Apixaban Versus Warfarin for Mechanical Heart Valve Thromboprophylaxis in a Swine Aortic Heterotopic Valve Model

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Objective—Warfarin is the current standard for oral anticoagulation therapy in patients with mechanical heart valves, yet optimal therapy to maximize anticoagulation and minimize bleeding complications requires routine coagulation monitoring, possible dietary restrictions, and drug interaction monitoring. As alternatives to warfarin, oral direct acting factor Xa inhibitors are currently approved for the prophylaxis and treatment of venous thromboembolism and reduction of stroke and systemic embolization. However, no in vivo preclinical or clinical studies have been performed directly comparing oral factor Xa inhibitors such as apixaban to warfarin, the current standard of therapy.

Approach and Results—A well-documented heterotopic aortic valve porcine model was used to test the hypothesis that apixaban has comparable efficacy to warfarin for thromboprophylaxis of mechanical heart valves. Sixteen swine were implanted with a bileaflet mechanical aortic valve that bypassed the ligated descending thoracic aorta. Animals were randomized to 4 groups: control (no anticoagulation; n=4), apixaban oral 1 mg/kg twice a day (n=5), warfarin oral 0.04 to 0.08 mg/kg daily (international normalized ratio 2–3; n=3), and apixaban infusion (n=4). Postmortem valve thrombus was measured 30 days post-surgery for control-oral groups and 14 days post-surgery for the apixaban infusion group. Control thrombus weight (mean) was significantly different (1422.9 mg) compared with apixaban oral (357.5 mg), warfarin (247.1 mg), and apixaban 14-day infusion (61.1 mg; P<0.05).

Conclusions—Apixaban is a promising candidate and may be a useful alternative to warfarin for thromboprophylaxis of mechanical heart valves. Unlike warfarin, no adverse bleeding events were observed in any apixaban groups.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:942-948. DOI: 10.1161/ATVBAHA.116.308649.)

Key Words: anticoagulant • aortic valve • atrial fibrillation • embolism • pharmacokinetics

In middle-aged adults, the decision to surgically implant a mechanical or a bioprosthetic valve balances the risk of bleeding, mainly associated with mechanical valves and requirement for long-term anticoagulation, with the risk of reoperation secondary to structural valve deterioration as reported with bioprosthetic valves. Because of the associated risk of thromboembolic events and stroke, the American College of Chest Physicians Evidence-Based Clinical Practice Guidelines recommend lifelong anticoagulation for patients with mechanical heart valves versus no long-term anticoagulation. Although low-molecular-weight heparin can be used to achieve anticoagulation, the vitamin K antagonist, warfarin, is currently the only oral anticoagulant recommended for patients with aortic and mitral mechanical heart valves. Moreover, achieving optimal anticoagulation with warfarin while minimizing the risk of major bleeding is challenged by the need for frequent monitoring and targeting of the international normalized ratio (INR), genetic polymorphism alterations in warfarin metabolism, multiple food and drug interactions, comorbid medical conditions, and age. In a recent study involving 546 patients treated with warfarin using targeted INR therapy after receiving aortic and mitral valves, the incidence of major bleeding was 4.4 and 4.6 per 100 patent-years, respectively. As alternatives to warfarin, multiple novel oral anticoagulants have been developed and are currently approved to reduce the risk of stroke, systemic embolism, and thromboembolic events secondary to atrial fibrillation and surgery, respectively, and for the treatment of venous thromboembolism.

See accompanying editorial on page 743

Because of the risk of bleeding and challenges associated with warfarin management, oral alternatives to vitamin K antagonists for mechanical valve anticoagulation are needed.
To date, only dabigatran, a direct thrombin inhibitor, has been evaluated and compared with the standard of care, warfarin, in human patients with mechanical aortic and mitral valves. The RE-ALIGN study (Randomized Phase II Study to Evaluate the Safety and Pharmacokinetics of Dabigatran Etxilate Oral in Patients after Heart Valve Replacement) was halted early, as dabigatran, although adjusted to a trough plasma level >50 ng/mL, was associated with an increased rate of thromboembolic events, valve thrombosis, and major bleeding complications compared with warfarin. \(^1\) Unlike direct thrombin inhibitors, factor Xa inhibitors have not been tested in a clinical setting for mechanical heart valve thromboprophylaxis. On the basis of its earlier sequence in the coagulation cascade, ability to greatly trigger the generation of thrombin, by 1000-fold, and limited platelet activation by factor Xa compared with thrombin, factor Xa may be a preferred anticoagulation target compared with factor IIa. \(^2\) However, clinical trials of oral factor Xa inhibitors compared directly to factor IIa inhibitors regarding thromboprophylaxis efficacy have not been performed.

Swine are commonly used animal models for research thromboembolism because of their anatomic, hemolologic, and coagulation similarities to humans. \(^3\) McKellar et al\(^4\) developed a heterotopic aortic valve replacement model in swine to evaluate thromboprophylaxis in mechanical heart valves. Using this model, previous studies have evaluated the thromboprophylaxis of oral dabigatran etexilate, a direct thrombin inhibitor, and oral rivaroxaban, a factor Xa inhibitor, with results demonstrating equal to superior thromboprophylaxis compared with enoxaparin, a low-molecular-weight heparin. \(^5\) However, there are no preclinical studies using a heterotopic aortic valve replacement model in swine comparing the thromboprophylaxis efficacy of a factor Xa inhibitor to warfarin, the mainstay of oral anticoagulant therapy in humans with mechanical heart valves.

Apixaban, a factor Xa inhibitor, is currently approved by the US Food and Drug Administration for the treatment of deep vein thrombosis, for the prevention of thromboembolism after knee and hip replacement surgery, and to reduce the risk of systemic embolism and stroke in patients with nonvalvular atrial fibrillation. Apixaban demonstrates >30 \(\mu M^{-1} \cdot s^{-1}\) and inhibits free and prothrombinase and clot-bound factor Xa activity in vitro. In addition, apixaban inhibits factor Xa from rabbits, rats, and dogs, an activity that parallels its antithrombotic potency in these species. \(^6\) In humans, apixaban oral T\(_{1/2}\) max is 3 hours, and its half-life is \(\approx 12\) hours (8–15 hours). Routine monitoring is generally not advocated during apixaban therapy, the ideal administration is twice a day orally, and in humans, it is predominantly eliminated by nonrenal excretion (73% fecal and 27% renal). \(^7\)

In this study, we tested the thromboprophylactic efficacy and incidence of major bleeding of apixaban compared with warfarin in a well-established aortic heterotopic valve swine model. On the basis of results in other preclinical models, we anticipated that the half-life of apixaban might be considerably shorter in swine than in humans. We, therefore, evaluated apixaban not only by oral dosing but also in a separate group of animals in which the concentration versus time profile approximated that observed in humans.

### Materials and Methods

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement. In summary, swine were randomized to the following groups: no anticoagulation-control, oral apixaban, oral warfarin, and apixaban continuous intravenous infusion. Animals were implanted with a bileaflet mechanical aortic valve that bypassed the ligated descending thoracic aorta. End points were as follows: no anticoagulation=30 days, oral apixaban and oral warfarin=30 days, and apixaban infusion=14 days. The apixaban infusion was modeled by correlating human clinical trial plasma concentrations to swine apixaban pharmacokinetic parameters. Swine administered warfarin were monitored via prothrombin time and INR (2–3) via a mechanical clot-detection system fibrometer and thromboplastin (International Sensitivity Index=0.92). Valves were harvested at group-specific end points. Valve weights were measured and compared for statistical significance.

### Results

**Pharmacokinetics: Apixaban Intravenous Bolus**

Raw plasma concentration per time data are outlined in Table 1. The curvilinear plasma concentration versus time was determined (Figure 1). Noncompartmental pharmacokinetic parameters (mean±SD) for intravenous 0.5 mg/kg bolus were as follows: \(t_{1/2}=6.2±3.8\) hours, area under the curve=6186.67±4981.81 ng∙h/mL, clearance=118.00±72.75 mL/h∙kg\(^{-1}\), volume of distribution=577.27±113.05 mL/kg, area under the first moment curve=88048±6219.36 ng h\(^{2}\)/mL, mean residual time=6.5±4.34 hours (Table 2).

**Pharmacokinetics: Apixaban Oral Bolus**

Raw plasma concentration per time data are outlined in Table 3. Noncompartmental pharmacokinetic parameters (mean±SD) for oral 0.5 mg/kg bolus were as follows: \(t_{1/2}=5.93±1.37\) hours, area under the curve=1390±564.02 ng h/mL, clearance=397.67±144.89 mL/h∙kg\(^{-1}\), area under the first moment curve=9286.33±2806.98 ng h\(^{2}\)/mL, mean residual time=6.93±0.72 hours, bioavailability=31.87±20.36% (Table 4). One animal (swine 5) developed a catheter-related complication during the crossover washout period, was removed from the study, and replaced with another animal (swine 6).

**Anti-Xa Low-Molecular-Weight Heparin Anticoagulation Measurement and International Normalized Ratio**

The curvilinear anti-Xa low-molecular-weight heparin (LMWH) activity versus time was determined for single intravenous apixaban 0.5 mg/kg bolus and single orally administered apixaban 0.5 mg/kg dose. Anti-Xa LMWH activity for early intravenous time points (≤2 hours) were associated with the upper limit of the LMWH calibrator (1.3 IU/mL). Both intravenous and oral apixaban exceeded the suggested lower therapeutic range for prophylaxis (0.5–1.2 IU/mL) at 4 hours up to =8 hours for each route of
administration (Figure 2). Plasma apixaban ranges significantly predicted anti-Xa LMWH activity for both intravenous and orally administered apixaban ($R^2=0.8; F=27.48; P<0.001$ and $R^2=0.84; F=126.1; P<0.0001$), respectively (Figure 3). Plasma samples >300 ng/mL were associated with the upper limit of LMWH calibrator (anti-Xa LMWH=1.3 IU/mL).

Swine administered warfarin were monitored via INR (Figure 4).

Valve Thrombus Weight and Postmortem Valve Evaluation

All control animals (no anticoagulation) had visible evidence of thrombus at 30 days postimplantation. Control valve thrombus weight (mean±SD) was significantly different (1422±676.4 mg) compared with apixaban 1 mg/kg twice a day oral (357.5±234.9 mg), warfarin (247.1±134.3 mg), and apixaban multistep 14-day infusion (61.1±47.2 mg; ANOVA $F=10.88; P=0.001$; Figure 5; Table 5). Three out of five swine within the apixaban oral group had evidence of clot. Two out of three swine within the warfarin group had evidence of clot. No animals in the apixaban infusion group had evidence of a film on the bileaflet mechanical valve at the time of harvest but no discernible thrombus. The film weight was included in the analysis. Histopathology demonstrated the structure to be composed almost exclusively of fibrin consistent with an early loosely organized thrombus with scattered regions of mineralization. There was no evidence of a biofilm or bacterial colonization. Photos representing postmortem bileaflet mechanical valve for all groups including control, apixaban oral, warfarin, and apixaban infusion are shown in Figure 6.

Complications

There was no evidence of thromboembolic events, severe or complete valve or graft thrombosis, or hemorrhage in the apixaban treatment groups. The majority of swine in the warfarin treatment group remained within a therapeutic INR without complications, with the exception of 2 animals that were removed from study because of major bleeding complications including pulmonary hemorrhage, hemothorax, and multifocal subcutaneous hemorrhage. A mechanical clot-detection system (fibrometer) was used

### Table 1. Concentration of Apixaban in Swine Plasma (ng/mL) After IV Administration

<table>
<thead>
<tr>
<th>Sample Time, h</th>
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<th>Animal Number 4</th>
<th>Animal Number 5</th>
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<th>SD</th>
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<td>127</td>
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</table>

BLQ indicates beyond level of detection; and NA, not applicable.

### Table 2. Selected Pharmacokinetic Parameters of Apixaban Intravenous in Swine

<table>
<thead>
<tr>
<th>Swine No.</th>
<th>$t_{1/2}$</th>
<th>AUC</th>
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<th>Clearance</th>
<th>AUMC</th>
<th>MRT</th>
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<td>4</td>
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<td>11899</td>
<td>483.5</td>
<td>42</td>
<td>136669</td>
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<tr>
<td>5</td>
<td>4.8</td>
<td>3992</td>
<td>545.5</td>
<td>125</td>
<td>17389</td>
<td>4.3</td>
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</tbody>
</table>

Mean: 6.20 6186.67 577.27 118.00 88048.00 6.50 SD: 3.80 4981.81 113.05 72.75 62619.36 4.34

AUC indicates area under the curve; AUMC, area under the first moment curve; MRT, mean residual time; and Vd, volume of distribution.

### Table 3. Concentration of Apixaban in Swine Plasma (ng/mL) After Oral Administration

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<th>Sample Time, h</th>
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<th>Animal Number 4</th>
<th>Animal Number 6</th>
<th>Mean</th>
<th>SD</th>
</tr>
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<td>BLQ</td>
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<td>NA</td>
</tr>
<tr>
<td>0.25</td>
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<td>7.82</td>
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<td>79.1</td>
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<td>73.4</td>
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<tr>
<td>24</td>
<td>7.32</td>
<td>7.44</td>
<td>8.38</td>
<td>7.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

BLQ indicates beyond level of detection; and NA, not applicable.
with a low International Sensitivity Index thromboplastin, which may have improved accuracy in monitoring INR values, thus reducing warfarin-associated bleeding complications.16

**Histopathology Evaluation**

There was no consistent pathological change noted across animals of all groups with tissues provided. Thrombosis was not observed, and hemorrhage was not a significant feature of any of the animals. Minimal to mild tubular basophilic area was noted in the kidneys of one animal within the apixaban oral treatment group and one animal in the apixaban infusion group. One animal in the warfarin oral group had a fibrinopurulent pleuritis. Minimal lymphocytic infiltrates were noted in the coronary artery adventitia in one animal in the apixaban oral treatment group and were considered a background change in this animal.

**Discussion**

The goal of this study was to determine the preclinical efficacy of apixaban regarding thromboprophylaxis of mechanical heart valves in a swine surgical model. Swine were chosen for this model because of their similar coagulation properties and cardiovascular anatomy and physiology compared with humans. The study was designed to provide a comparison to current standard therapy, warfarin, for anticoagulation and stroke prevention in human patients implanted with a mechanical heart valve. Results from this study demonstrated that apixaban has comparable efficacy to warfarin in reducing mechanical valve thrombosis in swine. This in vivo preclinical study demonstrates the thromboprophylactic efficacy of apixaban in direct comparison to warfarin for mechanical heart valves.

Apixaban has a high degree of selectivity for factor Xa, no active metabolites, a predictable dose response, and pharmacokinetic profile with limited renal excretion, minimal drug and food interactions, and a reduced need for titration and therapeutic monitoring, making it a favorable alternative to warfarin for the prevention of stroke in patients with atrial fibrillation.15 Its bioavailability in humans is ≈50%, which is greater than the mean bioavailability of 32% seen in swine with the current study. The half-life of apixaban in humans is ≈12 hours versus a 1.6-hour oral half-life in swine.

In the current study, an oral 0.5 mg/kg dose in swine was associated with anti-Xa LMWH plasma levels >0.6 IU/mL at 4 hours. However, we chose to use a higher dose (1 mg/kg) to ensure adequate anti-Xa activity when using twice a day oral dosing.

The use of orally administered direct factor Xa inhibitors for mechanical valve thromboembolic prophylaxis is promising especially when compared with the current standard of care, warfarin, which is associated with bleeding complications, frequent anticoagulation monitoring and titration, dietary restrictions, and drug interactions including associated healthcare costs for testing, monitoring, and treatment for complications. As a result, the decision to use a bioprosthetic valve with a risk of premature valve failure must be weighed versus lifelong warfarin therapy recommended in patients implanted with mechanical valves.

Dabigatran, an oral direct thrombin inhibitor, demonstrated favorable thrombotic reduction in a porcine heterotopic aortic mechanical valve model.12 In that study, dabigatran was as effective as enoxaparin dosed at 2.5 mg/kg SC BID (goal to maintain anti-Xa levels >0.6 IU/mL at 4 hours after administration) in preventing valve thrombosis compared with the no anticoagulation group.12 Dabigatran was also compared with warfarin in the RE-ALIGN study in 2 groups of patients implanted with aortic or mitral mechanical heart valves (implantation within 7 days or after 3 months).8 In the RE-ALIGN trial, dabigatran dosing was based on previous doses used in patients with atrial fibrillation to maintain a trough level >50 ng/mL, whereas warfarin was dosed to an INR (2–3 or 2.5–3.5) based on thromboembolic risk. The study was halted early because of a higher incidence

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### Table 4. Selected Pharmacokinetic Parameters of Apixaban Oral in Swine

<table>
<thead>
<tr>
<th>SwineNo.</th>
<th>$t_{1/2}$</th>
<th>AUC</th>
<th>Clearance</th>
<th>AUMC</th>
<th>MRT</th>
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<tbody>
<tr>
<td></td>
<td>h</td>
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<td>mL/h/kg</td>
<td>ng·h/mL</td>
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<td>%</td>
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<td>12472</td>
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<td>50.6</td>
</tr>
<tr>
<td>Mean</td>
<td>5.93</td>
<td>1390.00</td>
<td>397.67</td>
<td>9286.33</td>
<td>6.93</td>
<td>214.67</td>
<td>1.58</td>
<td>31.87</td>
</tr>
<tr>
<td>SD</td>
<td>1.37</td>
<td>564.02</td>
<td>144.89</td>
<td>2806.98</td>
<td>0.87</td>
<td>91.51</td>
<td>0.72</td>
<td>20.36</td>
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</table>

One animal was replaced and not used in crossover IV to PO pharmacokinetic analysis. AUC indicates area under the curve; AUMC, area under the first moment curve; and MRT, mean residual time.

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![Figure 2. Chromogenic assay anti-Xa low-molecular-weight heparin (LMWH) activity after a single apixaban 0.5 mg/kg IV dose (n=3) and single apixaban oral dose 0.5 mg/kg (n=3) vs time (mean±SEM).](http://atvb.ahajournals.org/Downloaded from)
of thromboembolic ischemia or stroke (5% patients receiving dabigatran versus no patients receiving warfarin) and major bleeding events (dabigatran 4% versus warfarin 2% in patients receiving dabigatran within 7 days of surgery). Of interest, the majority of major bleeding occurred in the immediate postsurgical period, which is generally characterized by inflammation, enhanced thrombogenicity, and platelet reactivity. In the RE-ALIGN trial, the authors hypothesized that the higher incidence of thromboembolic complications was possibly because of activation of the coagulation cascade secondary to tissue factor released during surgery and activation of the intrinsic pathway via contact with the sewing ring and valve leaflets of mechanical heart valves. In this setting, warfarin may have been more efficacious as it targets multiple pathways of the clotting cascade versus dabigatran, which is limited to thrombin inhibition.

Because factor Xa is an initial component of the coagulation common pathway where both the extrinsic and contact activation pathways converge, inhibition of factor Xa inhibits both coagulation pathways and the downstream exponential

<table>
<thead>
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<th>Table 5. Valve Thrombus Weight (mg) in Swine</th>
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<td>Control 30 d (n=4)</td>
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</tr>
<tr>
<td>Valve Thrombus Weight (mg)</td>
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<td>Control 30 d (n=4)</td>
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<tr>
<td>Warfarin Oral 30 d* (n=3)</td>
</tr>
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<td>Apixaban Infusion 14 d* (n=4)</td>
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</table>

Control (no anticoagulation), apixaban oral 1 mg/kg BID was given for 30 d, warfarin oral 0.04–0.08 mg/kg was given for 30 d, and apixaban multi-step infusion was given for 14 d.

*Valve thrombus weights in the anticoagulant groups were significantly different from control group valve thrombus weights. One-way ANOVA (F=10.88; P=0.001) and Tukey multiple comparison test (P<0.05).
amplification of thrombin. Furthermore, in vitro and in vivo animal studies have demonstrated hypercoagulability and thrombogenesis with subtherapeutic doses of direct thrombin inhibitors versus direct Xa inhibitors, resulting from inactivation of the protein kinase C system. Thus, factor Xa inhibitors such as apixaban may provide a greater reduction in mechanical heart valve thrombosis compared with direct thrombin inhibitors such as dabigatran with minimal antagonism of existing thrombin needed to maintain hemostasis via platelet thrombin receptor interactions. Theoretically, this may reduce major bleeding in the immediate postoperative period. In addition, the favorable pharmacokinetic and clearance profile of apixaban may lend to less permutations in plasma levels with increased anticoagulation stability. In these efforts, the apixaban infusion group dose was modeled after human pharmacokinetic data based on previous studies and the Apixaban trial pharmacokinetic data for the prevention of systemic emboli in mechanical heart valve leaflets at the 14-day postmortem end point.

One limitation of the study is translating and determining optimal dosing based on previous studies and indications for venous thrombosis and prevention of embolic disease and stroke. Doses of factor Xa or direct thrombin inhibitors with confirmed efficacy for low flow or stasis conditions may not correlate with similar efficacy when used in high flow and high shear environments associated with mechanical heart valves. In addition, the acute postoperative period is generally an inflammatory and prothrombotic environment characterized by a high degree of platelet activation. As a result, additional preclinical and appropriate controlled trials evaluating the use of a factor Xa inhibitor such as apixaban possibly in combination with an antiplatelet agent are needed to elucidate optimal dosing to minimize both thromboembolic complications and incidences of major bleeding.

Additional limitations to the study were small sample sizes mainly in part to complexity associated with this research model, which limited power to establish noninferiority of apixaban compared with warfarin for thromboprophylaxis efficacy. However, despite the small sample size, we demonstrated significant differences in thrombus deposition between control and treatment groups, emphasizing the anticoagulant efficacy of apixaban. In addition, we used a short-term 30-day time point to measure thromboprophylaxis based on previous publications and used a 14-day infusion time period to focus on the immediate postsurgical period in which the risk of thromboembolism would be greatest. With regard to major bleeding complications, intracranial hemorrhage is of great concern in human patients receiving anticoagulation. Postmortem evaluation of the central nervous system to completely confirm absence of intracranial hemorrhage was not performed as the study did not demonstrate clinical evidence of major bleeding or neurological signs within the apixaban groups. Two animals in the warfarin group were removed from study because of major bleeding complications including pulmonary hemorrhage, hemothorax, and multifocal subcutaneous hemorrhage (bleeding complication animal 1, last INR 3.85 on day 6 post-surgery; bleeding complication animal 2, last INR 5.35 on day 15 post-surgery).

Conclusions

This study was not powered to test for noninferiority, yet apixaban demonstrated promising efficacy comparable to oral warfarin for preventing short-term arterial thromboembolism in swine implanted with a heterotopic mechanical heart valve. Swine that received apixaban via infusion modeled after human clinical trial pharmacokinetic data for the prevention of systemic emboli had the smallest thrombus weights compared with all groups. Although the duration of the infusion was only 14 days compared with 30 days for the oral apixaban and warfarin groups, the smaller thrombus weights seem to correlate with apixaban’s rapid in vivo activity and predictable pharmacokinetic profile.

Figure 6. Postmortem photographs of valves removed after 30 (A–C) and 14 d (D–E). A, control (no anticoagulation); B, apixaban 1 mg/kg oral; C, warfarin oral (average international normalized ratio=2.6); D, apixaban 14-d infusion; E, apixaban 14-d infusion—fibrin film on graft; F, reverse side of graph (E) demonstrating film.
valves. These data provide support for additional dose–response studies powered to directly compare thromboprophylaxis efficacy of apixaban to warfarin in preclinical mechanical valve models. Data from such studies may provide the foundation for future clinical studies evaluating apixaban as an alternative to warfarin in select patients with mechanical heart valves.

Acknowledgments

We would like to thank the following individuals for the participation and assistance with this study: Drs Bo Wen, Ting Zhao, and Duxin Sun, Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan (NIH-NCI P30CA046592) for assistance with pharmacokinetic assays and Terry Majors for assistance with coagulation analysis. Dr Thomas R. Meier for assistance with development of a swine jacketed-tether infusion system. Lorie Gavulic for her medical illustrations. Finally, we would like to thank the Unit for Laboratory Animal Medicine Animal Care Supervisors, Scot A. Pittman and Michael J Ream and Veterinary Technicians, Lisa A. Burlingame and Laura B. Durham for their expert technical assistance and supportive veterinary care during the course of this study.

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Disclosures

Dr Diaz is member of the Board of Directors of the American Venous Forum. The other authors report no conflicts.

References


Highlights

• Despite small sample size, apixaban demonstrated significant reductions in mechanical valve thrombus weights, as oral warfarin, in swine implanted with a heterotopic mechanical heart valve. Compared with warfarin, swine in the apixaban groups showed no evidence of bleeding complications.
• Apixaban is a promising candidate and may be a useful alternative to warfarin for thromboprophylaxis of mechanical heart valves.
• Human plasma concentrations from apixaban clinical trials were used to develop a novel targeted infusion model for swine based on comparable pharmacokinetic parameters. Additional dose–response studies are warranted to fully elucidate the thromboprophylaxis efficacy of apixaban.
Apixaban Versus Warfarin for Mechanical Heart Valve Thromboprophylaxis in a Swine Aortic Heterotopic Valve Model

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Materials and Methods

Pharmacokinetics of Apixaban in Swine
All animal experiments were approved by the University of Michigan Institutional Animal Care and Use Committee and performed at the University of Michigan, an AAALAC accredited institution. All Landrace cross swine were acquired from the same vendor and had a similar pathogen free status and genetic background. Swine were implanted with vascular access ports to facilitate blood collection prior to drug administration. Apixaban (BMS562247, New Brunswick, New Jersey) was delivered IV over 10 minutes at 0.5 mg/kg. Following a two-week wash out period animals received apixaban orally (PO) at 0.5 mg/kg. The intravenous dose was formulated in 10% N,N-dimethylacetamide (Sigma-Aldrich, Catalog# 270555), 20% propylene glycol (Sigma-Aldrich, Catalog# P4347) and 70% dextrose 5% in water (v/v). The oral solution was solubilized in dimethylacetamide (50% w/v) with propylene glycol added to achieve 10mg/ml concentration (w/v). Whole blood was collected into EDTA plasma tubes at the following time points: (IV) baseline (prior to drug), 5 minute, 15 minute, 30 minute, 45 minute, 1h, 2h, 4h, 6h, 8h, and 24h; (PO) baseline, 15 minute, 30 minute, 45 minute, 1h, 2h, 4h, 6h, 8h, and 24h. Collected samples were immediately inverted to ensure mixing, and centrifuged at 4°C at 4000 rcf for 20 minutes. Platelet-free plasma was separated into 1 ml aliquots in 2 mL cryopreservation tubes and stored frozen at -80°C until use.

Sample Preparation for LC-MS/MS
Protein precipitation was used to prepare all apixaban swine plasma samples for LC-MS/MS analyses. Plasma samples were prepared by mixing plasma and methanol at 1:1 ratio, followed by the addition of 3 volume of acetonitrile containing internal standard (IS) (400ng/mL BMS562247-03). The mixture was vortexed for 1 min at high speed and centrifuged at 15000 rpm for 10 min to precipitate proteins. 5 µL of the supernatant was injected for LC-MS/MS analyses. To construct the calibration curve, a series of calibration standards (2.5 ng/mL-7500 ng/mL) of the test compound apixaban were prepared with blank plasma.

LC-MS/MS Conditions
Quantitative LC-MS/MS analyses of apixaban in swine plasma was performed on an Agilent 1200 HPLC system coupled to an API 3200 mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada). Chromatographic separation was achieved using an Agilent Zorbax Extend-C18 column (5 cm X 2.1 mm, 5 µm). Apixaban and internal standard were eluted at a flow rate of 1 mL/min using a rapid gradient of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile), beginning with a composition of 90% solvent A for 0.5 min, which was reduced to 10% solvent A at 0.51 min, then held for 2.5 min, and returned back to 90% for column equilibration at 3.1 min. The mass spectrometer was operated in ESI positive ion mode and the ions were selectively detected by the multiple reaction monitoring (MRM). MRM transitions
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at 460.2 → 199.1, and 464.3 → 447.2 were used to monitor apixaban and the internal standard BMS562247-03, respectively. During the pharmacokinetic study, plasma concentrations of the apixaban were determined by the LC-MS/MS method developed and validated for this study. Intra-assay accuracy (relative error within 15%) and precision (coefficient of variation within 15%) were assessed using at least two quality control samples. The linearity of the relationship between peak area ratio and concentration was demonstrated by the correlation coefficients (R) obtained for the linear regression analysis. All pharmacokinetic parameters were calculated by non-compartmental methods using WinNonlin® version 3.2 (Pharsight Corporation, Mountain View, CA, USA). Parameters are presented as a mean ± standard deviation (SD).

Animal Model – Vascular Access Port and Catheter Surgery

Swine were acclimated to the environment and laboratory staff three to seven days prior to surgery. 24 hours pre-surgery, swine received cefadroxil tablets (Cefa-Tabs, Boehringer Ingelheim Vetmedica) 33mg/kg orally twice daily. The night before surgery, animals were washed and bathed with a chlorhexidine scrub and a fentanyl 75 mcg/h (Duragesic®, Janssen) transdermal patch was applied to the shaved dorsal surface of the skin. Animals were fasted the night before surgery. On day of surgery, tiletamine-zolazepam (Telazol, Zoetis) 6 mg/kg IM + xylazine (Anased®, Lloyd Laboratories, Shenandoah, IA) 2 mg/kg + IM atropine (VetOne, Boise, ID) 0.04 mg/kg IM was administered to swine for pre-anesthetic sedation. Animals were supplied 100% oxygen via facemask then intubated with a 7-8 Fr endotracheal tube and ventilated (TV = 10-20ml/kg, RR 10-15) with isoflurane (1-3%) in 100% oxygen. Monitoring consisted of indirect and/or direct blood pressure, O₂ saturation monitoring, end-tidal CO₂ monitoring, electrocardiogram, and arterial blood gases. Pre-surgical antibiotic cefazolin 50 mg/kg IV and ceftiofur (Naxcel®, Zoetis) 5 mg/kg, IM were administered. In addition a preemptive dose of carprofen (Rimadyl®, Zoetis) 4 mg/kg SQ was administered. Intravenous isotonic fluids, lactated ringers solution (Hospira, Lake Forest, IL), 5-10 ml/kg were administered via a lateral ear vein catheter. The surgical site was shaved and prepped with alternating scrubs of povidone-iodine and 70% isopropyl alcohol. Ioban® drape (3M) material was placed over the surgical site. A four cm full-thickness incision was made in the left jugular groove (paramedian) at the mid cervical region. The left external or internal jugular vein were isolated and encircled with elastic vessel loops proximal and distal to the anticipated venotomy site. A 5-6 cm incision was made in the ipsilateral posterior auricular region to form a subcutaneous pocket. A sterilized stainless steel trocar was used to create a subcutaneous tunnel between the auricular incision and jugular furrow. The vascular access port (VAP) was secured to fascia within the auricular pocket with polyglactin 910 (Vicryl) sutures and the catheter was passed through the hollow stainless steel trocar and secured to VAP (Access Technologies, Ti solopart MAX; uncoated PU cath (7FR; attachable); round tip; 60 cm; 2 movable beads BN590 (CV), Item # MAX-PU-C70). Or central Line: Solomon Scientific, San Antonio, TX, Silicone Catheter; 7Frx60cm, .040”/1.0mm ID x .085”/2.2mm OD; female luer, clear, round tip, STERILE, Catalog # SIL-
C70). The VAP and catheter were flushed with 0.9% saline. A 2 to 3 mm venotomy was created sharply and the catheter was advanced 8 to 10 cm into the cranial vena cava and secured via ligatures using polydioxanone (PDS) suture placed caudal to the insertion site and on either side of the catheter retention beads. The port and catheter were flushed with 0.9% saline and locked with 3 mL heparin (Sagent Pharmaceuticals, Schaumburg, IL) 1000 USP units/ml. For the apixaban infusion aim of study, the external jugular catheter was tunneled subcutaneously exiting near the dorsal surface of the neck. The incisional subcutaneous tissues and skin were closed with PDS or Vicryl. Skin adhesive was applied to wound edges post closure.

Animal Model - Heterotopic Aortic Bileaflet Mechanical Valve Surgery
Heterotopic mechanical aortic valve placement was performed in adult male swine modified from previously publication. Under general anesthesia and in a lateral decubitus position, a left thoracotomy was performed through the fifth intercostal space and the descending thoracic aorta was exposed. Select lung lobe ventilation was performed to assist with surgical access to the descending thoracic aorta. A Cohen endobronchial blocker (Cook Medical, Catalog#G44122) was placed into the left main stem bronchus via a pediatric fiber optic scope and inflated. The inferior pulmonary ligament and the azygous vein were ligated to facilitate descending thoracic aortic exposure. The modified heterotopic valved conduit was pre-fabricated in preparation for the case during the port placement typically and constructed from a 19mm St. Jude Masters Series (bileaflet, aortic) Mechanical Heart Valve (St. Jude Medical, Minneapolis, MN) and composite aortic conduit (Gelsoft [knitted] vascular graft, Terumo Cardiovascular, Ann Arbor, MI). The aortic conduit was transected and reconstructed with the valve positioned in the middle using non-absorbable monofilament suture placed in a continuous fashion. The aorta was partially occluded proximally permitting the creation of an end-to-side anastomosis with the proximal modified valved conduit (Terumo Cardiovascular, Gelsoft (knitted) Vascular Graft, Vascular graft, usable length 25 cm, 1, Catalog # 632518). The graft was then occluded and flow restored to the native aorta in full. The distal descending thoracic aorta was partially occluded permitting the creation of the distal end-to-side anastomosis with the modified valved conduit. With flow restored through the native aorta and modified valved conduit, the intervening native aorta was then plicated to approximately one-half the diameter of the normal aorta using non-absorbable, monofilament suture and ultrasound guidance (Supplemental Figure I). With hemostasis confirmed, an intercostal nerve block was performed utilizing bupivacaine the chest wall was closed and a tube thoracostomy left in place (MILA International, Inc., Florence, Kentucky, Chest tube - 14Fr×38cm (15in), Catalog # CT1415). The incisional subcutaneous tissues and skin were closed with polydioxanone (PDS) or polyglactin 910 (Vicryl). Tissue glue was applied to wound edges post closure. The animal was recovered from anesthesia and received carprofen (Rimadyl®, Zoetis) 2 mg/kg orally twice a day for 3-4 days then as needed. If needed, buprenorphine (Buprenex®, Reckitt Benckister Healthcare, Hull, England) 0.01 mg/kg IV was utilized for additional analgesia
once fentanyl patches were removed at 72 h post-application. Post-operative pain was assessed during daily to twice daily monitoring including behavioral, physical examination, and use of a simple-descriptive pain scale (Pain Assessment in Swine, Sinclair Research). The chest tube was monitored twice daily for pleural air and/or fluid and removed in 3-4 days when withdrawal of air and/or fluid was minimal to absent. Animals received enrofloxacin (Bayer, Shawnee Mission, KS) 5 mg/kg oral daily and cefadroxil (Cefa-Tabs, Boehringer Ingelheim Vetmedica) 20mg/kg oral twice a day for 5 days post-operative.

Aim One: Comparison of oral apixaban to oral warfarin for thrombophrophylaxis of heterotopic aortic bileaflet mechanical valve.
Fourteen animals were randomized to one of three treatment groups. All animals had surgery to implant a vascular access port or exteriorized catheter immediately followed by heterotopic valve replacement surgery. The control group (n=4) received no anticoagulation following surgery. The apixaban group (n=5) received apixaban 1 mg orally twice a day. Apixaban was suspended in Ora-Blend SF (Perrigo, Allegan, MI) final concentration = 10mg/ml. The warfarin group (n=5) received oral warfarin adjusted to international normalized ratio (INR) 2-3. Administration of apixaban or warfarin was initiated on post-surgical day one.

Primary and Secondary Endpoints
Animals (Aim One) were euthanized at 30 days to determine the primary endpoint of valve thrombus burden. Animals were sedated with tiletamine-zolazepam (Telazol®, Zoetis) administered intravenous heparin 1000 USP unit/ml, and euthanized with intravenous sodium pentobarbital overdose (Beuthanasia-D, Scering-Plough, Union, NJ). The aortic heterotopic valve was harvested and and thrombotic material was removed and weighed (mg) (mean +/- SD). The graft neo-intima was excluded.

Secondary endpoints included post-operative hemorrhage and arterial thromboembolism. During their post-operative course, animals were monitored daily to twice a day for evidence of bleeding (gingival, suture site) hemothorax (until post-surgical chest tube removed), difficult breathing, lethargy, mucous membrane color, capillary refill time, hematemesis and hematochezia. In addition, animals were monitored daily for thromboembolic complications with a clinical assessment that targeted limb swelling, difficult ambulation, neurological abnormalities, respiratory difficulties, or gastrointestinal ischemia.

Post-mortem Tissue Evaluation - Histopathology
The following tissues were collected post-mortem and submitted for paraffin embedding and H/E staining: Stomach, diaphragm, skeletal muscle, liver, left lung lobe, right lung lobe, colon, azygous vein, lymph node, aorta, kidney, heart, duodenum, spleen, skin, thymus, esophagus, pancreas, and adrenal. Post-formalin fixation, tissues were trimmed into cassettes and processed into paraffin blocks and sectioned at 4 µm, stained with Hematoxylin and Eosin via a Leica
AutoStainer X automatic stainer, and coverslipped. Histology sections were analyzed via a board-certified veterinary pathologist.

**Blood Collection**
To protect against VAP and/or catheter related infections, the skin site above the subcutaneous VAP or catheter exit site was scrubbed with three alternating topical application of providone-iodine antimicrobial solution followed by 0.9% sterile saline or 70% isopropyl alcohol prior to blood withdrawal. For VAP blood withdrawal, a 22-gauge ¾”, non-coring Huber needle (Solomon Scientific, Catalog#HN22-750) attached to a 5 mL syringe was inserted through the skin and into the VAP septum. For exteriorized catheters the syringe was attached directly to catheter stopcock. 2 - 3 mL of blood was withdrawn and discarded. A new syringe was attached to withdraw test samples. The VAP was flushed with eight ml 0.9 % saline flush solution.

**Anti-Xa Low-Molecular Weight Heparin (LMWH) Assay**
Whole blood was collected from a non-treated animal into 3.2% sodium citrate blood collection tubes, immediately inverted to ensure complete mixing and centrifuged at 4°C at 4000 rcf for 20 minutes. Platelet-free plasma was separated into 1 mL aliquots in 2 mL cryopreservation tubes and stored frozen at -80°C until use. An apixaban standard curve 100 – 5000 ng/mL was generated by serial dilution. Results were calibrated against a LMWH standard curve (0.2 – 1.3 IU/mL) and reported as anti-Xa LMWH using a Siemens BCS coagulation analyzer in conjunction with Siemens Berichrom Heparin chromogenic assay.

**Warfarin and Enoxaparin Bridge Dosing**
Initial warfarin dosing was based upon previous publications.³,⁴ Initial warfarin (Jantoven®) = 0.07 mg/kg until prothrombin time (PT) was 1.3X baseline or INR within target range (2-3), maintenance dose = 0.04 – 0.08 mg/kg daily, maximum daily dose = 0.08 mg/kg. Blood was collected daily for first 4-6 days then twice a week into sodium citrate tubes and centrifuged at 1500 rcf for 10 minutes. 10uL samples were utilized for measurement of prothrombin time (PT) and International Normalized Ratio (INR). INR = [Patient’s PT/Mean PT of normal range]¹² (ISI = International Sensitivity Index) of the thromboplastin reagent. A mechanical-clot-detection fibrometer was used for analysis with prothrombin reagent Innovin® (Dade Behring, Catalog B4212-40) (ISI = 0.92) was utilized to determine prothrombin time. Target PT was 1.3 – 1.5X baseline. Target INR = 2-3. Warfarin dosing adjustments were made by altering the weekly warfarin dose by 5-20%. Animals within the warfarin group received enoxaparin sodium 2.5mg/kg SQ q12h post-operatively as bridging therapy for three to four days until PT and/or INR were within therapeutic range. Enoxaparin dosing was based upon previous publications and anti-Xa LMWH levels were confirmed with in house testing.¹,² Animals receiving warfarin were monitored daily for signs of bleeding or hemorrhage including: epistaxis, melena, subcutaneous hematomas, pulmonary hemorrhage, gastric ulceration, central nervous system changes (attitude, weakness, lethargy), gingival bleeding, and hematuria. Dose reduction,
withholding of daily dose, and/or oral phytonadione 2.5 – 5mg was utilized if PT or INR was elevated outside of target range.

**Aim Two: Comparison of continuous apixaban infusion to oral apixaban and oral warfarin for thrombophrophylaxis of heterotopic aortic bileaflet mechanical valve.**

The human pharmacokinetic parameters based upon apixaban 5mg oral twice a day dosing as outlined in the ARISTOTLE clinical trial where used to replicate a comparative AUC pharmacokinetic multi-step apixaban infusion in swine (Supplemental Figures II and III). Four different infusion rates were used to mimic the first 12 hours of an oral apixaban 5 mg dose in humans followed by four separate infusion rates during subsequent 12-hour interval dosing intervals (Supplemental Table I). The swine control group (n=4) received no anticoagulation following surgery (Aim One). The swine apixaban infusion group (n=4) received a continuous multi-step infusion immediately post-surgery until study endpoint at 14 days. Animals in the apixaban infusion group (Aim Two) were euthanized at 14 days to determine the primary endpoint of valve thrombus burden. The same surgical model and methods for implanting exteriorized jugular catheter and porcine heterotopic bileaflet aortic valve surgical model were utilized. The catheter tubing was connected to a large animal jacket-swivel tether system (SAI Infusions Technology, Catalog #LA T4, AS P, SS J1, EXT-PM40, AT E1). An extension set (Baxter Healthcare Corporation, Catalog# ACT 5612) was connected to an Alaris SE (Carefusion - Becton, Dickinson and Company, Franklin Lakes, NJ) multi-step infusion pump with a vented infusion set (Carefusion – Becton, Dickinson and Company, Franklin Lakes, NJ, Catalog #72953). Apixaban was solubilized in N,N-dimethylacetamide (Sigma-Aldrich, Catalog# 270555) to a final concentration 10mg/ml. Apixaban-N,N-dimethylacetamide solution was diluted in 0.9% sodium chloride (Hospira, Lake Forest, IL) to a final concentration 0.025mg/mL (e.g., 2.5 mL of 1% apixaban-N,N-dimethylacetamide in 1000 mL sterile saline). If the pump occluded or infusion stopped during evening (time without apixaban <12 hours), the infusion was restarted the next morning utilizing the loading dose infusion rates (infusion rates 1-4) before returning to cyclic q12h dosing (infusion rates 5-8).

**Histopathology**

Euthanasia and post-mortem tissue procurement proceeded as described in Aim One. Collected tissues were place in 10% neutral buffered formalin for a minimum of 48 hours. Post-formalin fixation, tissues were trimmed into cassettes and processed into paraffin blocks and sectioned at 4 µm, stained with Hematoxylin and Eosin via a Leica AutoStainer X automatic stainer, and coverslipped. Histology sections were analyzed via a board-certified veterinary pathologist.

**Statistics**

Thrombotic material from the heterotopic implanted aortic mechanical valve (mean +/- SD) per group was analyzed via one-way ANOVA with Tukey’s post-hoc analysis (p<0.05). Linear regression was utilized to analyze apixaban plasma
concentrations versus anti-Xa LMWH activity. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc.).

Supplemental References


SUPPLEMENTAL MATERIAL

Supplementary Figures and Tables:

**Supplemental Figure I: Heterotopic aortic bi-leaflet mechanical valve surgery in swine.** Aortic conduit was transected and reconstructed with an aortic bi-leaflet mechanical valve positioned in the middle of the graft. The distal descending thoracic aorta was partially occluded permitting the creation of an end-to-side anastomosis with the modified valved conduit. Flow was restored through the native aorta and modified valved conduit, the intervening native aorta was then plicated (surgically stenosed) to approximately one-half the diameter of the normal aorta using non-absorbable, monofilament suture and ultrasound guidance.

**Supplemental Figure II:** Pharmacokinetic plasma apixaban concentration versus time simulation (0-12 hours) between a human oral apixaban 5mg dose and a comparable continuous multi-step apixaban infusion based upon pharmacokinetic data in swine.

**Supplemental Figure III:** Pharmacokinetic apixaban plasma concentration versus time simulation between the human oral 5mg twice a day dosing approved for stroke prophylaxis and systemic emboli in patients with atrial fibrillation and a continuous multi-step infusion based upon pharmacokinetic data in swine. Four separate infusions were utilized in the first 12 hours as a loading dose followed by four separate infusions repeated every 12 hours to maintain comparable steady state plasma concentrations for 14 days.

**Supplemental Table I:** Multi-step infusion paradigm used to mimic apixaban area under the curve dosing in swine compared to human 5 mg twice a day oral dosing for stroke prophylaxis and systemic emboli in patients with atrial fibrillation. Multi-step infusions were administered over a 24 h period. Infusions 1-4 (red) were administered for the first 12 hours as a loading dose. Infusions 5-8 (black) were repeated every 12 hours to maintain comparable steady state plasma concentrations for 14 days.
SUPPLEMENTAL MATERIAL

Supplemental Figure I
Supplemental Figure II:

Simulated Swine vs Simulated Atrial Fibrillation Patients (5 mg BID)
SUPPLEMENTAL MATERIAL

Supplemental Figure III:
Supplemental Table I:

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