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Effects of Rivaroxaban on Platelet Activation and Platelet–Coagulation Pathway Interaction: In Vitro and In Vivo Studies

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Abstract

Introduction: Activation of coagulation and platelets is closely linked, and arterial thrombosis involves coagulation activation as well as platelet activation and aggregation. In these studies, we investigated the possible synergistic effects of rivaroxaban in combination with antiplatelet agents on thrombin generation and platelet aggregation in vitro and on arterial thrombosis and hemostasis in rat models. **Materials and Methods:** Thrombin generation was measured by the Calibrated Automated Thrombogram method (0.5 pmol/L tissue factor) using human platelet-rich plasma (PRP) spiked with rivaroxaban (15, 30, or 60 ng/mL), ticagrelor (1.0 μg/mL), and acetylsalicylic acid (ASA; 100 μg/mL). Tissue factor-induced platelet aggregation was measured in PRP spiked with rivaroxaban (15 or 30 ng/mL), ticagrelor (1 or 3 μg/mL), or a combination of these. An arteriovenous (AV) shunt model in rats was used to determine the effects of rivaroxaban (0.01, 0.03, or 0.1 mg/kg), clopidogrel (1 mg/kg), ASA (3 mg/kg), and combinations on arterial thrombosis. **Results:** Rivaroxaban inhibited thrombin generation in a concentration-dependent manner and the effect was enhanced with ticagrelor and ticagrelor plus ASA. Rivaroxaban and ticagrelor also concentration-dependently inhibited tissue factor-induced platelet aggregation, and their combination increased the inhibition synergistically. In the AV shunt model, rivaroxaban dose-dependently reduced thrombus formation. Combining subefficacious or weakly efficacious doses of rivaroxaban with ASA or ASA plus clopidogrel increased the antithrombotic effect. **Conclusion:** These data indicate that the combination of rivaroxaban with single or dual antiplatelet agents works synergistically to reduce platelet activation, which may in turn lead to the delayed/reduced formation of coagulation complexes and vice versa, thereby enhancing antithrombotic potency.

Keywords

antiplatelet agents, combination drug therapy, platelet aggregation, rivaroxaban, thrombosis

Introduction

Acute coronary syndrome (ACS) manifests clinically as either an acute myocardial infarction (with or without ST-segment elevation) or an unstable angina,[1,2] which involves the progression, instability, or rupture of atherosclerotic coronary plaques. If erosion or rupture of a plaque occurs, thrombogenesis takes place via platelet activation and aggregation and activation of coagulation, leading to complete or partial vessel occlusion.[3,4] Patients who experience ACS as a result of coronary thromboembolism are often at high risk of recurrent events.[2] The current standard of care for secondary prevention of cardiovascular events is dual antiplatelet therapy with acetylsalicylic acid (ASA)—an irreversible inhibitor of cyclooxygenase 1 that thereby inhibits thromboxane A2 synthesis[5,6]—in combination with an inhibitor of the adenosine diphosphate (ADP) platelet receptor $P2Y_{12}$ (such as clopidogrel, prasugrel, or ticagrelor).[2,3] The clinical benefit of these agents has been established, yet there remains a residual risk of recurrent events.[7,8] This risk may partially be due to the persistent excess of thrombin generation after myocardial infarction.[9] There is evidence that combining anticoagulants (such as unfractionated heparin, low-molecular-weight heparin, fondaparinux, or bivalirudin) with antiplatelet drugs is more effective than either treatment alone for secondary prevention after ACS. The 2012 European Society of Cardiology guidelines recommend this dual-pathway approach with anticoagulant and antiplatelet agents, primarily for the acute phase of treatment.[10]

The quest to improve long-term antithrombotic therapy has led to the development of novel oral anticoagulants that directly target specific proteases within the coagulation cascade, such as

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factor Xa and thrombin.[11] Rivaroxaban is an oral, direct factor Xa inhibitor that competitively binds to the active site of the serine protease (K_i of 0.4 nmol/L), thus preventing substrate binding.[12] It potently inhibits prothrombinase complex-bound factor Xa on the surface of activated platelets (half maximal inhibitory concentration [IC_{50}] of 2.1 nmol/L),[13] and thrombin generation was almost completely inhibited in platelet-rich plasma (PRP) at physiologically relevant concentrations of rivaroxaban (80-100 nmol/L).[14] In animal models of both venous and arterial thrombosis, rivaroxaban has shown potent antithrombotic efficacy.[13,15-18] Rivaroxaban has been found not to affect platelet aggregation induced by collagen, ADP, thromboxane A2, or thrombin.[19,20] However, it has been shown in vitro to potently inhibit tissue factor-mediated platelet aggregation in human plasma indirectly via inhibition of thrombin generation.[12,21]

Activated platelets provide a catalytic surface for initiating and sustaining coagulation, and the large amount of thrombin generated on the surface of activated platelets is responsible for producing a stable hemostatic clot.[22] Therefore, inhibiting platelet activation may affect thrombin generation, as has been shown with $P2Y_{12}$ antagonists such as clopidogrel ex vivo in rat PRP.[23] Another potential candidate for platelet activation is thrombin itself. Thus, reducing thrombin generation by inhibition of factor Xa, together with a reduction in platelet aggregation using antiplatelet agents, may result in a potentiation of antithrombotic efficacy. The aim of this study was to assess the effects of coadministration of rivaroxaban and antiplatelet agents (ASA and/or ticagrelor) on thrombin generation and tissue factor-induced platelet aggregation in human PRP. In addition, the effect of combinations of low-dose rivaroxaban, ASA, and clopidogrel was assessed in models of arterial thrombosis in rats.

Materials and Methods

Agents

In vitro studies. For both the thrombin generation and platelet aggregation studies, rivaroxaban 0.5 mg/mL was dissolved in 100% dimethylsulfoxide (DMSO). This stock solution was further diluted with either 25% DMSO (thrombin generation study) or 50% DMSO (aggregation study). In the thrombin generation study, ticagrelor 1 mg/mL was dissolved in 100% DMSO then diluted with a 25% solution. In the platelet aggregation study, ticagrelor 10 mg/mL was dissolved in 100% DMSO, followed by dilution with 50% DMSO. Ticagrelor was used in the in vitro studies because it does not require metabolic activation (unlike the thienopyridines clopidogrel and prasugrel). The final DMSO concentrations were 0.9% and 1.0% in the thrombin generation assay and the platelet aggregation assay, respectively. Lysine ASA (Aspisol; Bayer HealthCare Pharmaceuticals, Leverkusen, Germany) was dissolved in water for injection and further diluted with demineralized water.

In vivo studies. Rivaroxaban was dissolved in a solution of polyethylene glycol (996 g), water (100 g), and glycerol (60 g). Clopidogrel and ASA were dissolved in 0.5% Tylose.

Animals

Male Wistar rats (HSD CPB; WU, Harlan-Winkelmann, Borchen, Germany) were used in the arteriovenous (AV) shunt model. All animals were fasted overnight but water was available ad libitum. The animals were anesthetized with a mixture of xylazine (12 mg/kg bodyweight) and ketamine (50 mg/kg bodyweight). All procedures were conducted in accordance with the German Animal Protection Act (Deutsches Tierschutzgesetz).

Human Volunteers

For the thrombin generation and platelet aggregation studies, blood samples were obtained from healthy volunteers (age >18 years). The investigations were performed in the Institute of Cardiovascular Research, Acute Care, Bayer Research Centre, Wuppertal, Germany. Written informed consent was obtained from all patients. Exclusion criteria were any medication in the previous 10 days, pregnancy, and a high caloric diet over the last 12 hours prior to blood sampling.

Thrombin Generation

Blood was collected by venipuncture from 11 healthy male and female humans. Samples were placed into vacutainer tubes containing 1/10 volume of 3.12% trisodium citrate, and PRP was obtained by immediate centrifugation of the blood at 140*g* for 20 minutes at 20°C.

Thrombin generation was determined by using the Calibrated Automated Thrombogram (Stago, Paris, France) method in accordance with the manufacturer's instructions with minor modifications. The PRP (74 µL) was spiked with 2 µL (3 times) of each of the appropriate vehicle and/or rivaroxaban 15, 30, and 60 ng/mL; ticagrelor 1.0 µg/mL; ASA 100 µg/mL (plasma concentrations); rivaroxaban plus ticagrelor; or rivaroxaban plus ticagrelor and ASA. Thrombin generation was triggered using 0.5 pmol/L tissue factor (PRP reagent; Stago). Thrombin calibration curves were performed for each individual PRP sample spiked with the solvents. Thrombin generation curves were calculated using Thrombinoscope software (Thrombinoscope, Maastricht, the Netherlands). The following parameters were assessed: lag time, time to peak thrombin generation (T_{max}), peak thrombin generation (C_{max}), endogenous thrombin potential (ETP), and mean velocity ($C_{max}/[T_{max} - \text{lag time}]$).

Platelet Aggregation

Platelet-rich plasma was obtained from 13 healthy male and female humans as per the method described in the thrombin generation study. To adjust platelet count, PRP was diluted with platelet-poor plasma to 300 000–350 000 platelets/µL; platelet-poor plasma was obtained by centrifugation of PRP at 1000*g* for 20 minutes at 20°C. Pefabloc FG (Pentapharm, Basel, Switzerland) was dissolved in demineralized water and added (2 mg/mL; final concentration) to prevent fibrin polymerization. After the addition of $CaCl_2$ (7 mmol/L, final concentration), aliquots (176 µL) were immediately placed in an

aggregometer (Apact 4, DiaSys Greiner, Flacht, Germany). The samples were spiked with 2 µL of increasing concentrations of rivaroxaban (plasma concentrations 7.5-60 ng/mL), ticagrelor (plasma concentrations 0.3-30 µg/mL), or vehicle (for concentration–response curves to determine the appropriate rivaroxaban and ticagrelor concentrations for use during the combination study) and were incubated for 2 minutes at 37°C. Platelets were stored at room temperature in sealed plastic tubes and used within approximately 60 minutes as long as the aggregation response was stable. This resulted in different numbers of investigations between the treatment groups. The following arms were assessed: rivaroxaban 15 and 30 ng/mL, ticagrelor 1 and 3 µg/mL, rivaroxaban plus ticagrelor, and vehicle.

Platelet aggregation was induced by the addition of 20 µL of tissue factor (Néoplastine Plus; Stago), dissolved in an aqueous solution of 10 mmol/L $CaCl_2$ (as per the manufacturer's instructions). Individual tissue factor concentrations (dilution 1:20-1:100 with 10 mmol/L $CaCl_2$ solution) were used to achieve the minimum tissue factor concentration for each experiment, resulting in maximal aggregation. Aggregation was measured turbidimetrically and recorded over 5 minutes and the aggregation response was evaluated as the area under the concentration–time curve for 5 minutes. The IC_{50} values were calculated using the Boltzmann test (GraphPad Prism).

Arteriovenous Shunt Model

An AV shunt model in anesthetized rats was performed as described previously, with minor modifications.[24,25] The right common carotid artery and left jugular vein were isolated and cannulated with 2 catheters connected by Tygon tubing (Saint-Gobain Performance Plastics, Paris, France). The Tygon tubes contained a rough thrombogenic nylon thread (of known weight) folded into a double string. The right common carotid artery was cannulated with an 80-mm polyethylene catheter (internal diameter [ID] = 0.76 mm) connected to a 60-mm Tygon tube (ID = 3.2 mm) containing the nylon thread (60 × 0.26 mm^2). The left jugular vein was cannulated with a 20-mm polyethylene tube (ID = 0.76 mm) connected to a 60-mm polyethylene catheter (ID = 1.14 mm). The tubing was filled with saline and then connected. The extracorporeal shunt was opened for 15 minutes. Subsequently, the nylon thread covered with the thrombus was withdrawn and weighed immediately.

The rivaroxaban, ASA, and clopidogrel dose levels were chosen based on the results of prior dose-finding studies. Rats received intravenous (iv) rivaroxaban 0.01 mg/kg (group 1), 0.03 mg/kg (group 2), or 0.1 mg/kg (group 3); oral clopidogrel 1 mg/kg; or oral ASA 3 mg/kg either alone or in combination, or the appropriate vehicles. Rivaroxaban, ASA, and clopidogrel (or the appropriate vehicles) were given for 15, 40, and 120 minutes, respectively, before the shunt was opened. Rivaroxaban was administered as an iv bolus injection, whereas the other agents/vehicles were administered orally via a gastric tube.

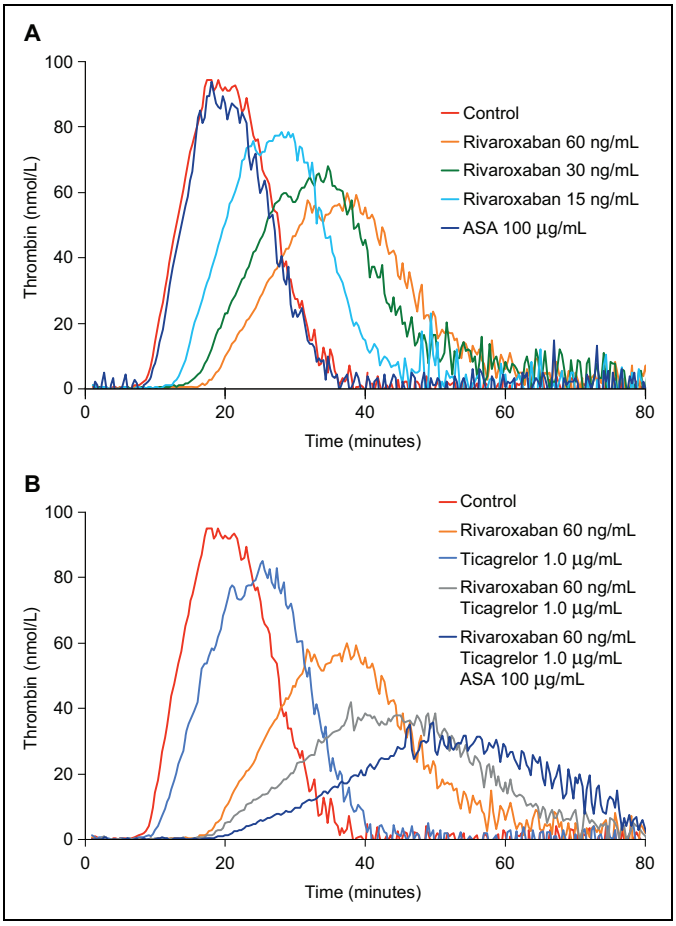

Figure 1. Representative thrombograms in platelet-rich plasma: (A) in the presence of a control, rivaroxaban (15, 30, or 60 ng/mL), or ASA (100 µg/mL); (B) in the presence of a control, rivaroxaban (60 ng/mL), ticagrelor (1.0 µg/mL), or in the combination of rivaroxaban (60 ng/mL) plus ticagrelor (1.0 µg/mL) or rivaroxaban (60 ng/mL) plus ticagrelor (1.0 µg/mL) and ASA (100 µg/mL). ASA indicates acetylsalicylic acid.

Statistical Analysis

Tukey multiple comparison test (1-way analysis of variance [ANOVA]) was used for the statistical analysis, with a significance level of $P < .05$. Results are shown as mean ± standard error of the mean.

Results

Thrombin Generation

Representative thrombograms after activation of the tissue factor pathway are presented in Figure 1, showing the kinetics of thrombin generation in the presence of rivaroxaban (15, 30, or 60 ng/mL) and ASA (100 µg/mL; Figure 1A), or in the presence of rivaroxaban (60 ng/mL), ticagrelor (1.0 µg/mL), or the combination of rivaroxaban (60 ng/mL) plus ticagrelor (1.0 µg/mL) or rivaroxaban (60 ng/mL) plus ticagrelor (1.0 µg/mL) and ASA (100 µg/mL; Figure 1B).

Rivaroxaban alone affected thrombin generation in a concentration-dependent manner, with decreases in C_{max} and

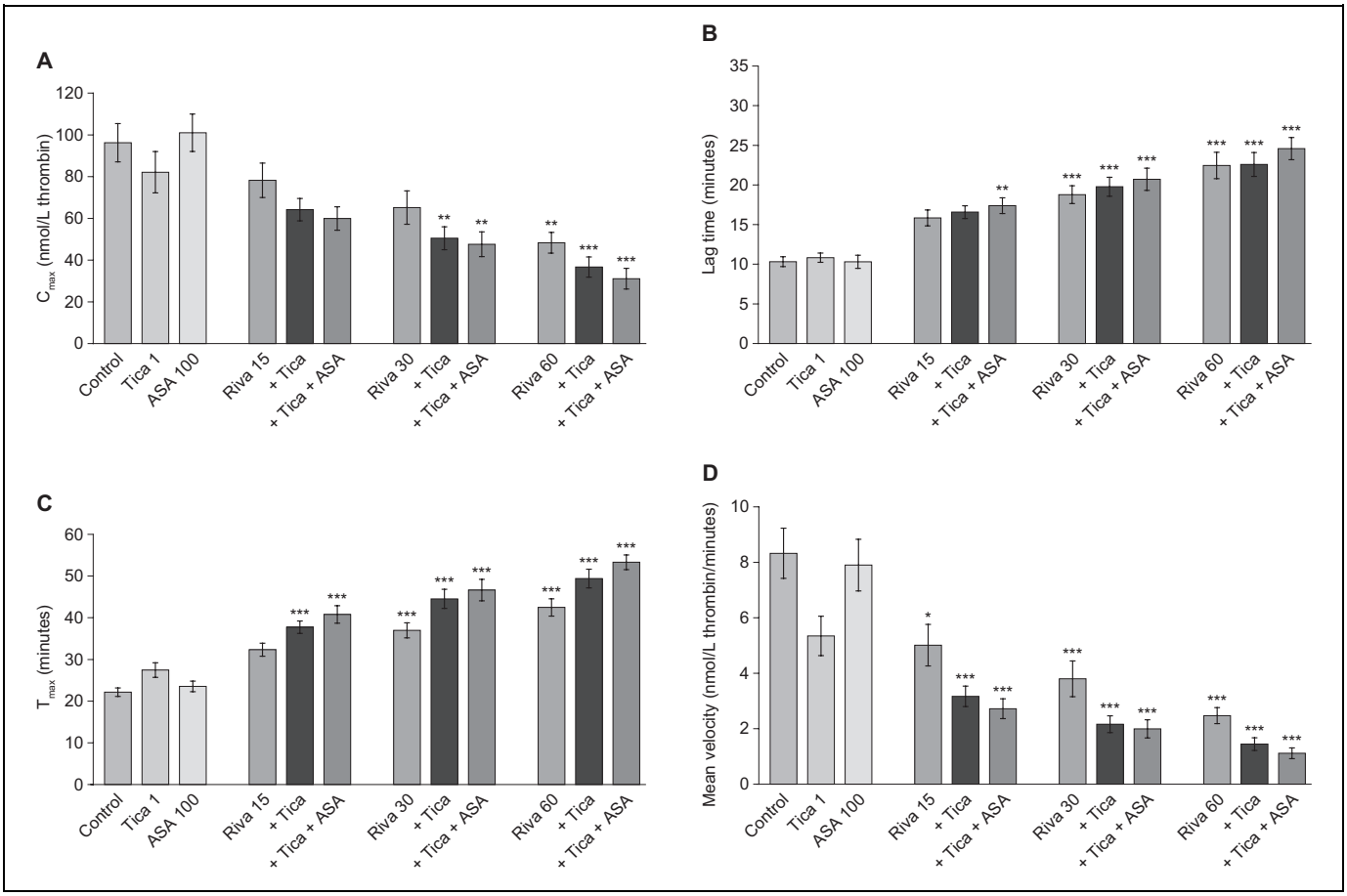

Figure 2. Thrombin generation in platelet-rich plasma in the presence of rivaroxaban (15, 30, or 60 ng/mL), ticagrelor (1 μg/mL), and ASA (100 μg/mL), and the combination of rivaroxaban plus ticagrelor or rivaroxaban plus ticagrelor and ASA. (A) C_{max}; (B) lag time; (C) T_{max}; and (D) mean velocity. Results are presented as mean values ± standard error of the mean (n = 11). *P < .05 versus control; **P < .01 versus control; ***P < .001 versus control. ASA indicates acetylsalicylic acid; C_{max}, peak thrombin generation; Riva, rivaroxaban; Tica, ticagrelor; T_{max}, time to peak thrombin generation.

mean velocity and an increase in lag time and T_{max} (Figure 2). The ETP was not significantly reduced by rivaroxaban at the low concentrations used (data not shown). By contrast, ASA alone had no influence on any parameter measured (Figure 2). Ticagrelor alone (1.0 μg/mL) had no effect on ETP (data not shown) and lag time, but reduced C_{max} and mean velocity, and prolonged T_{max}, although these effects did not reach statistical significance (Figure 2). There was no consistent effect on lag time. Combining low rivaroxaban concentrations (15, 30, and 60 ng/mL) with ticagrelor had an additive effect on reducing thrombin C_{max} and further decreased the mean velocity and prolonged T_{max}. These effects were slightly, but consistently, enhanced when the triple combination was used (rivaroxaban plus ticagrelor and ASA; Figure 2). For instance, C_{max} was reduced by rivaroxaban alone (60 ng/mL) by 50% and by ticagrelor alone by 16%, versus control; this reduction reached 62% with the combination of rivaroxaban and ticagrelor and 69% with the triple combination. Mean velocity was reduced by 70% with rivaroxaban alone, by 37% with ticagrelor alone, by 83% with the combination of rivaroxaban and ticagrelor, and by 87% with the triple combination. The T_{max} was delayed by the administration of rivaroxaban by 1.9-fold versus control but was more effectively delayed in the presence of ticagrelor (2.3-fold vs control) or with the triple combination of rivaroxaban plus ticagrelor and ASA (2.4-fold increase vs control). The dual combinations of ASA (100 μg/mL) and rivaroxaban (60 ng/mL) or ASA (100 μg/mL) and ticagrelor (1 μg/mL) had no obvious additional effect on any parameter measured compared with rivaroxaban or ticagrelor alone (data not shown).

Platelet Aggregation

Rivaroxaban potently inhibited tissue factor-induced platelet aggregation in a concentration-dependent manner with an IC_{50} of 34 ± 3 ng/mL (Figure 3). Ticagrelor also reduced tissue factor-induced platelet aggregation but the effect reached a plateau of ~50% inhibition at concentrations ≥3.0 μg/mL (Figure 3). The combination of different concentrations of rivaroxaban (15 or 30 ng/mL) and ticagrelor (1.0 or 3.0 μg/mL) resulted in a synergistically increased inhibition of platelet aggregation (Table 1). Rivaroxaban alone at a dose of 15 or 30 ng/mL inhibited platelet aggregation by 20% (nonsignificant)

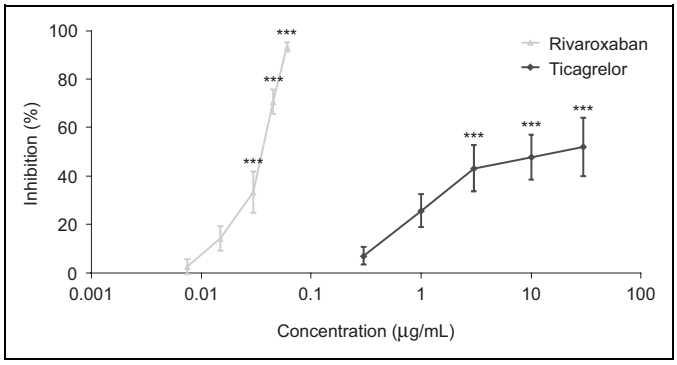

Figure 3. Inhibition of tissue factor-induced platelet aggregation in human platelet-rich plasma by rivaroxaban and ticagrelor. Results are presented as mean values ± standard error of the mean (ticagrelor, n = 9-13; rivaroxaban, n = 11-13). ***$P < .001$ versus control.

Table 1. Effects of Rivaroxaban, Ticagrelor, and Their Combination on Inhibition of Platelet Aggregation in Human Platelet-Rich Plasma.[a,b]

	Inhibition of Platelet Aggregation, % (n)		
	Rivaroxaban, ng/mL		
Ticagrelor, µg/mL	0	15	30
0	–	20 ± 11 (7)	37 ± 11[c] (9)
1	17 ± 6[d] (9)	52 ± 11[c] (6)	90 ± 4[c,e,f] (5)
3	31 ± 8[c] (11)	76 ± 4[c,e,g] (7)	90 ± 6[c,e,f] (6)

[a]Aggregation response was evaluated as area under the curve over 5 minutes.
[b]Data are mean ± standard error of the mean (n = 5-11).
[c]$P < .001$ versus control.
[d]$P < .05$ versus control.
[e]$P < .001$ versus rivaroxaban.
[f]$P < .001$ versus ticagrelor.
[g]$P < .05$ versus ticagrelor.

and 37%, respectively. Ticagrelor alone at a dose of 1.0 or 3.0 µg/mL inhibited platelet aggregation by 17% and 31%, respectively. The combination of rivaroxaban (15 or 30 ng/mL) with ticagrelor at 1.0 µg/mL resulted in inhibition levels of 52% and 90%, respectively, whereas the combination of rivaroxaban with ticagrelor at 3.0 µg/mL led to inhibition levels of 76% and 90%, respectively (Table 1). Acetylsalicylic acid was not included in this set of experiments owing to the short incubation time used; results from our previous studies using collagen as an agonist indicated that, to be effective, ASA might need longer incubation times than those feasible in the presence of DMSO (unpublished data).

Arterial Thrombosis

Rivaroxaban and clopidogrel dose-dependently reduced thrombus formation in the rat AV shunt model with a dose that reduced thrombus formation by 50% (ED_{50}) of 0.33 mg/kg after iv administration and 6.5 mg/kg after oral administration, respectively. Acetylsalicylic acid also demonstrated a dose-dependent inhibition of thrombus formation but reached a plateau effect at 10 mg/kg (which was not statistically different from the 30 mg/kg dose) and showed a maximal inhibition of 30%–47% (Figure 4).

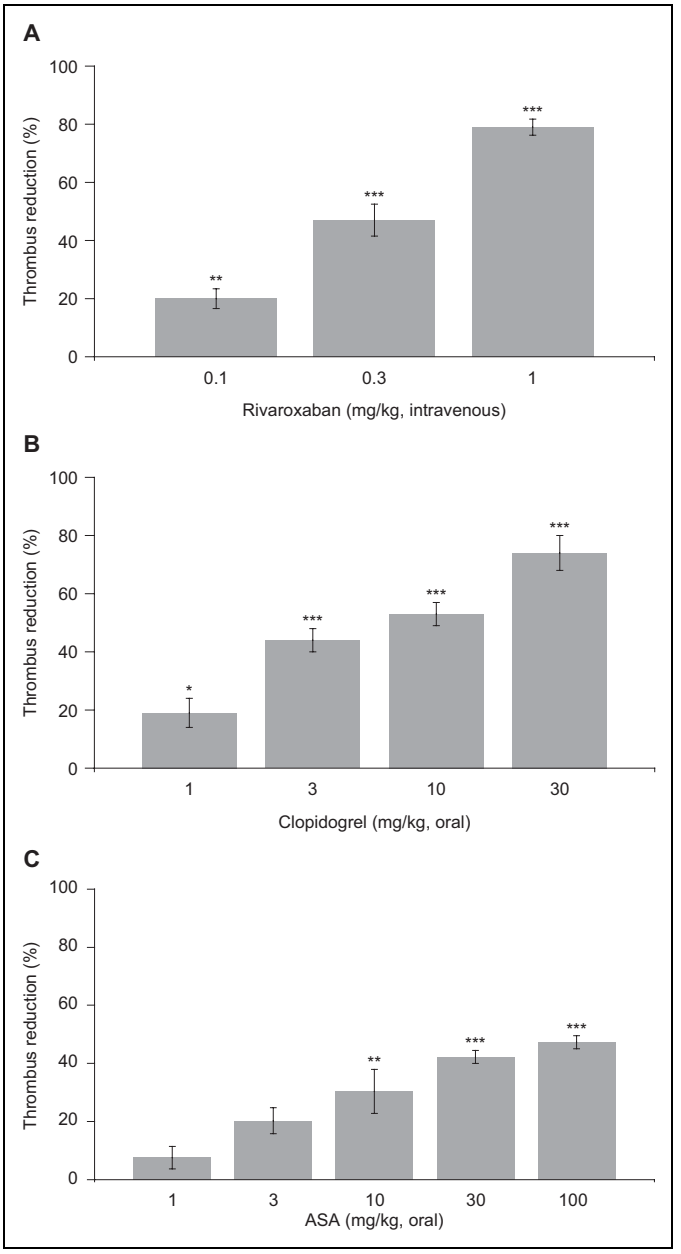

Figure 4. Inhibition of thrombus formation after administration of (A) rivaroxaban (0.1 mg/kg [n = 10], 0.3 mg/kg [n = 9], or 1.0 mg/kg [n = 10] intravenous); (B) clopidogrel (1.0, 3.0, 10, or 30 mg/kg oral; n = 6 per dosing group); or (C) ASA (1.0, 3.0, 10, 30, or 100 mg/kg oral; n = 6 per dosing group) in an arteriovenous shunt model in rats. Results are presented as mean values ± standard error of the mean. *$P < .05$ versus control; **$P < .01$ versus control; ***$P < .001$ versus control. ASA indicates acetylsalicylic acid.

In the combination studies, doses below the ED_{50} were used. Very low doses of rivaroxaban at 0.01 mg/kg (group 1) and 0.03 mg/kg (group 2) had no significant effect on thrombus formation, whereas the 0.1 mg/kg dose (group 3) significantly reduced thrombus formation by 28% versus control (Table 2).

Table 2. Effects of Rivaroxaban (0.01, 0.03, or 0.1 mg/kg Intravenous), Acetylsalicylic Acid (3 mg/kg Oral), or Clopidogrel (1 mg/kg Oral), and Their Combinations, on Thrombus Formation in Anesthetized Rats.[a,b,c]

	Rivaroxaban Alone	ASA Alone	Clopidogrel Alone	Rivaroxaban + ASA	Rivaroxaban + Clopidogrel	ASA + Clopidogrel	Rivaroxaban + ASA + Clopidogrel
Inhibition of thrombus formation, %							
Group 1	18 ± 4	15 ± 5	35 ± 4[d]	24 ± 6[e]	37 ± 7[d]	20 ± 2	43 ± 5[d,f,g]
Group 2	20 ± 7	17 ± 4	28 ± 3[h]	37 ± 4[d]	27 ± 7[h]	26 ± 5[h]	43 ± 3[d,f,g]
Group 3	28 ± 3[h]	22 ± 6	30 ± 5[h]	39 ± 7[d]	52 ± 4[d,f,g]	37 ± 7[d]	51 ± 2[d,f,g]

Abbreviation: ASA, acetylsalicylic acid.
[a]n = 6 per group.
[b]Group 1 received 0.01 mg/kg rivaroxaban, group 2 received 0.03 mg/kg rivaroxaban, and group 3 received 0.1 mg/kg rivaroxaban.
[c]Results are mean ± standard error of the mean (n = 6).
[d]$P < .001$ versus control.
[e]$P < .05$ versus control.
[f]$P < .05$ versus rivaroxaban.
[g]$P < .01$ versus ASA.
[h]$P < .01$ versus control.

Clopidogrel at 1 mg/kg also caused a significant, moderate inhibition of thrombus formation (28%-35% vs control), whereas ASA (3 mg/kg) failed to inhibit thrombus formation significantly (Table 2). When various combinations of rivaroxaban and the antiplatelet agents were investigated, synergistic effects were observed. Combining the subefficacious doses of rivaroxaban (0.01 or 0.03 mg/kg) and ASA (3 mg/kg) resulted in a modest but significant reduction in thrombus formation (24% and 37% vs control, respectively; Table 2). Likewise, the addition of subefficacious doses of rivaroxaban to ASA and clopidogrel (ie, triple combination) resulted in a further significant increase in the antithrombotic effect (43% vs control; Table 2). The addition of a moderately effective dose of rivaroxaban (0.1 mg/kg) to either ASA or clopidogrel, or both agents together, further increased the antithrombotic effect. Dual-pathway inhibition with rivaroxaban and clopidogrel alone was as effective as the triple combination of rivaroxaban, clopidogrel, and ASA. Compared with the control, the 0.1 mg/kg dose of rivaroxaban inhibited thrombus formation by 39%, 52%, and 51% when combined with ASA, clopidogrel, and ASA plus clopidogrel, respectively (Table 2). All triple combinations of rivaroxaban, clopidogrel, and ASA were more effective than dual antiplatelet combinations of ASA and clopidogrel (Table 2).

Discussion

To determine whether there was a synergistic effect with rivaroxaban in combination with antiplatelet therapy, the concentrations/doses selected for the combination studies were chosen in order to result in an inhibition of <50% based on the respective concentration/dose–response curves. The results of these studies indicated that the combination of rivaroxaban with single or dual antiplatelet therapy (ie, dual-pathway approach) resulted in improved antithrombotic activity compared with rivaroxaban or antiplatelet therapy alone, both in vitro and in vivo. Moreover, subefficacious doses of rivaroxaban in combination with antiplatelet drugs showed a significant antithrombotic effect. The data from the in vitro studies provide a rationale for the synergistic effect observed in vivo.

Rivaroxaban is a direct factor Xa inhibitor; it thereby inhibits thrombin generation and thus indirectly inhibits thrombin-induced platelet aggregation. In the present thrombin generation study, the effects of rivaroxaban were strongest on the dynamics of thrombin generation expressed by lag time, mean velocity, thrombin T_{max}, and thrombin C_{max}, representing the amount of thrombin generated at maximal velocity, compared with the ETP. Ticagrelor, which partly reduces tissue factor-induced platelet aggregation and thereby probably affects thrombin generation, affected T_{max} and mean velocity, as well as C_{max}, at a concentration of 1 μg/mL, but it did not influence lag time or ETP, whereas ASA alone did not affect any thrombin generation parameter. The combination of ticagrelor and rivaroxaban further reduced thrombin C_{max} and mean velocity, and prolonged thrombin T_{max}, compared with either agent alone. The combination of ASA, ticagrelor, and rivaroxaban slightly improved the effects compared with the dual rivaroxaban/ticagrelor combination on the same parameters, with the largest impact on mean velocity, thrombin T_{max}, and thrombin C_{max}. Similar synergistic effects were also seen with rivaroxaban and ticagrelor for the inhibition of tissue factor-induced platelet aggregation. These data suggest that dual or triple combination therapy further delayed the thrombin generation process, which may contribute to the enhanced antithrombotic effects observed in vivo. These findings could be considered clinically relevant because the low concentration of rivaroxaban used in the current study is equivalent to the trough plasma concentration after rivaroxaban 2.5 mg twice-daily dosing in humans.[26]

Other reports show that blocking the $P2Y_{12}$ ADP receptor with clopidogrel affects the amount of thrombin formed,[23] unlike ASA.[27] Activation of coagulation and platelets is closely linked in various ways, for example, many steps in the coagulation cascade are accelerated markedly on activated platelet membranes, which provide a catalytic surface for initiating and sustaining coagulation. Therefore, reduced platelet activation

may lead to the delayed/reduced formation of coagulation complexes, which affects thrombin generation in addition to the inhibition of prothrombinase activity by factor Xa inhibitors. This seems to be the case when considering the effects that combination therapy had on thrombin generation dynamics and platelet aggregation activity over and above monotherapy in the present in vitro studies. Both studies showed that $P2Y_{12}$ receptor blockers can enhance the inhibitory effects of rivaroxaban, suggesting the potential for an improved antithrombotic efficacy with this dual-pathway approach. It has also been shown that in blood samples from patients with known coronary artery disease who were receiving ASA, rivaroxaban inhibited tissue factor-induced (and to a lesser extent, thrombin-induced) platelet activation in vitro—effects that were accentuated when combined with a direct-acting $P2Y_{12}$ antagonist (cangrelor).[28]

In vitro studies measuring platelet aggregation and thrombin generation do not mirror the complex situation of thrombus development. Furthermore, the degree of aggregation inhibition by ASA is time dependent,[29] and the in vitro system used did not contain collagen as the activator of thromboxane A2 synthesis and platelet activation. Therefore, we investigated the effect of the combination of anticoagulation and inhibition of platelet aggregation in arterial thrombosis in vivo in a well-established AV shunt model. The antithrombotic efficacy of a subefficacious dose and a moderately effective dose of rivaroxaban was enhanced by the coadministration of antiplatelet agents (ASA and/or clopidogrel). This was expected in light of the observed effects of such a dual-pathway approach on thrombin generation and thrombin-induced platelet aggregation in the in vitro studies in human plasma.

A rat AV shunt model with specifically reduced arterial shear rates was used in this study for 2 reasons. The antithrombotic activity of ASA is lost at high shear rates, depending on the thrombogenic stimulus, in contrast to ADP antagonists or rivaroxaban.[13,30] Avoiding high shear rates allows investigation of the antithrombotic activity of ASA. In addition, the thrombus formed on the thread in the rat AV shunt has been described as a mixed thrombus composed of platelet aggregates surrounded by a red thrombus composed of red blood cells and fibrin.[25,31] It has been shown that fibrin is a major component of intracoronary thrombi, even at early stage in patients with ST-segment elevation myocardial infarction.[32] Our in vivo results are in agreement with those shown recently by Becker et al[33] in a porcine model of ex vivo stent thrombosis. Rivaroxaban reduced stent thrombosis, and rivaroxaban combined with ASA further reduced thrombus weight. The triple combination of rivaroxaban, ASA, and clopidogrel almost completely abolished stent thrombosis.[33] In another study, rivaroxaban administered to healthy volunteers inhibited ex vivo thrombus formation in a perfusion chamber system under low and high shear rates. A significantly greater reduction in thrombus D-dimer levels was observed with the coadministration of ASA and a low dose (5 mg) of rivaroxaban, compared with monotherapy, at low shear rates.[34,35] Furthermore, in healthy participants, a triple therapy of ticagrelor and ASA in combination with rivaroxaban (or dabigatran) effectively inhibited platelet activation and thrombin generation at the site of plug formation in an in vivo model in the skin microvasculature, by measuring shed blood β-thromboglobulin, prothrombin fragment 1 + 2, and thrombin–antithrombin complex concentrations.[34,35] Collectively, these findings further support the enhanced antithrombotic effect with the dual-pathway approach.

The efficacy and safety of low doses of rivaroxaban (2.5 or 5 mg twice daily) in combination with single or dual antiplatelet therapy have been demonstrated in the phase III ATLAS ACS 2 TIMI 51 study.[36] The majority of patients were receiving ASA plus a thienopyridine (either clopidogrel or ticlopidine). Compared with patients receiving antiplatelet therapy alone, rivaroxaban significantly reduced the composite primary end point of death from cardiovascular causes, myocardial infarction, or stroke, without an increase in fatal bleeding (although the risk of major bleeding and intracranial hemorrhage was significantly higher with rivaroxaban). The authors concluded that the addition of very low-dose anticoagulation with rivaroxaban to existing antiplatelet therapy may represent a new treatment strategy in patients with a recent ACS.[36] In addition, the ongoing phase III clinical study COMPASS is currently investigating rivaroxaban, low-dose rivaroxaban plus ASA, or ASA alone for reducing the risk of myocardial infarction, stroke, or cardiovascular death in coronary artery disease or peripheral artery disease (NCT01776424; www.clinicaltrials.gov). The data from the current studies provide a mechanistic rationale and further evidence that addition of a low dose of rivaroxaban to single or dual antiplatelet therapy could offer greater antithrombotic effects than antiplatelet therapy alone, for example, in patients with ACS. It would be interesting to test the effect of ticagrelor (as well as clopidogrel) in combination with rivaroxaban on the reduction of thrombus formation, but clopidogrel was the standard of care agent and is the most widely used $P2Y_{12}$ antagonist. Nevertheless, these studies provide a mechanistic rationale for the greater antithrombotic efficacy of combination therapy, that is, the dual-pathway approach. Future studies with newer antiplatelet agents in combination with low doses of rivaroxaban in different models may provide further insights into antithrombotic therapy in secondary prevention after an ACS.

Conclusion

The results of these studies indicate that, in addition to the direct inhibition of factor Xa and thrombin generation, the indirect effect of rivaroxaban on thrombin-mediated platelet aggregation may be particularly beneficial for the management of arterial thrombosis. A dual-pathway approach with a very low dose of rivaroxaban and single or dual antiplatelet agents could further improve antithrombotic therapy in ACS through a synergistic effect.

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Author Contribution

Elisbeth Perzborn contributed to conception and design; acquisition, analysis, and interpretation; drafted the article; critically revised the article; gave final approval; and agreed to be accountable for all aspects of work ensuring integrity and accuracy. Stefan Heitmeier and Volker Laux contributed to design, analysis and interpretation, critically revised the article, gave final approval, and agreed to be accountable for all aspects of work ensuring integrity and accuracy.

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