Journal of Thrombosis and Haemostasis, **6**: 820–829 DOI: 10.1111/j.1538-7836.2008.02939.x

ORIGINAL ARTICLE

Apixaban, an oral, direct and highly selective factor Xa inhibitor: *in vitro*, antithrombotic and antihemostatic studies

P. C. WONG, E. J. CRAIN, B. XIN, R. R. WEXLER, P. Y. S. LAM, D. J. PINTO, J. M. LUETTGEN and R. M. KNABB

Thrombosis Research, Bristol-Myers Squibb Company, Pennington, NJ, USA

To cite this article: Wong PC, Crain EJ, Xin B, Wexler RR, Lam PYS, Pinto DJ, Luettgen JM, Knabb RM. Apixaban, an oral, direct and highly selective factor Xa inhibitor: *in vitro*, antithrombotic and antihemostatic studies. *J Thromb Haemost* 2008; **6**: 820–9.

Summary. *Background*: Apixaban is an oral, direct and highly selective factor Xa (FXa) inhibitor in late-stage clinical development for the prevention and treatment of thromboembolic diseases. *Objective*: We evaluated the *in vitro* properties of apixaban and its *in vivo* activities in rabbit models of thrombosis and hemostasis. *Methods*: Studies were conducted in arteriovenous-shunt thrombosis (AVST), venous thrombosis (VT), electrically mediated carotid arterial thrombosis (ECAT) and cuticle bleeding time (BT) models. *Results*: *In vitro*, apixaban is potent and selective, with a K_i of 0.08 nM for human FXa. It exhibited species difference in FXa inhibition [FXa K_i (nM): 0.16, rabbit; 1.3, rat; 1.7, dog] and anticoagulation [$EC_{2\times}$ (μM, concentration required to double the prothrombin time): 3.6, human; 2.3, rabbit; 7.9, rat; 6.7, dog]. Apixaban at 10 μM did not alter human and rabbit platelet aggregation to ADP, γ-thrombin, and collagen. *In vivo*, the values for antithrombotic ED_{50} (dose that reduced thrombus weight or increased blood flow by 50% of the control) in AVST, VT and ECAT and the values for BT $ED_{3\times}$ (dose that increased BT by 3-fold) were 0.27 ± 0.03, 0.11 ± 0.03, 0.07 ± 0.02 and > 3 mg kg^{-1} h^{-1} i.v. for apixaban, 0.05 ± 0.01, 0.05 ± 0.01, 0.27 ± 0.08 and > 3 mg kg^{-1} h^{-1} i.v. for the indirect FXa inhibitor fondaparinux, and 0.53 ± 0.04, 0.27 ± 0.01, 0.08 ± 0.01 and 0.70 ± 0.07 mg kg^{-1} day^{-1} p.o. for the oral anticoagulant warfarin, respectively. *Conclusions*: In summary, apixaban was effective in the prevention of experimental thrombosis at doses that preserve hemostasis in rabbits.

Correspondence: Pancras C. Wong, Bristol-Myers Squibb Company, 311 Pennington-Rocky Hill Road, Pennington, NJ 08534, USA.
Tel.: +1 609 818 5572; fax: +1 609 818 7877.
E-mail: pancras.wong@bms.com

Presented in part at the 48th Annual Meeting of the American Society of Hematology, 9–12 December, 2006, Orlando, Florida (abstract 917) and at the XXI International Society on Thrombosis and Haemostasis Congress, 6–12 July 2007, Geneva, Switzerland (abstract no. P-W-656).

Received 31 October 2007, accepted 15 February 2008

Keywords: apixaban, blood coagulation, direct factor Xa inhibitor, hemostasis, oral anticoagulant, thrombosis.

Introduction

Thrombosis is a major cause of morbidity and mortality in the Western world and contributes to cardiovascular disorders. Despite recent advances in interventional and medical therapy for the prevention and treatment of a wide variety of thromboembolic events, the morbidity and mortality rates are still high [1]. Thus, there is a real unmet medical need for safer and more effective antithrombotic therapies. As thrombin plays a key role in the generation of an occlusive thrombus, inhibiting thrombin activity and/or its generation becomes a major drug target in the search of novel antithrombotic agents.

Because thrombin is the last serine protease in the blood coagulation cascade that causes fibrin clot formation, the early research approach has been focused on the inhibition of thrombin activity by direct thrombin inhibitors [2]. Another approach is the inhibition of thrombin production by blocking the upstream proteases in the blood coagulation cascade, such as factor Xa (FXa). Experimental evidence suggests that FXa inhibitors have antithrombotic efficacy with lower bleeding risk in animals when compared with thrombin inhibitors [3,4]. Thus, inhibition of FXa appears to be a promising mechanism for anticoagulant therapy, which prompted us to initiate a drug discovery program on small-molecule, direct FXa inhibitors [5,6].

Based on years of research and development, we recently identified a direct FXa inhibitor, apixaban (BMS-562247), which is highly potent and selective with a K_i of 0.08 nM for human FXa and with greater than 30 000-fold selectivity over other coagulation proteases [7]. Preliminary studies show that apixaban has high oral bioavailability in rats, dogs, and humans [8,9]. It prevented venous thromboembolic events with a favorable efficacy and safety profile in patients after knee replacement surgery [10]. Apixaban was also shown to be efficacious and safe in the treatment of patients with acute symptomatic deep vein thrombosis [11]. Currently, apixaban is in late-stage clinical development for the prevention and treatment of thromboembolic diseases.

In this study, we characterized the *in vitro* properties of apixaban and its antithrombotic profile for the prevention of thrombosis in rabbit models of arteriovenous-shunt thrombosis (AVST), venous thrombosis (VT) and electrolytically mediated carotid arterial thrombosis (ECAT), in comparison with the indirect FXa inhibitor fondaparinux and the oral anticoagulant warfarin. Antihemostatic effects of these agents were also studied in a well-characterized rabbit cuticle bleeding time (BT) model.

Materials and methods

Reagents

The following drugs and chemicals were used in this study: chromogenic substrates S-2222, S-2238 and S-2765 from Chromogenix AB (distributed by DiaPharma Group, Inc., West Chester, OH, USA); activated partial thromboplastin time (APTT) reagent Alexin from Trinity Biotech (St Louis, MO, USA); prothrombin time (PT) reagents Thromboplastin C Plus from Dade-Behring (Deerfield, IL, USA) or Thromboplastin® with calcium from Sigma Chemical Co (St Louis, MO, USA); HepTest reagent from American Diagnostica (Stamford, CT, USA); human FXa from Haematologic Technologies (Essex Junction, VT, USA); sodium warfarin, ADP and collagen from Sigma Chemical Co.; human γ-thrombin from ICN Biomedicals, Inc. (Costa Mesa, CA, USA); fondaparinux (Arixtra®) from GlaxoSmithKline (Research Triangle Park, NC, USA). Apixaban and DMP802 were synthesized at Bristol-Myers Squibb Company.

Animals

Studies were conducted in male New Zealand White rabbits weighing about 2–4 kg obtained from Covance (Denver, PA, USA). Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the regulations of the Animal Care and Use Committee of the Bristol-Myers Squibb Company.

In vitro *and* ex vivo *studies*

Enzyme assays Using established protein purification procedures, FX was isolated from citrated plasma obtained from healthy dogs, rats, and rabbits [12]. Purified FXa was obtained after activation with Russell's viper venom followed by affinity chromatography. The resulting FXa was > 95% pure as judged by sodium dodecylsulfate polyacrylamide gel electrophoresis. The substrate affinity values for FXa, expressed as the Michaelis–Menten–Henri constant (K_m), for human, rabbit, rat and dog FXa were determined using the chromogenic substrate S-2765, and were 36, 60, 240 and 70 μM, respectively. The substrate hydrolysis was monitored by measuring absorbance at 405 nm at 25 °C for up to 30 min using a SpectraMax 384 Plus plate reader and SoftMax (Molecular Devices, Sunnyvale, CA, USA). FXa activity for each substrate and inhibitor concentration pair was determined in duplicate. The K_i values were calculated by non-linear least-squares fitting of the steady-state substrate hydrolysis rates to the equation for competitive inhibition (Equation 1) using GRAFIT (Erithacus Software Ltd., Surrey, UK), where v equals reactions velocity in OD min^{-1}, V_{max} equals maxiumum reaction velocity, S equals substrate concentration, and I equals inhibitor concentration.

$$v = \frac{V_{max} S}{K_m(1 + \frac{I}{K_i}) + S} \quad (1)$$

Clotting assays Blood samples were collected in tubes containing 1/10 volume of 3.2% sodium citrate, and platelet-poor plasma was obtained after centrifuging at > 2000 × *g* for 10 min. Clotting times were measured with an automated coagulation analyzer (Sysmex®, Dade Behring Inc., Deerfield, IL, USA). PT, APTT, and HepTest reagents were reconstituted and assays were performed according to the manufacturer's instructions. The modified PT (mPT) assay was performed by diluting 1 mL of thromboplastin C Plus with 1.25 mL of 100 mM calcium chloride and using this diluted reagent in place of the normal PT reagent. For PT and mPT, plasma (50 μL) was warmed to 37 °C for 3 min before adding PT reagent (100 μL). For APTT, plasma (50 μL) was warmed to 37 °C for 1 min before adding APTT reagent (50 μL). After two more minutes, 25 mM calcium chloride (50 μL) was added. For HepTest, plasma (50 μL) was warmed to 37 °C for 2 min before bovine FXa (50 μL) was added. After two more minutes, HepTest ReCal mix (50 μL) was added. Determinations were performed in duplicate and expressed as a mean ratio of treated vs. baseline control. The concentrations required to prolong clotting time by 2-fold ($EC_{2\times}$) were expressed as total plasma concentrations, not final assay concentrations after addition of clotting assay reagents. For *in vitro* studies, apixaban was serially diluted into citrated plasma obtained from healthy dogs, rats, and rabbits, beginning with a 10 mM dimethylsulfoxide stock solution.

Platelet aggregation assays Platelet aggregation was measured in citrated human and rabbit platelet-rich plasma (PRP) *in vitro* with a platelet aggregometer (Model PAP-4D, BioData, Horsham, PA, USA). PRP was obtained from citrated blood after centrifuging at 250 × *g* for 6 min. Citrated PRP (250 μL) was mixed with 20 μL of vehicle, DMP802 at 3 μM or apixaban at 1–10 μM, and incubated for 3 min at 37 °C. DMP802, a glycoprotein (GP)IIb/IIIa receptor antagonist, was included as a positive control (IC_{50} = 29 nM against human platelet aggregation response to 10 μM ADP) [13]. Peak platelet aggregation was determined after the addition of 20 μL of the agonist (ADP at 10 μM, γ-thrombin at 35 nM, and collagen at 10 μg mL^{-1}, final concentration).

Anti-FXa and antithrombin assays Anti-FXa and antithrombin activities were determined using the chromogenic substrates S-2222 and S-2238, respectively, based on the method of Sato *et al.* [14] with modifications

[15]. The hydrolysis of the chromogenic substrates was assayed by measuring absorbance at 405 nm at 37 °C as described above. Anti-FXa or antithrombin activity was calculated by comparing the values of optical density from samples taken in the post-treatment period to those taken in the pretreatment period.

In vivo *studies*

AVST model The rabbit AVST model, described by Wong *et al.* [16], was used in this study. Briefly, male New Zealand White rabbits were anesthetized with ketamine (50 mg kg^{-1} i.m.) and xylazine (10 mg kg^{-1} i.m.), and their femoral artery, jugular vein and femoral vein were catheterized. These anesthetics were supplemented as needed. Thrombosis was induced by an arteriovenous (AV)-shunt device containing a silk thread. Blood flowed from the femoral artery via the AV shunt into the opposite femoral vein for 40 min. The shunt was then disconnected and the silk thread covered with thrombus was weighed.

As apixaban has an oral bioavailability of < 5% in rabbits (unpublished result), it was administered intravenously for *in vivo* studies. To achieve a stable plasma level with minimum experimental variability, apixaban, fondaparinux or vehicle was given by a continuous intravenous infusion 1 h prior to shunt placement. The infusion was continued throughout the experiment. Warfarin or vehicle was dosed orally once daily for 4 days. On the fourth day after the last oral dose of warfarin or vehicle, rabbits were anesthetized 1.5 h later, and the treatment effect was evaluated about 2 h postdose. Arterial blood samples for the determination of clotting times or plasma levels were collected 20 min after shunt placement. Plasma levels of apixaban were measured by a specific and sensitive liquid chromatographic mass spectrometry method (LC/MS/MS). In rabbits treated with apixaban, fondaparinux or warfarin, the antithrombotic effects of these agents were expressed as percentage inhibition of thrombus formation based on the treated vs. the corresponding mean vehicle. The ED_{50} value (dose that produced 50% inhibition of thrombus formation) was determined as described below.

The apixaban group treatment consisted of vehicle (10% *N*,*N*-dimethylacetamide; 30% 1,2-propanediol; 60% water) ($n = 4$), and apixaban (mg kg^{-1} h^{-1}) at 0.03 ($n = 7$), 0.1 ($n = 7$), 0.3 ($n = 7$), 1 ($n = 7$), and 3 ($n = 3$). The fondaparinux group treatment consisted of vehicle (saline) ($n = 6$), and fondaparinux (mg kg^{-1} h^{-1}) at 0.01 ($n = 5$), 0.03 ($n = 5$), 0.1 ($n = 5$), 0.3 ($n = 5$), and 1 ($n = 5$). The warfarin group treatment consisted of vehicle (water) ($n = 6$), and warfarin (mg kg^{-1} day^{-1}) at 0.1 ($n = 6$), 0.3 ($n = 6$), 1 ($n = 6$), and 3 ($n = 6$).

VT model The rabbit VT model, described by Hollenbach *et al.* [17], was used in this study with modifications. Briefly, rabbits were anesthetized as above. The left femoral vein was catheterized, using 11-cm IntraMedic polyethylene tubing (PE-200; Becton Dickinson, Sparks, MD, USA). A prosthetic device was passed through the PE-200 tubing into the abdominal vena cava. The prosthetic device consisted of a single strand of #10 awg braided copper wire (14 cm) terminated with eight pieces of 4-0 silk threads 3 cm in length. The silk threads were positioned in the abdominal vena cava by advancing the copper guide wire. Thrombi were formed on the silk threads in a time-dependent fashion.

Apixaban and fondaparinux were given intravenously as described above 1 h prior to the placement of the prosthetic VT device. Warfarin or its vehicle was dosed orally once daily for 4 days in rabbits as described above, and the prosthetic VT device was placed 2 h after the last oral dose. Ninety minutes after the placement of the prosthetic device, the abdominal vena cava was isolated through a midline abdominal incision. The vena cava was ligated just above and below the prosthetic device with 2-0 silk. The vena cava segment between the ligations was excised, and the threads with associated thrombus were removed, blotted twice on paper, and weighed. The weight of thrombus formed on the threads was calculated by subtracting the average weight of eight pieces of 4-0 silk threads 3 cm in length. Clotting times of apixaban, fondaparinux and warfarin in plasma samples collected during VT were measured as above.

In the VT study, the apixaban group treatment consisted of vehicle (10% *N*,*N*-dimethylacetamide; 90% of 5% dextrose) ($n = 6$), and apixaban (mg kg^{-1} h^{-1}) at 0.03 ($n = 6$), 0.1 ($n = 6$), 0.3 ($n = 6$), and 1 ($n = 6$). The fondaparinux group treatment consisted of vehicle (saline) ($n = 6$), and fondaparinux (mg kg^{-1} h^{-1}) at 0.01 ($n = 6$), 0.03 ($n = 6$), 0.1 ($n = 6$), and 0.3 ($n = 6$). The warfarin group treatment consisted of vehicle (water) ($n = 6$), and warfarin (mg kg^{-1} day^{-1}) at 0.1 ($n = 5$), 0.3 ($n = 5$), 1 ($n = 5$), and 3 ($n = 6$). The ED_{50} value (dose that produced 50% inhibition of mean vehicle thrombus weight) was determined as described below.

Arterial thrombosis model The rabbit ECAT model, described by Wong *et al.* [15], was used in this study. Briefly, male New Zealand White rabbits were anesthetized as above. An electromagnetic flow probe was placed on a segment of an isolated carotid artery to monitor blood flow. Thrombosis was induced by electrical stimulation of the carotid artery for 3 min at 4 mA, using an external stainless-steel bipolar electrode. Carotid blood flow was measured continuously over a 90-min period to monitor thrombosis-induced occlusion. Integrated carotid blood flow over 90 min was measured by the area under the flow–time curve, calculated using the trapezoidal rule, and expressed as percentage of total control carotid blood flow, which would result if control blood flow had been maintained continuously for 90 min. The administration of apixaban and fondaparinux was initiated intravenously as described above 1 h prior to the artery injury. Warfarin or its vehicle was dosed orally once daily for 4 days in rabbits, and thrombosis was initiated 2 h after the last oral dose. Clotting times of apixaban, fondaparinux and warfarin, and

concentrations of apixaban in plasma samples, taken during electrically induced arterial thrombosis, were measured as above. In addition, we also measured *ex vivo* anti-FXa and antithrombin activities.

In this study, the apixaban group treatment consisted of vehicle (10% *N*,*N*-dimethylacetamide; 90% of 5% dextrose), and apixaban (mg kg^{-1} h^{-1}) at 0.01, 0.03, 0.1, 0.3, and 1 (n = 6 per group). The fondaparinux group treatment consisted of vehicle (saline), and fondaparinux (mg kg^{-1} h^{-1}) at 0.1, 0.3, 1, and 3 (n = 6 per group). The warfarin group treatment consisted of vehicle (water) (n = 6), and warfarin (mg kg^{-1} day^{-1}) at 0.03, 0.1, 0.3, 1, and 3 (n = 6 per group). The ED_{50} (dose that increased carotid blood flow to 50% of the control) of compounds and the EC_{50} (plasma concentration that increased carotid blood flow to 50% of the control) of apixaban were estimated as described below.

Cuticle bleeding model The rabbit cuticle BT model [4] was used in this study. Briefly, rabbits were anesthetized as described above. A standard cut was made at the apex of the cuticle with a razor blade. Blood was allowed to flow freely by keeping the bleeding site in contact with 37 °C lactated Ringer's solution. BT was defined as the time after transection when bleeding ceased. It was measured by averaging the bleeding time of three nail cuticles. The maximum bleeding recorded was 20 min. Apixaban, fondaparinux and warfarin were administered as described above. In rabbits treated with anticoagulants, the BT effect was expressed as a ratio of treated vs. the mean vehicle value. The $ED_{3\times}$ (dose that increased BT 3-fold) values of compounds were estimated as described below.

The apixaban group treatment consisted of vehicle (10% *N*,*N*-dimethylacetamide; 30% 1,2-propanediol; 60% water) (n = 6), and apixaban (mg kg^{-1} h^{-1}) at 1 (n = 6) and 3 (n = 6). The fondaparinux group treatment consisted of vehicle (saline) (n = 6), and fondaparinux (mg kg^{-1} h^{-1}) at 0.3 (n = 6), 1 (n = 6), and 3 (n = 6). The warfarin group treatment consisted of vehicle (water) (n = 6), and warfarin (mg kg^{-1}) at 0.1 (n = 5), 0.3 (n = 5), 1 (n = 5), and 3 (n = 5).

Statistical analysis

The statistical analyses used were analysis of variance and the Student–Newman–Keuls test using the SAS system (SAS for Windows release 8.02A; Cary, NC, USA). ED_{50} doses were determined using the four-parameter logistic equation, $y = A + [(B - A)/(1 + [(C/x)^{D}])]$, where A = minimum y value, B = maximum, C = log ED_{50} and D = slope factor, and the logistic fit was analyzed by XLfit© (IDBS, Bridgewater, NJ, USA). Antithrombotic ED_{50} values were determined using a maximum value of 100 and a minimum value of zero, whereas BT $ED_{3\times}$ values were determined using a maximum value (BT treated/BT control) of seven and a minimum value of one. A value of $P < 0.05$ was considered statistically significant. All data are means ± SE.

Results

In vitro *studies*

Enzyme assays The Lineweaver–Burk plot of inhibition of human FXa by apixaban indicates that apixaban is a competitive inhibitor vs. the chromogenic peptide substrate S-2765, with a K_i of 0.08 nM (Fig. 1). Apixaban also inhibited FXa from rats, dogs, and rabbits (Table 1). In terms of FXa K_i at 25 °C, apixaban has similar potency in inhibiting human and rabbit FXa, but is 10–20 times less potent against rat and dog FXa (Table 1).

Clotting assays As expected for an inhibitor of FXa, addition of apixaban to normal human plasma prolonged clotting times, including APTT, PT, mPT, 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 1). 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 (Table 1).

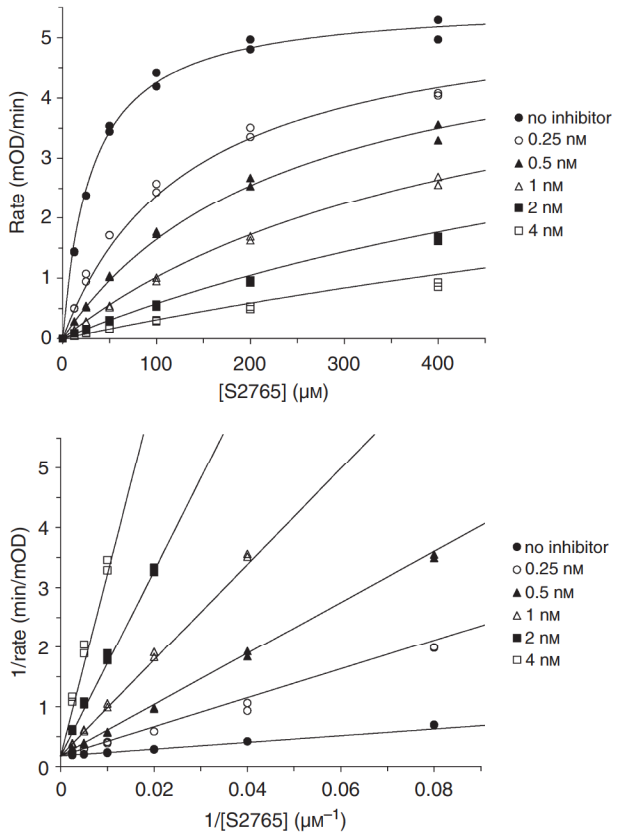

Fig. 1. Plot of apixaban inhibition of human FXa activity at different concentrations of the chromogenic peptide substrate S-2765. Top panel: Untransformed data. The solid lines are from non-linear fits of the data to the equation for competitive inhibition with K_i = 0.075 ± 0.0031 nM, S-2765 K_m = 31 ± 1.4 uM, and maximum rate = 5.6 ± 0.062 mOD min^{-1} (moD = OD × 1000 where OD is optical density). Bottom panel: Lineweaver–Burk plot of the data as in the top panel.

Table 1 *In vitro* potency (K_i) of apixaban against human, rabbit, rat and dog FXa and the concentrations ($EC_{2\times}$) required to double the prothrombin time (PT), modified prothrombin time (mPT), activated partial thromboplastin time (APTT) and HepTest in human, rabbit, dog and/or rat plasma

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	ND	20	ND
Dog	1.7 ± 0.2	6.7	ND	>20	ND

*Data from Pinto *et al.* [8]. ND, not determined.

Platelet aggregation *In vitro* platelet aggregation responses to ADP, γ-thrombin and collagen averaged 47 ± 5%, 53 ± 4% and 51 ± 5%, respectively in human PRP, and 50 ± 5%, 56 ± 5% and 60 ± 1%, respectively, in rabbit PRP. These platelet responses were not significantly changed by apixaban at 1, 3 and 10 μM, but were almost completely inhibited by the GPIIb/IIIa antagonist DMP802 at 3 μM (data not shown).

In vivo *studies*

AVST model Mean thrombus weights in the different vehicle-treated AVST rabbits were similar, and ranged from 290 ± 11 to 327 ± 15 mg (n = 6 per group). As shown in Fig. 2, apixaban, fondaparinux and warfarin were efficacious in the AVST rabbits and produced dose-dependent antithrombotic effects; their ED_{50} values are reported in Table 2. At their top doses studied in this model, apixaban at 3 mg kg^{-1} h^{-1} i.v., fondaparinux at 1 mg kg^{-1} h^{-1} i.v. and warfarin at 3 mg kg^{-1} day^{-1} p. o. reduced thrombus weight by 98%, 86% and 77%, respectively, relative to their corresponding vehicle group. We observed that apixaban at 0.03, 0.1, 0.3, 1 and 3 mg kg^{-1} h^{-1} i.v. produced linear dose-proportional increases in plasma levels of 34 ± 2, 121 ± 9, 490 ± 104, 1155 ± 153 and 3705 ± 525 nM, respectively (n = 3–7 per group). The EC_{50} for apixaban was estimated to be 357 ± 90 nM.

VT model Mean thrombus weights in the different vehicle-treated VT rabbits were similar, and ranged from 64 ± 2 to 79 ± 7 mg (n = 6 per group). In this model, apixaban, fondaparinux and warfarin produced dose-dependent antithrombotic effects (Fig. 2); their ED_{50} values are given in Table 2. At their top doses studied in this model, apixaban at 1 mg kg^{-1} h^{-1}, fondaparinux at 0.3 mg kg^{-1} h^{-1} and warfarin at 3 mg kg^{-1} day^{-1} reduced thrombus formation by 83%, 74% and 84%, respectively, relative to their corresponding vehicle group.

Arterial thrombosis model Figure 3 (top panel) shows the effects of vehicle and apixaban on carotid blood flow after electrical stimulation. Basal carotid blood flow in the vehicle-treated animals averaged 21 ± 4 mL min^{-1}. After the initiation of thrombosis, blood flow was gradually decreased, and the artery was totally occluded in about 35 min in vehicle-treated animals. Apixaban at 0.01–1 mg kg^{-1} h^{-1} i.v. produced

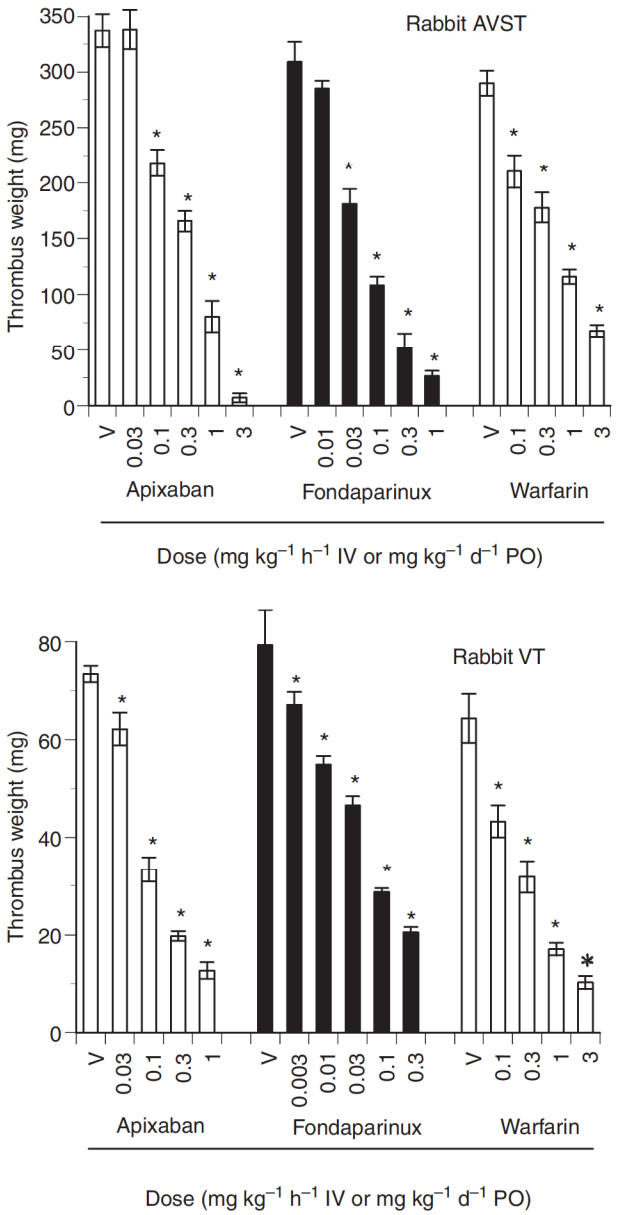

Fig. 2. Antithrombotic effects in the arteriovenous-shunt thrombosis (AVST) and venous thrombosis (VT) rabbit models. Top panel: Effects of apixaban, fondaparinux and warfarin on thrombus formation in AVST. Means ± SE, and n = 3–7 per group; *P < 0.05 vs. the corresponding vehicle. Preliminary AVST data for apixaban at 0.03–1 mg kg^{-1} h^{-1} were reported in Pinto *et al.* [7]. Bottom panel: Effects of apixaban, fondaparinux and warfarin on thrombus formation in VT. Mean ± SE, and n = 5–6 per group; *P < 0.05 vs. the corresponding vehicle.

Table 2 *In vivo* potency of apixaban, fondaparinux and warfarin in rabbit models of arteriovenous-shunt thrombosis (AVST), venous thrombosis (VT), electrically mediated carotid arterial thrombosis (ECAT) and bleeding time (BT)

Compound	AVST* $ED_{50}^{\dagger}$	VT $ED_{50}^{\dagger}$	ECAT $ED_{50}^{\dagger}$	BT $ED_{3\times}^{\dagger}$
Apixaban	0.27 ± 0.03	0.11 ± 0.03	0.07 ± 0.02	> 3
Fondaparinux	0.05 ± 0.01	0.05 ± 0.01	0.27 ± 0.08	> 3
Warfarin	0.53 ± 0.04	0.27 ± 0.01	0.08 ± 0.01	0.70 ± 0.07

Results are expressed as mean ± SE. *Preliminary AVST data for apixaban were reported in Pinto *et al.* [7]. †Expressed in mg kg^{-1} h^{-1} i.v. for apixaban and fondaparinux and in mg kg^{-1} day^{-1} p.o. for warfarin.

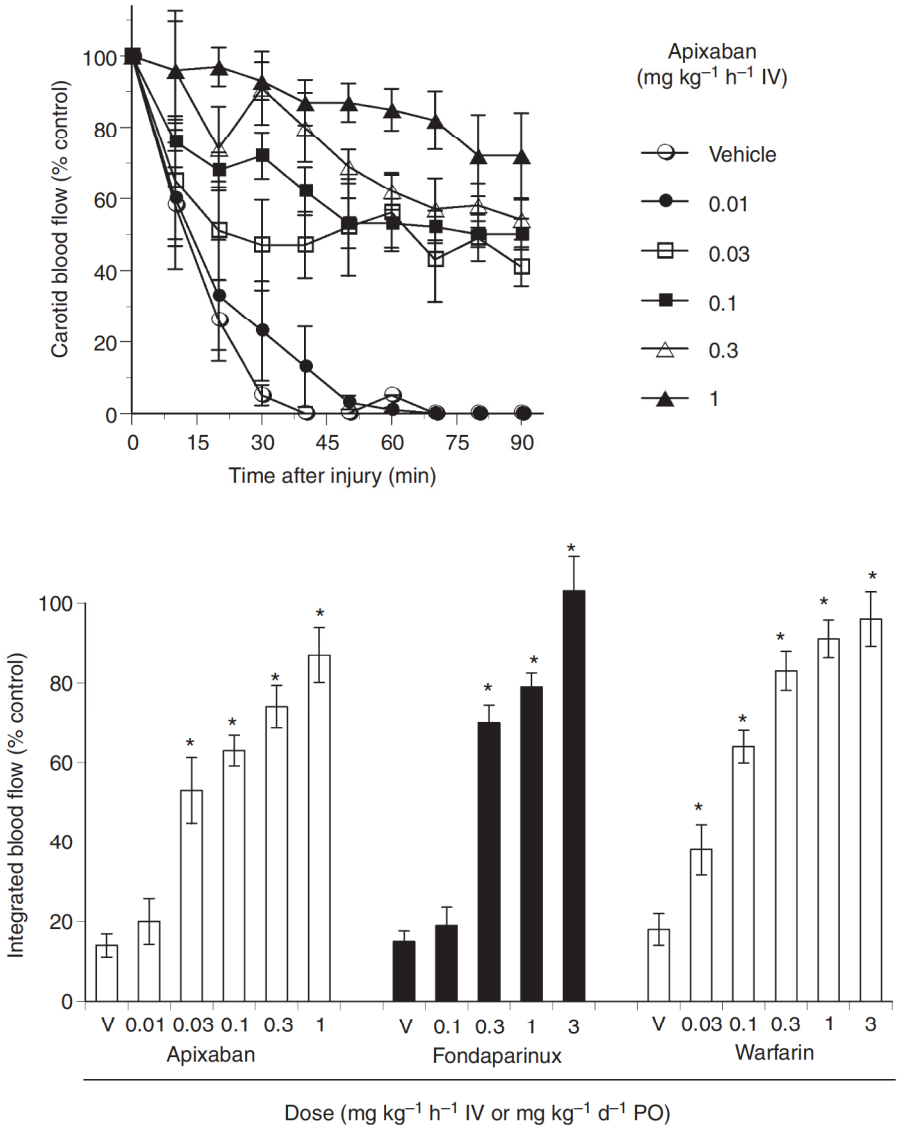

Fig. 3. Antithrombotic effects in the rabbit model of electrically mediated carotid arterial thrombosis. Top panel: Effects of vehicle and apixaban on carotid blood flow (expressed as % of control carotid blood flow) after artery injury. Means ± SE, and $n = 6$ per group. Bottom panel: Dose-dependent effects of apixaban, fondaparinux and warfarin on integrated blood flow. Mean ± SE, and $n = 6$ per group. *$P < 0.05$ vs. the corresponding vehicle.

a dose-dependent increase in duration of the patency of the injured artery. At 0.03–1 mg kg^{-1} h^{-1} i.v., there was no occlusion in any of the animals up to 90 min.

Figure 3 (bottom panel) also shows the effects of apixaban, fondaparinux and warfarin on integrated blood flow (ED_{50} values are given in Table 2). We observed that apixaban at

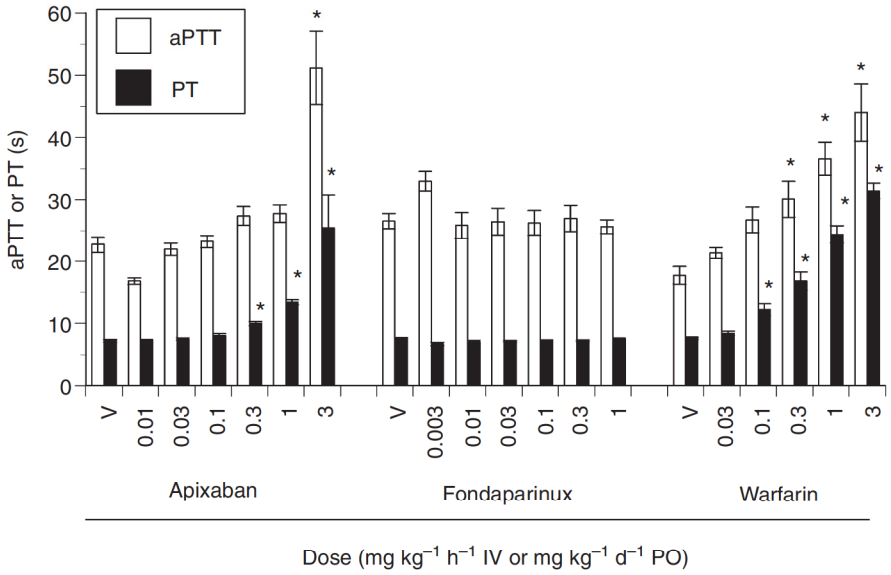

Fig. 4. *Ex vivo* activated partial thromboplastin time (APTT) and prothrombin time (PT) effects of apixaban, fondaparinux and warfarin in rabbits. Means ± SE, and n = 3–19 per group. *P < 0.05 vs. the corresponding vehicle.

0.01, 0.03, 0.1, 0.3 and 1 mg kg^{-1} h^{-1} i.v. produced linear dose-proportional increases in plasma levels of 13 ± 1, 45 ± 4, 146 ± 8, 435 ± 25 and 1495 ± 50 nM, respectively (n = 6 per group). The integrated blood flow EC_{50} for apixaban was estimated to be 106 ± 31 nM.

Ex vivo *coagulation markers* Figure 4 shows the summary of the *ex vivo* APTT and PT responses to apixaban, fondaparinux and warfarin obtained from the AVST, VT and ECAT studies. Apixaban significantly prolonged *ex vivo* APTT at 3 mg kg^{-1} h^{-1}, and PT at doses of 0.3 mg kg^{-1} h^{-1} and higher (Fig. 4). Fondaparinux at the doses studied did not have significant effects on *ex vivo* APTT and PT. Warfarin significantly prolonged *ex vivo* APTT at doses of 0.3 mg kg^{-1} day^{-1} and higher, and PT at doses of 0.1 mg kg^{-1} day^{-1} and higher (Fig. 4).

Figure 5 (top panel) shows the *ex vivo* effect of apixaban on FXa and thrombin activity. Apixaban produced a dose-dependent inhibition of *ex vivo* FXa activity and did not change *ex vivo* thrombin activity. A good correlation was observed between the antithrombotic effect of apixaban and its *ex vivo* anti-FXa activity (r^2 = 0.94), and also between the plasma concentrations and *ex vivo* anti-FXa activity (r^2 = 0.90).

Cuticle bleeding time model Mean cuticle BT in the vehicle-treated groups of apixaban, fondaparinux and warfarin were 172 ± 2, 181 ± 7 and 183 ± 7 s, respectively (n = 6 per group). Warfarin at 0.1, 0.3, 1 and 3 mg kg^{-1} day^{-1} p.o. produced dose-dependent increases in BT (228 ± 14, 371 ± 24, 929 ± 70 and 1129 ± 43 s, respectively), with an $ED_{3\times}$ of 0.70 mg kg^{-1} day^{-1} (Table 2). As compared to vehicle, warfarin at 1 and 3 mg kg^{-1} day^{-1} p.o. increased BT significantly by 5.1-fold and 6.2-fold, respectively (P < 0.05). In contrast, fondaparinux and apixaban at antithrombotic doses increased BT slightly (fondaparinux, 166 ± 4, 210 ± 12 and 213 ± 11 s at 0.3, 1 and 3 mg kg^{-1} h^{-1} i.v., respectively; apixaban, 191 ± 8 and 228 ± 14 s at 1 and 3 mg kg^{-1} h^{-1} i.v., respectively). At 3 mg kg^{-1} h^{-1} i.v., fondaparinux and apixaban increased BT by 1.3-fold and 1.2-fold relative to their vehicle, respectively with $ED_{3\times}$ of > 3 mg kg^{-1} h^{-1} for both compounds (Table 2).

Discussion

This study shows that apixaban is a potent, highly selective and direct inhibitor of FXa. It is also very efficacious for the prevention of arterial and venous thrombosis at doses that preserve hemostasis in rabbits.

Analysis of enzyme kinetics shows that apixaban is a direct and competitive inhibitor of free human FXa vs. a synthetic tripeptide substrate and does not need antithrombin III for its activity. It should be noted that apixaban is likely to behave as a mixed-type inhibitor of FXa activation of prothrombin in blood [18]. In addition, apixaban inhibited the prothrombinase-bound FXa activity *in vitro*, which resulted in the reduction of the conversion of prothrombin to thrombin with potency in the nanomolar range [18,19]. Together, these *in vitro* studies suggest that apixaban is a potent, selective, direct and effective inhibitor of both free and prothrombinase-bound FXa.

Previously, we, as well as others, reported a species difference in FXa inhibition in humans, rabbits, rats and dogs with small-molecule, direct FXa inhibitors [6,20,21]. This study extends these findings, and shows that apixaban was more potent at prolonging APTT and PT in human and rabbit plasma than in rat and dog plasma, which parallels its inhibitory potencies against human, rabbit, rat and dog FXa. Interestingly, apixaban was more potent in the prolongation of PT than

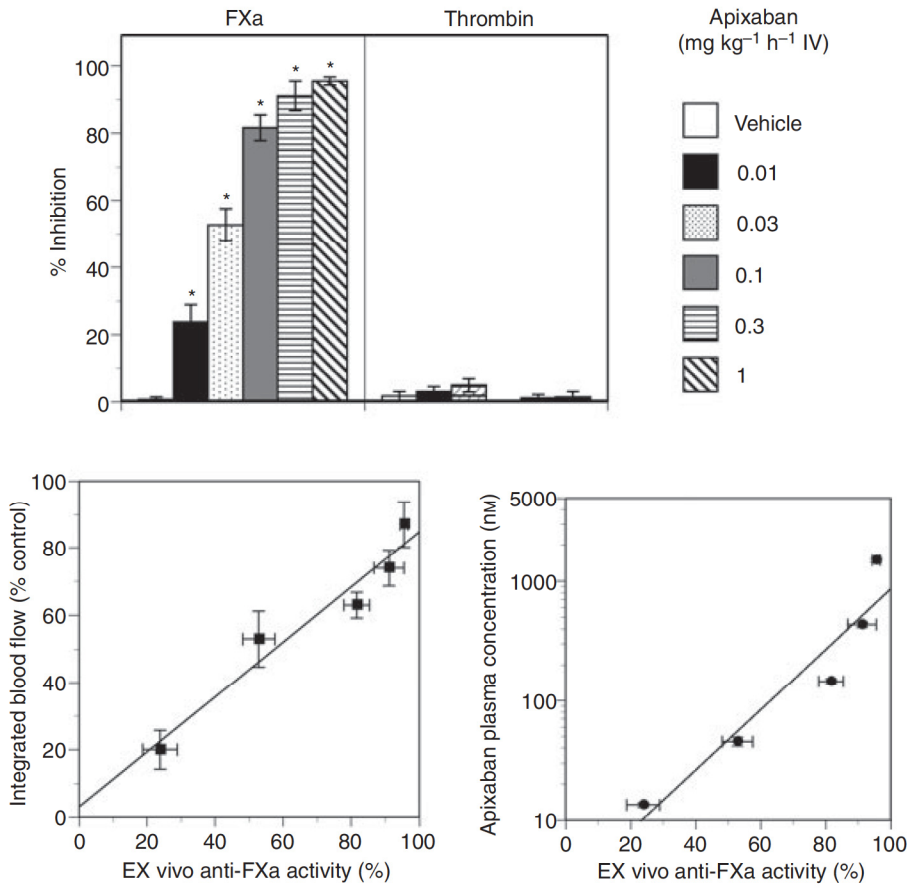

Fig. 5. *Ex vivo* anti-FXa and antithrombin activities. Top panel: *ex vivo* anti-FXa and antithrombin effects of apixaban in arterial thrombosis rabbits from Fig. 3. Bottom panel: Correlation of *ex vivo* anti-FXa with antithrombotic effects and plasma concentrations of apixaban in arterial thrombosis rabbits. *$P < 0.05$, compared with the vehicle. Mean ± SE, and $n = 6$ per group.

APTT *in vitro* and *ex vivo*. However, this effect may not extend to other direct FXa inhibitors, which may show varying effects on APTT and PT [3,4,21]. Among the clotting assays studied, the mPT and HepTest have the highest sensitivity to apixaban in human plasma. Although the HepTest and mPT appear to be promising clotting assays to monitor the anticoagulant activity of apixaban, further clinical validation is needed.

Because apixaban exhibited similar *in vitro* anti-FXa potency in humans and rabbits, we investigated its *in vivo* activity in well-characterized rabbit models of thrombosis and hemostasis. Although apixaban has high oral bioavailability in rats, dogs and humans [8,9], it has a low bioavailabiltiy of < 5% in rabbits (unpublished result). As a result, we studied apixaban *in vivo* in rabbits, using the intravenous route of administration, and evaluated its antithrombotic efficacy in different experimental models of thrombosis in rabbits. These were: (i) AVST – thrombosis on a silk thread in an AV shunt in which thrombus formation occurs due to the activation of platelets and blood coagulation, mimicking 'mixed thrombosis' [22]; (ii) VT – thrombosis on silk threads in the abdominal vena cava in which the thrombus formed is semiocclusive thrombus and consists of mainly fibrin and trapped red cells, mimicking venous thrombosis [17]; and (iii) ECAT – thrombosis in the electrolytically injured carotid artery in which the thrombus formed consists mainly of platelets and fibrin, mimicking arterial thrombosis [15].

Apixaban exhibited strong antithrombotic activity in these rabbit models, which compared well with previous results with unfractionated heparin, low molecular weight heparin, and direct thrombin inhibitors [4,15–17], as well as current results with fondaparinux and warfarin. Despite its chemical neutrality, apixaban displayed similar antithrombotic activity as the basic FXa inhibitors previously reported in these rabbit models [4,6,15,16]. Interestingly, apixaban was equally potent in the prevention of both arterial and venous thrombosis, whereas fondaparinux was about 5-fold less potent in the prevention of arterial than venous thrombosis in rabbits. A similar observation was also reported by Fukuda *et al.* [23] when they compared the antithrombotic properties of the direct FXa inhibitor DU-176b with fondaparinux in rat models of arterial and venous thrombosis. Brufatto *et al.* [24] showed that FXa when incorporated into the prothrombinase complex is highly protected from antithrombin-dependent FXa inhibitors such as fondaparinux. In contrast, small-molecule, direct FXa inhibitors are effective inhibitors of prothrombinase-bound FXa activity [19,21,25]. Whether the difference between fondaparinux and apixaban observed in this study is attributable to the ability of apixaban, but not fondaparinux, to inhibit

prothrombinase-bound FXa activity is not known. Furthermore, the clinical significance of this finding remains to be determined.

The antithrombotic effect of apixaban is consistent with its selective inhibition of FXa. *In vitro* studies show that apixaban was highly selective for FXa and did not directly alter platelet aggregation. *Ex vivo* studies show that apixaban inhibited *ex vivo* FXa activity but not *ex vivo* thrombin activity. However, by inhibiting FXa, apixaban is expected to inhibit thrombin generation *in vivo*, thus reducing thrombin-induced platelet activation and producing an indirect antiplatelet effect. We observed that apixaban prolonged *ex vivo* APTT and PT only modestly in rabbits. Both *ex vivo* APTT and PT were not significantly changed by apixaban at plasma levels of 0.15–0.5 μM, which were near the ED_{50}s of antithrombotic potency, and were only moderately increased at plasma levels > 1–3 μM. Similar to previous findings [26], fondaparinux at antithrombotic doses did not affect *ex vivo* APTT and PT in these rabbit models. Unlike apixaban and fondaparinux, warfarin, as expected, prolonged *ex vivo* APTT and PT in a parallel manner in rabbits. It should be noted that modest increases in *ex vivo* APTT and PT were also observed with other small-molecule, direct FXa inhibitors at antithrombotic doses in animal models of thrombosis [3,4,15,16]. Thus, laboratory coagulation tests with improved sensitivity, such as anti-FXa activity, mPT and HepTest assays, may be more suitable than APTT and PT for monitoring the anticoagulant and plasma levels of apixaban.

This study compared the bleeding potential of apixaban, fondaparinux and warfarin in the rabbit cuticle BT model. We showed that warfarin increased BT greatly at antithrombotic doses. In contrast, apixaban and fondaparinux achieved antithrombotic efficacy with a mild effect on BT in rabbits. The ratio of BT $ED_{3\times}$ to antithrombotic AVST ED_{50} is 1.3 for warfarin, but > 10 for fondaparinux and apixaban. At top efficacy doses, apixaban and fondaparinux both increased BT by 1.2-fold to 1.3-fold, whereas warfarin increased BT by 6.2-fold. These results show that apixaban and fondaparinux have a favorable efficacy/bleeding profile relative to warfarin in rabbits. As apixaban, but not fondaparinux, has oral bioavailability in humans, it may be a better choice for the long-term treatment of venous and arterial thrombosis.

In summary, apixaban was as effective as the current anticoagulant standard of care in the prevention of thrombosis at doses that preserve hemostasis in rabbits. HepTest, mPT and chromogenic anti-FXa assays were potential markers to monitor the anticoagulant and plasma levels of apixaban. Because of the favorable preclinical profile of apixaban, this compound was selected for clinical development. Similar to its favorable preclinical findings, initial phase II studies with apixaban have demonstrated its efficacy and safety in the prevention and treatment of venous thromboembolism [10,11]. Other potential indications for apixaban in the treatment and prevention of various life-threatening thromboembolic events are currently being elucidated in the clinic.

Acknowledgements

We thank M. J. Orwat for the synthesis of apixaban, F. A. Barbera for the purification of rabbit, rat and dog FXa, C. A. Watson for assistance in the animal studies, A. R. Rendina for the contribution to the data presented in Fig. 1, and W. Schumacher for critical review of this manuscript.

Disclosure of Conflict of Interests

The authors are employees of Bristol-Myers Squibb Company.

References

1 Thom T, Haase N, Rosamond W, Howard VJ, Rumsfeld J, Manolio T, Zheng ZJ, Flegal K, O'Donnell C, Kittner S, Lloyd-Jones D, Goff DC Jr, Hong Y, Adams R, Friday G, Furie K, Gorelick P, Kissela B, Marler J, Meigs J, *et al*. Heart disease and stroke statistics – 2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2006; **113**: e85–151.

2 Gustafsson D. Oral direct thrombin inhibitors in clinical development. *J Intern Med* 2003; **254**: 322–34.

3 Leadley RJ Jr. Coagulation factor Xa inhibition: biological background and rationale. *Curr Top Med Chem* 2001; **1**: 151–9.

4 Wong PC, Crain EJ, Watson CA, Zaspel AM, Wright MR, Lam PY, Pinto DJP, Wexler RR, Knabb RM. Nonpeptide factor Xa inhibitors III: effects of DPC423, an orally-active pyrazole antithrombotic agent, on arterial thrombosis in rabbits. *J Pharmacol Exp Ther* 2002; **303**: 993–1000.

5 Quan ML, Wexler RR. The design and synthesis of noncovalent factor Xa inhibitors. *Curr Top Med Chem* 2001; **1**: 137–49.

6 Wong PC, Pinto DJ, Knabb RM. Nonpeptide factor Xa inhibitors: DPC423, a highly potent and orally bioavailable pyrazole antithrombotic agent. *Cardiovasc Drug Rev* 2002; **20**: 137–52.

7 Pinto DJ, Orwat MJ, Koch S, Rossi KA, Alexander RS, Smallwood A, Wong PC, Rendina AR, Luettgen JM, Knabb RM, He K, Xin B, Wexler RR, Lam PY. Discovery of 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)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**: 5339–56.

8 He K, He B, Grace JE, Xin B, Zhang D, Pinto DJ, Luettgen JM, Knabb RM, Lam PYS, Wexler R, Humphrey WG, Shyu W-C, Grossman SJ. Preclinical pharmacokinetics and metabolism of apixaban, a potent and selective factor Xa inhibitor. *Blood* 2006; **108**: 273a (Abstract 910).

9 Frost C, Yu Z, Nepal S, Mosqueda-Garcia R, Shenker A. Apixaban, an oral direct, factor Xa inhibitor: single-dose safety, pharmacokinetics and pharmacodynamics in healthy subjects. *J Thromb Haemost* 2007; **5 Suppl. 2**: PM665.

10 Lassen MR, Davidson BL, Gallus A, Pineo G, Ansell J, Deitchman D. The efficacy and safety of apixaban, an oral, direct factor Xa inhibitor, as thromboprophylaxis in patients following total knee replacement. *J Thromb Haemost* 2007; **5**: 2368–75.

11 Büller HR. A dose finding study of the oral direct factor Xa inhibitor Apixaban in the treatment of patients with acute symptomatic deep-vein thrombosis – on behalf of the Botticelli investigators. *J Thromb Haemost* 2007; **5 Suppl. 2**: OS003.

12 Miletich JP, Broze GJ Jr, Majerus PW. Purification of human coagulation factors II, IX, and X using sulfated dextran beads. *Methods Enzymol* 1981; **80**: 221–8.

13 Mousa SA, Wityak J. Orally active isoxazoline GPIIb/IIIa antagonists. *Cardiovasc Drug Rev* 1998; **16**: 48–61.

14 Sato K, Taniuchi Y, Kawasaki T, Hirayama F, Koshio H, Matsumoto Y. Relationship between the antithrombotic effect of YM-75466, a novel factor Xa inhibitor, and coagulation parameters in rats. *Eur J Pharmacol* 1998; **347**: 231–6.

15 Wong PC, Crain EJ, Knabb RM, Meade RP, Quan ML, Watson CA, Wexler RR, Wright MR, Slee AM. Nonpeptide factor Xa inhibitors: II. Antithrombotic evaluation in a rabbit model of electrically induced carotid artery thrombosis. *J Pharmacol Exp Ther* 2002; **295**: 212–18.

16 Wong PC, Quan ML, Crain EJ, Watson CA, Wexler RR, Knabb RM. Nonpeptide factor Xa inhibitors: I. Studies with SF303 and SK549, a new class of potent antithrombotics. *J Pharmacol Exp Ther* 2000; **292**: 351–7.

17 Hollenbach S, Sinha U, Lin P-H, Needham K, Frey L, Hancock T, Wong A, Wolf D. A comparative study of prothrombinase and thrombin inhibitors in a novel rabbit model of non-occlusive deep vein thrombosis. *Thromb Haemost* 1994; **71**: 357–62.

18 Krishnaswamy S. Exosite-driven substrate specificity and function in coagulation. *J Thromb Haemost* 2005; **3**: 54–67.

19 Luettgen JM, Wang Z, Seiffert DA, Rendina AR, Knabb RM, Ogletree ML. Inhibition of measured thrombin generation in human plasma by apixaban: a predictive mathematical model based on experimentally determined rate constants. *J Thromb Haemost* 2007; **5** (Suppl. 2): PT633.

20 Hara T, Yokoyama A, Morishima Y, Kunitada S. Species differences in anticoagulant and anti-Xa activity of DX-9065a, a highly selective factor Xa inhibitor. *Thromb Res* 1995; **80**: 99–104.

21 Perzborn E, Strassburger J, Wilmen A, Pohlmann J, Roehrig S, Schlemmer KH, Straub A. *In vitro* and *in vivo* studies of the novel antithrombotic agent BAY 59-7939 – an oral, direct factor Xa inhibitor. *J Thromb Haemost* 2005; **3**: 514–21.

22 Peters RF, Lees CM, Mitchell KA, Tweed MF, Talbot MD, Wallis RB. The characterization of thrombus development in an improved model of arterio-venous thrombosis in the rat and the effects of recombinant desulphatohirudin (CGP 39393), heparin, and iloprost. *Thromb Haemost* 1991; **65**: 268–74.

23 Fukuda T, Matsumoto C, Honda Y, Sugiyama N, Morishima Y, Shibano T. Antithrombotic properties of DU-176b, a novel, potent and orally active direct factor Xa inhibitor in rat models of arterial and venous thrombosis: comparison with fondaparinux, an antithrombin dependent factor Xa inhibitor. *Blood* 2004; **104**: 11 (Abstract 1852).

24 Brufatto N, Ward A, Nesheim ME. Factor Xa is highly protected from antithrombin-fondaparinux and antithrombin-enoxaparin when incorporated into the prothrombinase complex. *J Thromb Haemost* 2003; **1**: 1258–63.

25 Gerotziafas GT, Elalamy I, Depasse F, Perzborn E, Samama MM. *In vitro* inhibition of thrombin generation, after tissue factor pathway activation, by the oral, direct factor Xa inhibitor rivaroxaban. *J Thromb Haemost* 2007; **5**: 886–8.

26 Walenga JM, Jeske WP, Samama MM, Frapaise FX, Bick RL, Fareed J. Fondaparinux: a synthetic heparin pentasaccharide as a new antithrombotic agent. *Expert Opin Investig Drugs* 2002; **11**: 397–407.