1000 were found to efficiently inhibit plasmin-mediated activation of pro-MMP-9 in HL60 cells as well as inhibit tube formation in HUVEC (IC_{50} 1.4 nM for DX-1000, 8.3 nM for 4PEG DX-1000) and LEII cells (IC_{50} 16.6 nM for DX-1000,15.8 nM for 4PEG DX-1000).[226] DX-1000 was rapidly cleared from plasma in mice and rabbits, however, 4PEG DX-1000 exhibited an extended plasma half-life and pharmacological profile.[226] 4PEG DX-1000 was found to block human breast cancer growth and tumor metastasis in vivo at a dose of 10 mg/kg. Furthermore, 4PEG DX-1000 treatment also led to a significant inhibition of tumor cell proliferation and a decrease of tumor vessel hot spots. Mechanistically, 4PEG DX-1000 inhibitory effects were found to be mediated by activating mitogen-activated protein kinase signaling pathways.[226] Interestingly, 4PEG GDX-1000 treatment also reduced uPA and plasminogen expression by 55% and 63%, respectively. Overall, 4PEG DX-1000 was found to have antiproliferative, antimetastatic, and antiangiogenic effects in vivo with improved bioavailability.

D. Sulfated Cholesterol (SC)

An important function in which plasmin is involved as a key enzyme is embryo implantation into maternal endometrium during pregnancy. The implantation process relies on the invasive capacity of the trophoblast cells, which is attributed to plasmin as well as MMPs. The proteases are required for the degradation of the matrix so that pregnancy can be established. While MMPs contribute to the process directly, plasmin contributes indirectly by converting the pro-MMP latent form to the active form. Matrix degradation is necessary but excessive degradation is detrimental because the damage can prevent or terminate pregnancy. Thus, regulation of plasmin proteolytic activities is important for embryo implantation.

It was shown that sulfated cholesterol (SC) content drastically increases in rabbit endometrium during the implantation period.[37] Koizumi et al. studied the inhibition of these enzymes by SC (**49**, Fig. 10).[37] In a concentration-dependent fashion, SC was found to reduce plasmin and MMP-3 activities to 14% and 26%, respectively, at a concentration of 30 μM in culture media of the extracellular matrix. Western blot analysis demonstrated that SC inhibited the plasmin-mediated conversion of MMP-3's latent form to its active form. Gelatin zymography analysis indicated inhibition of plasmin-mediated activation of MMP-9 by 10 μM SC.[37] In another study, Nakae et al. showed that SC directly inhibited plasmin activity in a noncompetitive manner, which led to reduction of invasion of a trophoblast cell

line.[49] In combination, the results appear to indicate that SC inhibition of plasmin may be essential for regulating the process of embryo implantation.

E. EACA, TXA, Aprotinin, Nafamostat, and Gabexate

Several reports present the potential benefit of using EACA (**1**), TXA (**2**), or aprotinin in managing, treating, and preventing disorders such as hereditary angioedema,[45, 46] traumatic bleeding,[268] chronic inflammation,[47, 48] and lymphoid malignancies.[50, 51] In particular, data from the Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage 2 (CRASH-2) study revealed that early administration of TXA reduced the risk of death in trauma patients in comparison to the placebo (4.9% vs. 5.7%, respectively; $p = 0.0077$).[269] Also, aprotinin's anticoagulant and antiinflammatory effects could further aid cardiopulmonary bypass success.[168, 170]

Recently, plasmin inhibitors have been reported to prevent leukocyte accumulation and remodeling events in the postischemic microvasculature,[270] which highlights their potential in the prevention of ischemia-reperfusion injury. Moreover, although not fully understood, TXA has been implicated in the treatment of melasma, prevention of UV-induced pigmentation, and the recovery of skin barrier.[270, 271] Finally, nafamostat and gabexate have been found useful for complex pathologies such as DIC,[7, 137, 148] pancreatitis,[138-144] and liver diseases.[272]

F. Upamostat (Mesupron)

Upamostat, inhibitor **50** (Fig. 10), is a hydroxyamidino prodrug of WX-UK1, a nonspecific inhibitor of several serine proteases.[273, 274] WX-UK1 was found to inhibit uPA (K_i 0.41 μM), plasmin (K_i 0.39 μM), thrombin (K_i 0.49 μM), factor Xa (K_i 1.7 μM), activated protein C (K_i 2.3 μM), plasma kallikrein (K_i 7.2 μM), trypsin (K_i 0.037 μM), and tryptase (K_i 6.3 μM). It appears that its direct and indirect inhibition of plasmin contributes to its activity against primary tumor growth and metastasis formation.[275, 276] Currently, upamostat is in phase II studies for patients with special forms of pancreatic cancer or breast cancer.[277] Interestingly, melagatran (**51**) and inhibitor H317/86 (**52**) (Fig. 10), related benzamidine derivatives to upamostat, were also found to inhibit plasmin with K_i values of 0.7 and 0.27 μM, respectively. Melagatran was approved as an anticoagulant direct thrombin inhibitor, yet it was withdrawn later from market due to hepatotoxicity.[278]

5. CONCLUSIONS

Plasmin is intravascularly formed upon activation of plasminogen by tPA, whereas uPA appears to be the major extravascular activator of plasminogen.[27, 52, 54] The resulting plasmin plays diverse physiological and pathophysiological roles, of which fibrinolysis is the key role for intravascular plasmin. Cell surface-derived plasmin is more involved in tissue remodeling, matrix degradation, and cell migration.[26-28] A couple of plasmin inhibitors are used clinically today including EACA (**1**) and TXA (**2**), which are lysine analogs. Despite their success in reducing blood loss associated with major surgeries, the two antifibrinolytics suffer from severe lack of efficacy. Large quantities need to be administrated and the therapeutic regimen has to be adjusted to account for every

patient.[171, 279-281] Further, specificity is also an issue as these lysine analogs may bind a nontarget protein possessing an appropriate negatively charged domain. An example of this is seizure attacks in some patients, which likely happen because of their action on GABA receptors.[91, 92, 231, 280, 282] A plasmin inhibitor in limited use in some countries is aprotinin. The controversy surrounding this polypeptide is an ongoing debate. Its disadvantage is its lack of specificity, which is the probable cause of associated morbidity and mortality.[83, 178-187]

The similarity of plasmin active site with several other serine proteases, e.g., coagulation enzymes, introduces considerable challenges in developing new potent and selective plasmin inhibitors. This problem is further compounded by the limited number of plasmin-ligand co-crystal structures resulting in considerable lack of understanding of interactions at the atomic level. This hampers progress in the direction of establishing key structural elements that enhance potency as well as selectivity. A thorough understanding of the spatiotemporal properties of extravascular and intravascular plasmin action is also required to develop novel plasmin inhibitors that selectively target fibrinolysis or tissue remodeling and cell migration processes.

Despite these difficulties, several novel plasmin inhibitors are emerging as antifibrinolytics. The molecules being developed are highly diverse spanning a wide range of size, scaffold, and physicochemical properties. Mechanistically, the group comprises competitive as well as noncompetitive inhibitors. Some exciting antiplasmin agents include textilinin-1,[196-202] KDI-L17R,[155] pegylated DX-1000,[154, 226] and the cyclic peptidomimetics **39** and **40**.[11] Majority of these molecules enjoy high affinity for plasmin. Yet, their specificity is not high enough and there is a possibility that the molecules may suffer unexpected consequences as they advance through the various stages of drug development.

An emerging trend in targeting plasmin is nonsaccharide GAG mimetics (NSGMs). Sulfated LMWLs, chemically modified sulfated dextrans, and sulfated PVA polymers are sulfated NSGMs that functionally mimic heparin/heparan sulfate and inhibit plasmin in an allosteric manner. This is worth investigating in detail. Allosteric inhibitors usually convey two advantages over orthosteric inhibitors. One, allosteric sites are typically less conserved among proteins of the same family leading to an enhanced specificity for molecules targeting these sites. Two, allosteric sites in principle afford the possibility of controlled reduction in enzyme function (<100% maximal inhibition), which may become critical for enzymes involved in multiple pathophysiological roles, such as plasmin.

The discovery of polymeric NSGMs as allosteric inhibitors may lead to the design of small NSGMs that specifically target plasmin. This is a relatively new concept and is in rapid development. For example, NSGMs of a specific type, tetrasulfated tetrahydroisoquinolines, were rationally designed to target the pentasaccharide-binding site of AT.[283] Likewise, several NSGMs including disulfated quinazolinone dimers[284] and polysulfated pentagalloyl glucosides[285] have been developed to target factor XIa, while monosulfated benzofuran dimers[286] and trimers[287] were designed to target exosite 2 of thrombin. Small NSGMs agents offer many advantages including (1) high water solubility, which is expected to help antifibrinolytic use during surgeries, (2) low cellular and central nervous system toxicity

arising from their highly charged nature, (3) good chemical stability, and (4) ease of chemical synthesis.[288] Yet, major efforts are needed to develop NSGMs as inhibitors of plasmin for clinical use.

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ABBREVIATIONS

6.

AaTI	*Aedes aegypti* thrombin inhibitor
APTT	activated partial thromboplastin times
AT	antithrombin
AvKTI	*Araneus ventricosus* spider is Kunitz-type serine protease inhibitor
BPTI	bovine pancreatic trypsin inhibitor
Bt-KTI	*Bombus terrestris* Kunitz-type serine protease inhibitor
BZA	*p*-Benzoylanilide
Cbz	benzyloxycarbonyl

clTI-1	chicken liver trypsin inhibitor-1
DIC	disseminated intravascular coagulation
DrKIn-II	*Daboia russelii* Kunitz Inhibitor-II
EACA	ε-Aminocaproic acid
GAGs	glycosaminoglycans
hTFPI-1	human tissue factor pathway inhibitor-1
K	kringle domain
KD APP	Kunitz domain of amyloid precursor protein
KD APPH	Kunitz domain of Alzheimer amyloid precursor protein homolog
KD hTFPI-1	Kunitz domain of human tissue factor pathway inhibitor-1
KD TFPI-2	Kunitz domain of tissue factor pathway inhibitor-2
KPI PN2	Kunitz protease inhibitor domain of protease nexin 2
LBS	lysine-binding site
LMWLs	low molecular weight lignins
MMPs	matrix metalloproteinases
NSGMs	nonsaccharide glycosaminoglycan mimetics
PT	prothrombin time
PVA	polyvinyl-acrylate
SAR	structure-activity relationship
SC	sulfated cholesterol
TdPI	tick-derived protease inhibitor
Tos	tosyl
tPA	tissue plasminogen activator
Tra	N^{α}-trans-4-aminomethylcyclohexanecarbonyl
TT	thrombin time
TXA	tranexamic acid
uPA	urokinase plasminogen activator

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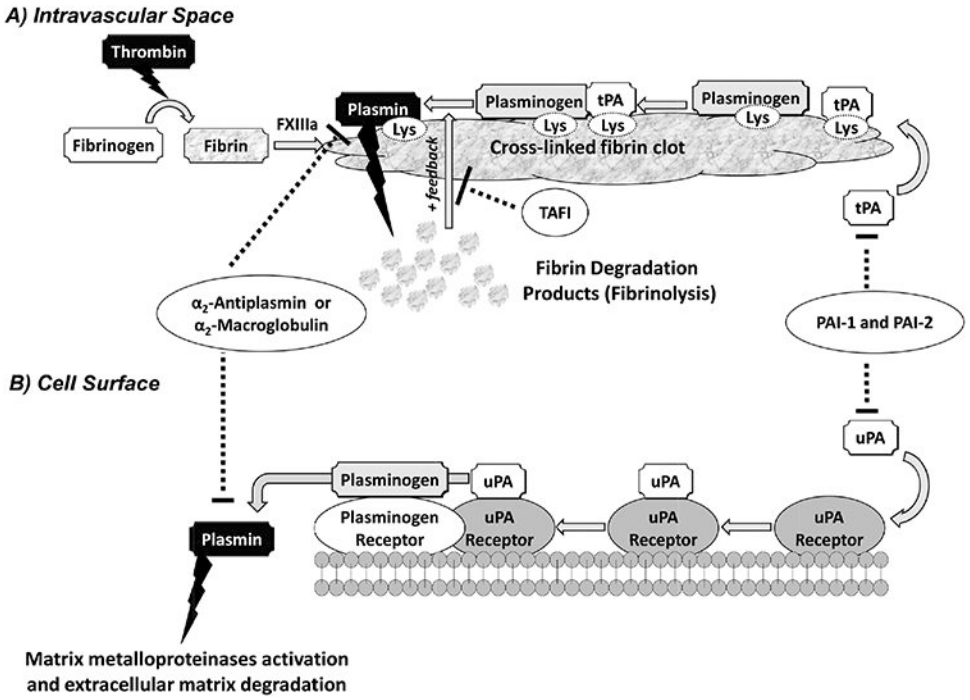

Figure 1.

A simplified schematic representation of the plasminogen-plasmin system. Plasminogen is activated in the intravascular space by tissue plasminogen activator (tPA) (A) or at the cell surface by urokinase plasminogen activator (uPA) (B). (A) During coagulation, thrombin converts fibrinogen into soluble fibrin monomers, which cross-link by the action of factor XIIIa resulting in formation of insoluble cross-linked fibrin clot. If the fibrinolysis is to be initiated, plasminogen and tPA bind to fibrin through their lysine-binding sites (LBSs) present on the kringle domains. Formation of such ternary complex activates plasminogen and releases plasmin, which hydrolyzes fibrin. In a positive feedback mechanism, plasmin promotes its own formation by exposing more C-terminal lysine residues of fibrin. Four physiologic inhibitors regulate fibrinolysis including plasminogen-activator inhibitor −1 and −2 (PAI-1 and PAI-2), which inhibit tPA and uPA, and α_2-antiplasmin and α_2-macroglobulin, which inactivate any unbound plasmin. In addition, activated thrombin-activatable fibrinolysis inhibitor, which upon activation by thrombin removes the C-terminal lysine residues of fibrin, also prevents plasmin generation. (B) Activator uPA binds to its receptor at the cell surface and activates plasminogen that is bound to its receptor nearby. This releases plasmin into the extracellular matrix. Plasmin generated at the cell surface is primarily regulated by the action of α_2-antiplasmin, PAI-1, and -2. Plasmin generated at the cell surface plays key roles in MMP activation and ECM degradation.

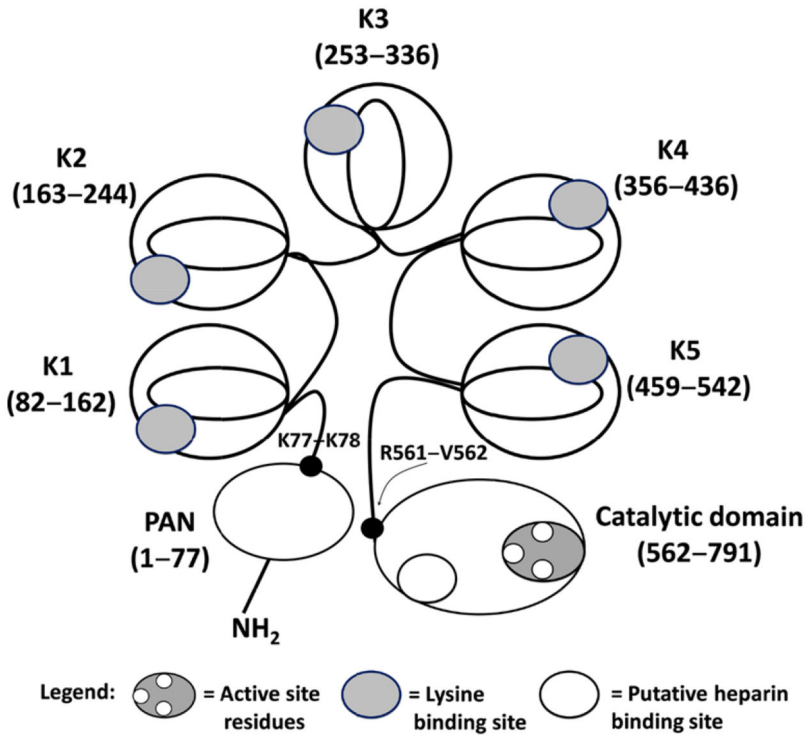

Figure 2.

A schematic depiction of the plasminogen structure. Plasminogen possesses an N-terminal plasmin-apple-nematode (PAN) domain (1–77), five kringle domains K1–K5 (residues 162–542), and a catalytic domain (562–791). K77-K78 and R561-V562 are two cleavage sites. Cleavage at the R561-V562 scissile bond by tPA and other activators produces full-length active plasmin. Cleavage at the K77-K78 bond can produce a shorter zymogen called Lys-plasminogen. Ligand- or substrate-binding sites are also shown (see legend). Competitive inhibitors such as the polypeptides aprotinin, DX-1000, KDI-L17R, and the cyclic peptidomimetics **39** and **40** bind to the active site, whereas lysine analogs such asTXAand EACA bind to the kringle domains. GAG mimetics bind to the putative heparin-binding site on the catalytic domain.

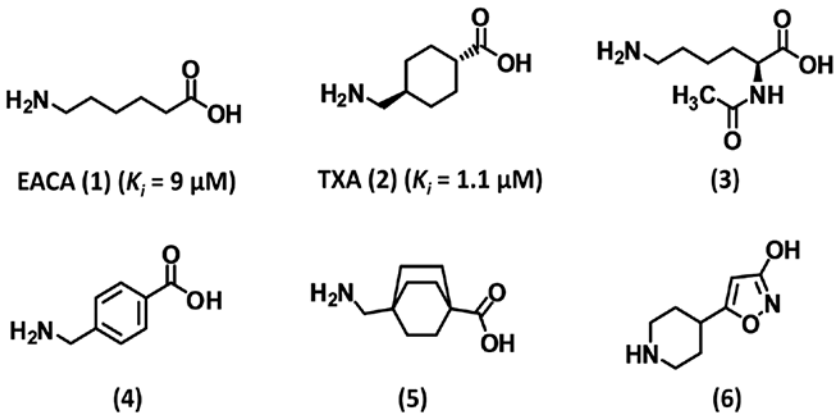

Figure 3.

Structures of lysine analogs 1–6. EACA (**1**) and TXA (**2**) are the most widely used antifibrinolytic agents. These agents are noncompetitive inhibitors of plasmin binding to kringle domains except inhibitor (**3**), which is a competitive inhibitor. Inhibitor **6** was recently identified by computational chemistry and exhibited fourfold better potency than TXA (**2**). K_i is the inhibition constant for plasmin.

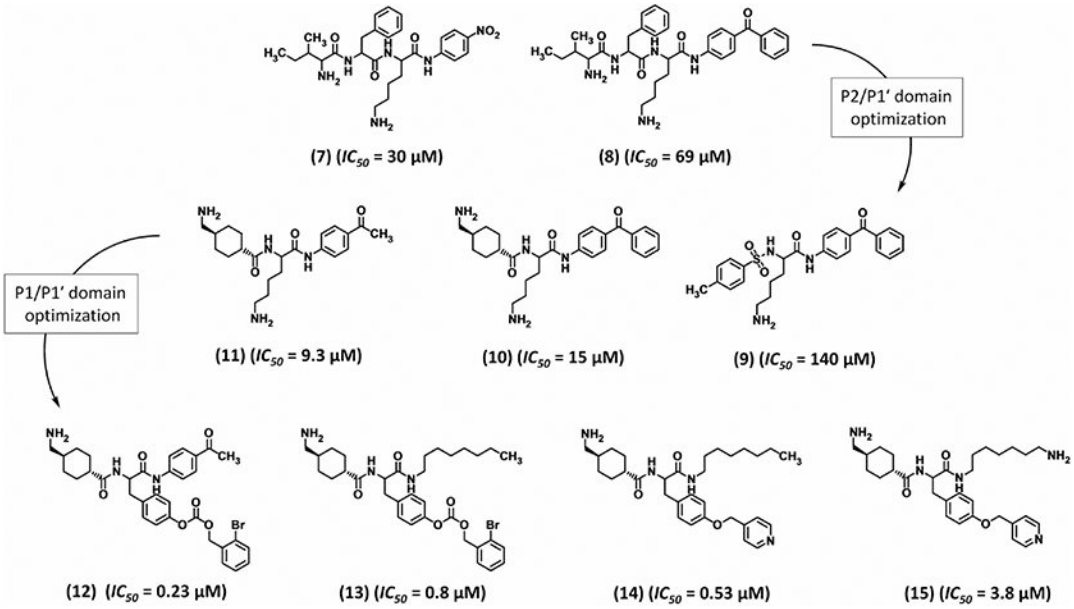

Figure 4.

Structures of trans-4-aminomethylcyclohexanecarbonyl-conjugated inhibitors **7–15**. These inhibitors are active site inhibitors. The development of inhibitor **15** was accomplished through two stages of P2/P1′ optimization followed by P1/P1′ optimization. IC_{50} refers to inhibition of plasmin amidolysis.

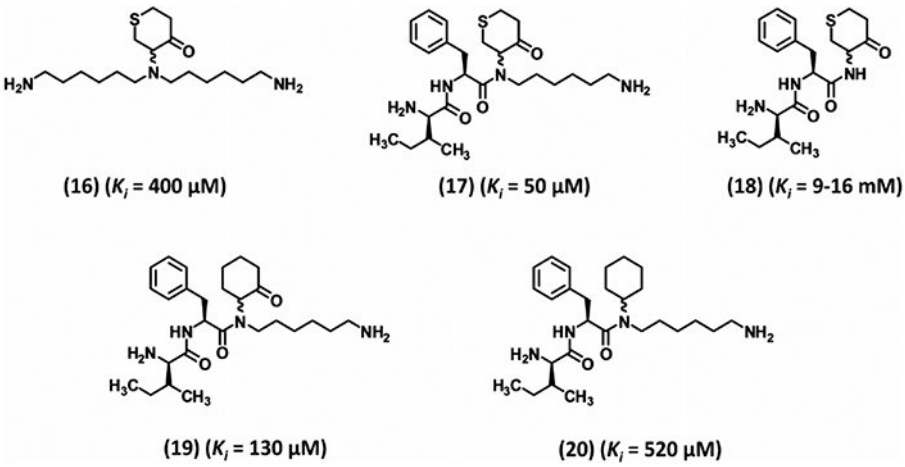

Figure 5.

Early development of cyclohexanone-based peptidomimetic inhibitors **16–20**. These inhibitors are reversible, covalent inhibitors (except inhibitor **20**) targeting the active site of human plasmin. Inhibition involves formation of hemiketal tetrahedral complex with the Ser residue of the catalytic triad. K_i is the inhibition constant for plasmin.

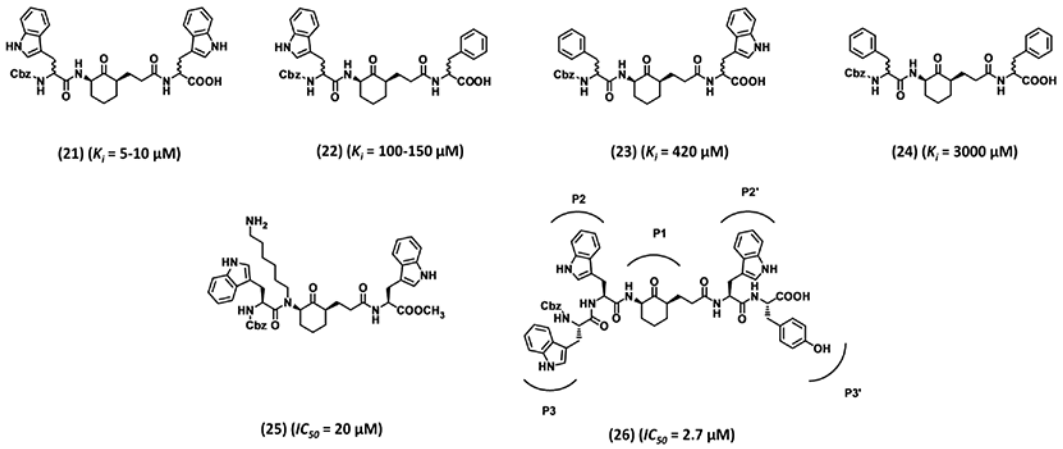

Figure 6.

Advanced cyclohexanone-based peptidomimetic inhibitors **21–26**. These inhibitors were developed by combinatorial chemistry approach to reversibly inhibit plasmin. Inhibitor **26** has six domains spaning plasmin active site from subsite S3-S3′. K_i is the inhibition constant for plasmin. IC_{50} refers to inhibition of plasmin amidolysis.

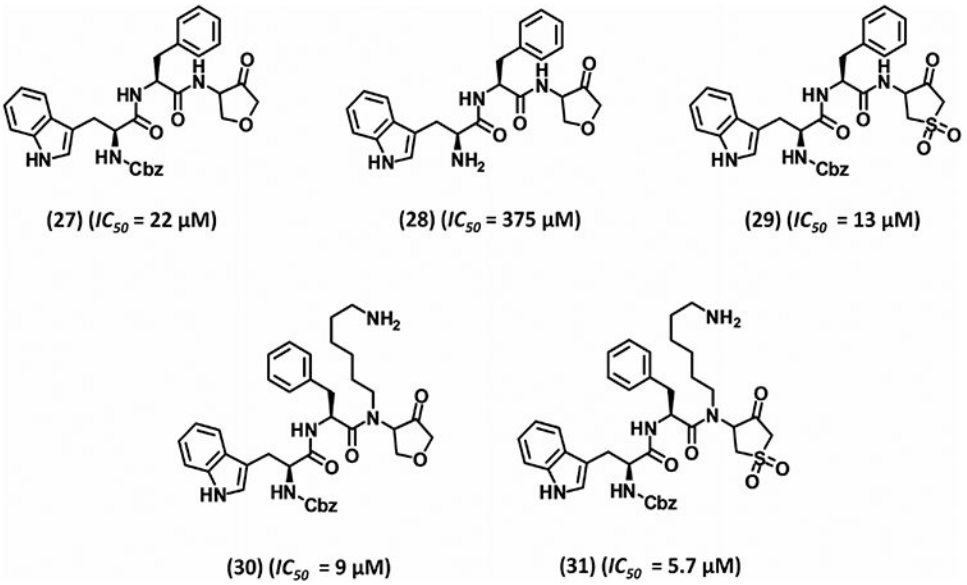

Figure 7.

Development of five membered heterocycle-based peptidomimtic inhibitors **27–31**. Inhibitors were developed by divalent classical bioisosterism replacement of position 4 methylene unit in previously designed inhibitors with either ether or sulfone moiety. IC_{50} refers to inhibition of plasmin amidolysis.

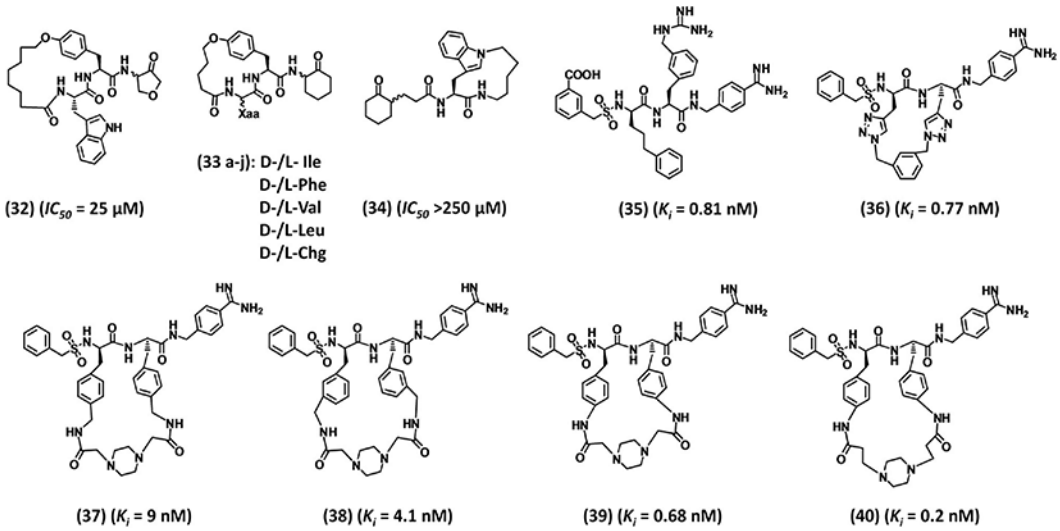

Figure 8.

Structures of various macrocyclic inhibitors **32–40**. Inhibitors **39** and **40** represent the most potent and selective small peptidomimetics reported in the literature. K_i is the inhibition constant for plasmin. IC_{50} refers to inhibition of plasmin amidolysis.

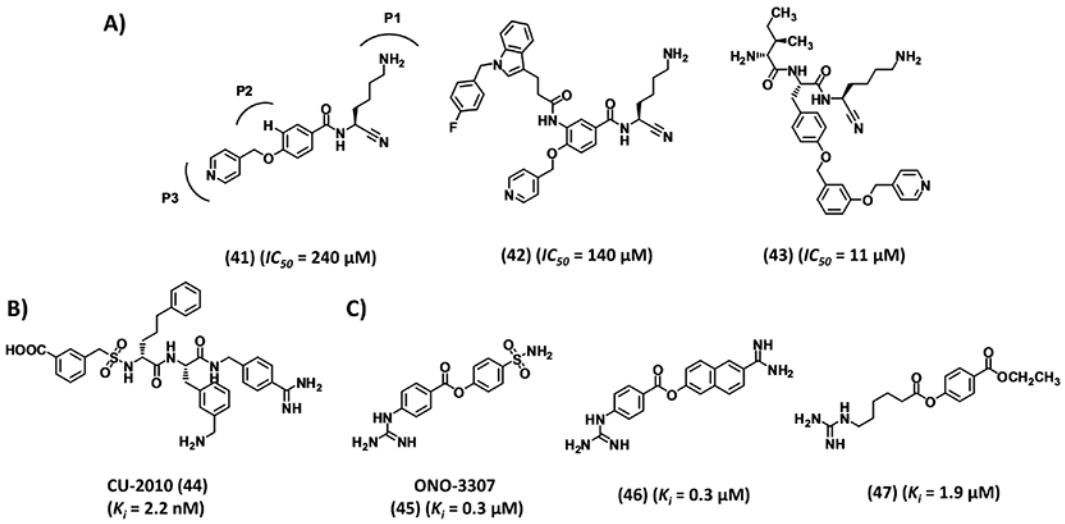

Figure 9.

Structures of diverse group of human plasmin inhibitors **41–49**. (A) Examples of nitrile warhead-based covalent inhibitors. (B) CU-2010 is amidinobenzyl-based peptidomimetic inhibitor that showed high promise, but its development was recently stopped. (C) Nonspecific inhibitors that are commonly used in complex pathologies such as DIC and pancreatitis. K_i is the inhibition constant for plasmin. IC_{50} refers to inhibition of plasmin amidolysis.

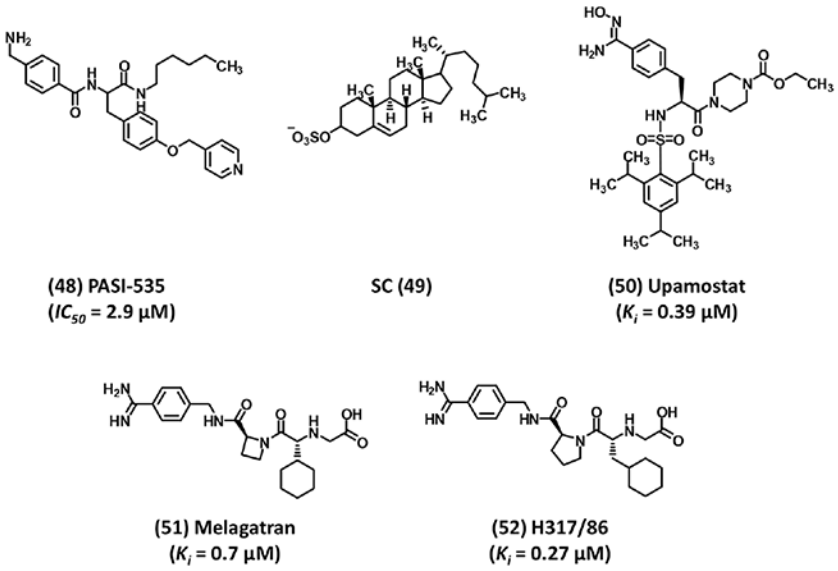

Figure 10.

Structures of plasmin inhibitors that show activities other than antifibrinolytic activity. Inhibitor **48** exhibits antitumor potential; SC **49** has been implicated in embryo implantation; Upamostat **50** is in pancreatic cancer Phase II studies; melagatran **51** was approved as a direct thrombin inhibitor; and H317/86 **52**, a benzamidine derivative related to upamostat, also inhibits plasmin. IC_{50} refers to inhibition of plasmin fibrinolysis.

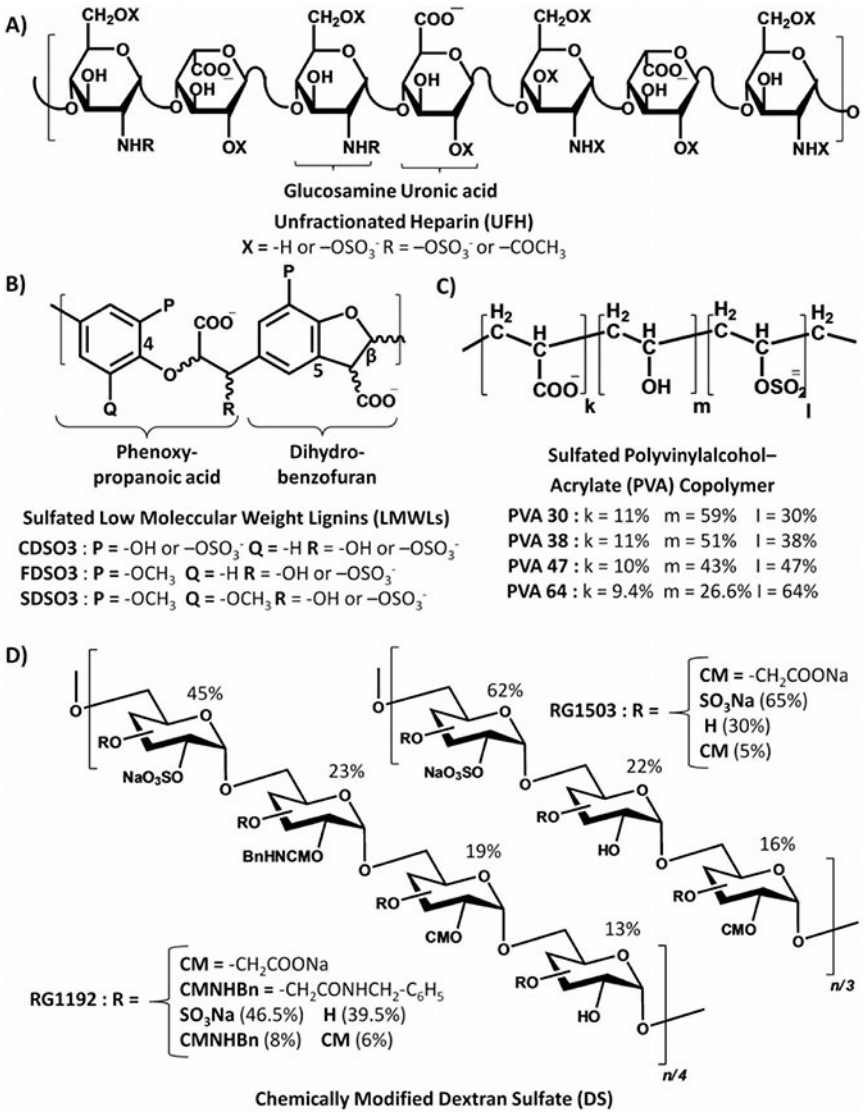

Figure 11.

Structures of glycosaminoglycan (GAG) mimetics. (A) Heparin is a polysaccharide that consists of variably sulfated glucosamine and uronic acid units. (B) Sulfated LMWLs are liginin-based polymers that have variable level of sulfation and hydrophobicity. (C) PVA copolymers. (D) Chemically modified dextran sulfates.

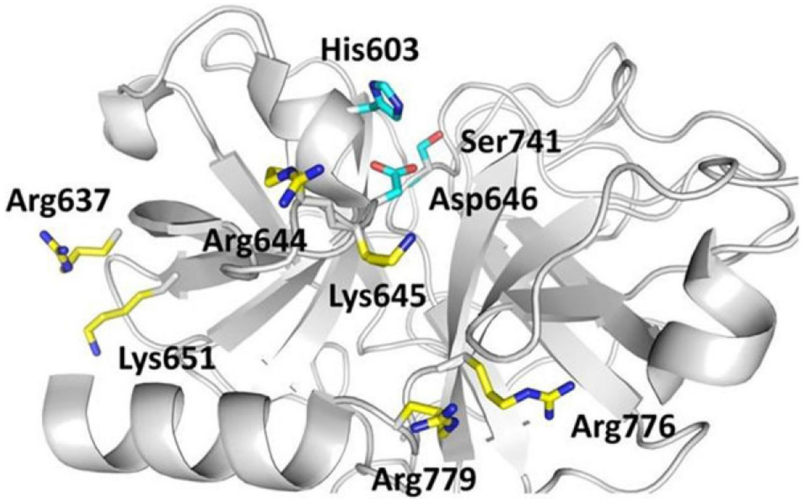

Figure 12.

A snapshot of the active site of human microplasmin (PDB ID: 3UIR) and the adjacent putative heparin-binding site. The pose shows basic amino acids that are likely to be targeted by GAGs mimetics of the sulfated LMWLs type. The putative heparin-binding site includes Arg637, Arg644, Lys645, Lys651, Arg776, and Arg779 in addition to other hydrophobic amino acid (see text). The catalytic triad of His603, Asp646, and Ser741 (His57, Asp102, and Ser195 in chymotrypsinogen numbering) is also shown. Colors used are gray for the protein backbone, cyan for carbons of the catalytic triad, yellow for the carbons of the putative heparin-binding site, red for oxygen, and blue for nitrogen atoms. The figure was generated in PyMOL (http://www.pymol.org/).

Table I.

Equilibrium Inhibition Constants (K_i) in Nanomolar for Selected Plasmin Inhibitors, Their Effect on APTT, PT, and Fibrinolysis

Protease	(40)[a]	CU-2010[b]	Aprotinin[c]	Textilinin-1[d]	DX-1000[e]	KD1-L17R[f]	$CDSO_3$[g]
Plasmin	0.2	2.2	0.18	0.11–3.5	0.087	0.9	242
Trypsin	38.3	ND	0.02	0.76	ND	ND	>10,000
Chymotrypsin	ND	ND	1.3	ND	~10,000	ND	>10,000
Plasma kallikrein	1000	0.019	13.4	4830	250	>3000	>10,000
Pancreatic kallikrein	ND	ND	0.023	ND	ND	ND	ND
Tissue kallikrein	ND	ND	0.004	12,900	ND	ND	ND
Urinary kallikrein	~10,000	ND	ND	ND	ND	ND	ND
Factor IXa	>5000	ND	>5000	ND	ND	ND	3380
Factor Xa	25,000	45	>30,000	NI	~1300	ND	34
Factor Xia	3370	18	288	ND	ND	>3000	22
Factor XIIa	>20,000	5200	6800	NI	ND	ND	>15,000
Factor VIIa/tissue factor	>50,000	ND	>1000	NI	ND	>3000	>29,000
Factor IIa	26,420	1700	>3000	NI	>>100,000	>3000	18
Factor IIa/thrombomodulin	ND	ND	~3000	ND	ND	>3000	ND
Activated protein C	>15,000	ND	~2000	>100,000	ND	>3000	>12,000
Tissue plasminogen activator	>40,000	ND	>3000	>500,000	ND	>3000	ND
Urokinase	ND	ND	~20,000	>100,000	>100,000	ND	ND
Cathepsin G	ND	ND	~4000	ND	ND	ND	232
Leukocyte elastase	ND	ND	~3500	>100,000	800	ND	11
Pancreatic elastase	ND	ND	>2,000,000	ND	ND	ND	>10,000
Clotting assay				*2 × clotting time (μM)*			
APTT	>100	1.4	11.9	>12	>0.7	>6	2.9
PT	>100	9.1	>100	>12	>5.6	>20	13.1
Fibrinolysis inhibition				*'Concentration (μM)*			
EC_{200}/IC_{50}	0.18	0.32	0.33	2—5	~0.56	0.36	ND

[a] Data from reference [11].

[b] Data from references [130, 132].

[c] Data from references [132, 155, 192].

[d] Data from references [197–199]. Factor VIIa data were reported without tissue factor. Concentration of fibrinolysis assay induces ~100% inhibition.

[e] Data from references[154, 226].

[f] Data from reference [155].

[g] Data from references [249, 250-251]. Factor VIIa data were reported without tissue factor and povine pancreatic elastase was used.

ND, not determined; NI, no significant inhibition.

Table II.

Polypeptide Inhibitor's Human Plasmin and Plasma Kallikrein Inhibition Constants (K_i, in nM) and Their Recognition Sequences

	K_i (nM)		Recognition sequence								
	Human plasmin	**Plasma kallikrein**	**P4**	**P3**	**P2**	**P1**	**P1′**	**P2′**	**P3′**	**P4′**	**References**
Natural Kunitz-type											
Aprotinin (BPTI)	0.18	13.4	Gly	Pro	Cys	**Lys**	Ala	Arg	Ile	Ile	160-165
KD APPH	81	86	Gly	Pro	Cys	**Arg**	Ala	Val	Met	Pro	188
KD APP (KPI PN2)	42	82	Gly	Pro	Cys	**Arg**	Ala	Met	Ile	Ser	188
KD TFPI-2	10	ND	Gly	Pro	Cys	**Arg**	Ala	Leu	Leu	Leu	190
KD1 rTFPI-1	26	ND	Gly	Pro	Cys	**Lys**	Ala	Ile	Met	Lys	191
Placental bikunin	0.5–1.0	~0.7	Gly	Pro	Cys	**Arg**	Ala	Phe	Ile	Gln	192, 193
L1TI	0.32	6.3	Ser	Pro	Tyr	**Arg**	Leu	Gly	Ser	Asn	194
TdPI	55	ND	Gly	Leu	Cys	**Lys**	Ala	Arg	Phe	Tyr	195
Textilinin-1	0.11–3.5	4830	Gly	Pro	Cys	**Arg**	Val	Arg	Phe	Pro	196-199
AvKTI	4.9	ND	Gly	Pro	Cys	**Lys**	Ala	Ser	Leu	Tyr	203
DrKIn-II	0.2	ND	Gly	Arg	Cys	**Arg**	Ala	His	Leu	Arg	204
Bt-KTI	2.01	ND	Gly	Thr	Cys	**Arg**	Gly	Tyr	Phe	Pro	205, 206
Tryptogalinin	5.83	NI	Gly	Pro	Cys	**Lys**	Ala	Met	Phe	Arg	207
Engineered Kunitz-type											
DX-1000	0.087	250	Gly	Pro	Cys	**Arg**	Ala	Arg	Phe	Asp	154, 224
4PEG DX-1000	0.232	ND	Gly	Pro	Cys	**Arg**	Ala	Arg	Phe	Asp	225
KD1-L17R	0.9	>3000	Gly	Pro	Cys	**Arg**	Ala	Arg	Leu	Leu	155
KPI PN2-R15K/M17K	8	NI	Gly	Pro	Cys	**Lys**	Ala	Lys	Ile	Ser	189
Natural Kazal-type											
Infestin-4	2.1	ND	Ala	Cys	Phe	**Arg**	Asn	Tyr	Val	Pro	210-214
Bdellin-KL	8.64	ND	Val	Cys	Thr	**Lys**	Glu	Leu	Leu	Arg	215
clTI-1	83	ND	Gly	Cys	Pro	**Arg**	Asp	Tyr	Ser	Pro	216, 217
Bikazin	2000	ND	Ala	Cys	Pro	**Arg**	Leu	His	Gln	Pro	217
AaTI	3.8	NI	Ala	Cys	Pro	**Arg**	Ile	Tyr	Met	Pro	218, 219
Natural Subtilisin-type											

	K_i (nM)		Recognition sequence								
	Human plasmin	**Plasma kallikrein**	**P4**	**P3**	**P2**	**P1**	**P1′**	**P2′**	**P3′**	**P4′**	**References**
Plasminostreptin	0.49	NI	Ala	Cys	Thr	**Lys**	Gln	Phe	Asp	Pro	[220]

ND, not determined or reported; NI, no inhibition at the highest concentration tested.