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Preclinical discovery of apixaban, a direct and orally bioavailable factor Xa inhibitor

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Abstract Apixaban (BMS-562247; 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-*c*]pyridine-3-carboxamide), a direct inhibitor of activated factor X (FXa), is in development for the prevention and treatment of various thromboembolic diseases. With an inhibitory constant of 0.08 nM for human FXa, apixaban has greater than 30,000-fold selectivity for FXa over other human coagulation proteases. It produces a rapid onset of inhibition of FXa with association rate constant of 20 $\mu M^{-1}/s$ approximately and inhibits free as well as prothrombinase- and clot-bound FXa activity in vitro. Apixaban also inhibits FXa from rabbits, rats and dogs, an activity which parallels its antithrombotic potency in these species. Although apixaban has no direct effects on platelet aggregation, it indirectly inhibits this process by reducing thrombin generation. Pre-clinical studies of apixaban in animal models have demonstrated dose-dependent antithrombotic efficacy at doses that preserved hemostasis. Apixaban improves pre-clinical antithrombotic activity, without excessive increases in bleeding times, when added on top of aspirin or aspirin plus clopidogrel at their clinically relevant doses. Apixaban has good bioavailability, low clearance and a small volume of distribution in animals and humans, and a low potential for drug–drug interactions. Elimination pathways for apixaban include renal excretion, metabolism and biliary/intestinal excretion. Although a sulfate conjugate of *O*-demethyl apixaban (*O*-demethyl apixaban sulfate) has been identified as the major circulating metabolite of apixaban in humans, it is inactive against human FXa. Together, these non-clinical findings have established the favorable pharmacological profile of apixaban, and support the potential use of apixaban in the clinic for the prevention and treatment of various thromboembolic diseases.

Keywords Apixaban · Factor Xa · Anticoagulants · Thrombosis · Atrial fibrillation

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Introduction

Thrombosis is a major cause of morbidity and mortality in the Western world and plays a pivotal role in the pathogenesis of numerous cardiovascular disorders, including acute coronary syndrome (ACS) (i.e. unstable angina and myocardial infarction), sudden cardiac death, peripheral arterial occlusion, ischemic stroke, deep vein thrombosis (DVT) and pulmonary embolism. Despite recent advances in interventional and drug therapy for thrombosis, the burden of thrombotic disease remains unacceptably high [1, 2]. There is therefore a significant need for new antithrombotic therapies that are more effective and provide improved safety profile compared with current treatments. This review focuses on the pre-clinical discovery of apixaban, a promising new oral antithrombotic agent that specifically targets activated factor X (FXa) of the blood coagulation cascade.

Drug discovery strategy—targeting factor Xa

As the last serine protease in the blood coagulation cascade, thrombin is the key enzyme responsible for physiological fibrin clot formation and platelet activation. Thrombin also plays a prominent role in the pathologic generation of occlusive thrombi in arteries or veins, a process that may lead to arterial or venous thrombotic disease. Thus, attenuation of the activity of thrombin—either via direct inhibition or via blockade of other proteases that lie upstream in the coagulation cascade and are intimately involved in thrombin generation (e.g. FXa)—has been intensively investigated as a novel means to prevent and treat thrombotic disease.

Three key observations supported our hypothesis that inhibition of FXa may represent an acceptable approach for effective and safe antithrombotic therapy. First, as the process of blood coagulation involves sequential activation and amplification of coagulation proteins, generation of one molecule of FXa can lead to the activation of hundreds of thrombin molecules [3]. In principle, therefore, inhibition of FXa may represent a more efficient way of reducing fibrin clot formation than direct inhibition of thrombin activity. This principle is consistent with an in vitro observation, suggesting that inhibition of FXa but not thrombin may result in a more effective sustained reduction of thrombus-associated procoagulant activity [4]. Second, inhibition of FXa is not thought to affect existing levels of thrombin. Further, reversible FXa inhibitors might not completely suppress the production of thrombin. These small amounts of thrombin might be sufficient to activate high affinity platelet thrombin receptors to permit physiological regulation of hemostasis. Indeed, experimental evidence from animal studies suggests that the antithrombotic efficacy of FXa inhibitors is accompanied by a lower risk of bleeding when compared with thrombin inhibitors [5–7] (for review, see Hauptmann and Stürzebecher [8] and Leadley [9]). Finally, the strongest evidence for FXa as an antithrombotic drug target is the clinical proof of concept studies of the indirect FXa inhibitor fondaparinux [10]. Taken together, these observations suggest that inhibition of FXa is a potentially attractive antithrombotic strategy.

We initiated a drug discovery program on small-molecule direct FXa inhibitors, with the goal of identifying novel oral anticoagulants not burdened by the well-known limitations of vitamin K antagonists such as warfarin, agents that remain the only oral anticoagulants approved for long-term use until very recently [11]. (On October 19, 2010, FDA approved the oral direct thrombin inhibitor dabigatran etexilate to prevent stroke and blood clots in patients with non-valvular atrial fibrillation [12].) These new FXa inhibitors would have the following target profile. First, they would be direct, highly selective and reversible inhibitors of FXa, with a rapid onset of action, and would demonstrate a relatively wide therapeutic index and few food and drug interactions (thereby avoiding the need for frequent coagulation monitoring and dose adjustment). Second, these FXa inhibitors would have predictable pharmacokinetic and pharmacodynamic profiles that allow fixed oral dosing, accompanied by low peak-to-trough plasma concentrations that provide high levels of efficacy and low rates of bleeding. Finally, as the FXa target resides in the central or blood compartment, the pharmacokinetic profile of these agents would also feature a low volume of distribution (to minimize off-target risks) and low systemic clearance (to reduce the potential for drug-drug interactions).

Based on many years of research and development, we have identified the potent, highly selective and direct FXa inhibitor, apixaban (BMS-562247) [13–15]. Apixaban is one of the most promising specific, single-target oral anticoagulants in late clinical development. In clinical trials, apixaban has been shown to provide predictable and consistent anticoagulation, accompanied by promising efficacy and safety profiles in the prevention and treatment of various thromboembolic diseases [16–22]. The pharmacological and clinical profiles of apixaban suggest that it has the potential to address many of the limitations of warfarin therapy, currently the standard of care in chronic oral anticoagulation. In this review, we summarize the chemistry and pre-clinical profile of apixaban.

Chemistry

Apixaban is a small-molecule, selective FXa inhibitor. It is chemically described as 1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl)phenyl]-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-*c*]pyridine-3-carboxamide. The molecular formula for apixaban is $C_{25}H_{25}N_5O_4$, which corresponds to a molecular weight of 459.5.

Discovery of apixaban

In the early 1990s, DuPont scientists invested a great amount of effort in the development of inhibitors of glycoprotein IIb/IIIa. These efforts resulted in several compounds that were advanced to clinical trials as potential anti-platelet agents. By the mid-1990s, scientists at DuPont had recognized similarities between the platelet glycoprotein GPIIb/IIIa peptide sequence Arg-Gly-Asp (RGD) and the prothrombin substrate FXa sequence, Glu-Gly-Arg (EGR). Consequently, a high-throughput lead evaluation program was initiated to screen the IIb/IIIa library for FXa inhibitory activity. This effort resulted in the identification

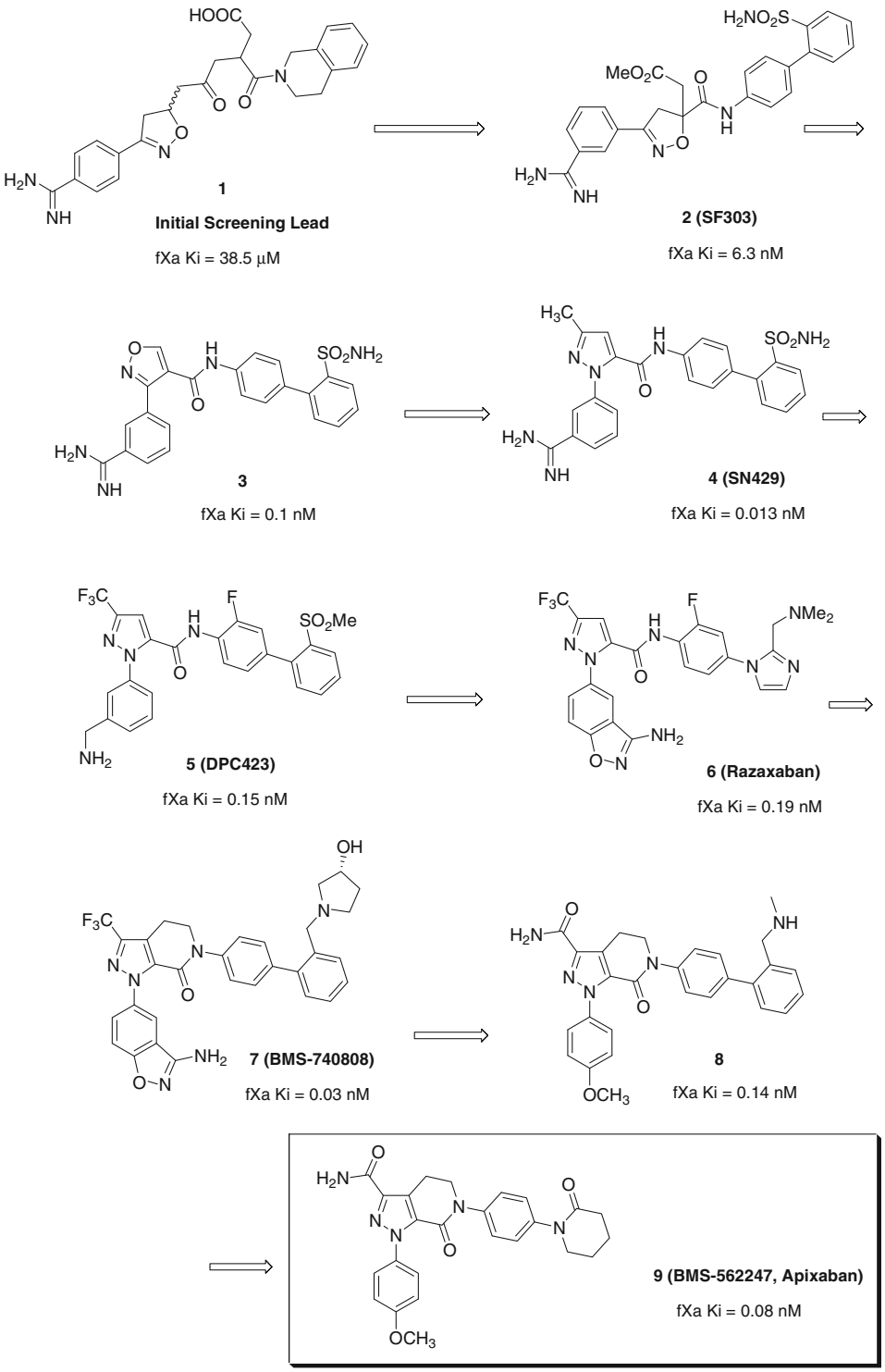

Fig. 1 The evolution of the pyrazole-based FXa inhibitors: the discovery of apixaban

of a small number of isoxazoline derivatives such as **1** (FXa K_i = 38.5 μM) (Fig. 1) [23]. Using molecular modeling and structure-based design, an optimization strategy resulted in the identification of a benzamidine containing FXa inhibitor **2** (SF303) with enhanced potency (FXa K_i = 6.3 nM) and potent antithrombotic activity in an experimental model of thrombosis [24–26]. Aside from the key amidine P1 and the enzyme Asp189 interaction, the biarylsulfonamide P4 moiety was designed to neatly stack in the S4 hydrophobic box of FXa, which contains the residues Tyr99, Phe174 and Trp215, with the terminal *O*-phenylsulfonamide ring making an edge-to-face interaction with Trp215. Subsequent re-optimizations led to vicinally substituted isoxazole analogs such as compound

3, which retained anti-FXa potency (FXa $K_i = 0.1$ nM) [27] and a pyrazole analog **4** (SN429), which demonstrated 13 pM binding affinity against FXa and good antithrombotic activity in a rabbit model of thrombosis [28, 29]. The discovery of SN429 was tremendously important in that it set the stage for an optimization strategy that led to the discovery of several important compounds, such as **5** (DPC423), a phase I clinical candidate with a long terminal half-life of approximately 30 h in humans [6, 28–30], and **6** (razaxaban) [31, 32], a compound that was advanced to a phase II proof-of-principle clinical trial. In fact, razaxaban was the first small molecule FXa inhibitor to provide clinical validation of the effectiveness of FXa inhibition strategies [33].

Development of razaxaban was quickly followed by the identification of a novel bicyclic tetrahydropyrazolo-pyridinone analog **7** (BMS-740808, FXa $K_i = 0.03$ nM) [34]. The evolution of the bicyclic pyrazole template allowed for the incorporation of a diverse set of P1 groups, the most important of which was the *p*-methoxyphenyl analog **8** ($K_i = 0.14$ nM) [13]. Compound **8** retained potent FXa affinity and good anticoagulant activity in vitro, was efficacious in in vivo rabbit antithrombotic models and showed high oral bioavailability in dogs. A significant breakthrough was subsequently achieved, via the incorporation of a pendent P4 lactam group and a carboxamido pyrazole moiety, that led to the discovery of **9** (BMS-562247, FXa $K_i = 0.08$ nM) [13], a highly potent and selective FXa inhibitor with good efficacy in various animal models of thrombosis. Importantly, compound **9** also showed an excellent pharmacokinetic profile in dogs, with low clearance, low volume of distribution and high oral bioavailability [13]. The superior pre-clinical profile demonstrated by **9** enabled its rapid progression into clinical development as apixaban [15]. Figure 2 illustrates the X-ray structure of apixaban bound to FXa and shows the *p*-methoxyphenyl P1 deeply inserted into the S1 pocket, with the aryllactam P4 moiety neatly stacked in the hydrophobic S4 pocket.

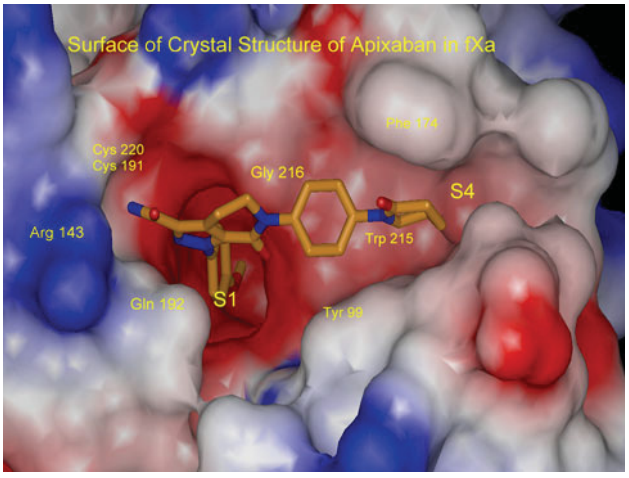

Fig. 2 X-ray structure of apixaban bound to factor Xa

In vitro pharmacology

Potency, selectivity and kinetic mode of inhibition

Apixaban is a highly potent, reversible, active-site inhibitor of human FXa, with a K_i of 0.08 nM at 25°C and 0.25 nM at 37°C in the FXa tripeptide substrate (*N*-α-benzyloxycarbonyl-D-Arg-Gly-Arg-pNA) assay [35]. Analysis of enzyme kinetics shows that apixaban acts as a competitive inhibitor of FXa versus the synthetic tripeptide substrate, indicating that it binds in the active site. Apixaban produces a rapid onset of inhibition under a variety of conditions with association rate constant of 20 ($\mu M^{-1}/s$ approximately, and shows competitive inhibition of FXa versus the synthetic tripeptide substrate. Reversibility of FXa inhibition is demonstrated by the recovery of FXa activity at 37°C upon 200-fold dilution of a pre-formed FXa:apixaban complex into tripeptide substrate, an effect associated with a dissociation rate constant of $\sim 0.0113\ s^{-1}$. Unlike indirect inhibitors of thrombin and FXa, such as heparin, the low molecular weight heparins and fondaparinux, apixaban, a direct FXa inhibitor, does not require the presence of antithrombin III to inhibit FXa. As shown in Table 1, apixaban has greater than 30,000-fold selectivity for FXa relative to other human coagulation proteases and structurally related enzymes involved in digestion and fibrinolysis [13].

In the prothrombinase assay, apixaban is an effective inhibitor of the action of human FXa on its physiological substrate, prothrombin, blocking the action of FXa on prothrombin within the prothrombinase complex with a K_i

Table 1 In vitro K_i values for inhibition of human enzymes by apixaban at 25°C [13]

Enzyme	K_i (nM)
Factor Xa	0.08 ± 0.03
Activated protein C	>30,000
Chymotrypsin	3,500
Factor IXa	>15,000
Factor VIIa	>15,000
Plasma kallikrein	3,700
Plasmin	>25,000
Thrombin	3,100
Tissue plasminogen activator	>40,000
Trypsin	>20,000

Table 2 In vitro potency (K_i) of apixaban against human, rabbit, rat and dog factor Xa (FXa) and the concentrations required to double ($EC_{2\times}$) the prothrombin time (PT), modified prothrombin time (mPT), activated partial thromboplastin time (aPTT) and HepTest in human, rabbit, rat or dog plasma [15]

Species	FXa K_i (nM)	PT $EC_{2\times}$ (μM)	mPT $EC_{2\times}$ (μM)	aPTT $EC_{2\times}$ (μM)	HepTest $EC_{2\times}$ (μM)
Human	0.081 ± 0.002	3.6	0.37	7.4	0.4
Rabbit	0.16 ± 0.01	2.3	0.6	4.8	1.8
Rat	1.3 ± 0.1	7.9	n.d.	20	n.d.
Dog	1.7 ± 0.2	6.7	n.d.	>20	n.d.

n.d. not determined

of 0.62 nM [35]. It should be noted that when apixaban was evaluated as an inhibitor of FXa versus the physiological substrate prothrombin in its prothrombinase state, non-competitive inhibition was observed. This finding is consistent with prothrombin binding being dictated primarily by interactions at exosites of FXa [36]. Apixaban also inhibits thrombus-associated FXa activity with a concentration causing 50% inhibition (IC_{50}) of 1.3 nM [37]. In summary, apixaban is capable of inhibiting the activity of free FXa, thrombus-associated FXa and FXa within the prothrombinase complex. Apixaban is a direct inhibitor of FXa from rats, rabbits and dogs, with K_i values of 1.3, 0.16 and 1.7 nM, respectively (Table 2 [15]). Previous studies involving other small molecule, direct FXa inhibitors have also reported a species difference in FXa inhibition among humans, rabbits, rats and dogs [29, 38, 39].

In vitro pharmacodynamic studies

To evaluate the in vitro pharmacodynamic activity of apixaban in human plasma, studies were undertaken to examine [1] thrombin generation, [2] anticoagulant activity and [3] platelet aggregation. By inhibiting FXa, apixaban prevents the conversion of prothrombin to thrombin, resulting in decreased generation of thrombin. Using the thrombogram method, apixaban was shown to inhibit tissue factor-initiated thrombin generation in human platelet-poor plasma in vitro. The IC_{50} of the rate of thrombin generation was 50 nM, and the IC_{50} for attenuation of the peak thrombin concentration was 100 nM [40]. In human platelet-rich plasma, apixaban inhibited tissue factor-induced thrombin generation, as measured by the release of prothrombin fragment 1 + 2, with an IC_{50} of 37 nM [41].

As expected for an inhibitor of FXa, addition of apixaban to normal human plasma prolonged clotting times, including activated partial thromboplastin time (aPTT), prothrombin time (PT), modified PT (mPT, using diluted PT reagent) and HepTest. Among the three clotting time assays, it appears that the mPT and HepTest are 10–20 times more sensitive than aPTT and PT in monitoring the in vitro anticoagulant effect of apixaban in human plasma (Table 2 [15]). In both the PT and aPTT assays, apixaban had the highest potency in human and rabbit plasma, but was less potent in rat and dog plasma, which parallels its inhibitory potencies (K_i) against human, rabbit, rat and dog FXa (Table 2 [15]).

In the human platelet aggregation assay, apixaban had no direct effects on platelet aggregation response to ADP, collagen, γ-thrombin, α-thrombin and TRAP [15, 41]. However, it indirectly inhibited platelet aggregation induced by thrombin derived from tissue factor-mediated coagulation pathway, with an IC_{50} of 4 nM [41]. The potent indirect antiplatelet effect of apixaban, together with its direct antithrombotic and anticoagulant activity, suggests that apixaban may possess dual mechanisms to prevent and treat both venous (platelet-poor and fibrin-rich) and arterial (platelet-rich and fibrin-poor) thrombosis. It should be noted that the in vitro tissue factor model of platelet aggregation is a useful tool for evaluation of the antiplatelet mechanisms of action of anticoagulants. However, caution should be exercised as in vitro antiplatelet potencies of compounds obtained in this model may not directly translate into antithrombotic potencies in patients in whom multiple prothrombotic mechanisms, complications of cardiovascular disease and polypharmacy are common.

In vivo pharmacology

The non-clinical pharmacology of apixaban has been studied in vivo in rats and rabbits. Its in vivo effects were assessed over a comprehensive dose range in various well-established non-clinical models of thrombosis and hemostasis. These non-clinical models have been well characterized with standard antiplatelet agents and anticoagulants, making them appropriate for evaluating the antithrombotic potential and bleeding liability of apixaban.

Antithrombotic and bleeding time effects in rats

Dose-dependent effects of apixaban were examined in a broad range of experimental models of thrombosis and hemostasis in rats [42]. Efficacy was evaluated using established models of thrombosis, including arterial-venous

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