Journal of Thrombosis and Haemostasis, **3**: 514–521

ORIGINAL ARTICLE

In vitro and *in vivo* studies of the novel antithrombotic agent BAY 59-7939—an oral, direct Factor Xa inhibitor

E. PERZBORN, J. STRASSBURGER, A. WILMEN, J. POHLMANN,† S. ROEHRIG,† K-H. SCHLEMMER* and A. STRAUB†

*Cardiovascular Research, *Preclinical Pharmacokinetics and †Chemical Research, Bayer HealthCare AG, Wuppertal, Germany*

To cite this article: 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 Haemost* 2005; **3**: 514–21.

Summary. BAY 59-7939 is an oral, direct Factor Xa (FXa) inhibitor in development for the prevention and treatment of arterial and venous thrombosis. BAY 59-7939 competitively inhibits human FXa (K_i 0.4 nM) with > 10 000-fold greater selectivity than for other serine proteases; it also inhibited prothrombinase activity (IC_{50} 2.1 nM). BAY 59-7939 inhibited endogenous FXa more potently in human and rabbit plasma (IC_{50} 21 nM) than rat plasma (IC_{50} 290 nM). It demonstrated anticoagulant effects in human plasma, doubling prothrombin time (PT) and activated partial thromboplastin time at 0.23 and 0.69 μM, respectively. *In vivo*, BAY 59-7939 reduced venous thrombosis (fibrin-rich, platelet-poor thrombi) dose dependently (ED_{50} 0.1 mg kg^{-1} i.v.) in a rat venous stasis model. BAY 59-7939 reduced arterial (fibrin- and platelet-rich) thrombus formation in an arteriovenous (AV) shunt in rats (ED_{50} 5.0 mg kg^{-1} p.o.) and rabbits (ED_{50} 0.6 mg kg^{-1} p.o.). Slight inhibition of FXa (32% at ED_{50}) reduced thrombus formation in the venous model; to affect arterial thrombosis in the rat and rabbit, stronger inhibition of FXa (74%, 92% at ED_{50}) was required. Calculated plasma levels in rabbits at the ED_{50} were 14-fold lower than in the rat AV shunt model, correlating with the 14-fold lower IC_{50} of FXa inhibition in rabbit compared with rat plasma; this may suggest a correlation between FXa inhibition and antithrombotic activity. Bleeding times in rats and rabbits were not significantly affected at antithrombotic doses (3 mg kg^{-1} p.o., AV shunt). Based on these results, BAY 59-7939 was selected for clinical development.

Correspondence: E. Perzborn, Cardiovascular Research, Bayer HealthCare AG, Building 500, Aprather Weg 18a, D-42096 Wuppertal, Germany.
Tel.: +49 202 36 8354; fax: +49 202 36 8009; e-mail: elisabeth.perzborn@bayerhealthcare.com

Received 3 March 2004, accepted 3 November 2004

Keywords: antithrombotic activity, Factor Xa inhibitor, oral anticoagulant.

Introduction

Anticoagulants in current clinical use comprise the vitamin K antagonists—such as warfarin—heparins (including low-molecular-weight heparins), and parenterally administered direct thrombin inhibitors. Warfarin can be administered orally; however, its major drawbacks include the need for monitoring—because of a narrow therapeutic window and large inter- and intraindividual variability in dose–response—a slow onset and offset of action, and extensive food and drug interactions [1–3]. Heparins have a rapid onset of action, but must be administered parenterally. Despite recent developments, there is still an unmet need for safe, oral anticoagulants for both short- and long-term use.

Factor Xa (FXa) has emerged as a particularly promising target for effective anticoagulation because it acts at the convergence point of the intrinsic and extrinsic coagulation pathways. FXa catalyzes the conversion of prothrombin to thrombin; one molecule of FXa results in the generation of more than 1000 thrombin molecules [4]. Thus, inhibiting FXa may block this burst of thrombin generation, thereby diminishing thrombin-mediated activation of coagulation and platelets.

Recent research has focused on the identification of small-molecule FXa inhibitors with good oral bioavailability and predictable pharmacokinetics. An oral, direct FXa inhibitor that does not require routine coagulation monitoring would offer significant advantages over current therapies. BAY 59-7939 belongs to a new class of small-molecule, active-site-directed FXa inhibitors. It is a non-basic compound with high oral bioavailability in rats and dogs (60–86%) [5]. Currently, BAY 59-7939 is in clinical development for the prevention and treatment of thromboembolic disorders.

We report the *in vitro* properties of BAY 59-7939, its antithrombotic efficacy in animal models of arterial and venous thrombosis, and its effect on hemostasis—the pharmacological profile on which BAY 59-7939 was chosen for clinical development.

© 2005 International Society on Thrombosis and Haemostasis

Materials and methods

Agents

BAY 59-7939 (5-chloro-*N*-({(5*S*)-2-oxo-3-[4-(3-oxomorpholin-4-yl)phenyl]-1,3-oxazolidin-5-yl}methyl)thiophene-2-carboxamide; Mr = 435.89 g mol^{-1}; Fig. 1) was synthesized by Bayer HealthCare AG (Wuppertal, Germany). Human, rat, and rabbit purified FXa, thrombin, and plasmin were obtained from Kordia (Leiden, The Netherlands); Factor XIa (FVIIa) from Calbiochem® (Schwalbach, Germany); trypsin and urokinase from Sigma (Taufkirchen, Germany); activated protein C (APC) from Haemochrom Diagnostica (Essen, Germany); Factor VIIa (FVIIa), Factor IXaβ (FIXaβ), FX, and prothrombin from Enzyme Research Laboratories (Swansea, UK); tissue factor from American Diagnostica Inc. (Stanford, USA). Chromogenic substrates (chromozym TH, X, U, trypsin, and plasmin) were from Roche Diagnostics (Mannheim, Germany); S 2366™ from Chromogenix Instrumentation Laboratory (Bubendorf, Switzerland); and Pefachrome® FXa from Pentapharm (Basel, Switzerland). Fluorogenic substrates (I-1100 and H-D-Phe-Pro-Arg-6-amino-1-naphthalene-benzylsulfonamide·H_2O) were from Bachem (Bubendorf, Switzerland); Russell's viper venom (RVV) from Pentapharm; Neoplastin® Plus (thromboplastin) and PTT-Reagent from Roche Diagnostics; hirudin (Refludan®) from Aventis (Strasbourg, France). Xylazine (Rompun®) was from Bayer HealthCare, ketamine (Ketavet®) from Pharmacia & Upjohn (Karlsruhe, Germany), and pentobarbital-Na (Nembutal®) from Richter Pharma (Wels, Austria).

In vitro *studies*

Enzyme assays The activity of BAY 59-7939 against purified serine proteases was measured using chromogenic or fluorogenic substrates in 96-well microtiter plates at 25 °C. The enzymes were incubated with BAY 59-7939 or its solvent, dimethyl sulfoxide (DMSO), for 10 min. The reactions were initiated by the addition of the substrate, and the color or fluorescence was monitored continuously at 405 nm using a Spectra Rainbow Thermo Reader (Tecan, Crailsheim, Germany), or at 630/465 nm using a SPECTRAfluor plus (Tecan), respectively, for 20 min (if not otherwise stated).

Enzymatic activity was analyzed in the following buffers (final concentrations): human FXa (0.5 nM), rabbit FXa (2 nM), rat FXa (10 nM), or urokinase (4 nM) in 50 mM Tris–HCl buffer, pH 8.3, 150 mM NaCl, and 0.1% bovine serum albumin (BSA); Pefachrome FXa (50–800 μM) or chromozym U (250 μM) with thrombin (0.69 nM), trypsin (2.2 nM), or plasmin (3.2 nM) in 0.1 μM Tris–HCl, pH 8.0, and 20 mM $CaCl_2$; chromozym TH (200 μM), chromozym plasmin (500 μM), or chromozym trypsin (500 μM) with FXIa (1 nM) or APC (10 nM) in 50 mM phosphate buffer, pH 7.4, 150 mM NaCl; and S 2366 (150 or 500 μM) with FVIIa (1 nM) and tissue factor (3 nM) in 50 mM Tris–HCl buffer, pH 8.0, 100 mM NaCl, 5 mM $CaCl_2$ and 0.3% BSA, H-D-Phe-Pro-Arg-6-amino-1-naphthalene-benzylsulfonamide·H_2O (100 μM) and measured for 3 h as described previously [6]. The FIXaβ/FX assay, comprising FIXaβ (8.8 nM) and FX (9.5 nM) in 50 mM Tris–HCl buffer, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$ and 0.1% BSA, was started by the addition of I-1100 (50 μM), and measured for 60 min.

The inhibitory constant (K_i) against FXa was calculated according to the Cheng–Prusoff equation ($K_i = IC_{50}/1 + [S]/K_m$), where [S] is the substrate concentration, and K_m is the Michaelis–Menten constant. K_m was determined from a Lineweaver–Burk plot. The IC_{50} was the amount of inhibitor required to diminish the initial velocity of the control by 50%.

Prothrombinase assay The effect of BAY 59-7939 on prothrombinase activity was measured via thrombin generation, as described previously with some modifications [7]. Briefly, human FXa (0.025 nM) was incubated in 10 mM HEPES buffer, pH 7.4, 2 mM $CaCl_2$ and washed human platelets (1×10^7 mL^{-1}) for 10 min at 37 °C. The reaction was initiated by adding prothrombin (1 μM) and BAY 59-7939 or DMSO. After 20 min, 20-μL aliquots were diluted with 160 μL buffer, and thrombin activity was measured using 20 μL chromozym TH (500 μM).

FXa activity in plasma Human, rat, or rabbit plasma (45 μL) was mixed with 5 μL hirudin (10 μg mL^{-1}), 5 μL BAY 59-7939 or DMSO, and 50 μL RVV (human, 0.7 mU mL^{-1}; rat/rabbit, 3.5 mU mL^{-1}), dissolved in 50 μM $CaCl_2$ at 37 °C. Chromozym X (50 μL; 600 μM) was added after 15 min. The increase in optical density was measured at 37 °C, as described above.

Coagulation assays Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were measured using commercially available kits. BAY 59-7939 or DMSO (3 μL) were added to 100 μL platelet-poor plasma (PPP) and incubated for 10 min at 37 °C. Clotting times were measured in a coagulometer (Biomatic 4000; Sarstedt, Nümbrecht, Germany), in accordance with the manufacturer's instructions (final volume 303 μL). Anticoagulant activity was defined as the concentration required to double the plasma clotting times [CT_2 (μM)].

Plasma preparation Human blood was collected by venipuncture from healthy subjects who had not been medicated during the last 10 days. Rabbit blood was obtained by puncture of the A. carotis, and rat blood was withdrawn from the abdominal aorta under anesthesia. Blood was collected into plastic tubes containing 1/10 volume of 3.8% trisodium citrate. PPP was obtained by immediate centrifugation at 2500 *g* for 10 min at 4 °C, and stored at − 20 °C.

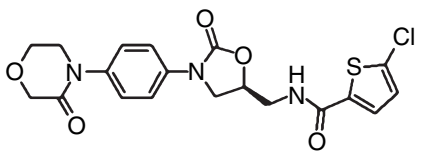

Fig. 1. Chemical structure of BAY 59-7939 (5-chloro-*N*-({(5*S*)-2-oxo-3-[4-(3-oxomorpholin-4-yl)phenyl]-1,3-oxazolidin-5-yl}methyl)thiophene-2-carboxamide).

© 2005 International Society on Thrombosis and Haemostasis

In vivo *studies*

Animals and anesthetics Fasted, male Wistar rats (HsdCpb:WU) were anesthetized by intraperitoneal injection of xylazine and ketamine (12 and 50 mg kg^{-1}, respectively); in the bleeding-time model, pentobarbital-Na (60 mg kg^{-1}) was used. Fasted, female New Zealand White rabbits (Esd:NZW) were anesthetized by intramuscular administration of xylazine and ketamine (5 and 40 mg kg^{-1}, respectively). All procedures were conducted in accordance with the German Animal Protection Act (Deutsches Tierschutzgesetz).

Rat venous stasis model Thrombus formation was induced in anesthetized rats ($n = 10$ per dose group) as described previously, with minor modifications [8]. The abdominal vena cava was exposed and two loose sutures (8–10 mm apart) were placed below the left renal venous branch. BAY 59-7939 dissolved in polyethylene glycol/H_2O/glycerol (996 g/100 g/60 g), or vehicle was given by intravenous (i.v.) bolus injection into a tail vein 15 min before thrombus induction. Thromboplastin (0.5 mg kg^{-1}) was injected into a femoral vein and, after 15 s, the proximal and distal sutures were tied. Fifteen minutes later, the ligated segment was removed, the thrombus withdrawn and weighed. Blood samples were obtained by cardiac puncture immediately before thrombus removal.

Arteriovenous shunt model in rats and rabbits An arteriovenous (AV) shunt in anesthetized rats and rabbits was performed as described previously, with minor modifications [8–10]. The right common carotid artery and the left jugular vein were cannulated with two 100-mm-long, saline-filled catheters. In rats ($n = 10$ per dose group), the polyethylene catheters (PE-60; Becton Dickinson, Sparks, MD, USA) were connected with a 30-mm-long polyethylene tube (PE-160; Becton Dickinson) containing a rough nylon thread (40×0.15 mm), folded into a double string. In rabbits ($n = 6$ per dose group), polyurethane vein catheters (outside diameter 2.1 mm; Braun, Melsungen, Germany) were connected with a 40-mm-long polyethylene tube (PE-240; Becton Dickinson), containing a rough nylon thread (60×0.15 mm), folded into a double string. BAY 59-7939, dissolved in solutol/ethanol/H_2O [40%/10%/50% (v/v/v)], or vehicle was given orally 90 min before the shunt was opened for 15 min. The nylon thread was then withdrawn and weighed. Blood samples were withdrawn from the carotid artery just after thrombus removal.

Rat tail-bleeding model BAY 59-7939 ($n = 10$ per dose group) or vehicle was given orally 90 min before the tails of anesthetized rats were transected 2 mm from the tip and vertically immersed in saline at 37 °C. The time until continuous blood flow ceased for > 30 s was measured, with a maximum observation time of 10 min (longer bleeding times were assigned a value of 10 min).

Rabbit ear-bleeding model Ear-bleeding time (EBT) was determined in anesthetized rabbits ($n = 5$ per dose group), as described previously [11]. A standardized 3-mm-long incision was made at different sites of the right ear in each animal 90 and 105 min after administration of oral BAY 59-7939 or vehicle. Blood from the incision was removed with filter paper every 30 s. The time until the bleeding stopped was measured.

Statistical analysis

Student's *t*-test (one-way ANOVA) was used for unpaired data, with a statistical significance level of $P < 0.05$. Data are expressed as mean ± SEM. IC_{50} values were calculated using Graph Pad Prism, version 3.02 (Graph Pad Software Inc., San Diego, CA, USA). ED_{50} values were calculated by linear regression analysis using Excel 97 (Microsoft®).

Results

In vitro *studies*

Enzyme assays BAY 59-7939 inhibited human FXa concentration dependently, with a K_i of 0.4 ± 0.02 nM (Fig. 2). It is a competitive inhibitor of the amidolytic activity of FXa, as demonstrated by Lineweaver–Burk analysis (Fig. 3). At concentrations up to 20 μM, BAY 59-7939 did not affect related serine proteases; selectivity was more than 10 000-fold greater for FXa (Table 1). BAY 59-7939 showed a similar affinity to purified human and rabbit FXa (IC_{50} 0.7 ± 0.01 and 0.8 ± 0.01 nM, respectively), but was less potent against purified rat FXa (IC_{50} 3.4 nM; Table 2).

Prothrombinase assay To determine whether BAY 59-7939 was an effective inhibitor of FXa complexed with Factor Va and Ca^{2+} on a phospholipid membrane, we reconstituted the prothrombinase complex on platelets. The generation of thrombin was inhibited concentration-dependently, with an IC_{50} of 2.1 ± 0.4 nM, as measured in an amidolytic assay (Fig. 2).

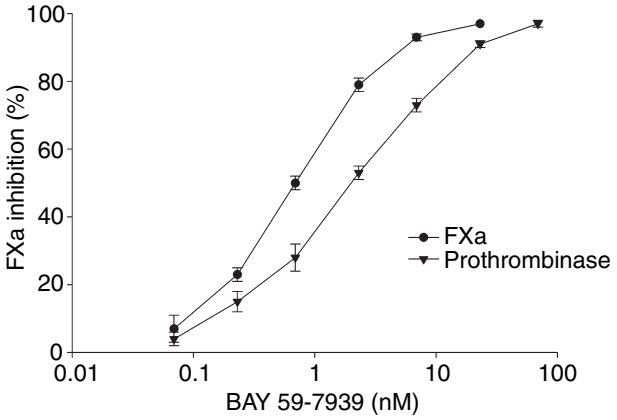

Fig. 2. Effect of BAY 59-7939 on purified human free Factor Xa (FXa) using a chromogenic substrate of FXa (●), and on prothrombinase activity on platelet surfaces using prothrombin as substrate (measuring generated thrombin; ▼). Each value represents the mean ± SEM of five measurements in triplicate.

© 2005 International Society on Thrombosis and Haemostasis

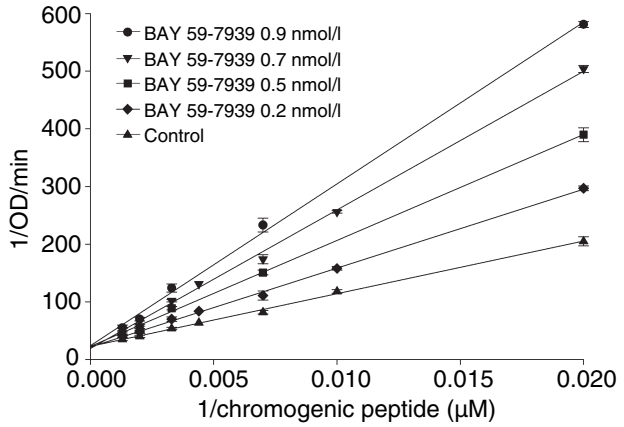

Fig. 3. Kinetic analysis of the inhibitory effect of BAY 59-7939 on Factor Xa (FXa). Lineweaver–Burk plots of the activity of 0.5 nM FXa against a chromogenic substrate in the absence or presence of 0.2, 0.5, 0.7, and 0.9 nM BAY 59-7939. Results are mean ± SD.

Table 1 Human protease selectivity profile of BAY 59-7939

Inhibition of	Concentration (nM)
Factor Xa	$K_i = 0.4 \pm 0.02$
Factor VIIa, Factor IXa, Factor XIa, thrombin, activated protein C, plasmin, urokinase, trypsin	$IC_{50} > 20\,000$

Table 2 Effect of BAY 59-7939 on inhibition of human, rabbit, and rat Factor Xa (FXa) in buffer, plasma FXa, and the concentrations required to double the prothrombin time (PT) and activated partial thromboplastin time (aPTT) *in vitro* (CT_2)

Species	FXa (buffer) IC_{50} (nM)	FXa (plasma) IC_{50} (nM)	PT CT_2 (μM)	APTT CT_2 (μM)
Human	0.7 ± 0.01	21 ± 1.0	0.23 ± 0.02	0.69 ± 0.09
Rabbit	0.8 ± 0.01	21 ± 2.0	0.12 ± 0.01	1.97 ± 0.49
Rat	3.4 ± 0.02	290 ± 20.0	0.30 ± 0.02	2.09 ± 0.19

Results expressed as mean ± SEM.

FXa activity in plasma In plasma, endogenous human and rabbit FXa, generated by RVV, was inhibited to a similar extent by BAY 59-7939 (IC_{50} 21 ± 0.001 and 21 ± 0.002 nM, respectively), whereas 14-fold higher concentrations were required in rat plasma (IC_{50} 290 ± 0.02 nM; Table 2).

Plasma clotting times BAY 59-7939 prolonged PT and aPTT concentration dependently; the PT assay was more sensitive than aPTT. In the PT assay, anticoagulant activity was greatest in the rabbit (CT_2 0.12 ± 0.01 μM), followed by human (CT_2 0.23 ± 0.02 μM), and then rat (CT_2 0.30 ± 0.02 μM; Table 2). In the aPTT assay, BAY 59-7939 was most potent in human plasma (CT_2 0.69 ± 0.09 μM) and less effective in rabbit and rat plasma (CT_2 1.97 ± 0.49 and 2.09 ± 0.19 μM, respectively).

In vivo *studies*

Rat venous stasis model In a venous thrombosis model, thrombi were obtained by employing a combination of stasis and injection of thromboplastin. BAY 59-7939, administered by i.v. bolus before thrombus induction, reduced thrombus formation (ED_{50} 0.1 mg kg^{-1}), inhibited FXa, and prolonged PT (Fig. 4A–C) dose dependently. PT and FXa were affected slightly at the ED_{50} (1.8-fold increase and 32% inhibition, respectively). At 0.3 mg kg^{-1} (dose leading to almost complete inhibition of thrombus formation), BAY 59-7939 moderately prolonged PT (3.2 ± 0.5-fold) and inhibited FXa activity (65 ± 3%).

Rat AV-shunt model Thrombosis was induced by exposure of a thrombogenic surface in an AV shunt. To evaluate its potential oral efficacy, BAY 59-7939 was given orally before blood was circulated in the shunt. BAY 59-7939 reduced thrombus formation dose dependently (ED_{50} 5.0 mg kg^{-1}; Fig. 5A). It also had a dose-dependent effect on

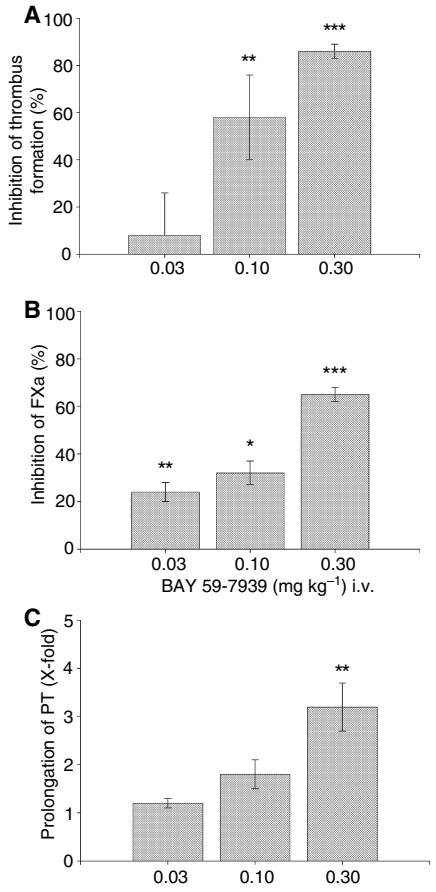

Fig. 4. Effect of BAY 59-7939 in a rat venous stasis model. BAY 59-7939 or the appropriate vehicle was given by i.v. bolus injection 15 min before thrombus induction. (A) Inhibition of thrombus formation. (B) Inhibition of endogenous Factor Xa (FXa) after activation by Russell's viper venom. (C) Prolongation of prothrombin time (PT). Blood samples were withdrawn by cardiac puncture immediately after removal of the thrombus. Results are mean ± SEM of 10 animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

© 2005 International Society on Thrombosis and Haemostasis

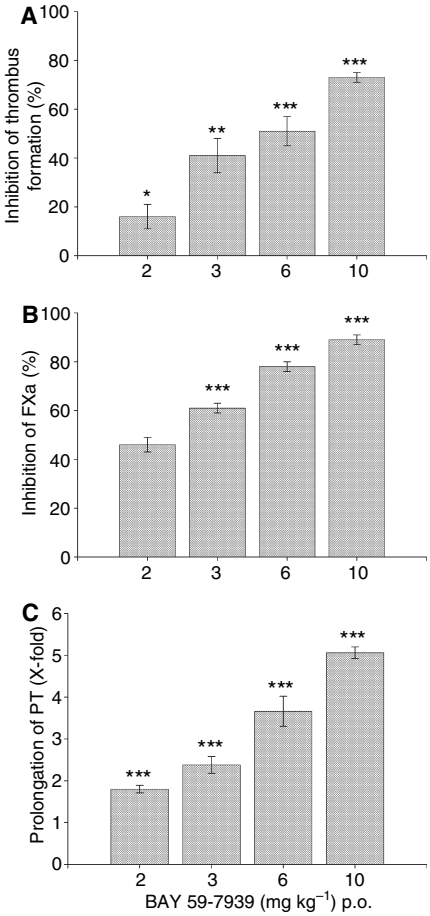

Fig. 5. Effect of BAY 59-7939 in a rat arteriovenous (AV)-shunt model. BAY 59-7939 or vehicle was given orally 90 min before blood was circulated in the shunt. (A) Inhibition of thrombus formation. (B) Inhibition of endogenous Factor Xa (FXa) after activation by Russell's viper venom. (C) Prolongation of prothrombin time (PT). Blood samples were withdrawn from the carotid artery catheter just after thrombus removal. Results are mean ± SEM of six animals. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

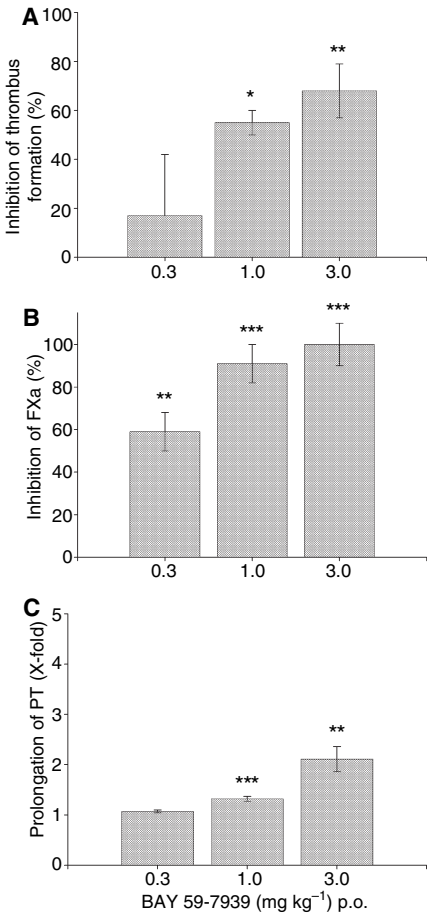

Fig. 6. Effect of BAY 59-7939 in a rabbit arteriovenous (AV)-shunt model. The extracorporeal circulation was opened 90 min after oral administration of BAY 59-7939 or vehicle. (A) Inhibition of thrombus formation. (B) Inhibition of endogenous Factor Xa (FXa) after activation by Russell's viper venom. (C) Prolongation of prothrombin time (PT). Blood samples were withdrawn from the carotid artery catheter just after removal of the thrombus. Each value represents the mean ± SEM of six animals. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

FXa activity and PT (Fig. 5B,C); at the ED_{50}, BAY 59-7939 inhibited FXa by 74% and prolonged PT 3.2-fold, as calculated from the dose–response curves.

Rabbit AV-shunt model Oral BAY 59-7939, given before opening the shunt, inhibited thrombus formation dose dependently (ED_{50} 0.6 mg kg^{-1}; Fig. 6A). It also had a dose-dependent effect on FXa activity and PT (Fig. 6B,C); at the ED_{50}, FXa was almost completely inhibited (92%), but PT was prolonged only slightly (1.2-fold), as calculated from the dose–response curves.

Rat tail-bleeding model Tail-bleeding time was evaluated at the antithrombotic-effective oral dose (minimal dose preventing thrombus formation in AV shunt model) of 3 mg kg^{-1} and multiples thereof. Bleeding time was not different from baseline at the antithrombotic-effective dose of BAY 59-7939 (Table 3). At doses greater than the ED_{50} (6 and 10 mg kg^{-1}), there was a dose-dependent, moderate prolongation of approximately 2- and 3-fold, respectively.

Rabbit ear-bleeding model EBT was assessed at 90 and 105 min in the same animal after oral administration of BAY 59-7939. At all doses tested, there was no significant increase of EBT, even at multiples of the ED_{50} in the AV-shunt model (Table 3).

Table 3 Effect of BAY 59-7939 on rat tail-transection bleeding time and rabbit ear-bleeding time measured 90 and 105 min after oral administration

	Prolongation of bleeding time (X-fold)		
		Ear-bleeding time, rabbit	
BAY 59-7939 (mg kg^{-1}) p.o.	Tail-bleeding time, rat	$t = 90$ min	$t = 105$ min
0.3	ND	1.4 ± 0.7	1.0 ± 0.5
1.0	ND	1.7 ± 0.9	1.1 ± 0.5
3.0	1.0 ± 0.1	1.6 ± 0.8	1.3 ± 0.7
6.0[a]	2.1 ± 0.2*	ND	ND
10.0[a]	2.7 ± 0.2***	ND	ND

ND, Not determined. $*P < 0.05$; $***P < 0.001$. Results are expressed as mean ± SEM. [a]Bleeding did not stop within the observation time of 10 min in two of 10 rats.

© 2005 International Society on Thrombosis and Haemostasis

Discussion

BAY 59-7939 is a highly potent, competitive, reversible, direct FXa inhibitor with a K_i of 0.4 nM for purified human FXa. *In vivo* results indicate that direct inhibition of FXa with BAY 59-7939 is a highly effective strategy for the prevention of both arterial and venous thrombosis. Bleeding times in rats and rabbits were not significantly prolonged at antithrombotic-effective doses.

BAY 59-7939 is highly selective for FXa: its inhibitory effect against FXa was > 10 000-fold higher than for other biologically relevant serine proteases. In contrast to several other FXa inhibitors, BAY 59-7939 does not inhibit trypsin [7,12,13] and therefore is not expected to interfere with this digestive enzyme in the gastrointestinal tract.

The potential of BAY 59-7939 to reduce prothrombinase activity was evaluated. BAY 59-7939, in the nanomolar range, effectively inhibited human FXa bound to the phospholipid surface of platelets (IC_{50} 2.1 nM). In the prothrombinase complex, the rate of prothrombin conversion is highly accelerated, approximately 280 000-fold [14]. This, in addition to the high concentrations of prothrombin used in our study (almost physiological concentrations), supports the high affinity of BAY 59-7939 for FXa within the prothrombinase complex. These data are further supported by recent results showing a concentration-dependent reduction in thrombin generation triggered by tissue factor in human platelet-rich plasma (IC_{50} 25 nM) [15]. Interestingly, in that study, almost complete inhibition of thrombin generation was observed with 80 nM BAY 59-7939. In contrast, maximum inhibition of thrombin generation of 60% was reported with the pentasaccharide fondaparinux, an antithrombin-dependent FXa inhibitor [15]. These results suggest that BAY 59-7939, a direct FXa inhibitor, could access the active site of FXa within the prothrombinase complex more effectively than indirect FXa inhibitors.

Because FXa is at the convergence point of the intrinsic and extrinsic coagulation pathways, direct inhibition of FXa by BAY 59-7939 is expected to prolong both PT and aPTT. The sensitivity of the PT and aPTT assays varies among different chemical classes of FXa inhibitors and may reflect differences in enzyme kinetics [16]. The PT assay was more sensitive to BAY 59-7939 than the aPTT assay. *In vivo*, we demonstrated a dose-dependent prolongation of PT in rats and rabbits. In the rabbit AV-shunt model, a strong correlation between PT and plasma concentrations of BAY 59-7939 ($r = 0.98$) was observed (data not shown), suggesting that PT can be used to characterize the anticoagulant efficacy of BAY 59-7939 in humans. In clinical phase I studies, good correlation between plasma levels of BAY 59-7939 and prolongation of clotting times was observed [17,18].

An antithrombotic effect was achieved with BAY 59-7939, even at low or moderate levels of anticoagulation: 1.8-, 3.2-, and 1.2-fold increases in PT at the ED_{50} in the rat venous model and the rat and rabbit AV shunt models, respectively. Other animal studies with direct FXa inhibitors have shown a moderate increase in PT and aPTT at antithrombotic doses (for review, see Leadly *et al.* [16]). These results suggest that the antithrombotic efficacy of direct FXa inhibitors can be achieved at doses that produced only a low to moderate increase in systemic anticoagulation.

In order to speculate on the effect of BAY 59-7939 in humans from the *in vivo* thrombosis results, it was necessary to elucidate the species differences between rats and rabbits. Species differences in FXa inhibition in humans, rabbits, and rats are well documented. Various compounds show similar inhibition of human and rabbit FXa, but are less potent against rat FXa [13,19,20]. We showed that the affinity of BAY 59-7939 for purified human and rabbit FXa was similar, but BAY 59-7939 has a 5-fold lower affinity for purified rat FXa. Under similar enzyme kinetic conditions, human, rabbit and rat plasma anti-FXa activity was significantly lower compared with the purified enzymes. This may be explained by non-specific plasma–protein binding, which is greatest in rats, and lowest in rabbits [5].

As potent FXa inhibition was demonstrated in both rats and rabbits, these animals were chosen for investigation of the ability of BAY 59-7939 to prevent thrombus formation in established venous and arterial thrombosis. Thrombi in the rat stasis model are fibrin-rich, platelet-poor, red thrombi (mimicking venous thrombosis), whereas thrombi in the AV shunt in rats and rabbits are considered 'mixed' thrombi, consisting mainly of platelets and fibrin—mimicking arterial thrombosis.

BAY 59-7939 showed dose-dependent antithrombotic activity in both venous and arterial thrombosis, with higher potency in the venous model. Compared with the rat arterial thrombosis model, lower inhibition of FXa (32% vs. 74%) and PT prolongation (1.8- vs. 3.2-fold) were required to reduce thrombus formation by 50% in the rat venous thrombosis model. This corresponds to 10-fold lower plasma concentrations of BAY 59-7939 in the venous stasis model ($\sim$ 0.1 μM) compared with the AV-shunt model ($\sim$ 1.0 μM), as estimated from *ex vivo* PT and anti-FXa activity. FXa activity and PT were measured after thrombus removal (15 min after thrombus induction); however, due to the pharmacokinetic profile of BAY 59-7939 in rats ($t_{1/2}$ 1–2 h), these values reflect conditions at thrombus induction. Higher potency in venous than arterial thrombosis has also been reported for other FXa inhibitors [20–22]. These differences probably reflect the greater platelet enrichment in arterial thrombosis.

In contrast to our results with BAY 59-7939, fondaparinux has lower efficacy in a rat AV-shunt model, resulting in maximal thrombus reduction of 50% [23]. Oral BAY 59-7939 reduced thrombus formation by 73% at 10 mg kg^{-1}. The higher plasma levels of BAY 59-7939 achieved after i.v. administration reduced thrombus formation almost completely (92%; data not shown). These data suggest that a direct FXa inhibitor, such as BAY 59-7939, may be more effective against platelet-rich arterial clots than an antithrombin-dependent FXa inhibitor.

In contrast to the rat model, in which PT was increased 3.2-fold, there was only a 1.2-fold increase in PT at the ED_{50} in the rabbit AV shunt, which does not support a strong

© 2005 International Society on Thrombosis and Haemostasis

correlation between anticoagulation and thrombus reduction. However, in both rat and rabbit AV-shunt models, strong inhibition of FXa activity (74% and 92%, respectively) was required to reduce thrombus formation by 50%. BAY 59-7939 plasma concentrations at the ED_{50} were 14-fold lower in the rabbit compared with the rat AV-shunt model (0.070 and 1 μM, respectively; extrapolated from the dose–response curve at the ED_{50}), which corresponded to the 14-fold lower IC_{50} values for the inhibition of FXa in rabbit vs. rat plasma *in vitro*. These data suggest that the antithrombotic efficacy may be predicted more precisely by the anti-FXa activity of BAY 59-7939 in plasma, rather than by PT.

The antithrombotic effect of BAY 59-7939 is primarily attributed to the inhibition of FXa: it does not directly affect platelet aggregation *in vitro* [24,25]. However, BAY 59-7939 may decrease platelet activation *in vivo* indirectly via inhibition of thrombin generation, and may thereby affect thrombin-induced aggregation [26].

In order to distinguish between the antithrombotic and accompanying antihemostatic effects of BAY 59-7939, we investigated bleeding times in well-characterized experimental models that measure bleeding from small vessels. At antithrombotic-effective doses, BAY 59-7939 did not prolong rat tail-bleeding time or rabbit EBT. Whether the significant increase in tail-bleeding time, but not EBT, observed at doses of BAY 59-7939 above the antithrombotic dose, correlates with the higher PT values in rats, or depends on the animal model used, warrants further evaluation. Although these results may not be directly applicable to humans, they may provide an estimation of bleeding tendency. In clinical studies in healthy male subjects, BAY 59-7939 did not increase bleeding times or signs or symptoms of bleeding across a wide range of oral doses [17,18].

BAY 59-7939 is a reversible inhibitor of FXa; therefore, it is conceivable that a minimal amount of thrombin could be produced even when FXa is strongly inhibited. Numerous studies have demonstrated antithrombotic efficacy with FXa inhibitors at doses that have little or no effect on template bleeding times, tail-bleeding time, or cuticle-bleeding times (for review, see Leadley *et al.* [16]), suggesting a relatively wide therapeutic window between antithrombotic efficacy and bleeding tendency.

In summary, BAY 59-7939 is an oral, direct FXa inhibitor that inhibited thrombus formation in established rat and rabbit thrombosis models at doses that did not significantly increase bleeding times. The clinical relevance of these data needs to be investigated. Based on its potency, selectivity and efficacy, BAY 59-7939 may offer a safe and effective oral therapy for the prevention and treatment of arterial and venous thrombosis; BAY 59-7939 is currently undergoing clinical evaluation.

Acknowledgements

The authors thank U. Buetehorn for measuring plasma levels, and B. Arndt, M. Harwardt, U. Lange, A. Trabandt, and M. Voegler for technical assistance.

References

1 Hirsh J, Dalen JE, Anderson DR, Poller L, Bussey H, Ansell J, Deykin D, Brandt JT. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest* 2001; **119**: 8S–21S.

2 Ansell J, Hirsh J, Dalen J, Bussey H, Anderson D, Poller L, Jacobson A, Deykin D, Matchar D. Managing oral anticoagulant therapy. *Chest* 2001; **119**: 22S–38S.

3 Wells PS, Holbrook AM, Crowther NR, Hirsh J. Interactions of warfarin with drugs and food. *Ann Intern Med* 1994; **121**: 676–83.

4 Mann KG, Brummel K, Butenas S. What is all that thrombin for? *J Thromb Haemost* 2003; **1**: 1504–14.

5 Weinz C, Buetehorn U, Daehler HP, Kohlsdoffer C, Pleiss U, Sandmann S, Schlemmer K-H, Schwarts T, Steinke W. Pharmacokinetics of BAY 59-7939—an oral, direct Factor Xa inhibitor—in rats and dogs. *Pathophysiol Haemost Thromb* 2004; **33** (Suppl. 2): PO054.

6 Butenas S, Ribarik N, Mann KG. Synthetic substrates for human factor VIIa and factor VIIa-tissue factor. *Biochemistry* 1993; **32**: 6531–8.

7 Taniuchi Y, Sakai Y, Hisamichi N, Kayama M, Mano Y, Sato K, Hirayama F, Koshio H, Matsumoto Y, Kawasaki T. Biochemical and pharmacological characterization of YM-60828, a newly synthesized and orally active inhibitor of human factor Xa. *Thromb Haemost* 1998; **79**: 543–8.

8 Berry CN, Girard D, Girardot C, Lochot S, Lunven C, Visconte C. Antithrombotic activity of argatroban in experimental thrombosis in the rabbit. *Semin Thromb Hemost* 1996; **22**: 233–41.

9 Berry CN, Girard D, Lochot S, Lecoffre C. Antithrombotic actions of argatroban in rat models of venous, 'mixed' and arterial thrombosis, and its effects on the tail transection bleeding time. *Br J Pharmacol* 1994; **113**: 1209–14.

10 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.

11 Herbert JM, Bernat A, Barthelemy G, Dol F, Rinaldi M. Antithrombotic activity of SR 46349, a novel, potent and selective 5-HT2 receptor antagonist. *Thromb Haemost* 1993; **69**: 262–7.

12 Hara T, Yokoyama A, Ishihara H, Yokoyama Y, Nagahara T, Iwamoto M. DX-9065a, a new synthetic, potent anticoagulant and selective inhibitor for factor Xa. *Thromb Haemost* 1994; **71**: 314–9.

13 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.

14 Nesheim ME, Taswell JB, Mann KG. The contribution of bovine Factor V and Factor Va to the activity of prothrombinase. *J Biol Chem* 1979; **254**: 10952–62.

15 Gerotziafas GT, Depasse F, Chakroun T, Elalamy I, Samama MM. Effect of the oral, direct Factor Xa inhibitor, BAY 59-7939, on thrombin generation after tissue factor pathway activation. ICT, Ljubljana, Slovenia 2004.

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

17 Kubitza D, Becka M, Wensig G, Voith B, Zuehlsdorf M. Single dose escalation study investigating the pharmacodynamics, safety, and pharmacokinetics of BAY 59-7939 an oral, direct Factor Xa inhibitor in healthy male subjects. *Blood* 2003; **102**: Abstract 3010.

18 Kubitza D, Becka M, Wensig G, Voith B, Zuehlsdorf M. Multiple dose escalation study investigating the pharmacodynamics, safety, and pharmacokinetics of BAY 59-7939 an oral, direct Factor Xa inhibitor in healthy male subjects. *Blood* 2003; **102**: Abstract 3004.

19 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.

20 Abendschein DR, Baum PK, Martin DJ, Vergona R, Post J, Rumennik G, Sullivan ME, Eisenberg PR, Light DR. Effects of

© 2005 International Society on Thrombosis and Haemostasis

ZK-807834, a novel inhibitor of factor Xa, on arterial and venous thrombosis in rabbits. *J Cardiovasc Pharmacol* 2000; **35**: 796–805.

21 Vogel GM, Meuleman DG, Bourgondien FG, Hobbelen PM. Comparison of two experimental thrombosis models in rats; effects of four glycosaminoglycans. *Thromb Res* 1989; **54**: 399–410.

22 Herbert JM, Bernat A, Dol F, Herault JP, Crepon B, Lormeau JC. DX 9065A a novel, synthetic, selective and orally active inhibitor of factor Xa: *in vitro* and *in vivo* studies. *J Pharmacol Exp Ther* 1996; **276**: 1030–8.

23 Herbert JM, Herault JP, Bernat A, van Amsterdam RG, Vogel GM, Lormeau JC, Petitou M, Meuleman DG. Biochemical and pharmacological properties of SANORG 32701. Comparison with the 'synthetic pentasaccharide' (SR 90107/ORG 31540) and standard heparin. *Circ Res* 1996; **79**: 590–600.

24 Perzborn EP, Strassburger J, Wilmen A, Lampe T, Pernerstorfer P, Pohlmann J, Roehrig S, Schlemmer K-H, Straub A. Biochemical and pharmacologic properties of BAY 59-7939, an oral, direct Factor Xa inhibitor. *Pathophysiol Haemost Thromb* 2004; **33** (Suppl. 2), Abstract PO079.

25 Hoppensteadt D, Neville B, Maddenini J, Perzborn E, Misselwitz F, Fareed J. Comparative anticoagulant and antiprotease actions of BAY 59–7939—an oral, direct Factor Xa inhibitor—and enoxaparin and fondaparinux. ICT, Ljubljana, Slovenia 2004.

26 Kaiser B, Jeske W, Walenga JM, Fareed J. Inactivation of factor Xa by the synthetic inhibitor DX-9065a causes strong anticoagulant and antiplatelet actions in human blood. *Blood Coagul Fibrinolysis* 1999; **10**: 495–501.

© 2005 International Society on Thrombosis and Haemostasis