Apixaban Inhibits Cerebral Microembolic Signals Derived from Carotid Arterial Thrombosis in Rabbits

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ABSTRACT

Cerebral microembolic signal (MES) is an independent predictor of stroke risk and prognosis. The objective of this study is to assess the effects of apixaban, as a representative of the novel oral anticoagulant class, on a rabbit model of cerebral MES. A clinical transcranial Doppler ultrasound instrument was used to assess MESs in the middle cerebral artery in a 30% FeCl₃-induced carotid arterial thrombosis model in male New Zealand White rabbits. Ascending doses of apixaban were evaluated as monotherapy and in combination with aspirin on both arterial thrombosis and MES. Pharmacokinetic and pharmacodynamic responses were also evaluated. The effective dose for 50% inhibition (ED₅₀) of thrombus formation for monotherapy was 0.04 mg/kg per hour apixaban, i.v. (0.03 μM plasma exposure) for the integrated blood flow, 0.13 mg/kg per hour apixaban (0.10 μM plasma exposure) for thrombus weight, and 0.03 mg/kg per hour apixaban (0.02 μM plasma exposure) for MES. Dual treatment with aspirin (5 mg/kg, PO) and apixaban (0.015 mg/kg per hour, i.v.) resulted in a significant reduction in cerebral MES (P < 0.05) compared with monotherapy with either agent. Pharmacokinetic analysis of apixaban and pharmacodynamic assays using activated partial thromboplastin time (aPTT) and prothrombin time (PT) for apixaban- and arachidonic acid-induced platelet aggregation for aspirin were used to confirm the exposure-response relationships. In summary, our study demonstrates that apixaban in a concentration-dependent manner inhibits both arterial thrombosis and MES, suggesting a potential association between factor Xa (FXa) blockade and the reduction in MES in patients at risk of ischemic stroke.

Introduction

Cerebral embolism is a common cause of ischemic stroke. Microembolic signals (MESs) in patients with carotid stenosis, myocardial infarction, atrial fibrillation, and mechanical cardiac valves are often detectable in the cerebral circulation by a transcranial Doppler (TCD; Levi et al., 1997; Purkayastha and Sorond, 2012). Clinical evidence has demonstrated that the presence of MES in the cerebral circulation is an independent predictor of the risk and prognosis of stroke (Gao et al., 2004; Markus et al., 2005) and recurrence in patients with previous stroke or transient ischemic attack (TIA) of presumed arterial origin (Valton et al., 1998). MES was also shown to be a clinically relevant biomarker for assessing the efficacy of antiplatelet agents (e.g., clopidogrel and aspirin) in stroke prevention (Markus et al., 2005; Wong et al., 2010). Despite clinical evidence for the correlation between MES and the risk of ischemic stroke, no animal model has been available to evaluate cerebral microembolism for translational research. Therefore, we recently developed a rabbit model of cerebral MES in the setting of ferric chloride (FeCl₃)-induced carotid arterial thrombosis and demonstrated a reduction of MES in response to clopidogrel and aspirin treatment in this model (Zhou et al., 2016).

In contrast to antiplatelet agents, fewer studies (Al-Atassi et al., 2012; Demir et al., 2015) have been devoted to investigation of the effects of anticoagulants on MES, and the results are inconclusive. Since warfarin and the novel oral anticoagulants (NOACs) are the currently available options for stroke prevention in atrial fibrillation (Lin et al., 2015) and recent phase III clinical studies indicate that NOACs resulted in an overall better efficacy-safety profile than warfarin in patients with nonvalvular atrial fibrillation (Lin et al., 2015; Morais and De Caterina, 2016), we aimed to investigate the effects of apixaban as a representative example for the NOAC class on cerebral MES in our recently established preclinical model of cerebral MES. Apixaban was selected for this study because its in vitro and in vivo properties have been extensively characterized, along with efficacy assessment in various rabbit models of thrombosis and hemostasis for translational research (Pinto et al., 2007; Wong et al., 2011). Thus, the effects of apixaban on both arterial thrombosis in the carotid artery and MES in the middle cerebral artery (MCA) were monitored simultaneously in the current study, as well as the pharmacokinetic and pharmacodynamic parameters in response to the treatment. Furthermore, since aspirin is used as a standard of care in patients with risk of ischemic stroke, the dual therapy of apixaban with aspirin was also assessed.

REFERENCES

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ABBREVIATIONS: AA, Arachidonic acid; aPTT, activated partial thromboplastin time; MCA, middle cerebral artery; MES, microembolic signals; NOACs, novel oral anticoagulants; PRP, platelet-rich plasma; PT, prothrombin time; TCD, transcranial Doppler.
Material and Methods

Animals. Studies were conducted in male New Zealand White rabbits weighing 2.4–3.0 kg (obtained from Charles River Canada). All the animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health, and were approved by the Institution’s Animal Care and Use Committee of Merck Research Laboratories.

FeCl₃-Induced Carotid Arterial Thrombosis and Cerebral Microembolic Signal Detection. The FeCl₃ injury model was established on the basis of procedures described previously (Marsh Lyle et al., 1998; Wang and Xu, 2005) with modifications to optimize study of MES as part of a terminal procedure in rabbits (Zhou et al., 2016). Briefly, animals were anesthetized with a cocktail (ketamine HCl, 50 mg/kg (Pfizer Inc., New York, New York) and xylazine, 5 mg/kg, i.m. (LLOYD, Inc., Shenandoah, IA)), and the left common carotid artery was surgically exposed. A Doppler flow probe (Model 1.5 PRB; Transonic Systems, Ithaca, NY) connected to a flowmeter (Model T403; Transonic Systems) was placed on the surface of the artery and blood flow was continuously measured using a PowerLab 16/35 and LabChart Pro data acquisition system (AD Instruments, Colorado Springs, CO). Thrombosis was induced by applying to the adventitial surface of the vessel two pieces of disc-shaped filter papers [7.4-mm diameter, 0.5-mm thick, one above and one beneath the vessel (Life Technologies/Thermo Fisher Scientific, Grand Island, NY)] saturated with 30%, mass/volume, FeCl₃ (anhydrous 98%; ACROS Organics/Thermo Fisher Scientific, Waltham, MA). A piece of parafilm (Fisher Scientific) was put underneath the vessel to protect the surrounding tissue from injury. The filter papers were applied for 5 minutes followed by washout of the residual FeCl₃ by sterile, warm saline. The carotid blood flow was monitored for 60 minutes from the application of FeCl₃ (as time zero). Integrated carotid blood flow over 60 minutes was measured by area under the curve (AUC) calculated by the trapezoidal rule, and expressed as percent of control blood flow as described previously (Wong et al., 2008b). At the end of study i.e., 60 minutes after FeCl₃ injury, a section of vessel at the injury site (~20 mm long) was cut and the thrombus was pushed out using a blunt forceps to a parafilm with a drop of saline. The thrombus was briefly washed in saline (to remove loosely trapped blood) and semidried on a piece of parafilm (to remove residual water). The wet weight of thrombus was measured using a balance with a detection limit of 0.001 mg (Mettler Toledo Excellence Plus XP Series Analytical Balances; Mettler-Toledo, LLC, Columbus, OH). Afterward, the vessel was cut to open/validate complete removal of thrombus.

The SONARA TCD system (Nicolet Natus Neurology Inc., Middleton, WI) was used to continuously monitor blood flow velocity and MES in the ipsilateral MCA. A pulsed-wave 2-MHz probe (OD = 11.3 mm, mean frequency (E) and incidence of MES (F) in MCA were also dose dependently inhibited. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus vehicle. Fig. 1. Dose-dependent effect of apixaban on FeCl₃-induced arterial thrombosis and cerebral MES. Apixaban dose dependently inhibited 30% FeCl₃-induced carotid arterial thrombosis (vehicle, n = 6; 0.015, n = 6; 0.05, n = 6; 0.15, n = 7; and 0.5 mg/kg per hour, i.v., n = 6) as illustrated using carotid blood flow within 60 minutes upon FeCl₃ injury (A) with AUC of the integrated blood flow (B), and reduction in clot weight (C). MES was monitored simultaneously in the MCA ipsilateral to the FeCl₃ injury. (D) Representative image for MES detection by TCD in a vehicle-treated animal. The snapshot of TCD recording in a period of 4 seconds, showing the calculated parameters of “Mean” for the mean blood flow velocity/frequency in units of cm/s or kHz, the “Peak” for the maximal systolic velocity/frequency in units of cm/s or kHz, “EDV” for the end diastolic velocity, “PI” for the Gosling pulsatility index, and “HR” for the heart rate. The MES was indicated in an arrow determined by the Sonora software and confirmed manually as described in details in Materials and Methods. Mean frequency (E) and incidence of MES (F) in MCA were also dose dependently inhibited. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 versus vehicle.
90-mm long, focused at 12–25 mm, customized by MTB Medizintechnik Basler AG (Regensdorf, Switzerland) was fixed by a flexible-arm magnetic-base holder (McMaster-CARR, Princeton, NJ) at the posterior end of zygomatic bone of the rabbits, at an angle of ~80 degrees against the buccal surface. The animal was lying supine on a warm pad during the entire period of the experiment. The proximal segment of the MCA was insonated at a depth between 19 and 22 mm, with gain at 1, power at 43%, scale at 77, sample volume of 5, and detection threshold at 3.0 using a unilateral monitory mode. During continuous monitoring, the blood flow profile and MES (defined as high-intensity transient signals, HTSs) were saved, and the frequency of MES was automatically determined using an algorithm designed by the Sonora software (Nicolet Natus Neurology Inc.). The recorded MES was further confirmed by an off-line manual analysis on the basis of the criteria defined by the International Consensus Committee (Consensus Committee, 1995; Ringelstein et al., 1998): 1) a unidirectional and short-lasting (<300 milliseconds) signal with an amplitude >3 dB above background, 2) with a traverse at a prespecified depth, and 3) a typical “snap,” “chirp,” or “moan” audible output.

**Drug Administration.** The structure and compound profiles on selectivity, potency, and pharmacokinetic and pharmacodynamic parameters of apixaban have been extensively investigated and reported previously (Pinto et al., 2007; Wong et al., 2011). The intravenous dosing regimen for apixaban in rabbit essentially followed previous reports (Wong et al., 2008a,b) with the following specification: Apixaban dosing solution was prepared in vehicle (35% hydroxypropyl β-cyclodextrin in 10 mM phosphate buffer, pH 7.0) and dosed using continuous intravenous infusion (starting 60 minutes prior to FeCl3 injury), 2 ml/kg. Our pilot pharmacokinetic study (blood samples at 0, 1, and 2 hours of intravenous dosing) for the use of 0.5 mg/kg per hour, demonstrated that plasma drug exposure for apixaban had achieved a steady-state level during the course of experiment (i.e., between 1 and 2 hours after dosing). For the dose-response study, apixaban (0, 0.015, 0.05, 0.15, and 0.5 mg/kg per hour) was intravenously infused at a volume of 2 ml/kg per hour for 2 hours. Aspirin (cat. no. A2093; Sigma-Aldrich, St. Louis, MO) was dissolved in vehicle (0.5% methyl cellulose) and dosed orally once daily for 3 days (2 ml/kg). Owing to rapid hydrolysis of aspirin in solution in a temperature-dependent manner, the dosing solution was kept at 4°C and dosed within 24 hours of preparation. Rabbits were subjected to FeCl3 injury 1 hour after apixaban infusion, or 2 hours after the last oral dosing of aspirin. For a dual combination study, a partially effective dose of both aspirin (5 mg/kg, p.o., for 3 days) and apixaban (0.015 mg/kg per hour, i.v.) was used to determine the combined efficacy on arterial thrombosis and cerebral MES. The 5-mg/kg aspirin, PO dose was selected on the basis of the dose-dependent results reported previously in the same model (Zhou et al., 2016) and confirmed immediately prior to our combination study using vehicle, 5 and 25 mg/kg aspirin, PO, daily for 3 days (n = 5 each). The results of this study are illustrated in the Supplemental Figs. 1 and 2. As in our previous report (Zhou et al., 2016), partial inhibition by 5 mg/kg aspirin of both arachidonic acid-induced platelet aggregation and serum thromboxane B2 levels and almost complete abolishment by 25 mg/kg aspirin were achieved.

**Pharmacokinetics and Ex Vivo Clotting Time Assays.** Blood samples were collected into sodium citrate (with 3.2% final concentration) Vacutainers (Becton Dickinson, Franklin Lakes, NJ) from either the central ear artery or the carotid artery at terminal bleed. Blood samples were centrifuged at 2000g for 15 minutes at 4°C for plasma preparation and evaluated for pharmacokinetic and ex vivo clotting time assays.

For pharmacokinetic analysis, the plasma samples and plasma standards and quality controls were assessed by protein precipitation with acetonitrile (0.5 ml plasma + 300 μl acetonitrile). The supernatant of the plasma was then analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) for apixaban using Waters Acquity UPLC system (Waters Corporation, Milford, MA) for liquid chromatography and Applied Biosystems/MSD Sciex API 5500 Q-Trap (Applied Biosystems, Forster City, CA) for mass spectrometry analyses. Specifically, water, 0.1% formic acid was used as mobile phase A solution, and acetonitrile, 0.1% formic acid as mobile phase B solution. Water/acetonitrile/formic acid (80:20:0.1, v/v/v) was used as autosampler wash 1, followed by acetonitrile/ isopropanol/acetone/formic acid (50:40:10:0.5, v/v/v/v) as autosampler wash 2. The MonChrom C18, 100 × 2.0-mm, 3-μm column was used at 50°C. A gradient solution containing 95–5% of mobile phase A solution in combination with 5–95% of mobile phase B solution was used to elute the column.

Ex vivo plasma activated partial thromboplastin time (aPTT) and prothrombin time (PT) were determined by standard methods using Pacific Hemostasis aPTT-XL (Thermo Fisher Scientific) and TriniCLOT PT Excel (Teog, Bray, Ireland) on a KC4 Delta coagulation analyzer (Teog). **Ex Vivo Platelet Aggregation Assay.** Platelet aggregation studies were performed ex vivo using citrated platelet-rich plasma
Platelet aggregation was monitored for 5 min at 300, 600, and 900 Paired Jolla, CA) was used for comparison among groups at different doses. Bonferroni post-hoc test in GraphPad Prism (Version 6; GraphPad, La Jolla, CA) was used for comparison among groups at different doses. The peak aggregation response was recorded in percentage agonist. Platelet-poor plasma was used as 100% transmittance for aggregation. 

Data Analysis and Statistics. All the data are presented as mean ± S.E. One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test in GraphPad Prism (Version 6; GraphPad, La Jolla, CA) was used for comparison among groups at different doses. Paired t test was applied for comparisons between paired groups. t test was applied for comparisons between paired groups. The peak aggregation response was recorded in percentage agonist. Platelet-poor plasma was used as 100% transmittance for aggregation. 

Effect of Apixaban on Cerebral MES Induced by Carotid Arterial Thrombosis in Rabbits. Following the pilot pharmacokinetic study (on a 0.5 mg/kg-per-hour dose) to confirm a steady-state level of drug exposure in rabbits between 1–2 hours after intravenous infusion, dose-dependent effects of apixaban on thrombus formation in the carotid artery and on MES in the MCA were assessed simultaneously in the 30% FeCl3-induced carotid arterial thrombosis model in rabbits. As shown in Fig. 1, the integrated blood flow (AUC, illustrated as percentage of blood flow relative to baseline) showed dose-dependent increase from 62.8 ± 6.2% in vehicle group (n = 6) to 91.2 ± 2.7% for the 0.5 mg/kg-per-hour apixaban group (n = 6, P < 0.001), with ED50 of 0.04 mg/kg per hour apixaban, i.v. (Fig. 1, A and B). Clot weight was reduced from 6.9 ± 0.8 mg (vehicle) to 0.8 ± 0.2 mg (0.5 mg/kg per hour apixaban, P < 0.05), with ED50 of 0.13 mg/kg per hour apixaban, i.v. (Fig. 1C).

Dose-dependent inhibition of MES by apixaban in the MCA was also demonstrated. Figure 1D illustrates a representative MES recording in the MCA in rabbits treated with vehicle and 0.15 mg/kg per hour apixaban. The mean MES frequency was reduced from 3.8 ± 0.6 (vehicle) to 0.0 ± 0.0 (0.5 mg/kg per hour apixaban, i.v., P < 0.001), with ED50 of 0.03 mg/kg per hour apixaban, i.v. (Fig. 1E). The incidence of MES detected in animals was also decreased by apixaban from 100% (vehicle) to 0% (0.5 mg/kg per hour apixaban, i.v., P < 0.001) (Fig. 1F).

Pharmacokinetic and Pharmacodynamic Analysis of Apixaban in the Rabbit MES Model. To correlate the in vivo efficacy of apixaban with the reduction of thrombus formation and MES, pharmacokinetic and pharmacodynamic (PT and aPTT) assays were performed using plasma samples obtained from each individual animal (Fig. 2). A dose-related increase in drug exposure was confirmed (Fig. 2A), with 0.01 ± 0.001, 0.03 ± 0.001, 0.1 ± 0.005, and 0.425 ± 0.04 μM for 0.015, 0.05, 0.15, and 0.5-mg/kg-per-hour, i.v. doses of apixaban at 1 hour, respectively, yielding concentrations of 0.01 ± 0.000, 0.02 ± 0.003, 0.091 ± 0.004, and 0.325 ± 0.082 μM, respectively, at 2 hours. The ED50 values were defined as 0.03 μM, 0.10 μM, and 0.02 μM apixaban plasma drug exposure for AUC of integrated blood flow, clot weight reduction, and inhibition of MES, respectively.

Ex vivo PT and aPTT analysis confirmed dose-dependent responses by these two pharmacodynamic markers (Fig. 2, B and C). As illustrated in Fig. 2, PT was increased for 2.2 ± 2.2%, 8.2 ± 2.0% (P < 0.05), 15.3 ± 2.0% (P < 0.01), and 42.1 ± 2.9% (P < 0.001). aPTT increased −0.8 ± 3.4%, 9.3 ± 10.4%, 37.8 ± 12% (P < 0.01), and 40.2 ± 5% (P < 0.001) for 0.015, 0.05, 0.15, and 0.5-mg/kg-per-hour, i.v. doses of apixaban for the 1-hour data point, respectively, versus vehicle (Fig. 2, B and C).

Robust correlations were observed between apixaban plasma drug exposure and inhibition of carotid arterial thrombosis and MES) or pharmacodynamic responses (PT and aPTT). Data on apixaban drug exposure and PT/aPTT use Fig. 2 as a basis and data on antithrombotic efficacy (in both arterial thrombosis and MES) are illustrated in Fig 1. (A, B) The correlations between drug exposure and efficacy (A) and integrated blood flow and clot weight (B). (C) The correlation of plasma drug levels with MES. (D) The correlation of plasma drug levels and PT/aPTT. Data were analyzed using the GraphPad Prism 6 software.

![Figure 3](https://example.com/fig3.png)
thrombosis (the integrated blood flow, $r^2 = 0.92$; clot weight, $r^2 = 0.90$), MES ($r^2 = 0.78$), and percentage increase in both PT ($r^2 = 0.99$) and aPTT ($r^2 = 0.87$) (Fig. 3).

**Effect of Dual Treatment with Aspirin and Apixaban on MES in Rabbit Arterial Thrombosis.** To assess the effects of dual combination with aspirin and apixaban on MES, partially effective doses of both, i.e., 5 mg/kg aspirin and 0.015 mg/kg per hour apixaban, were selected according to the monotherapy studies for aspirin as described previously (Zhou et al., 2016) and confirmed again (Supplemental Figs. 1 and 2) and for apixaban (Fig. 1). As illustrated in Fig. 4, only a marginal antithrombotic effect on arterial thrombosis was observed for either aspirin or apixaban alone over vehicle, with $65.1 \pm 3.9$, $73.8 \pm 6.9$, (13.3% increase over vehicle), and $69.4 \pm 4.7$, (6.5% increase) in AUC for the integrated blood flow, or $6.97 \pm 0.56$, $4.43 \pm 0.95$ (36.4% reduction), and $6.62 \pm 0.97$ (5.1% reduction) in thrombus weight (mg) for vehicle ($n = 13$), aspirin (5 mg/kg, $n = 6$), and apixaban (0.015 mg/kg per hour, $n = 6$), respectively. A stronger and significant antithrombotic efficacy was achieved for the dual combination with aspirin and apixaban, with $84.3 \pm 2.3$ (29.4% increase; $n = 5$, $P < 0.05$) on AUC of the integrated blood flow or $1.17 \pm 0.65$ mg (73.9% reduction; $P < 0.01$) in thrombus weight.

Mean frequency of MES (Fig. 4D) and animals with MES incidence (Fig. 4E) were found to be significantly lower in the combination therapy group [mean frequency $= 0.40 \pm 0.24$, %

**Fig. 4.** Effects of vehicle, aspirin (5 mg/kg), apixaban (0.015 mg/kg per hour), and combination of aspirin and apixaban on FeCl3-induced arterial thrombosis and cerebral MES. Combination treatment ($n = 5$) with aspirin and apixaban further reduced arterial thrombosis by an enhanced carotid blood flow (A, B) or reduction in clot weight (C) compared with aspirin (5 mg/kg, PO, $n = 6$) or apixaban (0.015 mg/kg per hour, $n = 6$) alone. Mean frequency (D) and incidence of MES (E) in ipsilateral MCA was further decreased by combined treatment with aspirin and apixaban. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$, versus vehicle.
Discussion

Our current study provided direct evidence for the first time that apixaban dose dependently inhibited cerebral MES in the rabbit model of MES in a setting of carotid arterial thrombosis (Fig. 1). Furthermore, this effect was additive with aspirin (Fig. 4). In our current preclinical model, a similar dose response was observed for apixaban in MES and arterial thrombosis, showing the ED$_{50}$ of 0.03 mg/kg per hour apixaban and 0.04 mg/kg per hour for MES and integrated blood flow, respectively. Pharmacokinetic and pharmacodynamic analyses demonstrated an excellent correlation between apixaban plasma drug exposure and efficacy (both thrombus formation and MES) and PT/aPTT.

Improved efficacy-safety profiles compared with warfarin of apixaban and other NOACs has been demonstrated recently in patients with nonvalvular atrial fibrillation in phase III clinical studies (Granger et al., 2011; Lin et al., 2015; Morais and De Caterina, 2016). The use of apixaban to reduce the risk of stroke and systemic embolism in patients with nonvalvular atrial fibrillation was approved by the US Food and Drug Administration in 2012.

Clinical evidence suggests that MES in the cerebral circulation might be an independent predictor of the risk and prognosis of stroke (Gao et al., 2004; Markus et al., 2005), and the propensity toward early ischemic recurrence of stroke in patients with a prior event or TIA of presumed arterial origin (Valton et al., 1998). In addition, MES was shown to be a valuable biomarker in response to treatment with aspirin and clopidogrel, in particular when dosed as combination therapy (Markus et al., 2005; Wong et al., 2010). In our previous study, dose-dependent inhibition of cerebral MES was demonstrated for both aspirin and clopidogrel, in particular with their combination, in the same model of cerebral MES in rabbits (Zhou et al., 2016). MES in this preclinical model were observed in the setting of carotid arterial thrombosis, which mimics the arterial origin of MES as one of the primary sources in patients (Bonati et al., 2010; Yavin et al., 2011). In addition, the relatively low frequency of MES in this preclinical model is similar to what has been observed in patients. Because of the low MES number, however, it might be difficult to accurately define a dose-dependent cure. Thus, one must be cautious in interpreting the ED$_{50}$ data for apixaban on MES. Furthermore, it would be more clinically relevant if MES was derived from atrial fibrillation when considering stroke prevention in atrial fibrillation as the clinical indication. Unfortunately, no preclinical model is available for consistent generation of thrombus or MES in atrial fibrillation setting (Nishida et al., 2012).

It should be pointed out that some limitations exist for the current animal model. First, since the clinical TCD is designed specifically for assessment of MES in patients (such as the software for MES identification and the threshold limit for...
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MES detection), thrombi less than 100 μM in diameter could not be detected. Therefore, it is unknown to what extent the frequency of smaller MES might be affected by apixaban in the current model. Second, the 30% FeCl₃-induced carotid arterial thrombosis was optimized for MES detection in MCA rather than arterial thrombosis (Zhou et al., 2016). Thus, the evaluation of antithrombotic efficacy for therapeutic agents should be carefully correlated clinically. Third, the mechanisms of MES derived from FeCl₃-induced thrombosis remain to be further explored. By means of an in vitro endothelialized microfluidic system, Ciciliano et al. (2015) demonstrated a two-phase mechanisms for FeCl₃-induced thrombus formation, i.e., the initial phase with binding of negatively charged blood cells and plasma proteins to positively charged iron species, followed by the second phase depending on the standard biologic clotting cascade, suggesting that antithrombotic agents might only be effective in the second phase of thrombus formation induced by FeCl₃. Thus, one must interpret the data with caution for the use of FeCl₃ injury model.

Of note, the ED₅₀ for apixaban was defined as 0.04 and 0.13 mg/kg per hour (or 30 and 100 nM) for the integrated blood flow and thrombus weight, respectively, and 0.03 mg/kg per hour (20 nM) for MES (Fig. 1). The reason for ~3-fold difference on the antithrombotic efficacy between the blood flow and clot weight readouts is unknown. The antithrombotic efficacy of apixaban has been previously reported in various rabbit models of thrombosis, including arterial-venous shunt thrombosis, deep vein thrombosis, and electrically induced thrombus weight, and MES, respectively, and the ED₅₀ for clopidogrel was 3.1, 4.2, and 12.7 mg/kg for integrated blood flow, thrombus weight, and MES, respectively, using the same rabbit model of MES (Zhou et al., 2016). No direct comparison could be made for the antithrombotic effects of aspirin in rabbits with others (owing to the use of different dosing regimens). Since the dose of aspirin in the current rabbit model appeared to be less potent than those used in patients (Markus et al., 2005; Wong et al., 2010), we conducted a head-to-head comparison using both human and rabbit PRPs for AA-induced platelet aggregation assays in response to various concentrations of aspirin (Supplemental Fig. 3). This in vitro platelet aggregation study suggests that aspirin might be more sensitive (~10–100×) in human PRP than in rabbit. Since rabbit platelets appear to be resistant to aspirin, the rabbit model might not be the best one to assess efficacy and/or safety for the aspirin and apixaban drug combination. In contrast to aspirin, the ED₅₀ value for clopidogrel on arterial thrombosis appeared to be more potent (about 3-fold) than those from a rabbit model of electrically induced carotid artery thrombosis (ED₅₀ = 0.8 mg/kg/d, PO) (Wong et al., 2008b).

In summary, our study demonstrated the dose-dependent inhibition of MES by apixaban, which could be further enhanced when apixaban is dosed in combination with aspirin. Our data provided direct evidence for the potential association between FXA blockade and MES in the preclinical experimental model.

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Authorship Contributions

Participated in research design: Zhou, Seiffert, Gutstein, Wang.
Conducted experiments: Zhou, Wu, and Chu.
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Supplemental Methods

Drug Administration

Aspirin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in vehicle (0.5% methyl cellulose) and dosed orally at 0, 5, and 25 mg/kg (n=5 each) once daily for three days (2 mL/kg) in male New Zealand White rabbits. Blood samples were collected 2 hours after the last day oral dosing for ex vivo platelet aggregation assays and thromboxane B2 analysis.

Ex Vivo Platelet Aggregation Assay

Ex vivo platelet aggregation studies in response to 0, 5 and 25 mg/kg aspirin oral dosing were performed using citrated platelet-rich plasma (PRP) as described in detail in Methods.

In Vitro Platelet Aggregation Assay and Aspirin Responses

In vitro platelet aggregation studies in rabbit were conducted using the same method for the ex vivo platelet aggregation assays except that blood samples were collected from untreated rabbits (n=3) and various concentrations of aspirin (0-300 μM) were incubated with PRP for 10 min prior to the assay. For the in vitro assay, aspirin was dissolved in 7.5% sodium bicarbonate (GIBCO/ Thermo Fisher Scientific, Waltham, MA, USA). Human venous blood was collected from healthy volunteers (n=3) into citrate Vacutainer tubes and processed for the platelet aggregation assays under the same conditions as for rabbit assay.

Ex Vivo Thromboxane B2 (TXB2) Assay

TXB2, a metabolite of TXA2 after rapid hydrolysis, was measured ex vivo using thromboxane B2 express EIA Kit (Kayman, Item # 10004023, Ann Arbor, MI, USA) performed on SpectraMax M2 (equipped with Softmax Pro6 software, Molecular Devices, Sunnyvale, CA,
USA). Citrated arterial blood sample (1 ml) was drawn from the middle ear artery in rabbits. Arachidonic acid (2000 μM) and CaCl2 (2 mM) was added to the blood, incubated for 90 min at 37 °C to allow for TXB2 production, and then stored at -80 °C until the assays after all the samples were collected. Serum was prepared by spinning down the clotted blood at 1500 x g for 15 min at 4 °C. TXB2 levels in serum were measured according to the instruction provided by the vendor.
Supplemental Figure Legends

Supplemental Figure 1. Ex vivo analysis for the effect of vehicle and aspirin (ASA) oral dosing on arachidonic acid (AA; at 100, 300, 600, and 900 μM)-induced maximum platelet aggregation of PRP. Animals (n=5) were treated with vehicle, 5, and 25 mg/kg aspirin, p.o., daily for three days. ***p<0.001 vs. vehicle.

Supplemental Figure 2. Ex vivo analysis for the effects of vehicle aspirin oral dosing on thromboxane B2 (TXB2) levels. Citrated arterial blood was collected from the same groups of animals (n=5) as illustrated in Supplemental Figure 1 and processed for serum TXB2 assays as described in Supplemental Methods. ***p<0.001 vs. vehicle.

Supplemental Figure 3. Head-to-head comparison for the effects of aspirin (ASA) on arachidonic acid (AA)-induced platelet aggregation between rabbit and human PRPs. To address potential species differences for ASA effects in rabbit vs. clinic, we conducted an in vitro study (under the same experimental condition except for the sources of blood, i.e., rabbit vs. human) for arachidonic acid (AA; at 250, 500 and 1000 μM)-induced platelet aggregation (n=3 each) in response to various concentrations of ASA (0-300 μM) spiked in. Panels A and B illustrate the results of the maximum platelet aggregation for rabbit and human, respectively. Spiking in of 100, 300 and >300 μM ASA almost completely inhibited 250, 500 and 1000 μM AA-induced platelet aggregation, respectively, in rabbit PRP, whereas 1, 3 and 100 μM ASA achieved similar levels of inhibition in 250, 500 and 1000 μM AA-induced platelet aggregation, respectively, in human PRP.
Supplemental Figure 1.

![Graph showing maximum aggregation (% of vehicle) against AA (µM) for different treatments: Vehicle, p.o.; 5 mg/kg ASA, p.o.; 25 mg/kg ASA, p.o.](image)
Supplemental Figure 2.

![Graph showing TxB2 levels in ng/ml across different doses of ASA (mg/kg, p.o., 3 days).]
Supplemental Figure 3.

A. Rabbit

B. Human