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

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aPTT: activated partial thromboplastin time; AF: atrial fibrillation; MES: microembolic signals; MCA: middle cerebral artery; NOACs: novel oral anticoagulants; PT: prothrombin time; SPAF: stroke prevention in atrial fibrillation; TCD: transcranial Doppler; TIA: transient ischemic attack

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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 MES 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/h apixaban, i.v. (0.03 μM plasma exposure) for the integrated blood flow, 0.13 mg/kg/h apixaban (0.10 μM plasma exposure) for thrombus weight, and 0.03 mg/kg/h apixaban (0.02 μM plasma exposure) for MES. Dual treatment with aspirin (5 mg/kg, p.o.) and apixaban (0.015 mg/kg/h, i.v.) resulted in a significant reduction in cerebral MES (p<0.05) compared to 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 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 (MES) 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 to assess 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 investigate anticoagulants on MES but the results are inconclusive. Since warfarin and the novel oral anticoagulants (NOACs) are the currently available options for stroke prevention in atrial fibrillation (SPAF) (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 non-valvular 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 since 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.
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 U.S. 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 based on procedures described previously (Marsh Lyle et al., 1998; Wang and Xu, 2005) with modifications optimized to study MES as part of a terminal procedure in rabbits (Zhou et al., 2016). Briefly, animals were anesthetized with a cocktail (ketamine HCl, Pfizer Inc., 50 mg/kg, and xylazine, LLOYD Inc., 5 mg/kg, IM) and the left common carotid artery was surgically exposed. A Doppler flow probe (Model 1.5 PRB, Transonic Systems, Ithaca, NY, USA) connected to a flow meter (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, USA). Thrombosis was induced by applying two pieces of disc-shaped filter papers (7.4 mm in diameter, 0.5 mm thick, one above and one beneath the vessel, respectively; Life Technologies, Grand Island, NY, USA) saturated with 30%, m/v, of FeCl₃ (Anhydrous 98%, Cat# 169430050, ACROS Organics/Thermo Fisher Scientific, Waltham, MA, USA) to the adventitial surface of the vessel. A piece of para-film (Fisher Scientific) was put underneath the vessel to protect the surrounding tissue from injury. The filter papers were applied for 5 min
followed by washout of the residual FeCl$_3$ by sterile, warm saline. The carotid blood flow was monitored for 60 min from the application of FeCl$_3$ (as time zero). Integrated carotid blood flow over 60 min 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 end of study (i.e., 60 min after FeCl$_3$ 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 para-film with a drop of saline. The thrombus was briefly washed in saline (to remove loosely trapped blood) and semi-dried on a piece of para-film (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, USA). Afterwards, the vessel was cut to open/validate complete removal of thrombus.

The SONARA™ TCD System (Nicolet Natus Neurology Inc., Middleton, WI, USA) was used to continuously monitor blood flow velocity and MES in the ipsilateral MCA. A PW 2MHz probe (OD=11.3 mm, 90 mm long, focused at 12 to 25 mm, customized by MTB Medizentechnik Basler AG, Switzerland) was fixed by a flexible-arm magnetic-base holder (McMASTER-CARR, Princeton, NJ, USA) at the posterior end of zygomatic bone of the rabbits, at an angle of ~80 degree 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 to 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, HITS) 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 based on the criteria defined by the International Consensus Committee (Symposium, 1995; Ringelstein et al., 1998): 1) A unidirectional and short-lasting (<300 ms) signal with an amplitude >3 dB above background, 2) with a traverse at a pre-specified depth, and 3) a typical “snap”, “chirp”, or “moan” audible output.

**Drug Administration**

The structure and compound profiles on selectivity, potency, pharmacokinetic and pharmacodynamics parameters of apixaban have been extensively investigated and reported previously. (Pinto et al., 2007; Wong et al., 2011) The i.v. dosing regimen for apixaban in rabbit was essentially followed previous reports (Wong et al., 2008a; Wong et al., 2008b) 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 i.v. infusion (starting 60 min prior to FeCl₃ injury), 2 mL/kg. Our pilot pharmacokinetic study (blood samples at 0, 1 and 2 hours of i.v. dosing) for the use of 0.5 mg/kg/h, demonstrated the 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/h) was i.v. infused at a volume of 2 ml/kg/h for two hours. Aspirin (Sigma-Aldrich, St. Louis, MO, USA, Cat#A2093) was dissolved in vehicle (0.5% methyl cellulose) and dosed orally once daily for three days (2 ml/kg). Due to rapid hydrolysis of aspirin in solution in a temperature-dependent manner, the dosing solution was kept at 4 °C and dosed within 24 hour after preparation. Rabbits were subjected to FeCl₃ injury 1 hour after apixaban infusion, or 2 hour 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/h, i.v.) was used to determine the combined efficacy on arterial thrombosis and cerebral MES. The 5 mg/kg aspirin,
p.o. dose was selected based on 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 ASA, p.o. daily for 3 days (n=5 each). The results of this study are illustrated in the Supplemental figures 1 and 2. Similar to our previous report (Zhou et al., 2016), the partial inhibition by 5 mg/kg aspirin on both arachidonic acid-induced platelet aggregation and serum thromboxane B2 levels, and almost complete abolishment by 25 mg/kg aspirin was 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 2000 x g for 15 min at 4 °C for plasma preparation and evaluated for PK and ex vivo clotting time assays.

For PK analysis, the plasma samples and plasma standards and quality controls were assessed with protein precipitation with acetonitrile (10 μL plasma + 300 μL acetonitrile). The supernatant of the precipitation was then analyzed by liquid chromatography (LC)-mass spectrometry (MS)/MS for apixaban using Waters Acquity UPLC system (Waters Corporation, Milford, MA) for LC and Applied Biosystems / MSD Sciex API 5500 Q-Trap (Applied Biosystems, Forster City, CA) for MS analysis. 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 (50/40/10/0.05, v/v/v/v) as autosampler wash 2. TheMonChrom C18, 100x2.0 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 aPTT-XL (Pacific Haemostasis, Waltham, MA, USA) and TriniCLOT PT Excel (Tcoag, Bray, Ireland) on a KC4 Delta coagulation analyzer (Tcoag, Bray, Ireland).

**Ex Vivo Platelet Aggregation Assay**

Platelet aggregation studies were performed *ex vivo* using citrated platelet-rich plasma (PRP) in an optical ChronoLog Aggregometer (Model 700; ChronoLog, Havertown, PA) as previously described. (Wong et al., 2007) Briefly, PRP was generated from citrated whole blood after centrifugation at 200 x g for 15 min at 22 ºC. 250 μL PRP was incubated in a cuvette containing a stir bar for 2–3 min. Arachidonic acid (AA; at 100, 300, 600, and 900 μM) was used as an agonist for platelet aggregation. Platelet aggregation was monitored for 5–7 min after addition of the agonist. Platelet-poor plasma was used as 100% transmittance for aggregation. The peak aggregation response was recorded in percentage of transmittance.

**Data Analysis and Statistics**

All the data are presented as mean ± standard error (SE). One-way 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. EC$_{50}$, defined as doses for half-maximal effect, was determined by a non-linear four-parameter dose-response curve fit using GraphPad Prism under the dose ranges described in each figure legend. A Chi-square and Fisher’s test was applied to determine the association.
between drug treatment at each individual dose and efficacy or pharmacodynamic read-out. Results were considered significant when $p < 0.05$. 
Results

Effect of apixaban on cerebral MES induced by carotid arterial thrombosis in rabbits

Following the pilot pharmacokinetic study (on a 0.5 mg/kg/h dose) to confirm a steady-state level of drug exposure in rabbits between 1-2 hours after i.v. infusion, dose-dependent effects of apixaban on thrombus formation in the carotid artery and 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 % 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/h apixaban group (n=6, p<0.001), with EC₅₀ of 0.04 mg/kg/h apixaban, i.v. (Fig. 1A and B). Clot weight was reduced from 6.9 ± 0.8 mg (vehicle) to 0.8 ± 0.2 mg (0.5 mg/kg/h apixaban, p<0.05), with ED₅₀ of 0.13 mg/kg/h apixaban, i.v. (Fig. 1C).

Dose-dependent inhibition of apixaban on MES in the MCA was also demonstrated. Fig. 1D illustrates a representative MES recording in the MCA in rabbits treated with vehicle and 0.15 mg/kg/h apixaban. The mean MES frequency was reduced from 3.8 ± 0.6 (vehicle) to 0.0 ± 0.0 (0.5 mg/kg/h apixaban, i.v., p<0.001), with ED₅₀ of 0.03 mg/kg/h 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/h 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/h, i.e., doses of apixaban at 1 hour, respectively, yielding concentrations of 0.01 ± 0.007, 0.02 ± 0.003, 0.091 ± 0.004 and 0.325 ± 0.082 µM, respectively, at 2 hours. The EC$_{50}$ 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 on these two pharmacodynamic markers (Fig. 2B 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), and aPTT increased for -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/h, i.e., doses of apixaban for the 1 hour data point, respectively, vs. vehicle (Fig. 2B and C).

Robust correlations were observed between apixaban plasma drug exposure and inhibition of carotid arterial thrombosis (the integrated blood flow, r$^2$ = 0.92; clot weight, r$^2$ = 0.90), MES ((r$^2$ = 0.78) and % 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, a partially effective dose of both, i.e., 5 mg/kg aspirin and 0.015 mg/kg apixaban, was selected according to the monotherapy studies for aspirin as described previously (Zhou et al., 2016) and confirmed again (Supplemental Figures 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 ± 3.9, 73.8 ± 6.9 (13.3% increase over vehicle) and 69.4 ± 4.7 (6.5% increase) on AUC for the integrated blood flow, or 6.97 ± 0.56, 4.43 ± 0.95 (36.4% reduction)
and 6.62 ± 0.97 (5.1% reduction) on thrombus weight (mg), for vehicle (n=13), aspirin (5 mg/kg, n=6) and apixaban (0.015 mg/kg/h, n=6), respectively. A stronger and significant antithrombotic efficacy was achieved for the dual combination with aspirin and apixaban, with 84.3 ± 2.3 (29.4% increase; n=5, p<0.05) on AUC of the integrated blood flow or 1.17 ± 0.65 mg (73.9% reduction; p<0.01) on 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 ± 0.24, % MES (+) =40%; p<0.01 vs. vehicle) compared with either aspirin (mean frequency=5.5 ± 1.91, % MES (+) =83%) or apixaban alone (mean frequency=3.3 ± 0.61, % MES (+) =100%).

Pharmacokinetic and pharmacodynamic analyses of the dual combination with aspirin and apixaban are shown in Figure 5. Pharmacokinetic analysis demonstrated similar levels of plasma drug exposure for apixaban between apixaban alone and the combination group. As expected, the low levels of drug exposure were not sufficient to trigger the prolongation of both PT and aPTT by apixaban (Fig. 5B and C). In contrast, the *ex vivo* platelet aggregation assays confirmed the effects of aspirin in either group of aspirin alone or its combination with apixaban, showing significant but only a partial inhibition of the maximum platelet aggregation induced by AA (100, 300, 600, 900 μM) compared to vehicle treatment (p<0.05, Fig. 5D).
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 the 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/h apixaban and 0.04 mg/kg/h 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.

Apixaban and other NOACs have been recently demonstrated for improved efficacy–safety profiles compared to warfarin in patients with non-valvular 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 non-valvular atrial fibrillation was approved by the U.S. 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 towards 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 the treatment of 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 was 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 with caution in interpreting the data such as ED\textsubscript{50} for apixaban on MES. Furthermore, it would be more clinical relevant if MES was derived from AF when considering SPAF as the clinical indication. Unfortunately, no preclinical model is available for consistent generation of thrombus or MES in AF 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 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, since the 30% FeCl\textsubscript{3}-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 FeCl\textsubscript{3}-induced thrombosis remain to be further explored. By means of an in vitro endothelialized microfluidic system, Ciciliano et al. demonstrated 2-phase mechanisms for FeCl\textsubscript{3}-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 biological clotting cascade, (Ciciliano et al., 2015) suggesting that antithrombotic agents might only be effective in the second phase of thrombus formation induced by FeCl\textsubscript{3}. Thus, one must interpret the data with caution for the use of FeCl\textsubscript{3} injury model.
Of note, the ED$_{50}$ for apixaban was defined as 0.04 and 0.13 mg/kg/h (or 30 and 100 nM) for the integrated blood flow and thrombus weight, respectively, and 0.03 mg/kg/h (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 carotid artery thrombosis, where the ED$_{50}$ was defined as 0.27 mg/kg/h, i.v. (or 370 nM plasma drug level), 0.11 mg/kg/h, i.v. (65 nM), and 0.07 mg/kg/h, i.v. (110 nM), respectively. (Wong et al., 2008a; Wong et al., 2009) Our current ED$_{50}$ data for apixaban on arterial thrombosis are in agreement with those previous reports in rabbits, and are around a clinical peak-trough (i.e., 174 nM and 37 nM, respectively) range for apixaban. (Frost et al., 2014)

In our previous study, the ED$_{50}$ for aspirin was defined as 3.1, 4.2 and 12.7 mg/kg for integrated blood flow and thrombus weight and MES, respectively, and the ED$_{50}$ for clopidogrel was 0.30, 0.28 and 0.25 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 (due 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 study using both human and rabbit PRPs for AA-induced platelet aggregation assays in response to various concentrations of aspirin (Supplemental Figure 3). This in vitro platelet aggregation study suggests that aspirin might be more sensitive (~10-100x) 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 ED$_{50}$ 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 (ED$_{50}$=0.8 mg/kg/day, p.o.). (Wong et al., 2008b)

In summary, our study demonstrated the dose-dependent inhibition of apixaban on MES, 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, and Wang.

Conducted experiments: Zhou, Wu, and Chu

Performed data analysis: Zhou, and Wang

Wrote or contributed to the writing of the manuscript: Zhou, Seiffert, Gutstein, and Wang
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(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide (apixaban, BMS-562247), a highly potent, selective, efficacious, and orally bioavailable inhibitor of blood coagulation factor Xa. J Med Chem 50:5339-5356.


Figure Legends

Figure 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/h, i.v., n=6) as illustrated using carotid blood flow within 60 min upon FeCl₃ injury (A) with AUC of the integrated blood flow (B), and reduction in clot weight (C). MES was monitored simultaneously in the ipsilateral MCA to the FeCl₃ injury. Representative image for MES detection by TCD in vehicle treated animal is illustrated (D). 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/sec or kHz, the “Peak” for the maximal systolic velocity/frequency in units of cm/sec or kHz, “EDV” for the end diastolic velocity, “PT” 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 Methods. Mean frequency (E) and incidence of MES (F) in MCA was also dose-dependently inhibited. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 vs. vehicle.

Figure 2. Pharmacokinetic and pharmacodynamics responses for apixaban treatment on FeCl₃-induced arterial thrombosis and cerebral MES. Plasma samples were prepared in animals of each experimental group as illustrated in Fig. 1 prior to, or 1 (time of FeCl₃ injury) and 2 hours after apixaban i.v. dosing. Plasma drug exposures (A) and pharmacodynamic responses including PT (B) and aPTT (C) are illustrated. *p<0.05, **p<0.01, and ***p<0.001 vs. vehicle.

Figure 3. Correlation between apixaban plasma drug exposure and efficacy (arterial thrombosis and MES) or pharmacodynamic responses (PT and aPTT). Data on apixaban drug exposure and PT/aPTT are based on Fig. 2 and data on antithrombotic efficacy (in both arterial thrombosis and MES) are illustrated in Fig 1. Panels A and B show the correlation between drug exposure and
efficacy on integrated blood flow and clot weight, respectively. Panel C depicts the correlation between plasma drug levels with MES. Panel D illustrates the correlation of plasma drug levels and PT/aPTT. Data were analyzed using the GraphPad Prism 6 software.

**Figure 4.** Effects of vehicle, aspirin (5 mpk), apixaban (0.015 mg/kg/h), 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, p.o., n=6) or apixaban (0.015 mg/kg/h, n=6) alone. Mean frequency (D) and incidence of MES (E) in ipsilateral MCA was further decreased by combined treatment of aspirin with apixaban. **p<0.01 vs. vehicle.

**Figure 5.** Pharmacokinetic and pharmacodynamic responses for the dual combination study with aspirin and apixaban. Plasma samples were prepared in animals of each experimental group as illustrated in Fig. 4 prior to, or 1 (time of FeCl₃ injury) and 2 hours after 0.015 mg/kg/h apixaban i.v. dosing. Plasma drug exposures (A) and pharmacodynamics responses shown as PT (B) and aPTT (C) are illustrated (except for the aspirin only group). Ex vivo analysis using arachidonic acid (AA, 100, 300, 600 and 900 μM)-induced maximum platelet aggregation (D) confirmed aspirin treatment in both aspirin alone and co-treatment with apixaban and aspirin (apixaban alone not shown). *p<0.05, vs. vehicle.
Fig. 2

A

Plasma apix (μM)

Vehicle 0.015 0.05 0.15 0.5

Baseline 1 hr 2 hr

B

PT (sec)

Vehicle 0.015 0.05 0.15 0.5

Baseline 1 hr 2 hr

C

aPTT (sec)

Vehicle 0.015 0.05 0.15 0.5

Baseline 1 hr 2 hr
Fig. 4

A) Blood Flow (% of baseline) over time (min) for different treatments:
- Vehicle
- ASA 5mg/kg
- Apix 0.015 mg/kg/h
- ASA+Apix

B) Integrated blood flow (% of control) for different treatments:
- Vehicle
- ASA
- Apix
- ASA+Apix

C) Clot weight (mg) for different treatments:
- Vehicle
- ASA
- Apix
- ASA+Apix

D) MES frequency for different treatments:
- Vehicle
- ASA
- Apix
- ASA+Apix

E) % Animals for MES (+) and MES (-) for different treatments:
- Vehicle
- ASA
- Apix
- ASA+Apix
Fig. 5

A) Plasma apix (nM)

B) PT (sec)

C) aPTT (sec)

D) Max. aggregation (%) vs. AA (μM)

* Indicates statistical significance.