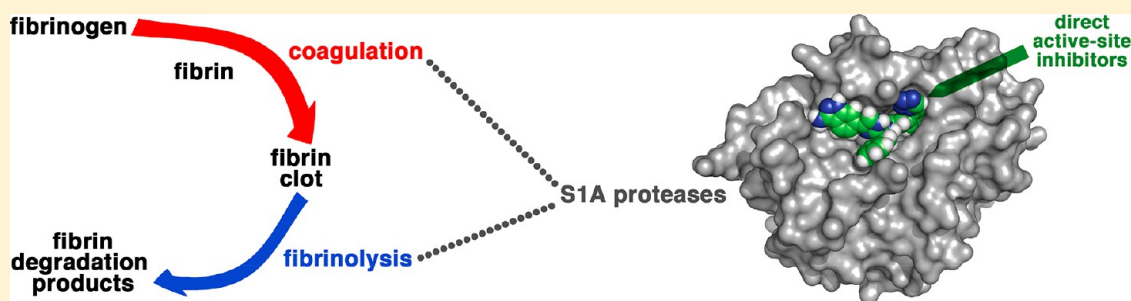


# Design of Small-Molecule Active-Site Inhibitors of the S1A Family Proteases as Procoagulant and Anticoagulant Drugs

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**ABSTRACT:** Vitamin K antagonists (VKA) have long been the default drugs for anticoagulant management in venous thrombosis. While efficacious, they are difficult to use due to interpatient dose–response variability and the risks of bleeding. The approval of fondaparinux, a heparin-derived factor Xa (fXa) inhibitor, provided validation for the development of direct oral anticoagulants (DOAC), and currently such inhibitors of thrombin and fXa are in clinical use. These agents can be used without regular coagulation monitoring, but the inherent risk of bleeding complications associated with blocking the common coagulation pathway remains. Efforts are now underway to develop DOACs that inhibit components of the intrinsic and extrinsic coagulation cascades upstream of thrombin and fX. Evidence from humans and from transgenic animal models suggests that this strategy may provide a better therapeutic margin between antithrombotic and antihemostatic effects. Here the design of active-site inhibitors of S1A proteases involved in coagulation and fibrinolysis is summarized.

## INTRODUCTION

**Hemostasis, Thrombosis, and Fibrinolysis.** Mammalian hemostasis, i.e., the formation of blood clots consisting of aggregated platelets and polymerized fibrin, is a physiological process that prevents bleeding upon injury to blood vessels. Thrombosis, on the other hand, is generally regarded as a pathological form of hemostasis, e.g., in arterial thrombosis as a result of atherosclerosis and in venous thrombosis due to interrupted blood flow, blood vessel irritation, and hypercoagulability.<sup>1</sup> However, thrombosis also plays an important role in the fight against pathogens, and such immunothrombosis, unless uncontrolled, is beneficial to the human host.<sup>2</sup> Formation of blood clots following vascular injury allows time for endothelial repair of the affected blood vessels. Once the vessel endothelium has been repaired, fibrinolysis sets in to remove the fibrin clot and to reinstate normal blood flow. Fibrinolysis is altered in some congenital disorders and especially during pathological overactivation (hyperfibrinolysis) upon severe injury or surgery.<sup>3</sup>

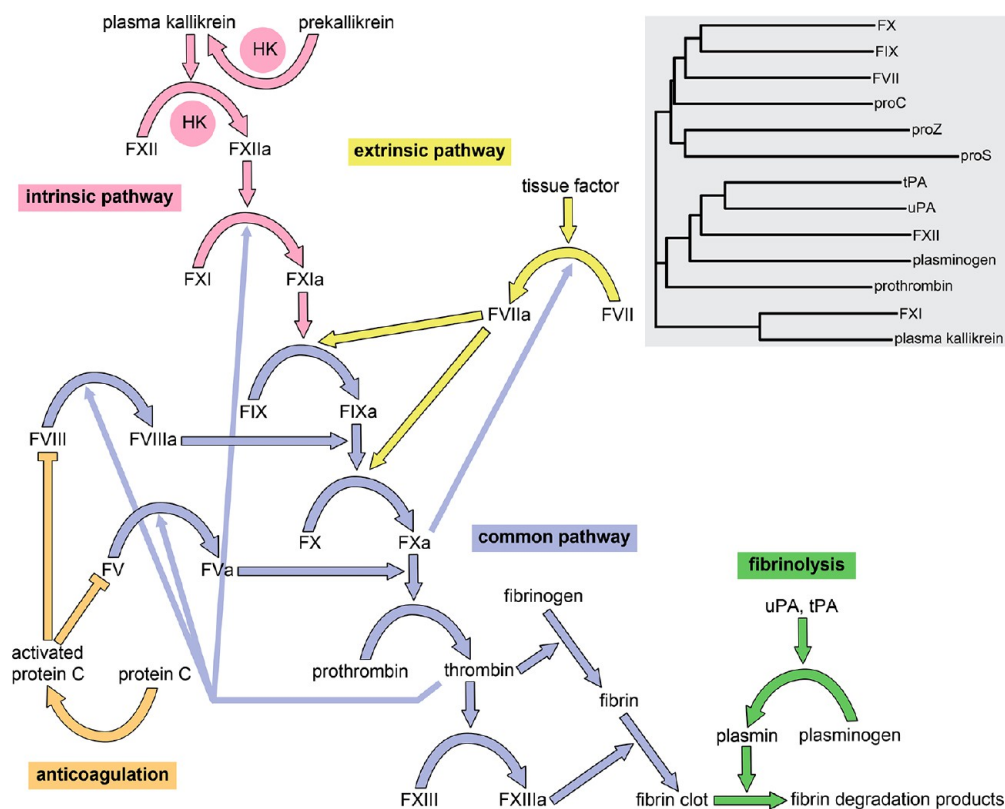
**Initiation of Coagulation through the Extrinsic Pathway.** Hemostasis is a complex process composed of two main components: platelet aggregation and platelet plug formation (primary hemostasis) and deposition of fibrin through the coagulation cascade (secondary hemostasis), although the interplay and spatiotemporal control of these components under pathophysiological conditions in vivo remain incompletely understood.<sup>4</sup> One hypothesis<sup>1</sup> holds that when a vascular

injury occurs, the local blood vessel endothelium is disturbed, leading to exposure of the blood to collagen, which, together with other factors, causes activation of circulating platelets. At the injury site these activated platelets adhere to exposed collagen below the endothelium through their van Willebrand factor (vWf) receptors and form an initial mechanical plug.<sup>5</sup> At the same time tissue factor (TF), which is expressed on subendothelial cells, is exposed to blood and interacts with circulating fVIIa and the resulting TF–fVIIa complex activates the coagulation cascade (Figure 1) through the extrinsic pathway. As a result, sequential activation of fX and prothrombin results in formation of thrombin, which in turn converts fibrinogen to polymerized fibrin. Additionally, thrombin activates several other coagulation factors, thus amplifying the coagulation cascade. Thrombin also further activates platelets, whose adherence to each other is promoted by the binding of platelet glycoprotein (Gp) IIb/IIIa to fibrinogen.<sup>6</sup> The combined actions of the platelet plug and the fibrin mesh then provide a mechanically stable thrombus, which is further sealed through fibrin polymer cross-linking by fXIIIa, the only coagulation factor that is not a protease but a transglutaminase.<sup>7</sup>

**Initiation of Coagulation through the Intrinsic Pathway.** Whereas activation of blood coagulation through the extrinsic pathway is understood well, the exact physiological

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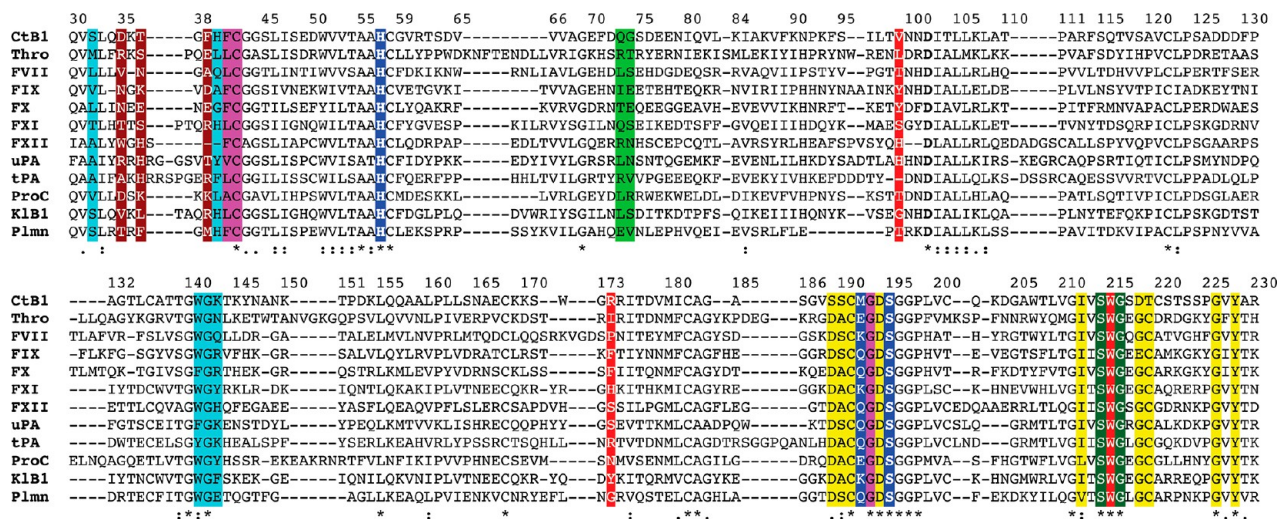
**Figure 1.** Roles of S1A proteases in coagulation and fibrinolysis (adapted from ref 8, <https://www.frontiersin.org/articles/10.3389/fcimb.2014.00128/full>). The extrinsic pathway of coagulation (TF pathway) mediates clot formation following vascular injury via *fVII*, whereas the intrinsic pathway (contact activation system) is initiated predominantly upon inflammation and activation of the innate immune system during infection by *fXII* in complex with prekallikrein and high-molecular weight kininogen (HK). The intrinsic and extrinsic pathways converge in the common coagulation pathway, which culminates in the formation of fibrin clots. Anticoagulation is mediated at the level of *fV* and *fVIII* by protein C (with the cofactors protein S and protein Z, which are not enzymatically active but are related to other coagulation factors). Fibrin clots are degraded through fibrinolysis, predominantly by activated plasmin. The inset is a cladogram showing the phylogenetic relationship between coagulation proteases based on multiple sequence alignment (Clustal Omega and ClustalW Phylogeny).<sup>9</sup>

relevance of the contact activation (intrinsic) pathway remains less clear. Originally it was thought that contact activation was necessary for initiation of coagulation, although the physiological activating surfaces had not been identified.<sup>10,11</sup> It has been known for a long time that in a test tube blood coagulates rapidly due to contact with glass and that many other anionic hydrophilic surfaces can initiate coagulation. The activated partial thromboplastin time (aPTT) assay, used as part of a series of medical blood coagulation screening tests, is based on this principle, where materials such as kaolin, micronized silica, or ellagic acid provide a surface for activation.<sup>12</sup> Following the proposal that coagulation in general is initiated through the intrinsic pathway, it later transpired that this was unlikely to be the case, since people deficient in *fXII*, prekallikrein, and HK, i.e., the components known to be involved in contact activation, were observed not to exhibit hemostatic defects, in stark contrast to people with deficiencies in factors of the extrinsic and common pathways, who do display such defects.<sup>13</sup> These observations led to the current belief that physiological hemostasis is initiated exclusively through the extrinsic pathway, although contact activation leading to coagulation and thrombosis plays a role in pathological states such as sepsis and acute respiratory distress syndrome and sometimes as a consequence of blood exposure to artificial surfaces of medical devices.<sup>14</sup>

The question of the role of contact activation *in vivo* has still not been fully answered, however. While a number of intrinsic materials capable of accelerating activation of *fXII* to

*fXIIa*, including polyphosphates, heparins, misfolded protein aggregates, and oligonucleotides, have been identified,<sup>15</sup> such activation may not involve classical contact activation.<sup>16</sup> Currently it is believed that the likely physiological roles of *fXIIa* include a support role in the maintenance of thrombus stability via polyphosphates from activated platelets, as well as local regulation of vascular permeability through activation of the kallikrein–kinin system (KKS) and formation of the proinflammatory peptide bradykinin.<sup>17</sup> What has recently become clear from animal studies, however, is an important and direct involvement of *fXIIa*-mediated coagulation in thrombosis rather than in hemostasis.<sup>15</sup> These recent findings are potentially of great importance in the development of new antithrombotics targeting intrinsic coagulation factors, which may provide anticoagulant activity devoid of bleeding side effects.<sup>14</sup>

**Anticoagulation and Fibrinolysis.** Because the maintenance of vascular homeostasis is of paramount importance in life, coagulation needs to be tightly controlled. This is probably the reason for the existence of a multilayered coagulation cascade, which allows fine control through crosstalk at multiple levels.<sup>18</sup> Under normal conditions anticoagulant signaling prevails over procoagulant activities. Anticoagulation pathways converge predominantly on protein C, which is activated to aPC on the endothelium by the thrombin–thrombomodulin–endothelial protein C receptor (EPCR) complex. Once formed, aPC counteracts coagulation in concert with its cofactor protein S by cleaving and inactivating *fV* and *fVIII*, i.e., the cofactor



**Figure 2.** Multiple sequence alignment (Clustal Omega) of the protease domains of the following human S1A proteases: chymotrypsin B1 (CtB1), thrombin (thro), fVII, fIX, fX, fXI, fXII, urokinase-like plasminogen activator (uPA), tissue plasminogen activator, (tPA), protein C (proC), kallikrein B1 (K1B1), and plasmin (Plmn). The residue numbering is that for CtB1. Subsites of the substrate recognition sites are colored as follows: S4, red; S3, dark green; S2, blue; S1, yellow; S1', pink; S2', cyan; S3', brown; S4', light green. The catalytic residues (H57, D102, S195) are indicated in bold.

precursors required for activation of prothrombin and fX, respectively.<sup>19</sup> Additional important physiological anticoagulation mechanisms include tissue factor pathway inhibitor, a Kunitz-type Ser protease inhibitor that operates at the level of fVIIa and fXa, and the serpin antithrombin that targets not only thrombin but several of the other coagulation Ser proteases. Both are endothelial surface proteins whose anticoagulant activity is enhanced by heparin.<sup>20,21</sup>

The other mechanisms that counteract coagulation when a thrombus has already been formed involve platelet disaggregation and fibrinolysis. The latter process is mediated by plasmin, which is formed from its precursor plasminogen by the action of tissue and urokinase-like plasminogen activators (tPA, uPA). Whereas tPA is released by endothelial cells, uPA is released predominantly by monocytes and macrophages. Plasmin may also have direct roles in anticoagulation at the level of fVa and fVIIIa and in platelet disaggregation at the level of vWf.<sup>22</sup>

**Structure and Functions of S1A Proteases.** The proteases responsible for the regulation of coagulation (Figure 1), as well as some proteases involved in digestion and complement activation, belong to the S1A family of the PA(S) clan of peptidases (MEROPS classification<sup>23</sup>), with chymotrypsin A as the prototype example. The S1A proteases contain His, Asp, and Ser as the catalytic triad<sup>24</sup> in a catalytic domain of ~220 amino acid residues, and this unit is extended N-terminally in many cases but rarely C-terminally (refer Figure 2 for sequence alignment). These proteases are expressed as single polypeptide chain zymogens that generally lack enzymatic activity, and they are activated through regulated proteolysis by other proteases or through autoproteolysis (Figure 3). In most cases activation entails proteolytic cleavage, which results in disulfide-bond-tethered two-chain protease forms consisting of a light chain that contains the protease catalytic domain and a heavy chain with several domains responsible for interactions with partner proteins.<sup>25</sup>

## ■ BLOOD COAGULATION AND FIBRINOLYSIS S1A PROTEASES AS PHARMACOLOGICAL TARGETS

**Introduction.** Whereas several of the factors in the coagulation cascade are being pursued as pharmacological targets for

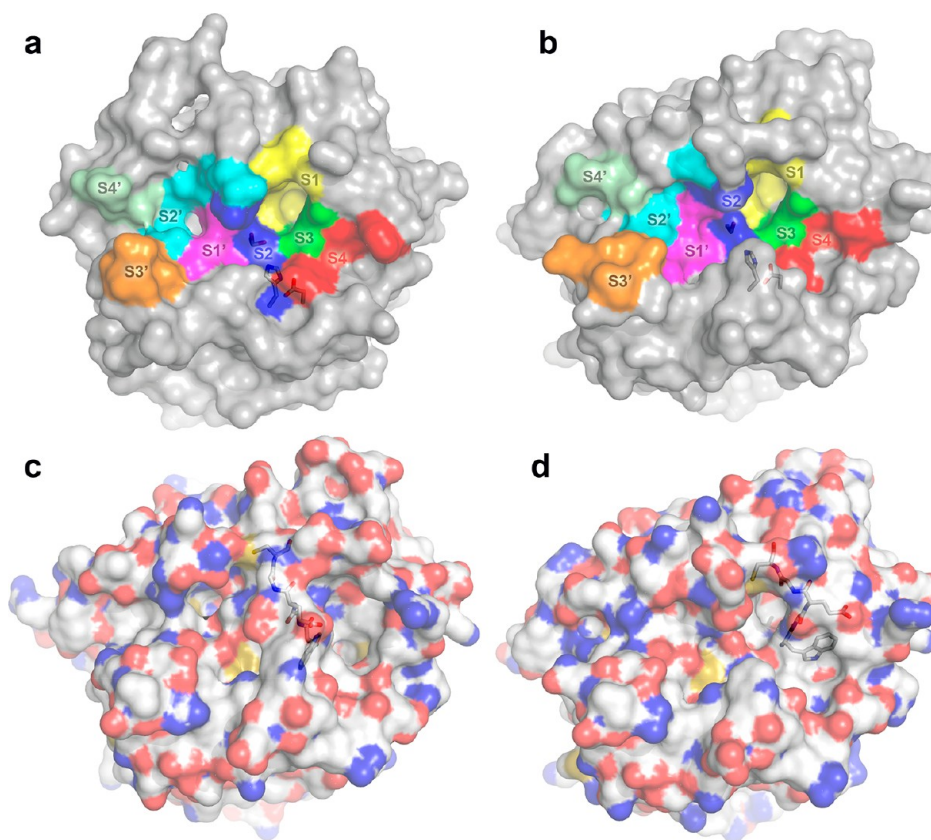
the development of new antithrombotic agents, the anticoagulant protein C is currently being investigated as a new inhibitor target for the treatment of hemophilias,<sup>26</sup> and certain proteases implicated in fibrinolysis provide a drug discovery rationale for the treatment of postoperative bleeding. The following discussion will be confined to proteases that are relevant to cardiovascular diseases, even though some S1A proteases involved in hemodynamic homeostasis, e.g., uPA and tPA, for which selective inhibitors are also being developed,<sup>27,28</sup> provide pharmacological rationales for other indications, such as tumor metastasis in the cases of uPA and tPA.<sup>29</sup>

**Thromboprophylaxis and Anticoagulants: Efficacy and Bleeding Side Effects.** Anticoagulant drugs are used for prophylaxis and treatment of thromboembolic disorders, such as deep-vein thrombosis, pulmonary and systemic embolisms, as well as coronary and cerebral ischemias. All of these disorders are characterized by the formation of blood clots in the vasculature. Anticoagulants such as heparin or warfarin, and more recently direct thrombin and fXa inhibitors, which target fibrin formation, are mostly used for conditions involving venous clots in deep-vein thrombosis and pulmonary embolism and especially in people with atrial fibrillation (AF) or transient ischemic attacks to prevent ischemic strokes. Antiplatelet agents such as aspirin, ADP receptor inhibitors, and GpIIb/IIIa inhibitors, on the other hand, are more effective for preventing arterial clots and are used to prevent thrombotic cerebrovascular and cardiovascular disease.<sup>36</sup>

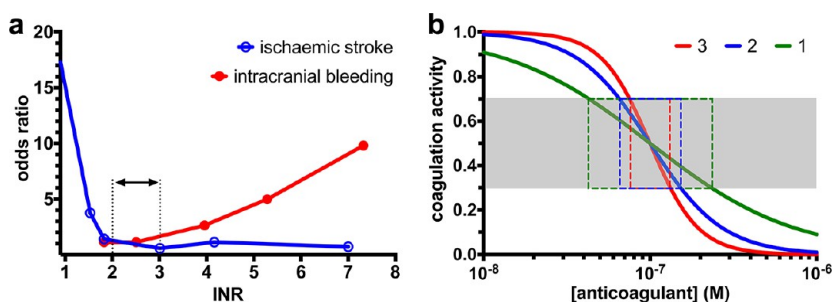
According to the statistics of the World Health Organization (WHO), ischemic heart disease and cerebrovascular disease, which in 2008 together accounted for a societal burden of over 100 million disability-adjusted life years (DALYs)<sup>37</sup> globally, are both expected to be among the four leading causes of DALYs by 2030 (together ~10% of all DALYs).<sup>38</sup> The latest global data from 2013 show that nearly a third of all deaths as a result of cardiovascular disease, especially ischemic heart disease and ischemic stroke and the increase in the total number of cardiovascular deaths, are believed to be due in large part to the aging and growth of populations.<sup>39</sup>

Until recently, the mainstays of thromboprophylaxis and anticoagulant therapy were VKAs, especially warfarin, and





**Figure 3.** Substrate (and inhibitor) binding sites of proteases can be divided into subsites based on how they recognize their macromolecular substrates. The substrate residues upstream (toward the N-terminus) and downstream (toward the C-terminus) of the scissile bond are designated P1, P2, etc. and P1', P2', etc., respectively.<sup>30</sup> In the protein surface representations of fXa (a; PDB code 1C5M<sup>31</sup>) and thrombin (b; PDB code 1SGI<sup>32</sup>) the subsites (S1, S2, etc.) that recognize the corresponding peptide substrate (P) residues are indicated.<sup>33</sup> The catalytic triad of residues in the enzymes that mediate P1–P1' peptide bond hydrolysis are H57, D102, and S195 (chymotrypsin numbering system; residue side chains shown as gray CPK-colored sticks). Following conversion of the zymogen forms of proteases to the apo-forms, two dominant forms of the enzymes exist in equilibrium: an inactive form (shown for thrombin in part c; PDB code 3BEI<sup>34</sup>) with the side chain of W215 and the entire segment 215–219 (stick models) collapsed in the active site, and the active form (d; PDB code 1SGI<sup>32</sup>), where the indole of W215 moves, together with the entire 215–219 segment, to open the active site and to form the S1 pocket.<sup>35</sup> This illustration and illustrations of protein 3D structures in all subsequent figures were created using the PyMOL Molecular Graphics System, version 1.7.6.0, Schrödinger, LLC, New York.



**Figure 4.** (a) Classical VKAs have a narrow therapeutic margin: adjusted odds ratios of ischemic stroke and intracranial bleeding as functions of anticoagulation intensity (expressed as prothrombin time international normalized ratio, INR) with warfarin are shown (adapted with permission from ref 43, Copyright 2006 Elsevier (<http://www.sciencedirect.com/science/journal/00493848/117/5?sdsc=2>)). Current guidelines recommend an INR target range of 2–3 during monitoring,<sup>43</sup> and it can be seen that INR < 2 will not provide protection from ischemic stroke, whereas INR > 3 represents a significant risk of intracranial bleeding. (b) A way of determining the therapeutic margin of anticoagulants based on the steepness of their dose–response curves in terms of clotting activity in plasma was proposed recently.<sup>44</sup> The steepness of a dose–response curve can be evaluated using the Hill coefficient from the Hill equation:  $\theta = 1 / ((K_d/[L])^n + 1)$ , where  $\theta$  is the fractional receptor occupancy (coagulation activity),  $K_d$  is the dissociation constant of the anticoagulant,  $L$  is the ligand (anticoagulant), and  $n$  is the Hill coefficient. For hypothetical anticoagulants with identical  $K_d$  ( $10^{-7}$  M) and  $n = 1, 2, \text{ or } 3$ , it can be seen (adapted from ref 44, <https://www.nature.com/articles/srep29387>) that variation in coagulation activity of, say,  $50 \pm 20\%$  (gray box) will result from dose variations as a percentage of  $K_d$  of 190% for  $n = 1$  (green box), 87% for  $n = 2$  (blue box), and 57% for  $n = 3$  (red box).

low-molecular weight heparins (LMWHs). Both LMWHs and VKAs inhibit blood coagulation indirectly. LMWHs enhance the interaction of fXa and to a lesser extent thrombin, with the

natural inhibitor antithrombin,<sup>40</sup> whereas VKAs interfere with the  $\gamma$ -carboxylation of glutamate residues in vitamin K-dependent proteins, including thrombin, fVII, fIX, and fX, which require

$\gamma$ -carboxylation for their procoagulant activity.<sup>41</sup> Because of the considerable difficulties associated with the clinical use and management of these drugs (Figure 4), extensive efforts have been underway over the past few decades in the pharmaceutical sector to find better anticoagulant drugs that can be given orally, have predictable dose–response and pharmacodynamics, have rapid onset and offset of action, display a wide therapeutic window, and are devoid of food–drug and drug–drug interactions. Such agents would hopefully obviate the cost and inconvenience of regular coagulation monitoring and dose adjustment, which are required for the safe and effective clinical use of VKAs and LMWHs.<sup>42</sup>

The first new generation anticoagulants (new oral anticoagulants, NOACs; now direct oral anticoagulants, DOACs), including the thrombin inhibitors argatroban (**2**; Figure 6)<sup>45</sup> and dabigatran etexilate (**4a**),<sup>46</sup> and the fXa inhibitors rivaroxaban (**22**; Figure 9),<sup>47</sup> apixaban (**23**),<sup>48</sup> and edoxaban (**24**),<sup>49</sup> are now approved for thromboprophylaxis and are being prescribed at fixed doses without the need for anticoagulant monitoring.<sup>50</sup> The main reason why these new agents are now often preferred is not because they necessarily have a wider therapeutic margin than VKAs and heparins (Figure 4) but because they generally have more predictable disposition properties. Since bleeding side effects are still limiting the use of DOACs,<sup>51–53</sup> highly selective agents are now sought that specifically target components of the coagulation system that are involved in thrombosis but that are not implicated in the induction of systemic hypocoagulation, thus avoiding bleeding complications.<sup>14,54,55</sup>

## ■ DRUG DISCOVERY OVERVIEW

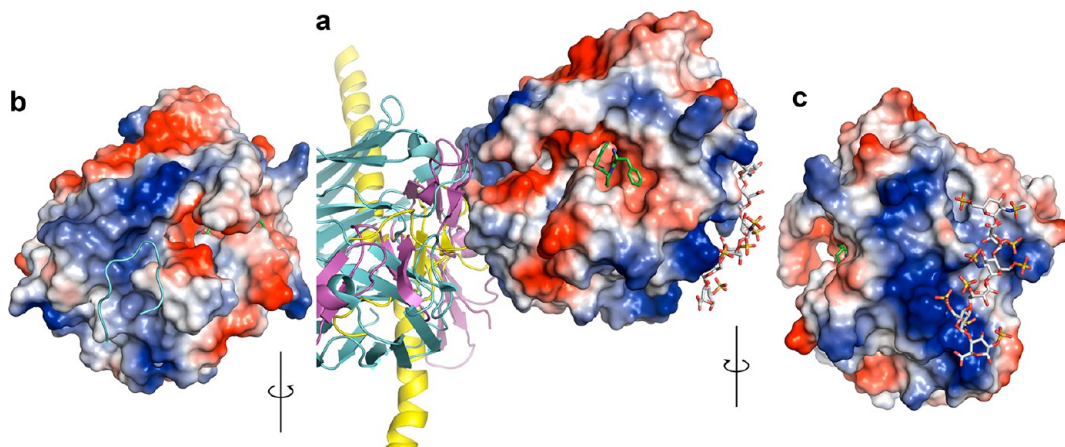
**Factor II (Thrombin).** *Functions and Biomedical Rationale.* Prothrombinase, i.e., the fXa–fV complex, converts prothrombin to thrombin, which in turn cleaves fibrinogen to fibrin (Figure 1). Fibrinogen consists of two identical subunits, each containing three nonidentical polypeptide chains, whose N-termini constitute the so-called E region. Two of the chains start with 16-residue fibrinopeptide sequences that are removed through thrombin proteolytic activity. Once the fibrinopeptide

portions have been cleaved, monomeric fibrin polymerizes spontaneously to insoluble fibrin. Thrombin recognizes the fibrinogen E region through exosite I (Figure 5a,b), which positions the fibrinopeptide portions of fibrinogen for productive proteolysis by thrombin.<sup>56</sup>

Complete deficiency of prothrombin is believed to be incompatible with life, and in mice it results in embryonic and neonatal lethality.<sup>57</sup> When prothrombin-null mice were reconstituted with human prothrombin to 5–10% of wild-type levels by transgene expression, this phenotype was observed to be reversed and animals developed normally and did not bleed spontaneously unless traumatized.<sup>58</sup> Because of the very low incidence of congenital prothrombin deficiencies in humans, a genotype–phenotype correlation has been difficult to ascertain, but defects in the prothrombin gene can lead to moderate or severe bleeding symptoms, including mucosal and surgical- or trauma-associated bleeding, joint bleeding, and intracranial hemorrhages, depending on prothrombin levels.<sup>59</sup>

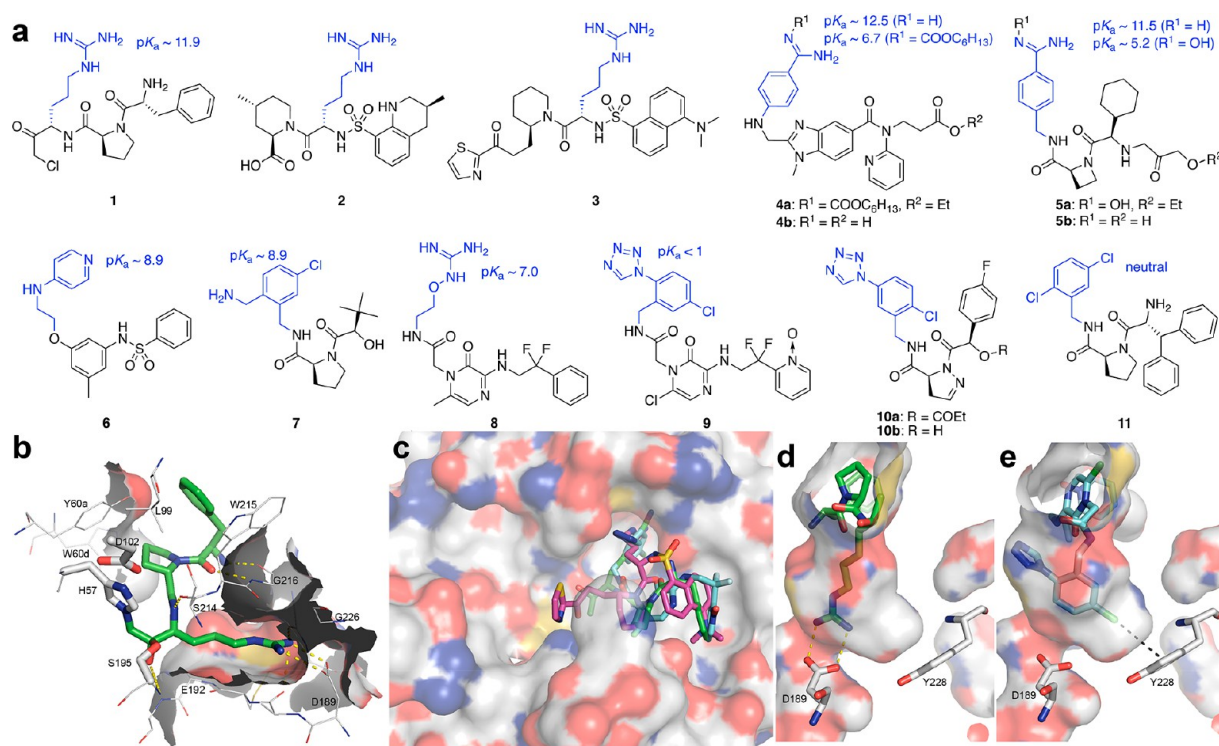
*Inhibitor Design.* Of all the S1A proteases, thrombin has been studied most intensively in terms of active-site inhibitor design. Already in the 1970s peptide aldehyde and other covalent and noncovalent inhibitors modeled on known scissile thrombin substrates were reported (reviewed in ref 64). Such compounds, e.g., **1** (H-D-Phe-Pro-Arg-CH<sub>2</sub>Cl, PPACK;<sup>65</sup> Figure 6a), permitted for the first time elucidation of the three-dimensional structure of thrombin by X-ray crystallography based on stable active site-inhibited enzyme forms,<sup>66,67</sup> thus opening the door to structure-based drug design.

Early covalent tripeptide arginal and arginyl chloromethyl ketone inhibitors were found to be too toxic for clinical use, and one of the earliest noncovalent reversible thrombin active site inhibitors is argatroban (**2**), a compound whose discovery was reported in 1984 and that possesses good potency against thrombin ( $K_i \approx 20$  nM) and selectivity with respect to trypsin, fXa, plasmin, and PK (>100-fold).<sup>68</sup> Argatroban (**2**) obtained its first approval in 2000 and remains in clinical use for anticoagulation in patients with heparin-induced thrombocytopenia type II who require parenteral antithrombotic treatment.<sup>45</sup>



**Figure 5.** Thrombin contains three distinct interactions sites for its binding partners. (a) Superposition of the X-ray crystal structures of the complexes between thrombin (electrostatic surface coloring) and an octasaccharide fragment of heparin (gray CPK stick model; PDB code 1XMN),<sup>60</sup> the fragment E<sub>ht</sub> of the central region of fibrinogen (yellow cartoon; PDB code 2A45),<sup>61</sup> the TME456 fragment of thrombomodulin (magenta cartoon; PDB code 1DX5),<sup>62</sup> and an anti-thrombin IgA paraprotein (cyan cartoon; PDB code 5E8E).<sup>63</sup> The thrombin structure shown is that from the 1XMN complex, including the active-site-bound **1** (green stick model; refer to Figure 6a). (b) View of the cationic thrombin exosite I, including the CDRH3 loop of the anti-thrombin IgA paraprotein that makes direct contact with this exosite (cyan cartoon). (c) View of the cationic thrombin exosite II with the bound heparin octasaccharide.





**Figure 6.** (a) Chemical structures of thrombin inhibitors; examples of inhibitors with more or less basic substituents (blue) that occupy the S1 site of the enzyme are shown: **2**, argatroban;<sup>67</sup> **3**, RWJ-S0215;<sup>72</sup> **4a**, dabigatran etexilate prodrug and **4b**, dabigatran;<sup>73</sup> **5a**, ximelagatran prodrug and **5b**, melagatran;<sup>74</sup> **6**, BM14.1248;<sup>75</sup> **7**, compound 14 from ref 76; **8**, RWJ-671818;<sup>77</sup> **9**, compound 34 from ref 78; **10a**, AZD8165 prodrug and its active parent compound **10b**;<sup>79</sup> **11**, compound 1u from ref 80. (b) The binding mode of **1** (green CPK stick model) in thrombin (PDB code 1PPB<sup>66</sup>) shows a covalent bond between one of the imidazole N atoms of H57 and the α-C of the methyl ketone as a result of nucleophilic attack and substitution of the Cl in the ligand, as well as a hemiketal bond between the S195 side chain O and the ligand methyl ketone carbonyl C. The Arg side chain of the ligand is buried in the deep S1 pocket (gray CPK surface) and makes polar interactions (yellow broken lines) with D189 at the base of the pocket. (c) Superposition of thrombin-bound conformations of **1** (green, PDB code 1PPB<sup>66</sup>), **3** (magenta, PDB code 1A4W<sup>72</sup>), and **9** (cyan, PDB code 1SL3<sup>78</sup>); the protein surface (gray CPK) of the complex with **9** is shown. Close-up views of the interactions of **1** (d; polar interactions in yellow) and **9** (e; Cl-π interaction in black) in the S1 site.

Thrombin is unusual among the S1A proteases in that it possesses a nine-residue insertion at position 60 (Figure 2). Two of the residues from this insertion, Y60a and W60d, can be seen to make van der Waals interactions with the ligand pyrrolidine ring in the thrombin complex with **1** (Figure 6b). Most S1A proteases can accommodate substrate and inhibitor peptides with comparatively large and varying P2 residues, whereas thrombin prefers Gly or Pro due to a constrained S2 site (Figure 3b). This difference is also relevant to small-molecule inhibitor selectivity design. Practically all known thrombin inhibitors do not occupy the S1'-S4' sites because of this restricted S2 site, which forms a bottleneck between the S1-S4 and the S1'-S4' subsite portions of the substrate recognition cleft. The peptidomimetic **3**, an extended derivative of **4b** in which the terminal thiazole group occupies part of the S1' site (Figure 6c), is one of the few exceptions.

From the outset in the 1980s, the design of active-site thrombin inhibitors aimed at oral agents, since warfarin and coumarin anticoagulants then used were also usually administered orally.<sup>69</sup> Early inhibitors were modeled on Phe-Pro-Arg tripeptides, and it was thought that a basic guanidine or benzamidine group was a prerequisite for high potency, due to a strong salt bridge interaction between such groups and the carboxyl function in the side chain of D189 at the base of the S1 pocket (Figure 6b,d) contributing significantly to binding. However, compounds with highly basic groups are fully ionized under physiological conditions of pH and therefore generally

possess poor membrane permeability and hence display low gastrointestinal absorption. This is the case with, for example, melagatran (**5b**), which has three ionizable groups (carboxylic acid, pK<sub>a</sub> = 2.0; 2° amine, pK<sub>a</sub> = 7.0; benzamidine, pK<sub>a</sub> = 11.5) and thus exists predominantly as the dibasic macroscopic species at the site of intestinal absorption (pH ≈ 6). As a consequence, **5b** displayed very low in vitro gastrointestinal permeability ( $P_{app} = 0.03 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  in a CaCo-2 in vitro permeability assay)<sup>70</sup> and low oral bioavailability in humans (3–7%).<sup>71</sup> The solution to this problem was a double prodrug, ximelagatran (**5a**), in which the benzamidine group was converted to the much less basic benzamidoxime function (pK<sub>a</sub> = 5.2) and the carboxylic acid was converted to the ethyl ester. Interestingly, the 2° amine in **5a** was also found to be significantly less basic (pK<sub>a</sub> = 4.5) than the corresponding amine in **5b**.<sup>71</sup> As a result, **5a** exists mainly as the neutral macroscopic species in vivo, which has better (albeit still limited) membrane permeability ( $P_{app} = 2.4 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$ ) and oral bioavailability in humans (18–24%). The prodrug **5a** itself has negligible thrombin-inhibitory activity but is rapidly converted to bioactive **5b** following absorption. Clinical trials with **5a** showed much improved predictability of disposition and a wider therapeutic window than warfarin. On this basis **5a** obtained early regulatory approvals as an anticoagulant in some territories and indications as the first non-VKA oral anticoagulant, although it was discontinued in 2004 due to liver toxicity concerns.<sup>54</sup>

Dabigatran (**4b**) is structurally related to **5b** but contains a benzimidazole unit in place of the proline-derived cores of earlier compounds. It was designed using the crystal structure complex of **39** (Figure 11) with thrombin as a starting point.<sup>67,73</sup> It potently inhibits thrombin ( $K_i = 4.5$  nM) and has much lower activity against other S1A proteases but also inhibits trypsin ( $K_i = 50$  nM).<sup>73</sup> As is observed with most thrombin inhibitors (Figure 6c), its affinity derives from polar interactions with the S1 site and (mostly lipophilic) interactions in the S2–S4 sites. Because of its high polarity ( $\log D_{7.4} = -2.4$ ) and extent of ionization at physiological pH values, **4b** is not orally bioavailable.<sup>81</sup> A double prodrug, dabigatran etexilate (**4a**), in which the benzimidazole was masked as a carbamate ester, was subsequently developed. This agent obtained its first approval in 2009 and is now used globally in deep venous thrombosis and pulmonary embolism, as well as for stroke prevention in AF.<sup>81</sup> This compound is still weakly basic (carbamic acid hexyl ester,  $pK_a = 6.7$ ; benzimidazole,  $pK_a = 4.0$ ) and has comparatively low oral bioavailability in humans (mean of 6.7%) and therefore has to be given at comparatively high doses.<sup>46</sup> However, low oral bioavailability appears to be mostly a result of P-gp-mediated efflux from epithelial cells upon absorption rather than low intrinsic permeability (a bidirectional CaCo-2 assay efflux ratio of 13.8 was reported and a  $P_{app,A-B}$  value of  $29 \times 10^{-6}$  cm·s<sup>-1</sup> in the presence of complete P-gp blockage).<sup>82</sup> This has been confirmed in clinical trials, where it was found that exposure to **4b** following coadministration of **4a** and the P-gp inhibitor verapamil was increased significantly.<sup>83</sup>

From the foregoing it is clear that the currently approved thrombin inhibitors **4a** and **5a** are not completely satisfactory from a pharmaceutical viewpoint, despite the fact that they represent a significant advance on earlier anticoagulants in terms of safety and efficacy. Much effort has been expended in the discovery of less basic and less polar thrombin inhibitors that would be orally bioavailable without the need for prodrug strategies. These studies showed that small ligand-efficient and potent inhibitors of much lower basicity than the early guanidine- and amidine-containing compounds could indeed be found and that these could still form productive polar interactions with D189 in the S1 pocket. Examples are the pyridine **6** ( $K_i = 23$  nM)<sup>75</sup> and the benzylamine **7** ( $K_i = 2.1$  nM and orally bioactive),<sup>76</sup> and especially the oxyguanidine **8** ( $K_i = 1.3$  nM and 100% orally bioavailable in dogs).<sup>77</sup>

Compounds with nonionizable groups that interact with the S1 site have also been reported, and an early example is the dichlorophenyl derivative **11** ( $K_i = 3$  nM),<sup>80</sup> as well as the extraordinarily potent chlorophenyltetrazole **9** ( $K_i = 1.4$  pM).<sup>78</sup> These compounds still interact with the S1 pocket, but the polar interactions of basic groups with D189 are replaced by electrostatic interactions in which the ligand Cl group interacts in an edge-to-face manner with the aromatic  $\pi$  system of Y228 (Figure 6d,e). Such halogen– $\pi$  interactions are still poorly understood<sup>84</sup> and appear to arise from long-range electrostatic and dispersion interactions, but it is clear that they can contribute significantly to binding affinity (in the order of  $\Delta\Delta G$  values of  $-10$  kJ·mol<sup>-1</sup>).<sup>85</sup> A number of thrombin inhibitors that do not contain highly basic S1-interacting groups have been evaluated in clinical trials, but none appear to have progressed to date. The latest addition to the list of these agents is **10a**, an ester prodrug of the carbinol **10b**, which retains the P1-interacting group of **9** ( $K_i = 0.3$  nM).<sup>79</sup> Despite its favorable pharmaceutical properties and performance in preclinical studies, this compound does not appear to have progressed.

**Indirect Inhibitors.** Fibrinogen is not the only substrate that is processed by thrombin, which also cleaves and activates fV, fVIII, fXI, and fXIII. Recognition of these substrates invariably involves one or both of the thrombin exosites (Figure 5), as well as the active site cleft.<sup>86</sup> Thrombomodulin, which switches thrombin specificity from procoagulant substrates to activation of anticoagulant protein C, is recognized by thrombin mainly through exosite I.<sup>62</sup> Exosite II is especially important for binding to platelet GpIb $\alpha$ , a component of the platelet receptor complex. In the bound complex GpIb $\alpha$  then functions as a cofactor and enhances cleavage of the platelet receptor PAR-1, thus promoting platelet activation.<sup>86</sup>

The pharmacological mode of action of the indirect thrombin inhibitors heparin and the LMWHs, as well as the bivalent direct inhibitors derived from hirudin, i.e., all anti-thrombin agents in current clinical use apart from the monovalent direct inhibitors argatroban (**2**) and dabigatran (**4b**), also involves interactions with the thrombin exosites. Hirudins (lepirudin, desirudin, bivalirudin) bind to both the catalytic cleft and exosite I,<sup>87</sup> whereas heparin and LMWH oligosaccharide drugs interact with thrombin (and fXa) through exosite II (Figure 5a,c).<sup>88</sup> The anticoagulant activity of heparin derivatives is due to their activity as cofactors for the serpin antithrombin (which also inhibits other coagulation proteases). Once the ternary thrombin–heparin–antithrombin complex is formed, thrombin inhibition ensues due to blocking of the thrombin active site by the so-called reactive center loop of antithrombin.<sup>89</sup>

A number of allosteric thrombin inhibitors acting at the exosites have been described, including aptamers targeting either exosite. Some of these oligonucleotide agents have undergone clinical development (reviewed in ref 90), although none appear to have progressed. Ichorcumab is an antibody being developed by Janssen,<sup>91</sup> it is an agent derived from an acquired antithrombin antibody isolated from a patient who was identified upon screening for coagulation abnormalities following a traumatic subdural hematoma.<sup>63</sup> This antibody was shown to interact with thrombin through exosite I (Figure 5a,b) and to dose-dependently increase clotting time in a thrombin time assay using human ex vivo blood, without evidence of inhibition of thrombin catalytic activity in biochemical assays. On the basis of the observation that the patient in question remained free of bleeding complications for an extended period, one might expect that ichorcumab (and perhaps other exosite I modulators) may provide a much wider anticoagulation versus bleeding therapeutic margin than is the case with other antithrombin agents, although further mechanistic and efficacy studies with ichorcumab are required.<sup>92</sup>

**Factor VII. Functions and Biomedical Rationale.** FVII is the key enzyme in the extrinsic coagulation pathway (Figure 1). Upon vascular injury the membrane protein TF becomes exposed on the vascular lumen, where it binds circulating procoagulant fVII or fVIIa. Formation of the activated TF–fVIIa complex then triggers the initiation of blood clotting through proteolytic activation of fIX to fIXa and fX to fXa.<sup>93</sup>

It has been reported that mice lacking fVII succumb perinatally due to bleeding from normal blood vessels,<sup>94</sup> whereas mice genetically engineered to express very low levels of fVII (~0.7% of normal) live to adulthood but develop cardiac fibrosis.<sup>95</sup> The International Registry on Congenital FVII Deficiency (IRF7) Study Group has been collecting extensive clinical data on fVII deficiency in people with rare bleeding disorders, and overall there is no consistent correlation between



bleeding symptoms and fVII levels, although surgery-related bleeding is frequent.<sup>96</sup> Compared to thrombin and fX deficiency, people with fVII deficiency have comparatively fewer bleeding events.<sup>97</sup> Although severe bleeding phenotypes typically occur for people with fVII coagulation activity of <2% of normal,<sup>98</sup> some individuals with fVII activity of <1% do not manifest any spontaneous or provoked bleeding, whereas many patients with fVII coagulation levels of >5% have severe bleeding symptoms.<sup>99</sup>

Collectively, the mouse knockout studies and the information available from fVII deficiency in humans suggest that therapeutic inhibition of fVIIa should be able to provide a margin between antithrombotic and bleeding effects. Studies with several biological agents that suppress fVII activity (including active site-inhibited fVIIa,<sup>100</sup> anti-TF antibodies, and TF mutants that bind fVII but form an enzymatically inactive complex<sup>101</sup>) in animal models of thrombosis also show that fVIIa inhibition can produce antithrombotic effects without severely disturbing hemostasis.

**Inhibitor Design.** Although a number of biologics targeting fVII activity, and small-molecule direct inhibitors of fVIIa, had been trialed in the past (reviewed in ref 102), none currently remain in clinical development as far as can be ascertained.

The substrate-binding site of fVIIa adopts a different overall shape compared with other related proteases. The main sequence differences are K192 (acidic or neutral residue in most proteases) and an insertion at position 170 (compare Figure 2). As can be observed in the experimental covalent complex between a chloromethyl ketone inhibitor (12, Figure 7a), fVIIa displays more extensive S2 and S3 sites compared to, for example, thrombin and fXa, whereas the S4 site is comparatively shallow and ill-defined (Figure 7b).

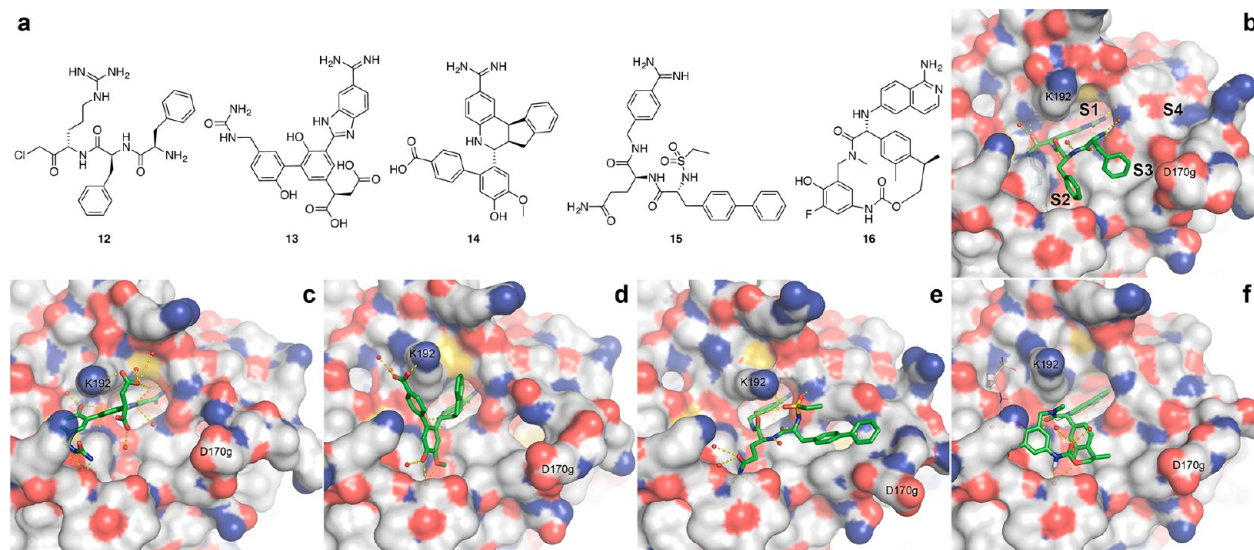
The benzimidazole amidine compound 13 is an example of a potent fVIIa inhibitor ( $K_i = 2$  nM) with high selectivity over most related proteases (including thrombin and fXa) except PK. It was designed to make multiple polar interactions with the fVIIa S2 site, including the comparatively unique K192 residue (Figure 7c), and is potentially suitable for once daily iv administration in humans.<sup>103</sup> The tetracyclic benzamidine 14 also interacts exclusively with the S1 and S2 sites of fVIIa,

including a salt bridge between its benzoate group with K192 and a hydrophobic interaction of the anisole group with a small S2 hydrophobic pocket (Figure 7d). Compound 14 is also highly potent (fVIIa  $K_i = 1.9$  nM) and selective (>1000-fold over thrombin, fXa, and trypsin) and was reported to show efficacy in a rabbit model of arterial thrombosis, with no significant prolongation of cuticle bleeding time.<sup>104</sup> Unlike 13 and 14, the peptidomimetic fVIIa inhibitor 15 (fVIIa  $IC_{50} = 93$  nM and >100-fold selective over thrombin)<sup>105</sup> extends into the enlarged S3 site of fVIIa, which undergoes further ligand-induced enlargement (biphenyl group, observe altered position of D170g region in Figure 7e compared to, for example, Figure 7d).

Like the other fVIIa inhibitors discussed, the recently reported macrocyclic carbamate 16 (fVIIa  $K_i = 1.4$  nM, highly selective over fXa, fXIa, thrombin, and trypsin but poorly selective over PK,  $K_i = 36$  nM)<sup>106</sup> also makes extensive interactions with the large S2 (and to some extent the S3) site of fVIIa (Figure 7f). Rather than a P1 benzamidine group, 16 contains an aminoisoquinoline, which is significantly less basic (1-aminoisoquinoline has a  $pK_a$  of 7.27<sup>108</sup>). However, permeability of macrocyclic compounds such as 16 was still observed to be low but could be improved upon further optimization<sup>109–111</sup> to afford derivatives of 16 with not only improved permeability and oral bioavailability (up to 40% in dog) but also selectivity over kallikreins.<sup>110</sup>

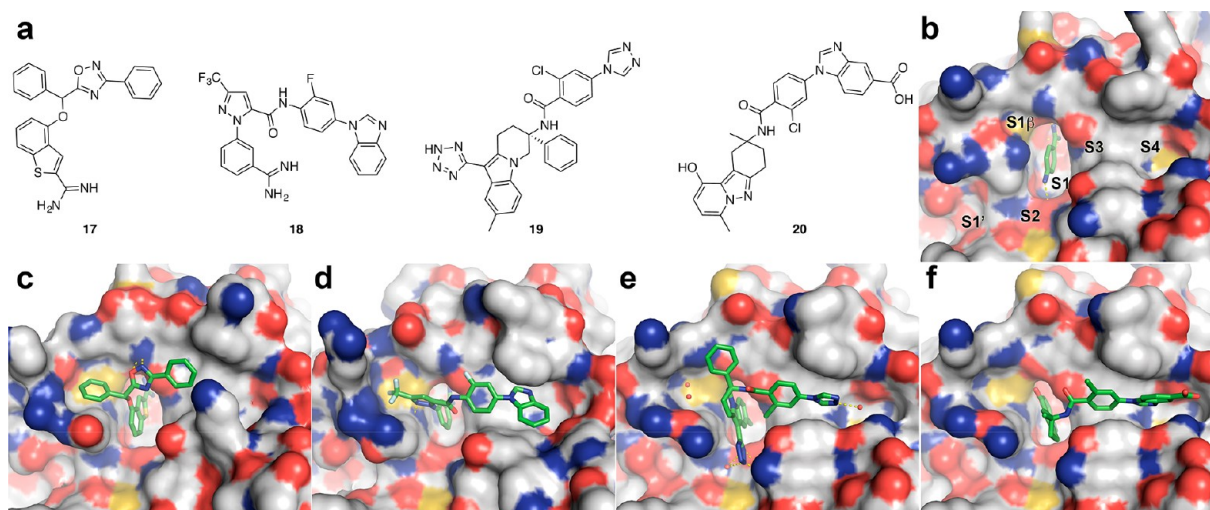
**Factor IX. Functions and Biomedical Rationale.** FIX is a key enzyme in the amplification of coagulation through the intrinsic pathway. Autoactivation of fVII upon binding of TF ultimately leads to the formation of thrombin, which feeds back to activate fXI on the surface of platelets. FXIa then initiates the intrinsic pathway by activating fIX, whose active form fIXa cleaves fX to fXa, thus generating more thrombin and accelerating fibrin formation (Figure 1).

FIX deficiency is the cause of the X-linked hereditary bleeding disorder hemophilia B, whereas hemophilia A results from deficient or defective fVIII. The severity of hemophilia B is correlated with plasma fIX levels and spontaneous bleeding occurs in individuals with <1% of normal fIX activity, whereas bleeding occurs only as a result of injury or surgery in those



**Figure 7.** (a) Chemical structures of fVIIa inhibitors: 12, H-d-Phe-Phe-Arg-CH<sub>2</sub>Cl; 13, compound 9 from ref 103; 14, BMS-593214;<sup>104</sup> 15, compound 5 from ref 105; 16, compound 27a from ref 106. Complexes of inhibitors (green CPK sticks) with fVIIa (gray CPK surface) are shown: 12 (a; PDB code 1DAN<sup>107</sup>), 13 (b; PDB code 2B7D), 14 (c; PDB code 4ISH), 15 (d; PDB code 1WTG), and 16 (e; PDB code 5I46).





**Figure 8.** (a) Chemical structures of fIX inhibitors: 17, compound 16 from ref 121; 18, compound 3b from ref 122; 19, compound 82 from ref 123; 20, compound 1 from ref 118. (b) *p*-Aminobenzamidine (green CPK sticks) bound in fIXa (gray CPK surface; PDB code 1RFN<sup>120</sup>). Binding modes of 17 (c; PDB code 3LCS), 18 (d; PDB code 1X7A), 19 (e; PDB code SEGM), and 20 (f; likely fIXa-binding mode from docking to the SEGM receptor) in fIXa. All docking results shown in this and subsequent illustrations were generated using the Glide application within the Maestro modeling suite (release 2016-2), Schrödinger, LLC, New York.

with 1–5% fIX activity, and the tendency to bleed from small wounds and during surgery decreases from patients with >5% fIX activity to hemophilia B carriers, who display 60% of normal median fIX activity.<sup>112</sup>

Inhibition of fIXa may be a good therapeutic strategy<sup>113</sup> because the fVIIIa–fIXa complex mediates propagation of coagulation upstream of the main thrombin-driven amplification at the fVa–fXa step and because fVIIIa–fIXa-catalyzed activation of fX appears to be the rate-limiting step for thrombin generation. Specific studies have shown that whereas reduction of fIX levels by up to 99% results in acceptable levels of bleeding, this provides a significantly reduced risk of thrombosis.<sup>114,115</sup> Potential efficacy and safety of anticoagulation at the level of fIX is suggested by studies with genetically engineered mice (reviewed in ref 116), and the safety aspect of such a strategy is also indicated by a recent clinical gene therapy study in people with severe hemophilia B, which showed that bleeding episodes were suppressed upon expression of as little as 1–6% of normal fIX.<sup>117</sup> Furthermore, in another recent study, in which a prototype small-molecule fIX inhibitor (20 in Figure 8a) was compared with the fXa inhibitor apixaban (23; Figure 9) using pharmacokinetics–pharmacodynamics analysis, a larger therapeutic window between antithrombotic and bleeding effects was predicted for the fIX inhibitor 20 compared to 23.<sup>118</sup>

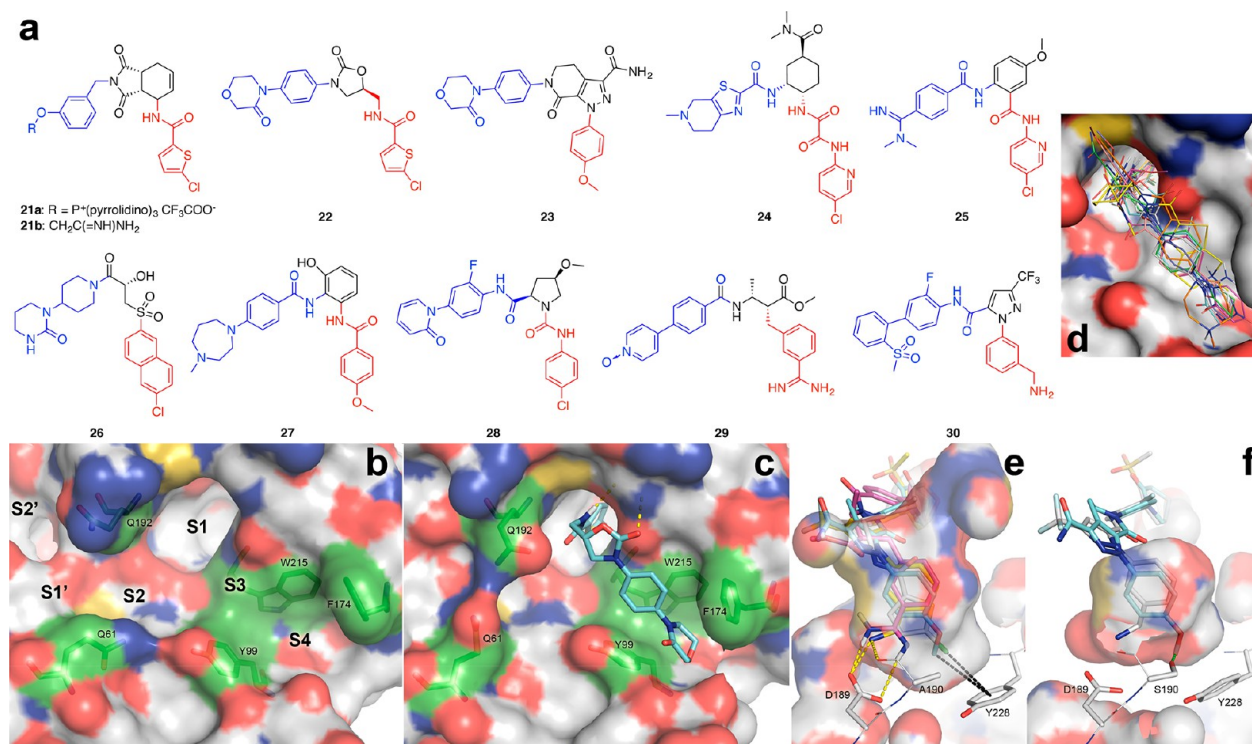
**Inhibitor Design.** There does not appear to be any small-molecule fIXa inhibitor currently under clinical development, although an oral fIXa inhibitory agent, TTP889 (structure not disclosed), was trialled unsuccessfully some years ago.<sup>119</sup>

An early X-ray crystal structure of the catalytic domain of fIXa with *p*-aminobenzamidine bound in the S1 site (Figure 8b) shows a similar substrate-recognition site as is observed in related Ser proteases, especially that of fXa, although the S3 and S4 sites adopt a somewhat different position and shape.<sup>120</sup> Most fIXa inhibitors were originally developed by reengineering fXa inhibitors; e.g., the oxadiazole 17 is an early redesigned fXa inhibitor with 15-fold selectivity for fIXa over fXa.<sup>121</sup> Its fIXa binding mode shows (Figure 8c) the usual interactions of the amidine function in the S1 site, with the phenyl substituent of

the oxadiazole ring occupying the S3 site, without extending into the S4 site. In this compound fIXa selectivity probably is derived from the interaction of the second phenyl group in an induced subpocket (compare Figure 8b) next to the S1 site and opposite the S3 site (sometimes referred to as the S1 $\beta$  site). Another dual fIXa and fXa inhibitor (fIXa  $K_i$  = 13 nM but 130-fold selective for fXa over fIXa) is the pyrazolobenzamidine 18.<sup>122</sup> It also binds into the S1 $\beta$  site (CF<sub>3</sub> group), but here the presumed fIXa selectivity gain from this interaction is counteracted by extension of the ligand into the conformationally flexible S4 site (benzimidazole group; Figure 8d) in a way reminiscent of what is observed with selective fXa inhibitors (see below).

An example of a more fIXa-selective compound is 19 ( $K_i$  = 2.4 nM and 210-fold selective over fXa), which makes only peripheral interactions with the S1 $\beta$  site (whose cavity is occupied by two crystallographically detectable water molecules) and extends into the S4 site (triazole ring), this site adopting a similar shape as in the uncomplexed form (compare Figure 8e,b).<sup>123</sup> Here the tetrazole group of the inhibitor also makes favorable polar interactions with the S2 site, and derivatives of 19 where the tetrazole is replaced with groups capable of charge-reinforced H-bonds in this region were reported to be even more fIXa-selective than 19.<sup>123</sup> As discussed in the thrombin and fXa sections, potent and selective inhibitors lacking a strongly basic group for interactions in the S1 site have been discovered, compound 19 is an analogous example of a fIXa inhibitor. Despite the lack of strongly ionized groups, triazole-containing compounds such as 19 were observed to lack oral bioavailability.<sup>123</sup> The structurally related compound 20 (fIXa  $K_i$  = 3.5 nM, 140-fold selective over fXa),<sup>118</sup> on the other hand, is predicted to interact only with the S1–S3–S4 sites, but here the carboxybenzimidazole group inserts deeply into the S4 cleft where it makes multiple favorable interactions (Figure 8f).

**Factor X. Functions and Biomedical Rationale.** The extrinsic and intrinsic coagulation pathways converge on fX, which is thus a key enzyme common to both pathways. fXa associates with fVa to form prothrombinase, which converts prothrombin to thrombin. fXa activity thus leads to blood clot



**Figure 9.** (a) Chemical structures of representative small-molecule direct fXa inhibitors: **21a** and **21b**, compounds **1** and **2** from ref 47; **22**, rivaroxaban;<sup>130</sup> **23**, apixaban;<sup>136</sup> **24**, edoxaban;<sup>137</sup> **25**, betrixaban;<sup>138</sup> **26**, letaxaban;<sup>139</sup> **27**, darexaban;<sup>140</sup> **28**, eribaxaban;<sup>141</sup> **29**, otamixaban;<sup>142</sup> **30**, DPC-423.<sup>143</sup> (b) The Ser protease S pockets (labeled) in the substrate-binding site (gray CPK surface) of the uncomplexed active form of fXa (PDB code 1CSM<sup>21</sup>) are shown, with a narrow channel (flanked by Q61 and Q192, green surface) between the S1 and S1' sites and a unique S4 pocket, lined by the aromatic residues Y99, F174, and W215 (green). (c) Complex between **22** and fXa (PDB code 2W26<sup>130</sup>). (d) Superposition of the fXa-binding modes of **22** (cyan), **23** (green, PDB code 2P16),<sup>136</sup> **24** (yellow, modeled), **25** (salmon, modeled), **26** (gray, PDB code 3KL6),<sup>139</sup> **27** (orange, modeled), **28** (magenta, PDB code 2PHB),<sup>141</sup> and **29** (blue, modeled). (e) Compounds such as **29** (magenta) and **30** (yellow; pose from PDB code 3M36<sup>143</sup>) form the usual H-bonding interactions with the S1 residue D189 (yellow broken lines), whereas compounds such as **22** (gray) and **23** (cyan) make edge-to-face interactions with Y228. (f) fXa has A190, which is S190 in trypsin. The fXa-binding mode of for example, **23** (cyan) is not compatible with this altered pocket: observe the steric clash (green broken line) between S190 of trypsin (structure from PDB code 3M35<sup>143</sup>) complex between **30** and trypsin) and the anisole methyl group of **23** (apixaban) upon structural superposition. Docked structures were obtained using the 2W26 receptor.

formation and wound closure, whereas fX deficiency disturbs hemostasis.

As is the case for prothrombin, fX deficiency is not compatible with life and complete fX deficiency does not occur in humans. In mice knockout of the *F10* gene causes partial embryonic lethality and animals that are born die from neonatal bleeding.<sup>124</sup> Mice with fX activity as low as 1–3%, however, are viable with a mild bleeding diathesis.<sup>125</sup> International registries of fX-deficient patients have been used to classify the bleeding severity associated with fX deficiency: at fX coagulation activity levels of <1% it is severe, at 1–5% it is moderate, and at 6–10% it is mild, whereas levels of >20% are not usually associated with bleeding.<sup>126</sup>

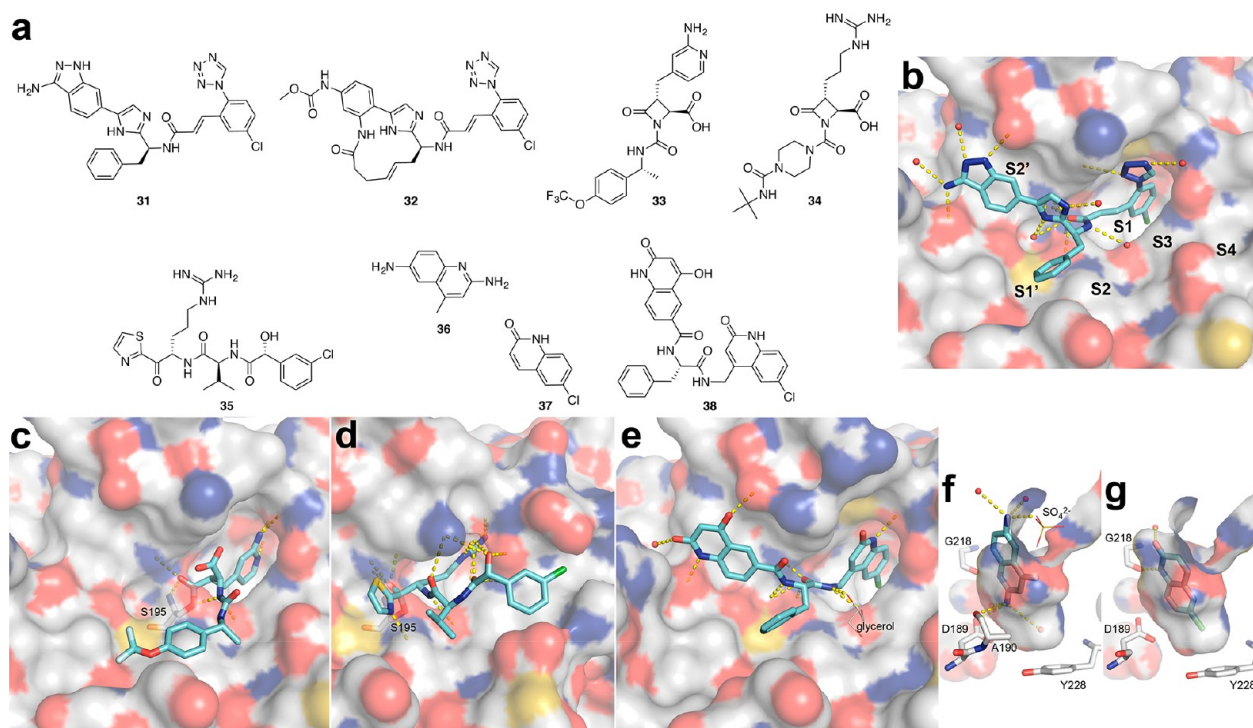
**Indirect Inhibition.** As mentioned in the thrombin section, formation of a ternary thrombin–heparin–antithrombin complex is necessary for heparins to block thrombin activity. Unfractionated heparin has a polysaccharide chain sufficiently long to form a ternary complex with thrombin, unlike LMWHs, which are too short to form this ternary complex and that are thus unable efficiently to inhibit thrombin. However, both heparin and LMWHs bind and activate antithrombin toward fXa inhibition, thus acting as indirect allosteric fXa inhibitors.<sup>127</sup> One such heparin derivative is the pentasaccharide fondaparinux, the first selective fXa inhibitor approved globally for the prevention and treatment of thrombosis (first approval in 2001).<sup>128</sup>

Fondaparinux is a parenteral agent that provided much of the initial clinical validation of fXa as an antithrombotic drug target that supported subsequent extensive efforts to develop oral direct fXa inhibitors, and fondaparinux remains in clinical use to this date.

**Inhibitor Design.** The discovery of fXa active-site inhibitors in many ways mirrors that of thrombin inhibitors, starting from early covalent peptide inhibitors targeting the protease S195 catalytic residue, progressing to reversible peptidomimetic agents with basic substituents that engage the fXa S1 subsite, and finally culminating with nonpeptidic, neutral, and orally bioavailable agents (reviewed in ref 129). In fact the first such agent (that is not a prodrug) approved for clinical use, rivaroxaban (**22**; Figure 9a), was a fXa rather than a thrombin inhibitor. Rivaroxaban was developed from high throughput fXa screening hits such as the tetrahydroisoindolediones **21** (fXa IC<sub>50</sub> ≈ 100 nM).<sup>47,130</sup> It was originally thought that the charged organophosphonium (**21a**) and acetimidamide (**21b**) substituents of these compounds acted as S1 ligands but subsequent SAR and X-ray crystallography studies revealed that in fact the chlorothieryl group present in both **21a** and **21b** (and retained in **22** and many other fXa inhibitors) fulfilled this function.<sup>47</sup>

Rivaroxaban (**22**) is a highly potent (fXa K<sub>i</sub> = 0.4 nM), selective (>20 000-fold with respect to IC<sub>50</sub> values against a





**Figure 10.** (a) Chemical structures of fXIa inhibitors: **31**, compound **33** from ref **153**; **32**, the closely related macrocyclic compound **16** from ref **154**; **33**,  $\beta$ -lactam covalent fXIa inhibitor related to clinical compound EP-7041 (compound **168** from ref **159**) and structurally and mechanistically related tryptase inhibitor BMS-262084 **34**,<sup>155</sup> **35**, irreversible  $\alpha$ -ketothiazole inhibitor (compound **11c** from ref **157**); **36** and **37**, S1-binding fragments elaborated into potent fXIa inhibitors such as **38** (compound **13** from ref **158**). (b) fXIa (gray CPK surface) binding mode of **31** (cyan sticks), showing interactions with the unique S1' and S2' subsites (labeled) of fXIa (PDB code 4X6P). (c) Likely covalent adduct formed between fXIa S195 catalytic residue and the  $\beta$ -lactam ring of **33** (modeled using covalent docking based on the 4X6P receptor). (d) Similar attack of the S195 alcohol side chain on the  $\alpha$ -ketothiazole of **35** leading to the covalent complex shown (PDB code 1ZPC). (e) Experimental binding pose of **38** in fXIa (PDB code 4CRF). (f) Interactions of fragment **36** and (g) fragment **37** in the S1 subsite (PDB codes 4CR5 and 4CR9).

range of related Ser proteases),<sup>131</sup> and orally bioavailable (>60% in preclinical species and >80% in humans)<sup>47</sup> compound and is now the most widely used of the new DOACs. Despite its comparatively short terminal half-life (6–9 h in humans),<sup>132</sup> **22** can be used in once- or twice-daily oral regimens in a number of licensed prevention (stroke and systemic embolism in patients with AF, venous thromboembolism after major surgery, recurrent venous thromboembolism, atherothrombotic events in patients with acute coronary syndrome) and treatment (deep vein thrombosis and pulmonary embolism) indications.<sup>133</sup> Apixaban (**23**)<sup>134</sup> and edoxaban (**24**)<sup>49</sup> are also highly potent and selective fXIa inhibitors that were approved for similar indications more recently. All three agents (**22**–**24**), as well as the thrombin inhibitor dabigatran etexilate (**4a**; see above), are used widely for the prevention of stroke in patients with AF, where the choice of the best agent for individual patients is still a developing area.<sup>135</sup>

A range of different fXIa inhibitors have been evaluated in clinical trials, but none of these appear to have progressed. Most recently betrixaban (**25**) did not perform significantly better than enoxaparin in terms of efficacy in deep-vein thrombosis and safety in terms of bleeding in a phase III trial.<sup>144</sup> This result followed on from another unsuccessful phase II trial with darexaban (**27**), which, when added to antiplatelet therapy after acute coronary syndrome, showed no efficacy but an increase in bleeding.<sup>145</sup> Letaxaban (**26**), eribaxaban (**28**), and otamixaban (**29**) are examples of other fXIa inhibitors that have been studied clinically.

The unique features of fXIa that have been widely exploited in the design of selective inhibitors are a narrow channel between the S1 and S1' sites and a unique S4 pocket (Figure 9a,b). For example, in the complex between **22** and fXIa the passage between S1 and S1' is obstructed through a rearrangement (with respect to the uncomplexed structure) of the Q61 and Q192 side chains, which can be seen to interact through H-bonds (Figure 9c). This has the effect of precluding ligand access to S1'–S4' and partially occluding S2; similar features are frequently observed in inhibitor–fXIa complexes. All selective fXIa inhibitors possess cyclic substituents (blue portions of structures in Figure 9a) that can interact with the S4 site by stacking between the aromatic residues Y99 and F174 (Figure 9d). The residues corresponding to Y99 and F174 in, for example, thrombin are L99 and I174, and the S4 site thus represents an important selectivity determinant between fXIa and thrombin. Structurally varied fXIa inhibitors invariably assume an “L”-shaped binding pose, where the short and long legs of the L (red and blue, respectively, in Figure 9a) bind into the S1 and S3–S4 pockets, respectively. As with thrombin inhibitors, compounds possessing basic groups in the substituents that interact with S1, such as **29** and **30**, form the usual H-bonding interactions with D189 at the base of the S1 site, whereas corresponding neutral substituents, such as those in **22** and **23**, make edge-to-face interactions with Y228 (Figure 9e). Unlike many other S1A proteases, fXIa possesses an Ala residue at position 190, and the fact that this residue is Ser in, for example, trypsin can be exploited for selectivity design. The presence of the S190 residue leads to a smaller volume of



the S1 pocket at its base in trypsin-like proteases. The FXa-binding mode of many nonbasic inhibitors, e.g., **23**, is not compatible with this contracted pocket (Figure 9f).

**Factor XI. Functions and Biomedical Rationale.** Following activation of the contact system FXIIa converts the zymogen FXI to active FXIa, which then promotes coagulation via Ca<sup>2+</sup>-dependent activation of fIX. People who lack FXI have a mild trauma-induced bleeding disorder, referred to as hemophilia C, but this is mainly restricted to tissues with high fibrinolytic activity. Even in individuals with severe FXI deficiency, serious spontaneous bleeding is uncommon, whereas these individuals have a high probability of postoperative hemorrhage; individuals with moderate levels and approaching the lower limit of the normal range generally have a lower risk of postoperative bleeding.<sup>146</sup> This mild clinical phenotype is mirrored by FXI deficiency mouse models, where it was observed that arterial thrombus formation was severely impaired but was not associated with excessive bleeding.<sup>147</sup>

These observations have led to the current belief that in vivo coagulation is mediated almost exclusively by the extrinsic rather than the intrinsic pathway.<sup>148</sup> Whereas FXIa appears to be dispensable for hemostasis, it clearly plays an important role in thrombosis, possibly through the thrombin–FXIa feedback loop (Figure 1). This is indicated by the findings that *fXI*<sup>-/-</sup> mice appear to be protected from carotid artery thrombus formation in a FeCl<sub>3</sub>-induced thrombosis model and that reconstitution of these animals with human FXI resolved resistance to thrombus formation.<sup>147</sup> Proof of concept for FXIa as a potentially valuable thrombosis drug target in humans has also recently been provided with a FXI antisense oligonucleotide,<sup>149</sup> d(P-thio)([2'-O-(2-methoxyethyl)]rA-[2'-O-(2-methoxyethyl)]m<sup>5</sup>rC-[2'-O-(2-methoxyethyl)]rG-[2'-O-(2-methoxyethyl)]rG-[2'-O-(2-methoxyethyl)]m<sup>5</sup>rC-A-T-T-G-G-T-G-m<sup>5</sup>C-A-m<sup>5</sup>C-[2'-O-(2-methoxyethyl)]rA-[2'-O-(2-methoxyethyl)]rG-[2'-O-(2-methoxyethyl)]m<sup>5</sup>rU-[2'-O-(2-methoxyethyl)]m<sup>5</sup>rU-[2'-O-(2-methoxyethyl)]-m<sup>5</sup>rU DNA, now being developed by Bayer and Ionis Pharmaceuticals as BAY 2306001 and IONIS-FXIRx<sup>150</sup>). In a clinical study with this agent it was found that postoperative venous thromboembolism could be prevented effectively by lowering FXI levels in patients undergoing knee arthroplasty and treatment appeared to be safe with respect to the risk of bleeding.<sup>151</sup>

**Inhibitor Design.** Although a number of advanced small-molecule direct FXIa inhibitors have been reported (reviewed in ref 152), currently the only clinical experimental drug with this mechanism is EP-7041 (structure not disclosed, probably related to **33** in Figure 10; ClinicalTrials.gov identifier NCT02914353), which is being developed by eXithera Pharmaceuticals as a parenteral agent.

Compared to most other Ser proteases, FXIa possesses a comparatively open S2–S1'–S2' binding region and most selective FXIa inhibitors for which experimental complexes are available can be observed to occupy this region, especially the S1' site (Figure 10). Compounds such as **31** and its macrocyclic derivative **32** are highly potent and selective (e.g., FXIa K<sub>i</sub> = 0.16 nM and 100-fold selectivity over PK and higher over a range of related proteases for **32**) but possess little oral bioavailability.<sup>153,154</sup> Covalent inhibitors are also known, e.g., β-lactams such as **33**, which presumably (Figure 10c) form similar covalent protein adducts as is known to occur with structurally related protease (trypsin and trypsin) inhibitors such as **34**.<sup>155,156</sup> Different irreversible FXIa inhibitors are also known,

e.g., the α-ketothiazole compound **35** (FXIa IC<sub>50</sub> = 0.12 μM),<sup>157</sup> whose covalent protein adduct structure has been determined experimentally (Figure 10d). This compound not only occupies the S1, S1', and S2 sites but also makes hydrophobic interactions with the S4 site, which assumes a different shape than the S4 sites observed in other FXIa complexes.

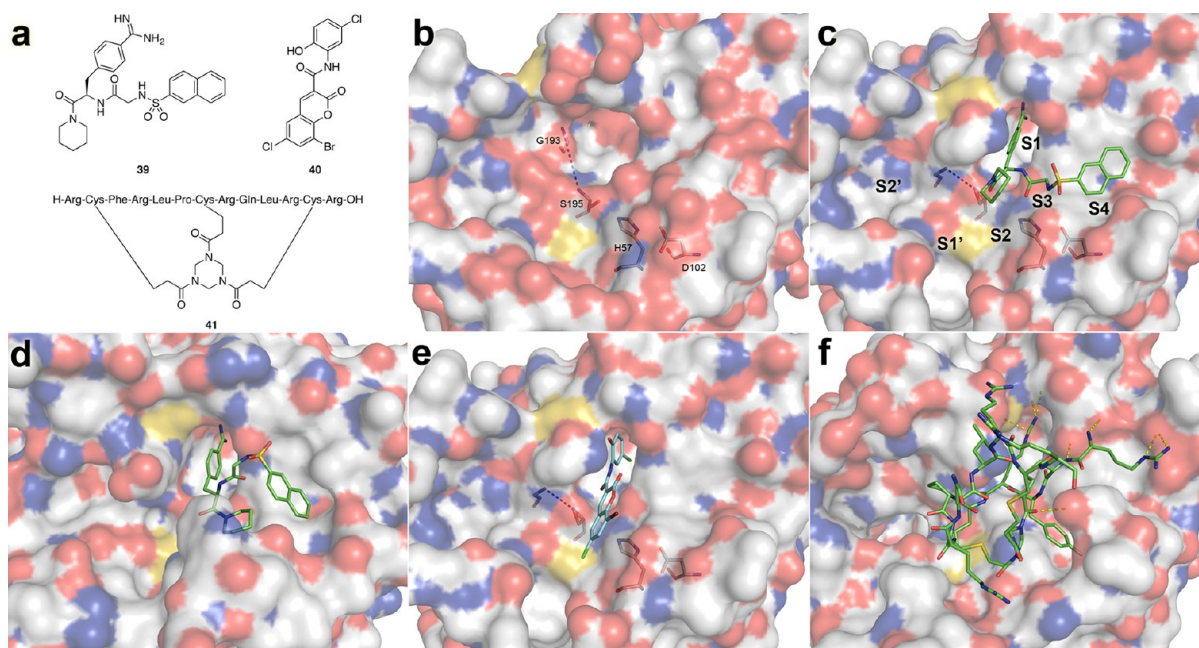
Fragment-based design has also been applied to the discovery of FXIa inhibitors.<sup>158</sup> By use of a screening cascade comprising biophysical (NMR, surface plasmon resonance, and X-ray crystallography) and biochemical assays, a number of S1-binding fragments such as **36** and **37** (high μM to mM affinity; Figure 10f,g) were identified, which were then elaborated into potent FXIa inhibitors such as the quinolinone **38** (Figure 10e), with a very similar binding mode to **31** (Figure 10b). Compound **38** was found to be highly potent (FXIa K<sub>i</sub> = 0.5 nM) and selective (again PK was the only off-target Ser protease, 10-fold higher K<sub>i</sub>) but showed low permeability (CaCo-2 P<sub>app</sub> < 0.28 × 10<sup>-6</sup> cm·s<sup>-1</sup>).<sup>158</sup>

**Factor XII. Functions and Biomedical Rationale.** The zymogen FXII can be activated in a number of ways.<sup>160</sup> This can occur by slow autoproteolysis or more efficiently by the action of PK upon surface-bound FXII to form the disulfide-tethered heavy (50 kDa) and light (30 kDa) chains of α-FXIIa.<sup>161</sup> This form of FXIIa then propagates the intrinsic coagulation pathway by activating FXI and reinforces the KKS by further activating prekallikrein. α-FXIIa can then be further cleaved by PK to form β-FXIIa, a 30-kDa fragment that contains the catalytic light chain and the C-terminal peptide of the heavy chain of α-FXIIa. β-FXIIa, which retains catalytic activity for prekallikrein but not FXI, is released from the activating surface as it has lost the portions of the heavy chain that mediate binding to other proteins and to surfaces. Apart from prekallikrein and FXI activation, FXIIa can also activate a range of different substrates, including the C1 esterase of the complement pathway, plasminogen, the uPA receptor of endothelial cells, and fVII.<sup>162,163</sup>

FXII-deficient mice were found to be protected against arterial thrombosis, collagen- and epinephrine-induced thromboembolism, and ischemic stroke.<sup>164,165</sup> In all these in vivo models, such protection was abolished by infusion of human FXII into FXII-null mice. Pharmacological target validation of FXIIa as a potential thrombosis drug target has also been provided with anti-FXIIa antibodies that provided thromboprotection without increasing bleeding risk in animal models (including primates) of thrombosis,<sup>166–168</sup> as well as with rHAI-4, a recombinant fusion protein between infestin-4 derived from *Triatoma infestans*, a blood-feeding insect, and human albumin, which was shown to improve outcomes in a rodent model of ischemic stroke.<sup>169</sup>

FXIIa-mediated activation of the intrinsic coagulation pathway has also recently been linked to Alzheimer's disease (AD).<sup>170,171</sup> It is believed that amyloid β can activate FXII directly or indirectly and that subsequent vessel occlusion and inflammation may contribute to cognitive decline in AD. FXIIa inhibitors may therefore offer a new treatment modality in neurodegeneration.<sup>172,173</sup>

**Inhibitor Design.** Apart from certain nonselective Ser protease inhibitors such as **39** (Figure 11a,c) and a number of high throughput FXIIa screening hits,<sup>174</sup> which do not appear to have been followed up, there are currently no known drug-like small-molecule FXIIa inhibitors. Structure-based design of such inhibitors is difficult because little is currently known about the active conformation of FXIIa (Figure 11b). Homology models (Figure 11c) suggest that FXIIa has the usual S1 subsite



**Figure 11.** (a) Chemical structures of fXIIa inhibitors: **39**, NAPAP; **40**, coumarin derivative **23** from ref 175; **41**, bicyclic tridecapeptide **61** from ref 176. (b) Currently available X-ray crystal structures of fXII (gray CPK surface) are of catalytically inactive conformations: although the catalytic residues S195, H57, and D102 (stick models) are positioned correctly, the oxyanion hole between the amino groups of G193 and S195 (broken line) is absent and the substrate binding pockets are incompletely formed (PDB code 4XDE<sup>179</sup>). (c) A homology model of fXIIa (similar to that described in ref 175 based on the inactive conformations of fXII and the known active conformation (1YCO<sup>180</sup>) of hepatocyte growth factor activator, the Ser protease most closely related to fXII, displays a fully formed S1 pocket and is consistent with binding of **39** (green CPK sticks)-derived compounds with known fXIIa-inhibitory activity.<sup>181</sup> The modeled binding mode is similar to that observed in the experimental binding mode of **39** in thrombin (d; PDB code 1DWD<sup>67</sup>). (e) Coumarin **40** (cyan CPK sticks) is likely to bind to fXIIa as shown.<sup>175</sup> (f) Highly potent and selective conformationally constrained peptide inhibitors of fXIIa have recently been reported, e.g., **41** (green CPK sticks; polar interactions with fXIIa as yellow broken lines) in a modeled bound conformation (according to ref 176).

but that S1', S2, S3, and S4 sites are more extensive than the corresponding sites observed in other Ser proteases.

Certain coumarin derivatives were recently reported as selective fXIIa inhibitors, although their potency is modest. For example, derivative **40** (fXIIa IC<sub>50</sub> = 5 μM) probably binds to fXIIa as shown in Figure 11e, engaging the S1 and S2 sites.<sup>175</sup> It is likely that these compounds are suicide inhibitors that form covalent adducts with the enzyme: the modeled bound pose places the pyrone ring of **40** in the vicinity of the catalytic S195, and one can imagine that nucleophilic attack of the Ser hydroxyl on the pyrone ring may ensue from the initial binding pose.

Very potent and highly selective peptide inhibitors of fXIIa have been reported.<sup>176–178</sup> These macrocyclic peptides were identified by selection against fXIIa binding and inhibition of phage-displayed peptide libraries of the form Cys-(Xaa)<sub>n</sub>-Cys-(Xaa)<sub>n</sub>-Cys, cyclized by reaction of the Cys thiol functions with trifunctional reagents such as 1,3,5-triacryloyl-1,3,5-triazinane in the case of **41** (Figure 11a,f). Some of the fXIIa-inhibitory peptides displayed high potency (low to sub-nM K<sub>i</sub> for fXIIa) and selectivity, as well as good plasma stability and the ability to block the activation of the intrinsic coagulation pathway in ex vivo human blood.

**Plasma Kallikrein. Functions and Biomedical Rationale.** Although here we only discuss plasma kallikrein (PK, KLKB1), this enzyme is actually part of the larger kallikrein family that also contains the functionally and structurally related<sup>182</sup> tissue kallikreins KLK1–KLK15. The tissue kallikreins are now beginning to be pursued as potential drug targets in a range of disorders,

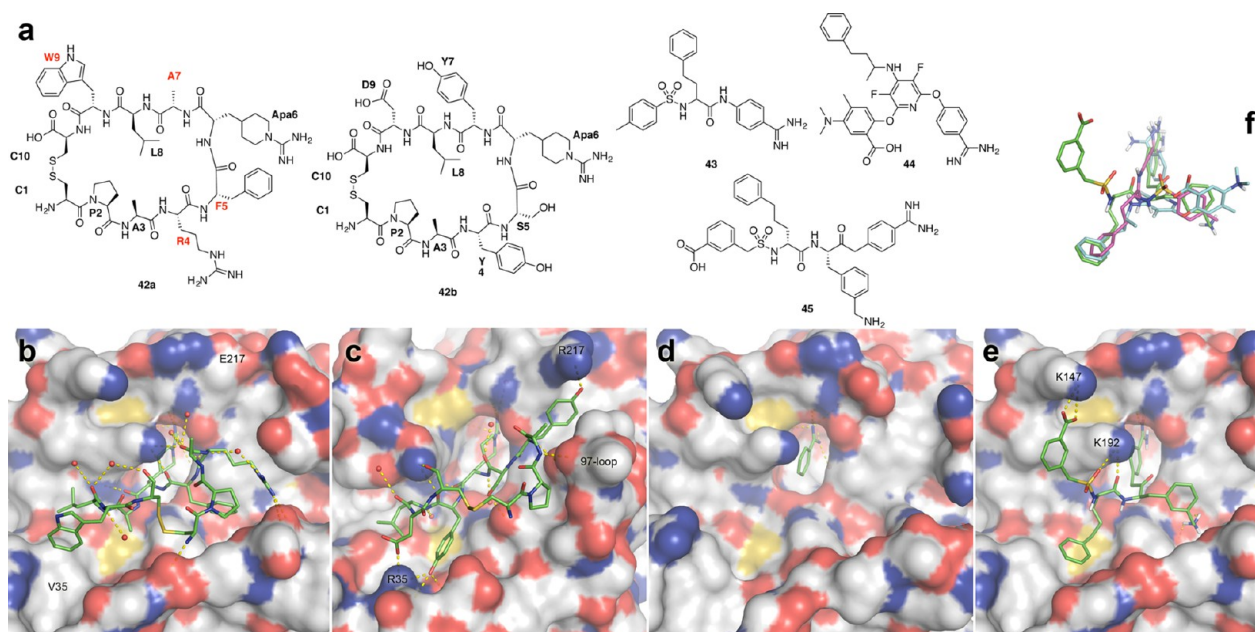
especially respiratory, cardiovascular, and dermatological diseases, as well as in cancer (reviewed in ref 183).

The zymogen of PK is prekallikrein, which circulates in the blood as a complex with HK. This zymogen is activated to PK by α-fXIIa on negatively charged surfaces (contact activation in pathobiological situations), by β-fXIIa in plasma,<sup>184</sup> and by prolylcarboxypeptidase on endothelial cells.<sup>185</sup> PK can activate the intrinsic pathway of coagulation through reciprocal activation of fXII, the KKS through cleavage of HK to generate bradykinin, the fibrinolytic system through activation of prourokinase and plasminogen, and the complement system via formation of β-fXIIa (reviewed in ref 13).

With roles in coagulation, fibrinolysis, and inflammation, PK has been proposed as a potential drug target in several diseases (reviewed in ref 186). Like other components of the contact activations system such as fXII (see above) and HK, congenital deficiency of prekallikrein in humans usually causes no bleeding or other health problems but manifests in long aPTT.<sup>187</sup> Similarly, PK-deficient mice (KLKB1<sup>-/-</sup>) show increased aPTT but without prolonged bleeding time.<sup>188</sup>

**Inhibitor Design.** In the past several peptide-based (including **42a** and unrelated bicyclic peptides with high potency and selectivity for PK)<sup>189</sup> and small-molecule direct PK inhibitors (Figure 12) have been reported. Some were evaluated in animal models of thrombosis, and the results from such studies suggest that PK inhibition may be a tractable anticoagulation strategy that provides a safety margin between antithrombotic and bleeding effects (reviewed in ref 186). However, no small-molecule direct PK inhibitors are currently under clinical trials as antithrombotics.





**Figure 12.** Progress has recently been made in the understanding of the structural biology of PK with the aid of cyclic peptides such as pkalin-3 (**42a** in panel a).<sup>27</sup> This cyclic decapeptide, which is selective for PK, was identified through re-engineering of a related peptide (**42b** in panel a), mupain-1–16, which is selective for uPA. The complexes of **42a** with PK and **42b** with murine uPA are shown in panel b (PDB code 4ZJ6<sup>27</sup>) and panel c (PDB code 4X1N<sup>190</sup>), respectively. In both complexes the side chain of the ligand L-3-(*N*-amidino-4-piperidinyl)alanine (Apa6) residue is bound in the S1 site. uPA contains a lengthened 97-loop compared to PK, and the presence of an Arg (R217) residue in uPA (E217 in PK) renders the S2 and S3 sites significantly different between the two proteases. This was exploited by replacement of Y4 and F5 in **42b** with R4 and F5 in **42a**. An additional difference occurs in the S3' site, where PK contains V35 instead of the R35 residue in uPA. This difference was exploited by replacement of Y7 and D9 in **42b** with A7 and W9 in **42a**. Comparison of the complexes of PK with **42a** (b) and benzamidine (d; PDB code 2ANY<sup>191</sup>) shows that overall the substrate-binding sites are similar with comparatively little induced fit in the complex with **42a**. Peptidomimetic (kallistop, **43**;<sup>192</sup> MDCO-2010, **45**<sup>193</sup>) and nonpeptidic (PF-04886847, **44**<sup>194</sup>) PK inhibitors are also known, and a likely PK binding mode for **43** (based on docking to the 4ZJ6 receptor) is shown in panel e. This compound makes many similar contacts with PK as the peptide **42a** and additionally forms polar interactions between the ligand benzoic acid group and the K147 residue. (f) The pharmacophoric similarity between the small-molecule PK inhibitors is evident from flexible alignment of **43** (magenta) and **44** (cyan) with the modeled bioactive conformation of **45** (green).

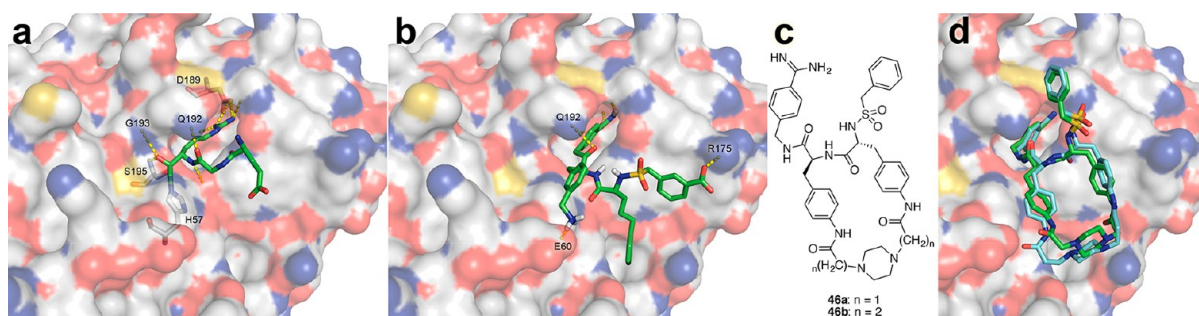
**PK Inhibitors in Perioperative Bleeding.** Since PK plays a role in activation of both coagulation and fibrinolysis pathways, PK inhibitors may paradoxically display pro- or anticoagulant activity, depending on pathological context. Small-molecule PK inhibitors are also potentially attractive for the treatment of bleeding during cardiac surgery and cardiopulmonary bypass, since coagulation, fibrinolysis, and inflammation all play a role in these indications. Aprotinin (bovine pancreatic trypsin inhibitor) and ecallantide (a recombinant peptide) are Kunitz-type Ser protease inhibitors. Aprotinin inhibits PK, as well as a number of other Ser proteases (including plasmin and thrombin).<sup>195</sup> This agent had been in successful clinical use to reduce blood loss and the need for transfusion associated with heart surgery. Aprotinin was withdrawn in 2007, but marketing authorization was reinstated in 2013 in the European Union.<sup>196</sup> Ecallantide, on the other hand, is a selective PK inhibitor and is currently approved for the treatment of hereditary angioedema.<sup>197</sup> This agent has also been trialed in surgical blood loss indications in comparison with the plasmin inhibitor tranexamic acid but was found to be less effective than tranexamic acid.<sup>198</sup> This result may indicate that combined PK and plasmin inhibition is desirable to prevent perioperative bleeding. The small-molecule direct dual PK and plasmin inhibitor **45** (Figure 12a,e), was evaluated in a phase II study in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass and was found to reduce chest tube drainage and transfusions.<sup>199</sup> However, a subsequent multicenter study was terminated due to unexpected patient safety

issues.<sup>200</sup> Whether or not these were associated with the incomplete protease selectivity of **45**, which apart from PK ( $K_i = 0.02$  nM) and plasmin ( $K_i = 2.2$  nM) also inhibits fXa ( $K_i = 45$  nM) and fXIa ( $K_i = 18$  nM),<sup>201</sup> is not clear.

**PK Inhibitors in Ophthalmic Indications.** Drugs targeting vascular endothelial growth factor (VEGF) signaling are currently used to treat loss of vision associated with macular edema and retinal vein occlusion.<sup>202</sup> VEGF-induced macular edema occurs as a result of increased retinal vascular permeability, which in turn allows influx of plasma components, including those of the KKS. This system is believed to mediate many of the inflammatory aspects of diabetic retinopathy, and the enzymatic activity of PK, which produces bradykinin from HK, is important in these processes.<sup>203</sup> KalVista Pharmaceuticals is currently developing a PK inhibitor (KVD001, structure not disclosed) as an intravitreal agent in subjects with diabetic macular edema (ClinicalTrials.gov identifier NCT02193113). KalVista originally acquired PK inhibitors from Vantia Therapeutics,<sup>204</sup> and a recent report including authors from Vantia showed that VEGF-induced retinal vascular permeability and retinal thickening in *KLKB1*<sup>-/-</sup> mice were reduced significantly in comparison to wild-type animals upon systemic administration of VA999272, a compound with high potency and selectivity for PK versus tissue kallikrein (KLK1) and a number of other related Ser proteases.<sup>205</sup> It is not clear if VA999272 and KVD001 refer to the same compound structure.

**Plasmin. Functions and Biomedical Rationale.** Under hemostatic conditions fibrinolysis is required in order to





**Figure 13.** (a) The substrate-recognition site of plasmin (gray CPK surface) presents a significantly different shape compared to other Ser proteases, as can be seen from an experimental complex with the covalent inhibitor H-Glu-Gly-Arg-CH<sub>2</sub>Cl (green CPK sticks), which forms an adduct with the catalytic residues H57 and S195 (gray CPK sticks) and in which the Glu side chain of the inhibitor projects into the extensive and comparatively flat S4 site (PDB code 1BUI<sup>213</sup>). (b) Modeled (docking against 1BUI receptor) binding mode of 45 (Figure 12a). (c) Macrocyclic benzamidine plasmin inhibitor compounds 46 (46a, compound 4 from ref 210; 46b, compound 8 from ref 210). (d) Predicted plasmin binding modes (modeled poses from ref 210) of 46a (green) and 46b (cyan).

prevent excessive clot formation. This process is self-regulated at the level of fibrin, which binds plasminogen, the circulating plasma zymogen of plasmin, as well as tPA. The activity of tPA is enhanced significantly upon binding to fibrin, and together with uPA, tPA activates plasminogen to plasmin. Plasmin then cleaves fibrin and activates tPA and uPA in a positive feedback loop. Because plasmin prefers a Lys residue at the P1 position in its substrates, the action of plasmin generates soluble fibrin degradation products with C-terminal Lys residues. The Kringle domains of both plasminogen and tPA contain Lys-binding sites, leading to further recruitment of plasminogen and uPA to fibrin, thus enhancing plasmin formation and fibrin cleavage.<sup>206</sup>

Increased fibrinolysis occurs in some forms of disseminated intravascular coagulation, in chronic liver disease, and in some leukemias and is frequently induced upon major cardiac surgery.<sup>207</sup> Furthermore, pathological plasmin generation can also occur during, for example, chronic inflammation and tumor metastasis.<sup>208</sup>

**Inhibitor Design.** Plasmin inhibitors derived from the amino acid lysine, such as 6-aminohexanoic acid and tranexamic acid, are commonly used as antifibrinolytic agents. These compounds block plasmin activity indirectly by engaging Lys-binding sites in the Kringle domain of plasminogen, thus preventing tPA- and uPA-mediated conversion of plasminogen to plasmin. Apart from broad-spectrum Ser protease inhibitors such as aprotinin (refer to PK section), there are currently no reversible active-site directed plasmin inhibitors approved or under clinical evaluation, although such agents would be desirable for improved antifibrinolysis treatments and other indications where plasmin activity is implicated.

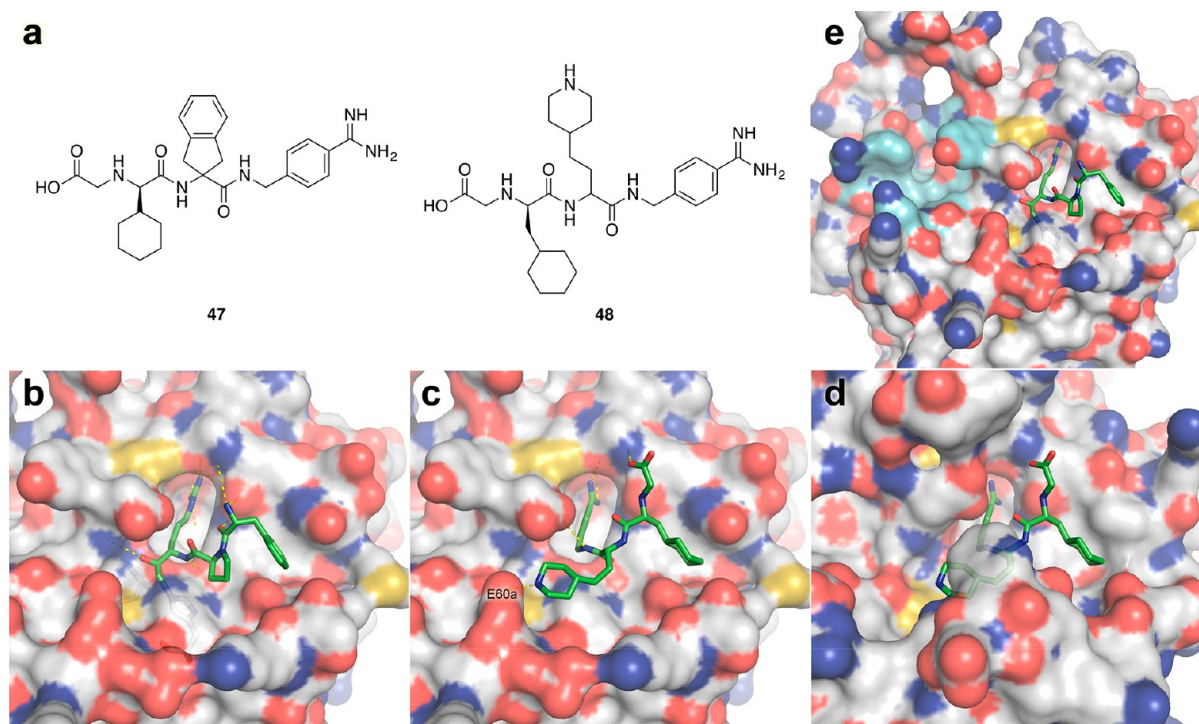
Numerous plasmin inhibitors have been described (reviewed in ref 208), but development has not progressed as far as for other Ser proteases discussed here, although structural selectivity rationales and medicinal chemistry starting points are available (Figure 13). The main structural difference between plasmin and its closely related Ser proteases is a six-residue deletion (with respect to chymotrypsin; Figure 2) and hence absence of the conserved 99- $\beta$ -hairpin loop,<sup>209</sup> resulting in a very extensive S4 site. The dual PK and plasmin inhibitor (45) discussed in the PK section is likely to bind to plasmin in a different conformation (Figure 13b) than to PK (Figure 12e). In plasmin the bulk of the inhibitor occupies the S4 site and forms a polar interaction from the benzoate group to the guanidine of R175. The most potent and selective plasmin inhibitors reported to date are macrocyclic benzamidine

compounds such as 46 (Figure 13c), which are predicted to make extensive interactions with the unique S4 site of plasmin (Figure 13d). Compound 46a was reported to inhibit plasmin with a  $K_i$  value of 0.68 nM and to be highly selective over a range of Ser proteases, except trypsin, over which it is moderately selective (100-fold).<sup>210</sup>

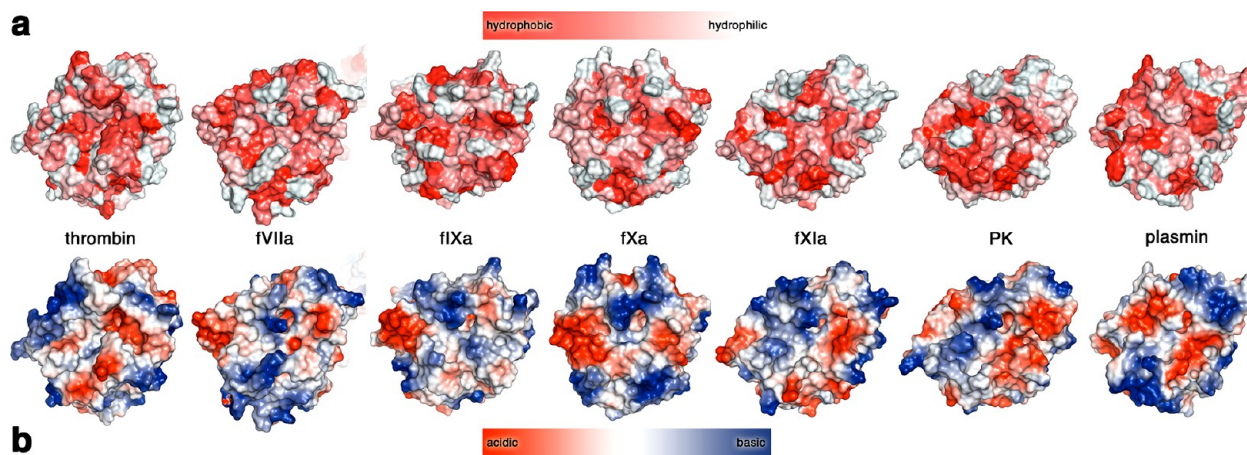
Like thrombin (Figure 5), fX, and fXI, plasmin contains a cationic exosite II that recognizes heparin, which is known to modulate plasmin activity allosterically.<sup>211</sup> It has been shown recently that certain dimeric sulfated polyphenols (termed nonsaccharide glycosaminoglycan mimetics) selectively bind to the plasmin exosite II, inhibit plasmin catalytic activity, and block clot lysis in vitro.<sup>212</sup>

**Activated Protein C. Functions and Biomedical Rationale.** Under hemostatic conditions blood clotting is held in check through anticoagulant mechanisms, in which protein C plays a dominant role. The circulating protein C zymogen is converted to aPC by thrombin upon association with the cell membrane receptors thrombomodulin and endothelial protein C receptor. In this process the procoagulant activity of thrombin is suppressed, since its exosite I, which otherwise engages procoagulant thrombin-binding proteins, is occupied by thrombomodulin. aPC, whose activity is further enhanced by the cofactor protein S, exerts its anticoagulant activity predominantly at the levels of fVa and fVIIa, which are degraded through proteolytic activity of aPC. These proteases form part of the prothrombinase (fXa–fVa) and tenase (fIXa–fVIIIa) complexes, which in the absence of aPC activate prothrombin and fX, respectively (Figure 1).<sup>19</sup>

As discussed earlier, thrombin generation is impaired in the common recessive X-linked genetic disorders hemophilia A and B, as well as the rare autosomal genetic disorder parahemophilia, due to deficiencies in functional fVIII, fIX, and fV, respectively. Most cases of hemophilia A and B are severe and require preventive treatment, which currently involves predominantly the use of octocog alfa (hemophilia A) or nonacog alfa (hemophilia B), i.e., engineered versions of the deficient clotting factors fVIII and fIX, but the economic burden of these treatments on healthcare systems is high.<sup>214</sup> For this reason alternative strategies for the preventative treatments of hemophilia A and B are sought,<sup>26</sup> and one potential approach is to enhance the lifetime of the prothrombinase complex through inhibition of aPC catalytic activity, which should enhance procoagulant activity. The potential safety of this approach is indicated by the findings that while individuals



**Figure 14.** (a) Chemical structures of aPC inhibitors: 47, compound 1 in ref 218; 48, compound 29 in ref 219. The only X-ray crystal structure of aPC (b; protein surface in gray CPK and catalytic residues as sticks) is one of a complex (PDB code 1AUT<sup>221</sup>) with 1 (green CPK sticks). A likely binding mode of 48 in aPC (based on docking to the 1AUT receptor) is shown in panel c. Superposition of the aPC-docked conformation of 48 onto an aPC-aligned structure of thrombin (d; PDB code 1PPB<sup>66</sup>) shows that the ethylpiperidine group of 48 occupies the wide S2 subsite in aPC (c), which is not consistent with binding to thrombin (d), which possess a 60-loop insertion (refer thrombin section). FV-binding exosite of aPC (cyan CPK surface in panel e).



**Figure 15.** S1A proteases differ in shape, hydrophobicity (a; surface coloring based on the normalized consensus hydrophobicity scale of amino acids<sup>230</sup> from least hydrophobic (pure white for Arg) to most hydrophobic (dark red for Ile)), and electrostatic potential (b; surface coloring from most negative (dark red) to most positive (dark blue), calculated using the APBS program<sup>231</sup>). Shown are views into the substrate-binding site of representative S1A protease structures from superpositions of PDB entries 1PPB (thrombin),<sup>66</sup> 2FIR (fVIIa),<sup>232</sup> 1RFN (fIXa),<sup>120</sup> 1C5M (fIXa),<sup>31</sup> 1ZPC (fIXa),<sup>157</sup> 5TJX (PK),<sup>233</sup> and 1BUI (plasmin).<sup>213</sup>

with resistance to aPC, protein S deficiency, or protein C deficiency are at increased risk of thromboembolic disease, many remain asymptomatic and do not have a substantially increased risk of thrombosis.<sup>215</sup>

**Inhibitor Design.** Using peptidomimetic aPC inhibitors based on the scissile fV substrate sequence, it was shown that such compounds could restore thrombin generation in TF-triggered contact pathway-inhibited fresh blood from hemophilia patients.<sup>216,217</sup> The first small-molecule aPC inhibitor to

be reported is the benzamidine derivative 47 (Figure 14a), which inhibits aPC with an  $IC_{50}$  value of  $0.8 \mu M$ , is  $\sim 60$ -fold selective over thrombin, and significantly restored thrombin generation in hemophilic plasma.<sup>218</sup> More recently benzamidines such as 48 were described, with similar potency (aPC  $IC_{50} = 0.9 \mu M$  for 48) as 47 but better-defined selectivity (over thrombin, fXa, and fXIa).<sup>219</sup>

Allosteric aPC inhibitors have also recently been reported.<sup>220</sup> These were discovered using structure-based virtual screening



and are thought to block an exosite adjacent to the catalytic site and known to be involved in the recognition of fV. Although the compounds have modest affinity for aPC (high  $\mu\text{M}$   $K_d$  values), they were shown to suppress aPC-mediated fVa inactivation in vitro.

## CONCLUSIONS

As we have seen, structure-based design has played a major role in the discovery of protease inhibitors, but rational design of selectivity remains challenging. In some cases unique features in the substrate-recognition sites of different proteases have been exploited successfully, but conformation flexibility and selection that are known to be responsible in large measure for protease substrate selectivity are also evident in the case of small-molecule inhibitor selectivity.<sup>75,222</sup> A comparison of representative complex structures of S1A proteases in terms of substrate-binding site hydrophobicity (Figure 15a) and electrostatic potential (Figure 15b) shows that these enzymes differ not only in their shape but also as far as their respective surface polarity is concerned. These differences are evident in all subsites of the binding site, even the well-conserved S1 pocket, and are frequently evident from 3D protein structure comparisons but not necessarily from enzyme substrate specificity differences alone.<sup>33,223</sup> On the basis of the extensive structural biology information that has been amassed, it can be expected that highly selective inhibitors for many of the S1A proteases can be designed. Such compounds not only will have potential uses as specific and safe new drugs as we have discussed in the individual sections above but will also help to unravel the complex biology of the regulatory processes that these enzymes are involved in.

Despite the improved utility in terms of predictability of pharmacokinetics and pharmacodynamics of the current DOACs that target thrombin and fXa, these are still associated with significant bleeding risks when compared with warfarin.<sup>51</sup> It is hoped that new agents targeting components of the coagulation cascade upstream of thrombin and fXa will provide anticoagulation with fewer bleeding complications.<sup>54</sup> In the meantime reversal agents for the DOACs are being developed.<sup>224,225</sup> Traditional indirect anticoagulants have well established antidotes, e.g., protamine sulfate in the case of heparin and vitamin K-containing preparations in the case of VKAs.<sup>226</sup> However, these antidotes do not reverse the effects of DOACs, whose use without antidote is therefore associated with a certain risk in cases of spontaneous or trauma-induced bleeding, as well as in the case of overdosing. This risk is exacerbated by the comparatively long half-lives (7–17 h) of DOACs.<sup>36</sup> Although there is debate regarding the importance of reversal agents for these drugs, the current lack of reversal agents prevents wider use of DOACs.<sup>224</sup> At present the only approved DOAC reversal agent is idarucizumab, an antibody fragment that binds and neutralizes both free and thrombin-bound dabigatran (4b).<sup>227</sup> Andexanet alfa is a modified and inactivated form of fX specifically designed to reverse the anticoagulant activity of both direct and indirect fXa inhibitors.<sup>228</sup> This reversal agent is currently in late-stage development.<sup>229</sup>

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## Notes

The author declares no competing financial interest.

## Biography

Peter M. Fischer was educated in Switzerland and Australia and has been active in drug research and development for over 25 years in both the pharmaceutical industry and in academia. He has held a Chair in Medicinal Chemistry in the School of Pharmacy at the University of Nottingham since 2005. His research focuses on chemical biology and structure-based design and optimization of peptide, peptidomimetic, and small-molecule inhibitors of biomedically relevant enzymes, including proteases, kinases, and nucleases, as well as modulators of G-protein-coupled receptors, protein–protein interactions, and protein–oligonucleotide interactions. He has authored over 150 original research reports and review papers, as well being a nominated inventor in over 80 patent documents.

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## ABBREVIATIONS USED

AD, Alzheimer's disease; AF, atrial fibrillation; aPC, activated protein C; aPTT, activated partial thromboplastin time; CPK, Corey–Pauling–Koltun; DALY, disability-adjusted life year; DOAC, direct oral anticoagulant; EPCR, endothelial protein C receptor; Gp, glycoprotein; HK, high-molecular weight kininogen; INR, international normalized ratio; KKS, kallikrein–kinin system; LMWH, low molecular weight heparin; NOAC, new oral anticoagulant; PDB, Protein Data Bank; PK, plasma kallikrein; TF, tissue factor; tPA, tissue plasminogen activator; uPA, urokinase-like plasminogen activator; VEGF, vascular endothelial growth factor; VKA, vitamin K antagonist; vWF, von Willebrand factor

## REFERENCES

- (1) Gale, A. J. Current understanding of hemostasis. *Toxicol. Pathol.* **2011**, *39*, 273–280.
- (2) Engelmann, B.; Massberg, S. Thrombosis as an intravascular effector of innate immunity. *Nat. Rev. Immunol.* **2013**, *13*, 34–45.
- (3) Moore, H. B.; Moore, E. E.; Gonzalez, E.; Chapman, M. P.; Chin, T. L.; Silliman, C. C.; Banerjee, A.; Sauaia, A. Hyperfibrinolysis, physiologic fibrinolysis, and fibrinolysis shutdown: The spectrum of postinjury fibrinolysis and relevance to antifibrinolytic therapy. *J. Trauma Acute Care Surg.* **2014**, *77*, 811–817.
- (4) Ivanciu, L.; Stalker, T. J. Spatiotemporal regulation of coagulation and platelet activation during the hemostatic response in vivo. *J. Thromb. Haemostasis* **2015**, *13*, 1949–1959.
- (5) Yau, J. W.; Teoh, H.; Verma, S. Endothelial cell control of thrombosis. *BMC Cardiovasc. Disord.* **2015**, *15*, 130.
- (6) Brass, L. F. Thrombin and platelet activation. *Chest* **2003**, *124*, 18s–25s.
- (7) Bagoly, Z.; Koncz, Z.; Harsfalvi, J.; Muszbek, L. Factor XIII, clot structure, thrombosis. *Thromb. Res.* **2012**, *129*, 382–387.
- (8) Loof, T. G.; Deicke, C.; Medina, E. The role of coagulation/fibrinolysis during *Streptococcus pyogenes* infection. *Front. Cell. Infect. Microbiol.* **2014**, *4*, 128.
- (9) McWilliam, H.; Li, W.; Uludag, M.; Squizzato, S.; Park, Y. M.; Buso, N.; Cowley, A. P.; Lopez, R. Analysis tool web services from the EMBL-EBI. *Nucleic Acids Res.* **2013**, *41*, W597–W600.
- (10) Macfarlane, R. G. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature* **1964**, *202*, 498–499.



- (11) Davie, E. W.; Ratnoff, O. D. Waterfall sequence for intrinsic blood clotting. *Science* **1964**, *145*, 1310–1312.
- (12) Favalaro, E. J.; Lippi, G.; Koutts, J. Laboratory testing of anticoagulants: the present and the future. *Pathology* **2011**, *43*, 682–692.
- (13) Schmaier, A. H. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. *J. Thromb. Haemostasis* **2016**, *14*, 28–39.
- (14) Weitz, J. I.; Fredenburgh, J. C. Factors XI and XII as targets for new anticoagulants. *Front. Med.* **2017**, *4*, 19.
- (15) Kenne, E.; Nickel, K. F.; Long, A. T.; Fuchs, T. A.; Stavrou, E. X.; Stahl, F. R.; Renne, T. Factor XII: a novel target for safe prevention of thrombosis and inflammation. *J. Intern. Med.* **2015**, *278*, 571–585.
- (16) Golas, A.; Yeh, C.-H. J.; Pitakjakpipop, H.; Siedlecki, C. A.; Vogler, E. A. A comparison of blood factor XII autoactivation in buffer, protein cocktail, serum, and plasma solutions. *Biomaterials* **2013**, *34*, 607–620.
- (17) de Maat, S.; Maas, C. Factor XII: form determines function. *J. Thromb. Haemostasis* **2016**, *14*, 1498–1506.
- (18) Diamond, S. L. Systems biology of coagulation. *J. Thromb. Haemostasis* **2013**, *11* (Suppl. 1), 224–232.
- (19) Dahlbäck, B.; Villoutreix, B. O. Regulation of blood coagulation by the protein C anticoagulant pathway. *Arterioscler., Thromb., Vasc. Biol.* **2005**, *25*, 1311–1320.
- (20) Maroney, S. A.; Mast, A. E. New insights into the biology of tissue factor pathway inhibitor. *J. Thromb. Haemostasis* **2015**, *13*, S200–S207.
- (21) Roemisch, J.; Gray, E.; Hoffmann, J. N.; Wiedermann, C. J. Antithrombin: a new look at the actions of a serine protease inhibitor. *Blood Coagulation Fibrinolysis* **2002**, *13*, 657–670.
- (22) Chapin, J. C.; Hajjar, K. A. Fibrinolysis and the control of blood coagulation. *Blood Rev.* **2015**, *29*, 17–24.
- (23) Rawlings, N. D.; Waller, M.; Barrett, A. J.; Bateman, A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* **2014**, *42*, D503–D509.
- (24) Hedstrom, L. Serine protease mechanism and specificity. *Chem. Rev.* **2002**, *102*, 4501–4524.
- (25) Walsh, P. N.; Ahmad, S. S. Proteases in blood clotting. *Essays Biochem.* **2002**, *38*, 95–111.
- (26) Shetty, S.; Ghosh, K. Novel therapeutic approaches for haemophilia. *Haemophilia* **2015**, *21*, 152–161.
- (27) Xu, P.; Xu, M.; Jiang, L.; Yang, Q.; Luo, Z.; Dauter, Z.; Huang, M.; Andreasen, P. A. Design of specific serine protease inhibitors based on a versatile peptide scaffold: conversion of a urokinase inhibitor to a plasma kallikrein inhibitor. *J. Med. Chem.* **2015**, *58*, 8868–8876.
- (28) Gladysz, R.; Adriaenssens, Y.; De Winter, H.; Joossens, J.; Lambear, A.-M.; Augustyns, K.; Van der Veken, P. Discovery and SAR of novel and selective inhibitors of urokinase plasminogen activator (uPA) with an imidazo[1,2-*a*]pyridine scaffold. *J. Med. Chem.* **2015**, *58*, 9238–9257.
- (29) Tyndall, J. D. A.; Kelso, M. J.; Ranson, M. Inhibitors of the plasminogen activation system - promising new agents for suppressing breast cancer metastasis. *Front. Anti-Cancer Drug Discovery* **2010**, *1*, 55–78.
- (30) Schechter, I.; Berger, A. On the active site of proteases. 3. Mapping the active site of papain; specific peptide inhibitors of papain. *Biochem. Biophys. Res. Commun.* **1968**, *32*, 898–902.
- (31) Katz, B. A.; Mackman, R.; Luong, C.; Radika, K.; Martelli, A.; Sprengeler, P. A.; Wang, J.; Chan, H.; Wong, L. Structural basis for selectivity of a small molecule, S1-binding, submicromolar inhibitor of urokinase-type plasminogen activator. *Chem. Biol.* **2000**, *7*, 299–312.
- (32) Pineda, A. O.; Carrell, C. J.; Bush, L. A.; Prasad, S.; Caccia, S.; Chen, Z.-W.; Mathews, F. S.; Di Cera, E. Molecular dissection of Na<sup>+</sup> binding to thrombin. *J. Biol. Chem.* **2004**, *279*, 31842–31853.
- (33) Waldner, B. J.; Fuchs, J. E.; Huber, R. G.; von Grafenstein, S.; Schauerperl, M.; Kramer, C.; Liedl, K. R. Quantitative correlation of conformational binding enthalpy with substrate specificity of serine proteases. *J. Phys. Chem. B* **2016**, *120*, 299–308.
- (34) Gandhi, P. S.; Chen, Z.; Mathews, F. S.; Di Cera, E. Structural identification of the pathway of long-range communication in an allosteric enzyme. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 1832–1837.
- (35) Vogt, A. D.; Pozzi, N.; Chen, Z.; Di Cera, E. Essential role of conformational selection in ligand binding. *Biophys. Chem.* **2014**, *186*, 13–21.
- (36) Weitz, J. I.; Eikelboom, J. W.; Samama, M. M. New antithrombotic drugs: antithrombotic therapy and prevention of thrombosis, 9th ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* **2012**, *141*, e120S–e151S.
- (37) Murray, C. J.; Acharya, A. K. Understanding DALYs (disability-adjusted life years). *J. Health Econ.* **1997**, *16*, 703–730.
- (38) [http://www.who.int/healthinfo/global\\_burden\\_disease/2004\\_report\\_update/en/index.html](http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/index.html) (accessed August 9, 2017).
- (39) Global Burden of Disease Study 2013 Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* **2015**, *386*, 743–800.
- (40) Canales, J. F.; Ferguson, J. J. Low-molecular-weight heparins: mechanisms, trials, and role in contemporary interventional medicine. *Am. J. Cardiovasc. Drugs* **2008**, *8*, 15–25.
- (41) Ansell, J.; Hirsh, J.; Hylek, E.; Jacobson, A.; Crowther, M.; Palareti, G. Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians evidence-based clinical practice guidelines (8th Edition). *Chest* **2008**, *133*, 160S–198S.
- (42) Kucher, N.; Connolly, S.; Beckman, J. A.; Cheng, L. H.; Tsilimingras, K. V.; Fanikos, J.; Goldhaber, S. Z. International normalized ratio increase before warfarin-associated hemorrhage - Brief and subtle. *Arch. Intern. Med.* **2004**, *164*, 2176–2179.
- (43) Oden, A.; Fahlen, M.; Hart, R. G. Optimal INR for prevention of stroke and death in atrial fibrillation: a critical appraisal. *Thromb. Res.* **2006**, *117*, 493–499.
- (44) Chang, J. B.; Quinnes, K. M.; Realubit, R.; Karan, C.; Rand, J. H.; Tatonetti, N. P. A novel, rapid method to compare the therapeutic windows of oral anticoagulants using the Hill coefficient. *Sci. Rep.* **2016**, *6*, 29387.
- (45) Saugel, B.; Schmid, R. M.; Huber, W. Safety and efficacy of argatroban in the management of heparin-induced thrombocytopenia. *Clin. Med. Insights: Blood Disord.* **2011**, *4*, 11–19.
- (46) Stangier, J.; Clemens, A. Pharmacology, pharmacokinetics, and pharmacodynamics of dabigatran etexilate, an oral direct thrombin inhibitor. *Clin. Appl. Thromb./Hemostasis* **2009**, *15*, 9S–16S.
- (47) Perzborn, E.; Roehrig, S.; Straub, A.; Kubitz, D.; Misselwitz, F. The discovery and development of rivaroxaban, an oral, direct factor Xa inhibitor. *Nat. Rev. Drug Discovery* **2011**, *10*, 61–75.
- (48) Roser-Jones, C.; Becker, R. C. Apixaban: an emerging oral factor Xa inhibitor. *J. Thromb. Thrombolysis* **2010**, *29*, 141–146.
- (49) Minguet, J.; Sims, H. M.; Smith, K. H.; Bramlage, P. The factor Xa inhibitor edoxaban for the prevention of stroke and systemic embolism in patients with atrial fibrillation. *Expert Rev. Clin. Pharmacol.* **2017**, *10*, 5–15.
- (50) Ufer, M. Comparative efficacy and safety of the novel oral anticoagulants dabigatran, rivaroxaban and apixaban in preclinical and clinical development. *Thromb. Haemostasis* **2010**, *103*, 572–585.
- (51) Yao, X.; Abraham, N. S.; Sangaralingham, L. R.; Bellolio, M. F.; McBane, R. D.; Shah, N. D.; Noseworthy, P. A. Effectiveness and safety of dabigatran, rivaroxaban, and apixaban versus warfarin in nonvalvular atrial fibrillation. *J. Am. Heart Assoc.* **2016**, *5*, e003725.
- (52) Levy, J. H.; Spyropoulos, A. C.; Samama, C. M.; Douketis, J. Direct oral anticoagulants. New drugs and new concepts. *JACC: Cardiovasc. Interventions* **2014**, *7*, 1333–1351.
- (53) Ruff, C. T.; Giugliano, R. P.; Braunwald, E.; Hoffman, E. B.; Deenadayalu, N.; Ezekowitz, M. D.; Camm, A. J.; Weitz, J. I.; Lewis, B. S.; Parkhomenko, A.; Yamashita, T.; Antman, E. M. Comparison of the efficacy and safety of new oral anticoagulants with warfarin in patients with atrial fibrillation: a meta-analysis of randomised trials. *Lancet* **2014**, *383*, 955–962.

- (54) Gulpen, A. J.; Ten Cate-Hoek, A. J.; Ten Cate, H. Upstream versus downstream thrombin inhibition. *Expert Rev. Cardiovasc. Ther.* **2016**, *14*, 1273–1282.
- (55) Gailani, D.; Bane, C. E.; Gruber, A. Factor XI and contact activation as targets for antithrombotic therapy. *J. Thromb. Haemostasis* **2015**, *13*, 1383–1395.
- (56) Blombäck, B. Fibrinogen and fibrin - proteins with complex roles in hemostasis and thrombosis. *Thromb. Res.* **1996**, *83*, 1–75.
- (57) Sun, W. Y.; Witte, D. P.; Degen, J. L.; Colbert, M. C.; Burkart, M. C.; Holmback, K.; Xiao, Q.; Bugge, T. H.; Degen, S. J. Prothrombin deficiency results in embryonic and neonatal lethality in mice. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 7597–7602.
- (58) Sun, W. Y.; Coleman, M. J.; Witte, D. P.; Degen, S. J. Rescue of prothrombin-deficiency by transgene expression in mice. *Thromb. Haemostasis* **2002**, *88*, 984–991.
- (59) Meeks, S. L.; Abshire, T. C. Abnormalities of prothrombin: a review of the pathophysiology, diagnosis, and treatment. *Haemophilia* **2008**, *14*, 1159–1163.
- (60) Carter, W. J.; Cama, E.; Huntington, J. A. Crystal structure of thrombin bound to heparin. *J. Biol. Chem.* **2005**, *280*, 2745–2749.
- (61) Pechik, I.; Yakovlev, S.; Mosesson, M. W.; Gilliland, G. L.; Medved, L. Structural basis for sequential cleavage of fibrinopeptides upon fibrin assembly. *Biochemistry* **2006**, *45*, 3588–3597.
- (62) Fuentes-Prior, P.; Iwanaga, Y.; Huber, R.; Pagila, R.; Rumennik, G.; Seto, M.; Morser, J.; Light, D. R.; Bode, W. Structural basis for the anticoagulant activity of the thrombin-thrombomodulin complex. *Nature* **2000**, *404*, 518–525.
- (63) Baglin, T. P.; Langdown, J.; Frasson, R.; Huntington, J. A. Discovery and characterization of an antibody directed against exosite I of thrombin. *J. Thromb. Haemostasis* **2016**, *14*, 137–142.
- (64) Markwardt, F. Historical perspective of the development of thrombin inhibitors. *Pathophysiol. Haemostasis Thromb.* **2002**, *32* (Suppl. 3), 15–22.
- (65) Kettner, C.; Shaw, E. D-Phe-Pro-Arg-CH<sub>2</sub>Cl - A selective affinity label for thrombin. *Thromb. Res.* **1979**, *14*, 969–973.
- (66) Bode, W.; Mayr, I.; Baumann, U.; Huber, R.; Stone, S. R.; Hofsteenge, J. The refined 1.9 Å crystal-structure of human alpha-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J.* **1989**, *8*, 3467–3475.
- (67) Banner, D. W.; Hadvary, P. Crystallographic analysis at 3.0-Å resolution of the binding to human thrombin of four active site-directed inhibitors. *J. Biol. Chem.* **1991**, *266*, 20085–20093.
- (68) Kikumoto, R.; Tamao, Y.; Tezuka, T.; Tonomura, S.; Hara, H.; Ninomiya, K.; Hijikata, A.; Okamoto, S. Selective inhibition of thrombin by (2*R*,4*R*)-4-methyl-1-[*N*<sup>2</sup>-[1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid. *Biochemistry* **1984**, *23*, 85–90.
- (69) Gustafsson, D.; Bylund, R.; Antonsson, T.; Nilsson, I.; Nystrom, J.-E.; Eriksson, U.; Bredberg, U.; Teger-Nilsson, A.-C. A new oral anticoagulant: the 50-year challenge. *Nat. Rev. Drug Discovery* **2004**, *3*, 649–659.
- (70) Lee, J. B.; Zgair, A.; Taha, D. A.; Zang, X.; Kagan, L.; Kim, T. H.; Kim, M. G.; Yun, H. Y.; Fischer, P. M.; Gershkovich, P. Quantitative analysis of lab-to-lab variability in Caco-2 permeability assays. *Eur. J. Pharm. Biopharm.* **2017**, *114*, 38–42.
- (71) Gustafsson, D.; Nystrom, J. E.; Carlsson, S.; Bredberg, U.; Eriksson, U.; Gyzander, E.; Elg, M.; Antonsson, T.; Hoffmann, K. J.; Ungell, A. L.; Sorensen, H.; Nagard, S.; Abrahamsson, A.; Bylund, R. The direct thrombin inhibitor melagatran and its oral prodrug H 376/95: Intestinal absorption properties, biochemical and pharmacodynamic effects. *Thromb. Res.* **2001**, *101*, 171–181.
- (72) Matthews, J. H.; Krishnan, R.; Costanzo, M. J.; Maryanoff, B. E.; Tulinsky, A. Crystal structures of thrombin with thiazole-containing inhibitors: probes of the S1' binding site. *Biophys. J.* **1996**, *71*, 2830–2839.
- (73) Huel, N. H.; Nar, H.; Priepeke, H.; Ries, U.; Stassen, J.-M.; Wienen, W. Structure-based design of novel potent nonpeptide thrombin inhibitors. *J. Med. Chem.* **2002**, *45*, 1757–1766.
- (74) Gerlach, C.; Smolinski, M.; Steuber, H.; Sottriffer, C. A.; Heine, A.; Hangauer, D. G.; Klebe, G. Thermodynamic inhibition profile of a cyclopentyl and a cyclohexyl derivative towards thrombin: the same but for different reasons. *Angew. Chem., Int. Ed.* **2007**, *46*, 8511–8514.
- (75) Engh, R. A.; Brandstetter, H.; Sucher, G.; Eichinger, A.; Baumann, U.; Bode, W.; Huber, R.; Poll, T.; Rudolph, R.; von der Saal, W. Enzyme flexibility, solvent and 'weak' interactions characterize thrombin-ligand interactions: implications for drug design. *Structure* **1996**, *4*, 1353–1362.
- (76) Morrisette, M. M.; Stauffer, K. J.; Williams, P. D.; Lyle, T. A.; Vacca, J. P.; Krueger, J. A.; Lewis, S. D.; Lucas, B. J.; Wong, B. K.; White, R. B.; Miller-Stein, C.; Lyle, E. A.; Wallace, A. A.; Leonard, Y. M.; Welsh, D. C.; Lynch, J. J.; McMasters, D. R. Low molecular weight thrombin inhibitors with excellent potency, metabolic stability, and oral bioavailability. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4161–4164.
- (77) Lu, T.; Markotan, T.; Ballentine, S. K.; Giardino, E. C.; Spurlino, J.; Brown, K.; Maryanoff, B. E.; Tomczuk, B. E.; Damiano, B. P.; Shukla, U.; End, D.; Andrade-Gordon, P.; Bone, R. F.; Player, M. R. Discovery and clinical evaluation of 1-{*N*-[2-(amidinoaminoxy)-ethyl]amino}carbonylmethyl-6-methyl-3-[2,2-difluoro-2-phenylethylamino]pyrazinone (RWJ-671818), a thrombin inhibitor with an oxyguanidine P1 motif. *J. Med. Chem.* **2010**, *53*, 1843–1856.
- (78) Young, M. B.; Barrow, J. C.; Glass, K. L.; Lundell, G. F.; Newton, C. L.; Pellicore, J. M.; Rittle, K. E.; Selnick, H. G.; Stauffer, K. J.; Vacca, J. P.; Williams, P. D.; Bohn, D.; Clayton, F. C.; Cook, J. J.; Krueger, J. A.; Kuo, L. C.; Lewis, S. D.; Lucas, B. J.; McMasters, D. R.; Miller-Stein, C.; Pietrak, B. L.; Wallace, A. A.; White, R. B.; Wong, B.; Yan, Y.; Nantermet, P. G. Discovery and evaluation of potent P<sub>1</sub> aryl heterocycle-based thrombin inhibitors. *J. Med. Chem.* **2004**, *47*, 2995–3008.
- (79) Abrahamsson, K.; Andersson, P.; Bergman, J.; Bredberg, U.; Braanal, J.; Egnell, A. C.; Eriksson, U.; Gustafsson, D.; Hoffman, K. J.; Nielsen, S.; Nilsson, I.; Pehrsson, S.; Polla, M. O.; Skjaeret, T.; Strimfors, M.; Wern, C.; Oelwegaard-Halvarsson, M.; Oertengren, Y. Discovery of AZD8165 - a clinical candidate from a novel series of neutral thrombin inhibitors. *MedChemComm* **2016**, *7*, 272–281.
- (80) Lumma, W. C.; Witherup, K. M.; Tucker, T. J.; Brady, S. F.; Sisko, J. T.; Naylor-Olsen, A. M.; Lewis, S. D.; Lucas, B. J.; Vacca, J. P. Design of novel, potent, noncovalent inhibitors of thrombin with nonbasic P-1 substructures: Rapid structure–activity studies by solid-phase synthesis. *J. Med. Chem.* **1998**, *41*, 1011–1013.
- (81) Eisert, W. G.; Huel, N.; Stangier, J.; Wienen, W.; Clemens, A.; van Ryn, J. Dabigatran: An oral novel potent reversible nonpeptide inhibitor of thrombin. *Arterioscler., Thromb., Vasc. Biol.* **2010**, *30*, 1885–1889.
- (82) Ishiguro, N.; Kishimoto, W.; Volz, A.; Ludwig-Schwelling, E.; Ebner, T.; Schaefer, O. Impact of endogenous esterase activity on in vitro P-glycoprotein profiling of dabigatran etexilate in Caco-2 monolayers. *Drug Metab. Dispos.* **2014**, *42*, 250–256.
- (83) Hartter, S.; Sennewald, R.; Nehmiz, G.; Reilly, P. Oral bioavailability of dabigatran etexilate (Pradaxa(R)) after co-medication with verapamil in healthy subjects. *Br. J. Clin. Pharmacol.* **2013**, *75*, 1053–1062.
- (84) Baum, B.; Mohamed, M.; Zayed, M.; Gerlach, C.; Heine, A.; Hangauer, D.; Klebe, G. More than a simple lipophilic contact: A detailed thermodynamic analysis of nonbasic residues in the S1 pocket of thrombin. *J. Mol. Biol.* **2009**, *390*, 56–69.
- (85) Matter, H.; Nazaré, M.; Güsregen, S.; Will, D. W.; Schreuder, H.; Bauer, A.; Urmann, M.; Ritter, K.; Wagner, M.; Wehner, V. Evidence for C-Cl/C-Br... $\pi$  interactions as an important contribution to protein–ligand binding affinity. *Angew. Chem., Int. Ed.* **2009**, *48*, 2911–2916.
- (86) Adams, T. E.; Huntington, J. A. Thrombin-cofactor interactions: structural insights into regulatory mechanisms. *Arterioscler., Thromb., Vasc. Biol.* **2006**, *26*, 1738–1745.
- (87) Warkentin, T. E. Bivalent direct thrombin inhibitors: hirudin and bivalirudin. *Best Pract. Res., Clin. Haematol.* **2004**, *17*, 105–125.
- (88) Harenberg, J.; Cimminiello, C.; Agnelli, G.; Di Minno, G.; Polo Friz, H.; Prandoni, P.; Scaglione, F. Biosimilars of low-molecular-

weight heparin products: fostering competition or reducing “biodiversity”? *J. Thromb. Haemostasis* **2016**, *14*, 421–426.

(89) Dementiev, A.; Petitou, M.; Herbert, J. M.; Gettins, P. G. The ternary complex of antithrombin-anhydrothrombin-heparin reveals the basis of inhibitor specificity. *Nat. Struct. Mol. Biol.* **2004**, *11*, 863–867.

(90) Li, W. B.; Wang, K. Y.; Zhao, M.; Yang, X. Y.; Chen, M.; Lan, X. P. Development of aptamer oligonucleotides as anticoagulants and antithrombotics for cardiovascular diseases: Current status. *Thromb. Res.* **2014**, *134*, 769–773.

(91) <https://www.enterprise.cam.ac.uk/case-studies/xo1-and-a-ground-breaking-drug-candidate/> (accessed August 8, 2017).

(92) Ten Cate, H. Challenging the anticoagulant paradigm? *J. Thromb. Haemostasis* **2016**, *14*, 134–136.

(93) Girard, T. J.; Nicholson, N. S. The role of tissue factor/factor VIIa in the pathophysiology of acute thrombotic formation. *Curr. Opin. Pharmacol.* **2001**, *1*, 159–163.

(94) Rosen, E. D.; Chan, J. C. Y.; Idusogie, E.; Clotman, F.; Vlasuk, G.; Luther, T.; Jalbert, L. R.; Albrecht, S.; Zhong, L.; Lissens, A.; Schoonjans, L.; Moons, L.; Collen, D.; Castellino, F. J.; Carmeliet, P. Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. *Nature* **1997**, *390*, 290–294.

(95) Rosen, E. D.; Xu, H.; Liang, Z.; Martin, J. A.; Suckow, M.; Castellino, F. J. Generation of genetically-altered mice producing very low levels of coagulation factor VII. *Thromb. Haemostasis* **2005**, *94*, 493–497.

(96) Lapecorella, M.; Mariani, G. Factor VII deficiency: defining the clinical picture and optimizing therapeutic options. *Haemophilia* **2008**, *14*, 1170–1175.

(97) Acharya, S. S.; Coughlin, A.; Dimichele, D. M. Rare Bleeding Disorder Registry: deficiencies of factors II, V, VII, X, XIII, fibrinogen and dysfibrinogenemias. *J. Thromb. Haemostasis* **2004**, *2*, 248–256.

(98) McVey, J. H.; Boswell, E.; Mumford, A. D.; Kembal-Cook, G.; Tuddenham, E. G. Factor VII deficiency and the FVII mutation database. *Hum. Mutat.* **2001**, *17*, 3–17.

(99) Giansily-Blaizot, M.; Verdier, R.; Biron-Adreani, C.; Schved, J.-F. Analysis of biological phenotypes from 42 patients with inherited factor VII deficiency: Can biological tests predict the bleeding risk? *Haematologica* **2004**, *89*, 704–709.

(100) Harker, L. A.; Hanson, S. R.; Wilcox, J. N.; Kelly, A. B. Antithrombotic and antilesion benefits without hemorrhagic risks by inhibiting tissue factor pathway. *Pathophysiol. Haemostasis Thromb.* **1996**, *26* (Suppl. 1), 76–82.

(101) Himber, J.; Refino, C. J.; Burcklen, L.; Roux, S.; Kirchhofer, D. Inhibition of arterial thrombosis by a soluble tissue factor mutant and active site-blocked factors IXa and Xa in the guinea pig. *Thromb. Haemostasis* **2001**, *85*, 475–481.

(102) Priestley, E. S. Tissue factor-FVIIa inhibition: update on an unfinished quest for a novel oral antithrombotic. *Drug Discovery Today* **2014**, *19*, 1440–1444.

(103) Young, W. B.; Mordenti, J.; Torkelson, S.; Shrader, W. D.; Kolesnikov, A.; Rai, R.; Liu, L.; Hu, H.; Leahy, E. M.; Green, M. J.; Sprengler, P. A.; Katz, B. A.; Yu, C.; Janc, J. W.; Elrod, K. C.; Marzec, U. M.; Hanson, S. R. Factor VIIa inhibitors: chemical optimization, preclinical pharmacokinetics, pharmacodynamics, and efficacy in an arterial baboon thrombosis model. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2037–2041.

(104) Priestley, E. S.; De Lucca, I.; Zhou, J.; Zhou, J.; Saiah, E.; Stanton, R.; Robinson, L.; Luetzgen, J. M.; Wei, A.; Wen, X.; Knabb, R. M.; Wong, P. C.; Wexler, R. R. Discovery and gram-scale synthesis of BMS-593214, a potent, selective FVIIa inhibitor. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 2432–2435.

(105) Kadono, S.; Sakamoto, A.; Kikuchi, Y.; Oh-Eda, M.; Yabuta, N.; Yoshihashi, K.; Kitazawa, T.; Suzuki, T.; Koga, T.; Hattori, K.; Shiraishi, T.; Haramura, M.; Kodama, H.; Ono, Y.; Esaki, T.; Sato, H.; Watanabe, Y.; Itoh, S.; Ohta, M.; Kozono, T. Novel interactions of large P3 moiety and small P4 moiety in the binding of the peptide mimetic factor VIIa inhibitor. *Biochem. Biophys. Res. Commun.* **2005**, *326*, 859–865.

(106) Glunz, P. W.; Mueller, L.; Cheney, D. L.; Ladziata, V.; Zou, Y.; Wurtz, N. R.; Wei, A.; Wong, P. C.; Wexler, R. R.; Priestley, E. S. Atropisomer control in macrocyclic factor VIIa inhibitors. *J. Med. Chem.* **2016**, *59*, 4007–4018.

(107) Banner, D. W.; D’Arcy, A.; Chène, C.; Winkler, F. K.; Guha, A.; Konigsberg, W. H.; Nemerson, Y.; Kirchhofer, D. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature* **1996**, *380*, 41–46.

(108) Zielinski, W.; Kudelko, A. Acid-base interactions in some isoquinoline and quinazoline amino derivatives. *ARKIVOC* **2005**, 66–82.

(109) Richter, J. M.; Cheney, D. L.; Bates, J. A.; Wei, A.; Luetzgen, J. M.; Rendina, A. R.; Harper, T. M.; Narayanan, R.; Wong, P. C.; Seiffert, D.; Wexler, R. R.; Priestley, E. S. Design and synthesis of novel meta-linked phenylglycine macrocyclic FVIIa inhibitors. *ACS Med. Chem. Lett.* **2017**, *8*, 67–72.

(110) Zhang, X.; Glunz, P. W.; Johnson, J. A.; Jiang, W.; Jacutin-Porte, S.; Ladziata, V.; Zou, Y.; Phillips, M. S.; Wurtz, N. R.; Parkhurst, B.; Rendina, A. R.; Harper, T. M.; Cheney, D. L.; Luetzgen, J. M.; Wong, P. C.; Seiffert, D.; Wexler, R. R.; Priestley, E. S. Discovery of a highly potent, selective, and orally bioavailable macrocyclic inhibitor of blood coagulation factor VIIa-tissue factor complex. *J. Med. Chem.* **2016**, *59*, 7125–7137.

(111) Ladziata, V.; Glunz, P. W.; Zou, Y.; Zhang, X.; Jiang, W.; Jacutin-Porte, S.; Cheney, D. L.; Wei, A.; Luetzgen, J. M.; Harper, T. M.; Wong, P. C.; Seiffert, D.; Wexler, R. R.; Priestley, E. S. Synthesis and P1’ SAR exploration of potent macrocyclic tissue factor-factor VIIa inhibitors. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 5051–5057.

(112) Plug, I.; Mauser-Bunschoten, E. P.; Broecker-Vriendts, A. H. J. T.; van Amstel, A. H. K. P.; van der Bom, J. G.; van Diemen-Homan, D.-H. J. E. M.; Willemse, J.; Rosendaal, F. R. Bleeding in carriers of hemophilia. *Blood* **2006**, *108*, 52–56.

(113) Eikelboom, J. W.; Zelenkofske, S. L.; Rusconi, C. P. Coagulation factor IXa as a target for treatment and prophylaxis of venous thromboembolism. *Arterioscler., Thromb., Vasc. Biol.* **2010**, *30*, 382–387.

(114) Sramek, A.; Kriek, M.; Rosendaal, F. R. Decreased mortality of ischaemic heart disease among carriers of haemophilia. *Lancet* **2003**, *362*, 351–354.

(115) Darby, S. C.; Kan, S. W.; Spooner, R. J.; Giangrande, P. L.; Hill, F. G.; Hay, C. R.; Lee, C. A.; Ludlam, C. A.; Williams, M. Mortality rates, life expectancy, and causes of death in people with hemophilia A or B in the United Kingdom who were not infected with HIV. *Blood* **2007**, *110*, 815–825.

(116) Monahan, P. E. Factor IX: insights from knock-out and genetically engineered mice. *Thromb. Haemostasis* **2008**, *100*, 563–575.

(117) Nathwani, A. C.; Reiss, U. M.; Tuddenham, E. G.; Rosales, C.; Chowdhary, P.; McIntosh, J.; Della Peruta, M.; Lheriteau, E.; Patel, N.; Raj, D.; Riddell, A.; Pie, J.; Rangarajan, S.; Bevan, D.; Recht, M.; Shen, Y. M.; Halka, K. G.; Basner-Tschakarjan, E.; Mingozzi, F.; High, K. A.; Allay, J.; Kay, M. A.; Ng, C. Y.; Zhou, J.; Cancio, M.; Morton, C. L.; Gray, J. T.; Srivastava, D.; Nienhuis, A. W.; Davidoff, A. M. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N. Engl. J. Med.* **2014**, *371*, 1994–2004.

(118) Ankrom, W.; Wood, H. B.; Xu, J.; Geissler, W.; Bateman, T.; Chatterjee, M. S.; Feng, K.-L.; Metzger, J. M.; Strapps, W. R.; Tadin-Strapps, M.; Seiffert, D.; Andre, P. Preclinical and translational evaluation of coagulation factor IXa as a novel therapeutic target. *Pharmacol. Res. Perspect.* **2016**, *4*, e00207.

(119) Eriksson, B. I.; Dahl, O. E.; Lassen, M. R.; Ward, D. P.; Rothlein, R.; Davis, G.; Turpie, A. G. G. Partial factor IXa inhibition with TTP889 for prevention of venous thromboembolism: an exploratory study. *J. Thromb. Haemostasis* **2008**, *6*, 457–463.

(120) Hopfner, K.-P.; Lang, A.; Karcher, A.; Sichler, K.; Kopetzki, E.; Brandstetter, H.; Huber, R.; Bode, W.; Engh, R. A. Coagulation factor IXa: the relaxed conformation of Tyr99 blocks substrate binding. *Structure* **1999**, *7*, 989–996.



- (121) Wang, S.; Beck, R.; Burd, A.; Blench, T.; Marlin, F.; Ayele, T.; Buxton, S.; Dagostin, C.; Malic, M.; Joshi, R.; Barry, J.; Sajad, M.; Cheung, C.; Shaikh, S.; Chahwala, S.; Chander, C.; Baumgartner, C.; Holthoff, H.-P.; Murray, E.; Blackney, M.; Giddings, A. Structure based drug design: development of potent and selective factor IXa (FIXa) inhibitors. *J. Med. Chem.* **2010**, *53*, 1473–1482.
- (122) Smallheer, J. M.; Alexander, R. S.; Wang, J.; Wang, S.; Nakajima, S.; Rossi, K. A.; Smallwood, A.; Barbera, F.; Burdick, D.; Luettgen, J. M.; Knabb, R. M.; Wexler, R. R.; Jadhav, P. K. SAR and factor IXa crystal structure of a dual inhibitor of factors IXa and Xa. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5263–5267.
- (123) Meng, D.; Andre, P.; Bateman, T. J.; Berger, R.; Chen, Y.-H.; Desai, K.; Dewnani, S.; Ellsworth, K.; Feng, D.; Geissler, W. M.; Guo, L.; Hruza, A.; Jian, T.; Li, H.; Metzger, J.; Parker, D. L.; Reichert, P.; Sherer, E. C.; Smith, C. J.; Sonatore, L. M.; Tschirret-Guth, R.; Wu, J.; Xu, J.; Zhang, T.; Campeau, L.-C.; Orr, R.; Poirier, M.; McCabe-Dunn, J.; Araki, K.; Nishimura, T.; Sakurada, I.; Hirabayashi, T.; Wood, H. B. Development of a novel tricyclic class of potent and selective FIXa inhibitors. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 5437–5443.
- (124) Rosen, E. D. Gene targeting in hemostasis. Factor X. *Front. Biosci., Landmark Ed.* **2002**, *7*, d1915–1925.
- (125) Tai, S. J.; Herzog, R. W.; Margaritis, P.; Arruda, V. R.; Chu, K.; Golden, J. A.; Labosky, P. A.; High, K. A. A viable mouse model of factor X deficiency provides evidence for maternal transfer of factor X. *J. Thromb. Haemostasis* **2008**, *6*, 339–345.
- (126) Brown, D. L.; Kouides, P. A. Diagnosis and treatment of inherited factor X deficiency. *Haemophilia* **2008**, *14*, 1176–1182.
- (127) Hirsh, J.; Bauer, K. A.; Donati, M. B.; Gould, M.; Samama, M. M.; Weitz, J. I. Parenteral Anticoagulants: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* **2008**, *133*, 141S–159S.
- (128) Turpie, A. G. G. Selective factor Xa inhibition with fondaparinux: from concept to clinical benefit. *Eur. Heart J. Suppl.* **2008**, *10*, C1–C7.
- (129) Pinto, D. J. P.; Smallheer, J. M.; Cheney, D. L.; Knabb, R. M.; Wexler, R. R. Factor Xa inhibitors: Next-generation antithrombotic agents. *J. Med. Chem.* **2010**, *53*, 6243–6274.
- (130) Roehrig, S.; Straub, A.; Pohlmann, J.; Lampe, T.; Pernerstorfer, J.; Schlemmer, K.-H.; Reinemer, P.; Perzborn, E. Discovery of the novel antithrombotic agent 5-chloro-N-((5S)-2-oxo-3-[4-(3-oxomorpholin-4-yl)phenyl]-1,3-oxazolidin-5-yl)methylthiophene-2-carboxamide (BAY 59-7939): An oral, direct factor Xa inhibitor. *J. Med. Chem.* **2005**, *48*, 5900–5908.
- (131) Perzborn, E.; Strassburger, J.; Wilmen, A.; Pohlmann, J.; Roehrig, S.; Schlemmer, K. H.; Straub, A. In vitro and in vivo studies of the novel antithrombotic agent BAY 59-7939 -an oral, direct factor Xa inhibitor. *J. Thromb. Haemostasis* **2005**, *3*, 514–521.
- (132) Kubitzka, D.; Becka, M.; Wensing, G.; Voith, B.; Zuehlsdorf, M. Safety, pharmacodynamics, and pharmacokinetics of BAY 59-7939—an oral, direct Factor Xa inhibitor—after multiple dosing in healthy male subjects. *Eur. J. Clin. Pharmacol.* **2005**, *61*, 873–880.
- (133) Trujillo, T.; Dobesh, P. P. Clinical use of rivaroxaban: Pharmacokinetic and pharmacodynamic rationale for dosing regimens in different indications. *Drugs* **2014**, *74*, 1587–1603.
- (134) Budovich, A.; Zargarova, O.; Nogid, A. Role of apixaban (Eliquis) in the treatment and prevention of thromboembolic disease. *Pharm. Ther.* **2013**, *38*, 206–231.
- (135) Schaefer, J. K.; McBane, R. D.; Wysokinski, W. E. How to choose appropriate direct oral anticoagulant for patient with nonvalvular atrial fibrillation. *Ann. Hematol.* **2016**, *95*, 437–449.
- (136) Pinto, D. J. P.; Orwat, M. J.; Koch, S.; Rossi, K. A.; Alexander, R. S.; Smallwood, A.; Wong, P. C.; Rendina, A. R.; Luettgen, J. M.; Knabb, R. M.; He, K.; Xin, B.; Wexler, R. R.; Lam, P. Y. S. Discovery of 1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxo-1-piperidinyl)phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide (apixaban, BMS-562247), a highly potent, selective, efficacious, and orally bioavailable inhibitor of blood coagulation factor Xa. *J. Med. Chem.* **2007**, *50*, 5339–5356.
- (137) Furugohri, T.; Isobe, K.; Honda, Y.; Kamisato-Matsumoto, C.; Sugiyama, N.; Nagahara, T.; Morishima, Y.; Shibano, T. DU-176b, a potent and orally active factor Xa inhibitor: in vitro and in vivo pharmacological profiles. *J. Thromb. Haemostasis* **2008**, *6*, 1542–1549.
- (138) Zhang, P.; Huang, W.; Wang, L.; Bao, L.; Jia, Z. J.; Bauer, S. M.; Goldman, E. A.; Probst, G. D.; Song, Y.; Su, T.; Fan, J.; Wu, Y.; Li, W.; Woolfrey, J.; Sinha, U.; Wong, P. W.; Edwards, S. T.; Arfsten, A. E.; Clizbe, L. A.; Kanter, J.; Pandey, A.; Park, G.; Hutchaleelaha, A.; Lambing, J. L.; Hollenbach, S. J.; Scarborough, R. M.; Zhu, B.-Y. Discovery of betrixaban (PRT054021), N-(5-chloropyridin-2-yl)-2-(4-(N,N-dimethylcarbamimidoyl)benzamido)-5-methoxybenzamide, a highly potent, selective, and orally efficacious factor Xa inhibitor. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2179–2185.
- (139) Fujimoto, T.; Imaeda, Y.; Konishi, N.; Hiroe, K.; Kawamura, M.; Textor, G. P.; Aertgeerts, K.; Kubo, K. Discovery of a tetrahydropyrimidin-2(1H)-one derivative (TAK-442) as a potent, selective, and orally active factor Xa inhibitor. *J. Med. Chem.* **2010**, *53*, 3517–3531.
- (140) Hirayama, F.; Koshio, H.; Ishihara, T.; Hachiya, S.; Sugawara, K.; Koga, Y.; Seki, N.; Shiraki, R.; Shigenaga, T.; Iwatsuki, Y.; Moritani, Y.; Mori, K.; Kadokura, T.; Kawasaki, T.; Matsumoto, Y.; Sakamoto, S.; Tsukamoto, S.-i. Discovery of N-[2-hydroxy-6-(4-methoxybenzamido)phenyl]-4-(4-methyl-1,4-diazepan-1-yl)benzamide (darexaban, YM150) as a potent and orally available factor Xa inhibitor. *J. Med. Chem.* **2011**, *54*, 8051–8065.
- (141) Kohrt, J. T.; Bigge, C. F.; Bryant, J. W.; Casimiro-Garcia, A.; Chi, L.; Cody, W. L.; Dahring, T.; Dudley, D. A.; Filipinski, K. J.; Haarer, S.; Heemstra, R.; Janiczek, N.; Narasimhan, L.; McClanahan, T.; Peterson, J. T.; Sahasrabudhe, V.; Schaum, R.; Van Huis, C. A.; Welch, K. M.; Zhang, E.; Leadley, R. J.; Edmunds, J. J. The discovery of (2R,4R)-N-(4-chlorophenyl)-N-(2-fluoro-4-(2-oxopyridin-1(2H)-yl)-phenyl)-4-methoxy-pyrrolidine-1,2-dicarboxamide (PD 0348292), an orally efficacious factor Xa inhibitor. *Chem. Biol. Drug Des.* **2007**, *70*, 100–112.
- (142) Guertin, K. R.; Choi, Y. M. The discovery of the factor Xa inhibitor otamixaban: from lead identification to clinical development. *Curr. Med. Chem.* **2007**, *14*, 2471–2481.
- (143) Pruitt, J. R.; Pinto, D. J.; Galembo, R. A., Jr.; Alexander, R. S.; Rossi, K. A.; Wells, B. L.; Drummond, S.; Bostrom, L. L.; Burdick, D.; Bruckner, R.; Chen, H.; Smallwood, A.; Wong, P. C.; Wright, M. R.; Bai, S.; Luettgen, J. M.; Knabb, R. M.; Lam, P. Y.; Wexler, R. R. Discovery of 1-(2-aminomethylphenyl)-3-trifluoromethyl-N-[3-fluoro-2'-(aminosulfonyl)[1,1'-biphenyl]-4-yl]-1H-pyrazole-5-carboxamide (DPC602), a potent, selective, and orally bioavailable factor Xa inhibitor. *J. Med. Chem.* **2003**, *46*, 5298–5315.
- (144) Cohen, A. T.; Harrington, R. A.; Goldhaber, S. Z.; Hull, R. D.; Wiens, B. L.; Gold, A.; Hernandez, A. F.; Gibson, C. M. Extended thromboprophylaxis with betrixaban in acutely ill medical patients. *N. Engl. J. Med.* **2016**, *375*, 534–544.
- (145) Steg, P. G.; Mehta, S. R.; Jukema, J. W.; Lip, G. Y. H.; Gibson, C. M.; Kovar, F.; Kala, P.; Garcia-Hernandez, A.; Renfurm, R. W.; Granger, C. B. RUBY-1: a randomized, double-blind, placebo-controlled trial of the safety and tolerability of the novel oral factor Xa inhibitor darexaban (YM150) following acute coronary syndrome. *Eur. Heart J.* **2011**, *32*, 2541–2554.
- (146) Gomez, K.; Bolton-Maggs, P. Factor XI deficiency. *Haemophilia* **2008**, *14*, 1183–1189.
- (147) Renne, T.; Oschatz, C.; Seifert, S.; Muller, F.; Antovic, J.; Karlman, M.; Benz, P. M. Factor XI deficiency in animal models. *J. Thromb. Haemostasis* **2009**, *7* (Suppl. 1), 79–83.
- (148) Muller, F.; Gailani, D.; Renne, T. Factor XI and XII as antithrombotic targets. *Curr. Opin. Hematol.* **2011**, *18*, 349–355.
- (149) Yoonis, H. S.; Crosby, J.; Huh, J.-I.; Lee, H. S.; Rime, S.; Monia, B.; Henry, S. P. Antisense inhibition of coagulation factor XI prolongs APTT without increased bleeding risk in cynomolgus monkeys. *Blood* **2012**, *119*, 2401–2408.
- (150) <http://pharma.bayer.com/en/innovation-partnering/development-pipeline/> (accessed October 10, 2017).

- (151) Buller, H. R.; Bethune, C.; Bhanot, S.; Gailani, D.; Monia, B. P.; Raskob, G. E.; Segers, A.; Verhamme, P.; Weitz, J. I. Investigators, F.-A. T. Factor XI antisense oligonucleotide for prevention of venous thrombosis. *N. Engl. J. Med.* **2015**, *372*, 232–240.
- (152) Al-Horani, R. A.; Desai, U. R. Factor XIa inhibitors: A review of the patent literature. *Expert Opin. Ther. Pat.* **2016**, *26*, 323–345.
- (153) Pinto, D. J. P.; Smallheer, J. M.; Corte, J. R.; Austin, E. J. D.; Wang, C.; Fang, T.; Smith, L. M.; Rossi, K. A.; Rendina, A. R.; Bozarth, J. M.; Zhang, G.; Wei, A.; Ramamurthy, V.; Sheriff, S.; Myers, J. E.; Morin, P. E.; Luettgen, J. M.; Seiffert, D. A.; Quan, M. L.; Wexler, R. R. Structure-based design of inhibitors of coagulation factor XIa with novel P1 moieties. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 1635–1642.
- (154) Corte, J. R.; Fang, T.; Osuna, H.; Pinto, D. J.; Rossi, K. A.; Myers, J. E., Jr.; Sheriff, S.; Lou, Z.; Zheng, J. J.; Harper, T. W.; Bozarth, J. M.; Wu, Y.; Luettgen, J. M.; Seiffert, D. A.; Decicco, C. P.; Wexler, R. R.; Quan, M. L. Structure-based design of macrocyclic factor XIa inhibitors: Discovery of the macrocyclic amide linker. *J. Med. Chem.* **2017**, *60*, 1060–1075.
- (155) Sutton, J. C.; Bolton, S. A.; Hartl, K. S.; Huang, M. H.; Jacobs, G.; Meng, W.; Ogletree, M. L.; Pi, Z.; Schumacher, W. A.; Seiler, S. M.; Slusarchyk, W. A.; Treuner, U.; Zahler, R.; Zhao, G.; Bisacchi, G. S. Synthesis and SAR of 4-carboxy-2-azetidinone mechanism-based trypsin inhibitors. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3229–3233.
- (156) Wilmoth, R. C.; Kassamally, S.; Westwood, N. J.; Sheppard, R. J.; Claridge, T. D. W.; Aplin, R. T.; Wright, P. A.; Pritchard, G. J.; Schofield, C. J. Mechanistic insights into the inhibition of serine proteases by monocyclic lactams. *Biochemistry* **1999**, *38*, 7989–7998.
- (157) Deng, H.; Bannister, T. D.; Jin, L.; Babine, R. E.; Quinn, J.; Nagafuji, P.; Celatka, C. A.; Lin, J.; Lazarova, T. I.; Rynkiewicz, M. J.; Bibbins, F.; Pandey, P.; Gorga, J.; Meyers, H. V.; Abdel-Meguid, S. S.; Strickler, J. E. Synthesis, SAR exploration, and X-ray crystal structures of factor XIa inhibitors containing an  $\alpha$ -ketothiazole arginine. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3049–3054.
- (158) Fjellström, O.; Akkaya, S.; Beisel, H.-G.; Eriksson, P.-O.; Erixon, K.; Gustafsson, D.; Jurva, U.; Kang, D.; Karis, D.; Knecht, W.; Nerme, V.; Nilsson, I.; Olsson, T.; Redzic, A.; Roth, R.; Sandmark, J.; Tigerström, A.; Öster, L. Creating novel activated factor XI inhibitors through fragment based lead generation and structure aided drug design. *PLoS One* **2015**, *10*, e0113705.
- (159) Chrusciel, R. A.; Gadwood, R. C.; Hayward, N. J.; Melnick, M. J.; Navia, M.; Poel, T. J.; Stewart, C. A.; Stassen, F. L. Preparation of  $\beta$ -Lactam Derivatives as Inhibitors of Factor X<sub>ia</sub> or Kallikrein. PCT Int. Pat. Appl. Publ. WO2015120062, 2015; Exithera Pharmaceuticals Inc., U.S.
- (160) Cool, D. E.; Edgell, C. J.; Louie, G. V.; Zoller, M. J.; Brayer, G. D.; MacGillivray, R. T. Characterization of human blood coagulation factor XII cDNA. Prediction of the primary structure of factor XII and the tertiary structure of beta-factor XIIa. *J. Biol. Chem.* **1985**, *260*, 13666–13676.
- (161) Ivanov, I.; Matafonov, A.; Sun, M. F.; Cheng, Q. F.; Dickeson, S. K.; Verhamme, I. M.; Emsley, J.; Gailani, D. Proteolytic properties of single-chain factor XII: a mechanism for triggering contact activation. *Blood* **2017**, *129*, 1527–1537.
- (162) Long, A. T.; Kenne, E.; Jung, R.; Fuchs, T. A.; Renne, T. Contact system revisited: an interface between inflammation, coagulation, and innate immunity. *J. Thromb. Haemostasis* **2016**, *14*, 427–437.
- (163) Renne, T.; Schmaier, A. H.; Nickel, K. F.; Blomback, M.; Maas, C. In vivo roles of factor XII. *Blood* **2012**, *120*, 4296–4303.
- (164) Kleinschnitz, C.; Stoll, G.; Bendszus, M.; Schuh, K.; Pauer, H.-U.; Burfeind, P.; Renne, C.; Gailani, D.; Nieswandt, B.; Renne, T. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J. Exp. Med.* **2006**, *203*, 513–518.
- (165) Pham, M.; Kleinschnitz, C.; Helluy, X.; Bartsch, A. J.; Austinat, M.; Behr, V. C.; Renne, T.; Nieswandt, B.; Stoll, G.; Bendszus, M. Enhanced cortical reperfusion protects coagulation factor XII-deficient mice from ischemic stroke as revealed by high-field MRI. *NeuroImage* **2010**, *49*, 2907–2914.
- (166) Matafonov, A.; Leung, P. Y.; Gailani, A. E.; Grach, S. L.; Puy, C.; Cheng, Q.; Sun, M.-f.; McCarty, O. J. T.; Tucker, E. I.; Kataoka, H.; Renné, T.; Morrissey, J. H.; Gruber, A.; Gailani, D. Factor XII inhibition reduces thrombus formation in a primate thrombosis model. *Blood* **2014**, *123*, 1739–1746.
- (167) Larsson, M.; Rayzman, V.; Nolte, M. W.; Nickel, K. F.; Björkqvist, J.; Jämsä, A.; Hardy, M. P.; Fries, M.; Schmidbauer, S.; Hedenqvist, P.; Broomé, M.; Pragst, I.; Dickneite, G.; Wilson, M. J.; Nash, A. D.; Panousis, C.; Renné, T. A factor XIIa inhibitory antibody provides thromboprotection in extracorporeal circulation without increasing bleeding risk. *Sci. Transl. Med.* **2014**, *6*, 222ra17.
- (168) Worm, M.; Kohler, E. C.; Panda, R.; Long, A.; Butler, L. M.; Stavrou, E. X.; Nickel, K. F.; Fuchs, T. A.; Renne, T. The factor XIIa blocking antibody 3F7: a safe anticoagulant with anti-inflammatory activities. *Ann. Transl. Med.* **2015**, *3*, 247.
- (169) Krupka, J.; May, F.; Weimer, T.; Pragst, I.; Kleinschnitz, C.; Stoll, G.; Panousis, C.; Dickneite, G.; Nolte, M. W. The coagulation factor XIIa inhibitor rHA-infestin-4 improves outcome after cerebral ischemia/reperfusion injury in rats. *PLoS One* **2016**, *11*, e0146783.
- (170) Zamolodchikov, D.; Strickland, S.; Renne, T. The Alzheimer's disease peptide  $A\beta$  promotes thrombin generation through activation of coagulation factor XII. *J. Thromb. Haemostasis* **2016**, *14*, 995–1007.
- (171) Zamolodchikov, D.; Chen, Z. L.; Conti, B. A.; Renne, T.; Strickland, S. Activation of the factor XII-driven contact system in Alzheimer's disease patient and mouse model plasma. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, 4068–4073.
- (172) Zamolodchikov, D.; Strickland, S. A possible new role for  $A\beta$  in vascular and inflammatory dysfunction in Alzheimer's disease. *Thromb. Res.* **2016**, *141*, S59–S61.
- (173) Chen, Z. L.; Revenko, A. S.; Singh, P.; MacLeod, A. R.; Norris, E. H.; Strickland, S. Depletion of coagulation factor XII ameliorates brain pathology and cognitive impairment in Alzheimer disease mice. *Blood* **2017**, *129*, 2547–2556.
- (174) Factor XIIa 1536 HTS Dose Response Confirmation. PubChem AID 852. <https://pubchem.ncbi.nlm.nih.gov/bioassay/852> (accessed August 8, 2017).
- (175) Bouckaert, C.; Serra, S.; Rondelet, G.; Dolusic, E.; Wouters, J.; Dogne, J. M.; Frederick, R.; Pochet, L. Synthesis, evaluation and structure-activity relationship of new 3-carboxamide coumarins as FXIIa inhibitors. *Eur. J. Med. Chem.* **2016**, *110*, 181–194.
- (176) Middendorp, S. J.; Wilbs, J.; Quarroz, C.; Calzavarini, S.; Angelillo-Scherrer, A.; Heinis, C. Peptide macrocycle inhibitor of coagulation factor XII with subnanomolar affinity and high target selectivity. *J. Med. Chem.* **2017**, *60*, 1151–1158.
- (177) Baeriswyl, V.; Calzavarini, S.; Chen, S.; Zorzi, A.; Bologna, L.; Angelillo-Scherrer, A.; Heinis, C. A synthetic factor XIIa inhibitor blocks selectively intrinsic coagulation initiation. *ACS Chem. Biol.* **2015**, *10*, 1861–1870.
- (178) Baeriswyl, V.; Calzavarini, S.; Gerschheimer, C.; Diderich, P.; Angelillo-Scherrer, A.; Heinis, C. Development of a selective peptide macrocycle inhibitor of coagulation factor XII toward the generation of a safe antithrombotic therapy. *J. Med. Chem.* **2013**, *56*, 3742–3746.
- (179) Pathak, M.; Wilmann, P.; Awford, J.; Li, C.; Hamad, B. K.; Fischer, P. M.; Dreveny, I.; Dekker, L. V.; Emsley, J. Coagulation factor XII protease domain crystal structure. *J. Thromb. Haemostasis* **2015**, *13*, 580–591.
- (180) Shia, S.; Stamos, J.; Kirchofer, D.; Fan, B.; Wu, J.; Corpuz, R. T.; Santell, L.; Lazarus, R. A.; Eigenbrot, C. Conformational lability in serine protease active sites: Structures of hepatocyte growth factor activator (HGFA) alone and with the inhibitory domain from HGFA inhibitor-1B. *J. Mol. Biol.* **2005**, *346*, 1335–1349.
- (181) Sturzebecher, J.; Prasa, D.; Hauptmann, J.; Vieweg, H.; Wikstrom, P. Synthesis and structure-activity relationships of potent thrombin inhibitors: piperazides of 3-amidinophenylalanine. *J. Med. Chem.* **1997**, *40*, 3091–3099.
- (182) Pathak, M.; Wong, S. S.; Dreveny, I.; Emsley, J. Structure of plasma and tissue kallikreins. *Thromb. Haemostasis* **2013**, *110*, 423–433.



- (183) Prassas, I.; Eissa, A.; Poda, G.; Diamandis, E. P. Unleashing the therapeutic potential of human kallikrein-related serine proteases. *Nat. Rev. Drug Discovery* **2015**, *14*, 183–202.
- (184) Revak, S. D.; Cochrane, C. G.; Bouma, B. N.; Griffin, J. H. Surface and fluid phase activities of two forms of activated Hageman factor produced during contact activation of plasma. *J. Exp. Med.* **1978**, *147*, 719–729.
- (185) Wang, J.; Matafonov, A.; Madkhali, H.; Mahdi, F.; Watson, D.; Schmaier, A. H.; Gailani, D.; Shariat-Madar, Z. Prolylcarboxypeptidase independently activates plasma prekallikrein (Fletcher Factor). *Curr. Mol. Med.* **2014**, *14*, 1173–1185.
- (186) Kolte, D.; Shariat-Madar, Z. Plasma kallikrein inhibitors in cardiovascular disease: An innovative therapeutic approach. *Cardiol. Rev.* **2016**, *24*, 99–109.
- (187) Girolami, A.; Scarparo, P.; Candeo, N.; Lombardi, A. M. Congenital prekallikrein deficiency. *Expert Rev. Hematol.* **2010**, *3*, 685–695.
- (188) Bird, J. E.; Smith, P. L.; Wang, X.; Schumacher, W. A.; Barbera, F.; Revelli, J. P.; Seiffert, D. Effects of plasma kallikrein deficiency on haemostasis and thrombosis in mice: murine ortholog of the Fletcher trait. *Thromb. Haemostasis* **2012**, *107*, 1141–1150.
- (189) Baeriswyl, V.; Rapley, H.; Pollaro, L.; Stace, C.; Teufel, D.; Walker, E.; Chen, S.; Winter, G.; Tite, J.; Heinis, C. Bicyclic peptides with optimized ring size inhibit human plasma kallikrein and its orthologues while sparing paralogous proteases. *ChemMedChem* **2012**, *7*, 1173–1176.
- (190) Zhao, B.; Xu, P.; Jiang, L.; Paaske, B.; Kromann-Hansen, T.; Jensen, J. K.; Sorensen, H. P.; Liu, Z.; Nielsen, J. T.; Christensen, A.; Hosseini, M.; Sorensen, K. K.; Nielsen, N. C.; Jensen, K. J.; Huang, M.; Andreasen, P. A. A cyclic peptidic serine protease inhibitor: increasing affinity by increasing peptide flexibility. *PLoS One* **2014**, *9*, e115872.
- (191) Tang, J.; Yu, C. L.; Williams, S. R.; Springman, E.; Jeffery, D.; Sprengeler, P. A.; Estevez, A.; Sampang, J.; Shrader, W.; Spencer, J.; Young, W.; McGrath, M.; Katz, B. A. Expression, crystallization, and three-dimensional structure of the catalytic domain of human plasma kallikrein. *J. Biol. Chem.* **2005**, *280*, 41077–41089.
- (192) Sturzebecher, J.; Svendsen, L.; Eichenberger, R.; Markwardt, F. A new assay for the determination of factor XII in plasma using a chromogenic substrate and a selective inhibitor of plasma kallikrein. *Thromb. Res.* **1989**, *55*, 709–715.
- (193) Van de Loch, A.; Dietrich, W.; Englberger, L.; Villiger, P.; Villiger, J. Methods of Treating or Preventing Blood Loss During Surgery Using the Serine Protease Inhibitor MDCO-2010. PCT Int. Pat. Appl. Publ. WO2013052736, 2013; The Medicines Company, U.S.
- (194) Kolte, D.; Bryant, J. W.; Gibson, G. W.; Wang, J.; Shariat-Madar, Z. PF-04886847 (an inhibitor of plasma kallikrein) attenuates inflammatory mediators and activation of blood coagulation in rat model of lipopolysaccharide (LPS)-induced sepsis. *Cardiovasc. Hematol. Agents Med. Chem.* **2012**, *10*, 154–166.
- (195) Fritz, H.; Wunderer, G. Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs. *Arzneim. Forsch.* **1983**, *33*, 479–494.
- (196) McMullan, V.; Alston, R. P. III. Aprotinin and cardiac surgery: a sorry tale of evidence misused. *Br. J. Anaesth.* **2013**, *110*, 675–678.
- (197) Stolz, L. E.; Horn, P. T. Ecallantide: a plasma kallikrein inhibitor for the treatment of acute attacks of hereditary angioedema. *Drugs Today* **2010**, *46*, 547–555.
- (198) Bokesch, P. M.; Szabo, G.; Wojdyga, R.; Grocott, H. P.; Smith, P. K.; Mazer, C. D.; Vetticaden, S.; Wheeler, A.; Levy, J. H. A phase 2 prospective, randomized, double-blind trial comparing the effects of tranexamic acid with ecallantide on blood loss from high-risk cardiac surgery with cardiopulmonary bypass (CONSERV-2 Trial). *J. Thorac. Cardiovasc. Surg.* **2012**, *143*, 1022–1029.
- (199) Englberger, L.; Dietrich, W.; Eberle, B.; Erdoes, G.; Keller, D.; Carrel, T. A novel blood-sparing agent in cardiac surgery? First in-patient experience with the synthetic serine protease inhibitor MDCO-2010: a phase II, randomized, double-blind, placebo-controlled study in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass. *Anesth. Analg.* **2014**, *119*, 16–25.
- (200) Faraoni, D.; Levy, J. H. Development of a novel blood-sparing agent in cardiac surgery: do we need another agent? *Anesth. Analg.* **2014**, *119*, 11–12.
- (201) Dietrich, W.; Nicklisch, S.; Koster, A.; Spannagl, M.; Giersiefen, H.; van de Loch, A. CU-2010-A novel small molecule protease inhibitor with antifibrinolytic and anticoagulant properties. *Anesthesiology* **2009**, *110*, 123–130.
- (202) Bahrami, B.; Zhu, M.; Hong, T.; Chang, A. Diabetic macular oedema: pathophysiology, management challenges and treatment resistance. *Diabetologia* **2016**, *59*, 1594–1608.
- (203) Kita, T.; Clermont, A. C.; Murugesan, N.; Zhou, Q.; Fujisawa, K.; Ishibashi, T.; Aiello, L. P.; Feener, E. P. Plasma kallikrein-kinin system as a VEGF-independent mediator of diabetic macular edema. *Diabetes* **2015**, *64*, 3588–3599.
- (204) [http://www.kalvista.com/assets/docs/press/11\\_KVP\\_01%20Series%20A%20Final1.pdf](http://www.kalvista.com/assets/docs/press/11_KVP_01%20Series%20A%20Final1.pdf) (accessed August 8, 2017).
- (205) Clermont, A.; Murugesan, N.; Zhou, Q.; Kita, T.; Robson, P. A.; Rushbrooke, L. J.; Evans, D. M.; Aiello, L. P.; Feener, E. P. Plasma kallikrein mediates vascular endothelial growth factor-induced retinal dysfunction and thickening. *Invest. Ophthalmol. Visual Sci.* **2016**, *57*, 2390–2399.
- (206) Cesarman-Maus, G.; Hajjar, K. A. Molecular mechanisms of fibrinolysis. *Br. J. Haematol.* **2005**, *129*, 307–321.
- (207) Ranucci, M. Hemostatic and thrombotic issues in cardiac surgery. *Semin. Thromb. Hemostasis* **2015**, *41*, 84–90.
- (208) Al-Horani, R. A.; Desai, U. R. Recent advances on plasmin inhibitors for the treatment of fibrinolysis-related disorders. *Med. Res. Rev.* **2014**, *34*, 1168–1216.
- (209) Parry, M. A. A.; Fernandez-Catalan, C.; Bergner, A.; Huber, R.; Hopfner, K.-P.; Schlott, B.; Guhrs, K.-H.; Bode, W. The ternary microplasmin-staphylokinase-microplasmin complex is a proteinase-cofactor-substrate complex in action. *Nat. Struct. Biol.* **1998**, *5*, 917–923.
- (210) Saupé, S. M.; Leubner, S.; Betz, M.; Klebe, G.; Steinmetzer, T. Development of new cyclic plasmin inhibitors with excellent potency and selectivity. *J. Med. Chem.* **2013**, *56*, 820–831.
- (211) Abdel Aziz, M. H.; Mosier, P. D.; Desai, U. R. Identification of the site of binding of sulfated, low molecular weight lignins on thrombin. *Biochem. Biophys. Res. Commun.* **2011**, *413*, 348–352.
- (212) Afosah, D. K.; Al-Horani, R. A.; Sankaranarayanan, N. V.; Desai, U. R. Potent, selective, allosteric inhibition of human plasmin by sulfated non-saccharide glycosaminoglycan mimetics. *J. Med. Chem.* **2017**, *60*, 641–657.
- (213) Parry, M. A. A.; Fernandez-Catalan, C.; Bergner, A.; Huber, R.; Hopfner, K. P.; Schlott, B.; Guhrs, K. H.; Bode, W. The ternary microplasmin-staphylokinase-microplasmin complex is a proteinase-cofactor-substrate complex in action. *Nat. Struct. Biol.* **1998**, *5*, 917–923.
- (214) Nicholson, A.; Berger, K.; Bohn, R.; Carcao, M.; Fischer, K.; Gringeri, A.; Hoots, K.; Mantovani, L.; Schramm, W.; van Hout, B. A.; Willan, A. R.; Feldman, B. M. Recommendations for reporting economic evaluations of haemophilia prophylaxis: a nominal groups consensus statement on behalf of the Economics Expert Working Group of The International Prophylaxis Study Group. *Haemophilia* **2008**, *14*, 127–132.
- (215) Murin, S.; Marelich, G. P.; Arroliga, A. C.; Matthay, R. A. Hereditary thrombophilia and venous thromboembolism. *Am. J. Respir. Crit. Care Med.* **1998**, *158*, 1369–1373.
- (216) Brummel-Ziedins, K. E.; Whelihan, M. F.; Rivard, G. E.; Butenas, S. Activated protein C inhibitor for correction of thrombin generation in hemophilia A blood and plasma. *J. Thromb. Haemostasis* **2011**, *9*, 2262–2267.
- (217) Butenas, S.; Orfeo, T.; Kalafatis, M.; Mann, K. G. Peptidomimetic inhibitors for activated protein C: implications for hemophilia management. *J. Thromb. Haemostasis* **2006**, *4*, 2411–2416.
- (218) De Nanteuil, G.; Gloanec, P.; Béguin, S.; Giesen, P. L. A.; Hemker, H. C.; Mennecier, P.; Rupin, A.; Verbeuren, T. J. Low

molecular weight activated protein C inhibitors as a potential treatment for hemophilic disorders. *J. Med. Chem.* **2006**, *49*, 5047–5050.

(219) Bach, P.; Knerr, L.; Fjellstrom, O.; Hansson, K.; Mattsson, C.; Gustafsson, D. Design, synthesis, and SAR of a series of activated protein C (APC) inhibitors with selectivity against thrombin for the treatment of haemophilia. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 821–827.

(220) Sperandio, O.; Wildhagen, K. C. A. A.; Schrijver, R.; Wielders, S.; Villoutreix, B. O.; Nicolaes, G. A. F. Identification of novel small molecule inhibitors of activated protein C. *Thromb. Res.* **2014**, *133*, 1105–1114.

(221) Mather, T.; Oganessyan, V.; Hof, P.; Huber, R.; Foundling, S.; Esmon, C.; Bode, W. The 2.8 Å crystal structure of Gla-domainless activated protein C. *EMBO J.* **1996**, *15*, 6822–6831.

(222) Fuchs, J. E.; Huber, R. G.; Waldner, B. J.; Kahler, U.; von Grafenstein, S.; Kramer, C.; Liedl, K. R. Dynamics govern specificity of a protein-protein interface: Substrate recognition by thrombin. *PLoS One* **2015**, *10*, e0140713.

(223) Gosalia, D. N.; Salisbury, C. M.; Ellman, J. A.; Diamond, S. L. High throughput substrate specificity profiling of serine and cysteine proteases using solution-phase fluorogenic peptide microarrays. *Mol. Cell. Proteomics* **2005**, *4*, 626–636.

(224) Ansell, J. E. Universal, class-specific and drug-specific reversal agents for the new oral anticoagulants. *J. Thromb. Thrombolysis* **2016**, *41*, 248–252.

(225) Hu, T. Y.; Vaidya, V. R.; Asirvatham, S. J. Reversing anticoagulant effects of novel oral anticoagulants: role of ciraparantag, andexanet alfa, and idarucizumab. *Vasc. Health Risk Manage.* **2016**, *12*, 35–44.

(226) Christos, S.; Naples, R. Anticoagulation reversal and treatment strategies in major bleeding: Update 2016. *West. J. Emerg. Med.* **2016**, *17*, 264–270.

(227) Pollack, C. V.; Reilly, P. A.; Eikelboom, J.; Glund, S.; Verhamme, P.; Bernstein, R. A.; Dubiel, R.; Huisman, M. V.; Hylek, E. M.; Kamphuisen, P. W.; Kreuzer, J.; Levy, J. H.; Sellke, F. W.; Stangier, J.; Steiner, T.; Wang, B.; Kam, C.-W.; Weitz, J. I. Idarucizumab for dabigatran reversal. *N. Engl. J. Med.* **2015**, *373*, 511–520.

(228) Lu, G.; DeGuzman, F. R.; Hollenbach, S. J.; Karbarz, M. J.; Abe, K.; Lee, G.; Luan, P.; Hutchaleelaha, A.; Inagaki, M.; Conley, P. B.; Phillips, D. R.; Sinha, U. A specific antidote for reversal of anticoagulation by direct and indirect inhibitors of coagulation factor Xa. *Nat. Med.* **2013**, *19*, 446–451.

(229) Connolly, S. J.; Milling, T. J. J.; Eikelboom, J. W.; Gibson, C. M.; Curnutte, J. T.; Gold, A.; Bronson, M. D.; Lu, G.; Conley, P. B.; Verhamme, P.; Schmidt, J.; Middeldorp, S.; Cohen, A. T.; Beyer-Westendorf, J.; Albaladejo, P.; Lopez-Sendon, J.; Goodman, S.; Leeds, J.; Wiens, B. L.; Siegal, D. M.; Zotova, E.; Meeks, B.; Nakamya, J.; Lim, W. T.; Crowther, M. Andexanet alfa for acute major bleeding associated with factor Xa inhibitors. *N. Engl. J. Med.* **2016**, *375*, 1131–1141.

(230) Eisenberg, D.; Schwarz, E.; Komaromy, M.; Wall, R. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* **1984**, *179*, 125–142.

(231) Baker, N. A.; Sept, D.; Joseph, S.; Holst, M. J.; McCammon, J. A. Electrostatics of nanosystems: Application to microtubules and the ribosome. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 10037–10041.

(232) Bajaj, S. P.; Schmidt, A. E.; Agah, S.; Bajaj, M. S.; Padmanabhan, K. High resolution structures of *p*-aminobenzamidine- and benzamidine-VIIa/soluble tissue factor: unpredicted conformation of the 192–193 peptide bond and mapping of Ca<sup>2+</sup>, Mg<sup>2+</sup>, Na<sup>+</sup>, and Zn<sup>2+</sup> sites in factor VIIa. *J. Biol. Chem.* **2006**, *281*, 24873–24888.

(233) Li, Z.; Partridge, J.; Silva-Garcia, A.; Rademacher, P.; Betz, A.; Xu, Q.; Sham, H.; Hu, Y.; Shan, Y.; Liu, B.; Zhang, Y.; Shi, H.; Xu, Q.; Ma, X.; Zhang, L. Structure-guided design of novel, potent, and selective macrocyclic plasma kallikrein inhibitors. *ACS Med. Chem. Lett.* **2017**, *8*, 185–190.