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Rivaroxaban – an oral, direct Factor Xa inhibitor – has potential for the management of patients with heparin-induced thrombocytopenia

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Summary

Rivaroxaban is an oral, direct activated Factor Xa (FXa) inhibitor in advanced clinical development for the prevention and treatment of thromboembolic disorders. Currently available anticoagulants include unfractionated heparin (UFH) and low molecular weight heparins (LMWHs); however, their use can be restricted by heparin-induced thrombocytopenia (HIT). HIT is usually caused by the production of antibodies to a complex of heparin and platelet factor-4 (PF4). This study was performed to evaluate, *in vitro*, the potential of rivaroxaban as an anticoagulant for the management of patients with HIT. UFH, the LMWH enoxaparin, fondaparinux and the direct thrombin inhibitor argatroban were tested to enable comparative analyses. Rivaroxaban did not cause platelet activation or aggregation in the presence of HIT antibodies, unlike UFH and enoxaparin, suggesting that rivaroxaban does not cross-react with HIT antibodies. Furthermore, rivaroxaban did not cause the release of PF4 from platelets and did not interact with PF4, unlike UFH and enoxaparin. These findings suggest that rivaroxaban may be a suitable anticoagulant for the management of patients with HIT.

Keywords: oral anticoagulant, rivaroxaban, Factor Xa inhibitor, heparin-induced thrombocytopenia, heparin-induced thrombocytopenia antibodies.

Unfractionated heparin (UFH) and the low molecular weight heparins (LMWHs) are the current standard of care for patients requiring short-term anticoagulation. However, approximately 1–5% of patients receiving heparins develop heparin-induced thrombocytopenia (HIT) – a severe and potentially life-threatening complication (Warkentin & Greinacher, 2004; Franchini, 2005). HIT is associated with a substantially increased risk of both venous and arterial thrombosis (Warkentin & Greinacher, 2004).

Heparin-induced thrombocytopenia is an immune-mediated response, usually initiated by the binding of heparins to platelet factor-4 (PF4), a glycoprotein released into the circulation upon platelet activation (Amiral *et al*, 1992). Formation of the heparin–PF4 complex triggers the production of anti-heparin–PF4 antibodies (HIT antibodies) (Greinacher *et al*, 1994, 1995; Suh *et al*, 1998; Kelton, 2005). HIT antibodies react with heparin–PF4, and the resulting immunocomplex binds to circulating platelets (Amiral *et al*, 2000; Newman & Chong, 2000; Kelton, 2005). This interaction triggers activation and aggregation of platelets, leading to thrombin generation via the coagulation cascade, a further release of PF4 (thus perpetuating the cycle of heparin-induced platelet activation), and the production of prothrombotic platelet microparticles. The overall result of this cascade of events is the generation of a hypercoagulable state, and, eventually, thrombocytopenia and thrombosis (Walenga *et al*, 2000, 2004; Franchini, 2005). If untreated, HIT leads to thrombotic complications in more than 50% of affected patients (Warkentin & Kelton, 1996), and to limb amputation or death in more than 35% (Wallis *et al*, 1999; Warkentin & Greinacher, 2004; Kelton, 2005).

The management of HIT remains a difficult task despite guidelines having recently been made available (Warkentin & Greinacher, 2004; Keeling *et al*, 2006). Clearly, heparin should be stopped immediately in patients who develop HIT. However, heparin cessation alone is not a viable option for these patients, given their exceptionally elevated risk of thrombosis (Wallis *et al*, 1999). An alternative mode of anticoagulation is required for the initial acute episode in patients with HIT and also for the subsequent prevention and

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treatment of thrombosis, should this be necessary. Warfarin, a vitamin K antagonist, does not interact with HIT antibodies; however, it can cause venous limb gangrene or skin necrosis during the extreme hypercoagulable stage of HIT because of its slow onset of action and inhibition of activated protein C, a natural anticoagulant (Srinivasan *et al*, 2004). Therefore, co-administration with another anticoagulant is necessary until patients are adequately anticoagulated.

It has been proposed that a HIT 'reactive' heparin has a molecular weight >2·4 kDa and a degree of sulphation >0·6 per carbohydrate unit (Greinacher *et al*, 1995). By these standards, fondaparinux (1·7 kDa), a synthetic pentasaccharide that indirectly inhibits activated Factor Xa (FXa), may be a suitable anticoagulant in patients with HIT. Fondaparinux does not cross-react with HIT antibodies (Greinacher *et al*, 1995; Jeske *et al*, 1999), and isolated case reports have demonstrated the efficacy and safety of this drug in patients with HIT (Jeske & Walenga, 2002; D'Amico *et al*, 2003; Parody *et al*, 2003; Bradner *et al*, 2004). However, fondaparinux does generate HIT antibodies in treated patients (Pouplard *et al*, 2005; Warkentin *et al*, 2005) and may not be inert in a subset of patients sensitive to heparin (Utikal *et al*, 2005). Furthermore, there is one report of fondaparinux-induced HIT (Warkentin *et al*, 2007).

Several studies have demonstrated that direct thrombin inhibitors (DTIs), such as argatroban, lepirudin and bivalirudin, do not cross-react with HIT antibodies (Bartholomew *et al*, 2005). Guidelines currently recommend the use of a DTI for the prevention and treatment of thrombosis associated with HIT (Warkentin & Greinacher, 2004). However, although DTIs are effective in patients with HIT (Greinacher *et al*, 1999a,b; Lewis *et al*, 2001, 2003), they are associated with a high bleeding risk, as well as other drug-specific limitations (Jeske & Walenga, 2002; Greinacher *et al*, 2003; Messmore *et al*, 2003; Hursting *et al*, 2005; Tardy *et al*, 2006). Furthermore, currently approved DTIs are only available as parenteral formulations, making them inconvenient for outpatient use, particularly in the long term.

There is increasing interest in the use of small molecule, direct FXa inhibitors as an alternative to DTIs for the management of patients with HIT. This is because active site-directed FXa inhibitors are structurally unrelated to heparin and therefore should not interact with PF4 and promote HIT (Turpie, 2007).

The oral, direct FXa inhibitor rivaroxaban is one such agent with potential for use in patients with HIT. Because rivaroxaban does not require a cofactor and is able to inhibit prothrombinase activity and clot-associated FXa activity (Depasse *et al*, 2005; Perzborn *et al*, 2005), it may be particularly suitable for use in patients with HIT. Rivaroxaban was significantly more effective than enoxaparin in phase III studies for the prevention of venous thromboembolism (VTE) after major orthopaedic surgery, with a similar, low rate of bleeding (Eriksson *et al*, 2007; Kakkar *et al*, 2007; Lassen *et al*, 2007). Based on promising results in phase II studies for the treatment of proximal deep vein thrombosis (Buller, 2006; Agnelli *et al*, 2007), rivaroxaban is now in phase III investigation for the treatment of VTE and the prevention of stroke in patients with atrial fibrillation. Rivaroxaban has also entered phase II studies for the prevention of fatal and non-fatal cardiovascular events in patients with acute coronary syndromes.

The objective of this study was to evaluate the potential of rivaroxaban as an alternative anticoagulant for the management of patients with HIT. Specifically, the aims of the study were to assess: (1) whether rivaroxaban cross-reacts with HIT antibodies, (2) whether rivaroxaban promotes the release of PF4 from platelets, and (3) whether rivaroxaban interacts with PF4. Several heparin- and other non-heparin-based drugs were also tested to enable comparative analyses.

Materials and methods

Study drugs

Analyses were performed using the following drugs: rivaroxaban (Xarelto®, Bayer HealthCare AG, Wuppertal, Germany), UFH (Baxter Healthcare, Deerfield, IL, USA), the LMWH enoxaparin (Lovenox®, Sanofi-aventis, Paris, France), fondaparinux (Arixtra®, Sanofi-Synthelabo, Paris, France) and argatroban (Mitsubishi Pharma Corp., Tokyo, Japan). For each drug, a range of concentrations covering the clinical dosing range was evaluated.

HIT antibodies

Serum from patients with HIT was obtained from the Serotonin Release Assay Laboratory of the Loyola University Medical Center (Maywood, IL, USA). Criteria for serum collection were: clinically suspected HIT following recent exposure to UFH or LMWH, ≥30% decrease in platelet count, a HIT antibody titre >0·8 by enzyme-linked immunosorbent assay (ELISA; GTI, Brookfield, WI, USA), and a positive response in the ^{14}C-serotonin release assay (see below). Serum was collected with the approval of the Ethical Committee of the Loyola University Medical Center. In all assays, serum was heat-inactivated at 56°C for 45 min before use.

Cross-reactivity with HIT antibodies

^{14}C-serotonin release assay. The ^{14}C-serotonin release assay was based on a method described previously (Sheridan *et al*, 1986). Washed platelets were prepared from whole blood collected from prescreened, healthy human subjects known to give a positive response in the ^{14}C-serotonin release assay. Platelets were loaded with ^{14}C-serotonin (Amersham, Piscataway, NJ, USA) and incubated with a mixture of patient serum and study drug or saline control (22–24°C, 60 min). The reaction was terminated by addition of EDTA, and platelets were pelleted by centrifugation (6000 rpm; 1500 ***g***). Radioactivity of the

supernatant (background activity and total ^{14}C-serotonin release) was determined in a scintillation counter (LKB 1209 RackBeta, Perkin-Elmer, Waltham, MA, USA). Serotonin release was then calculated for each sample and compared with saline control.

Heparin-induced platelet aggregation assay. The heparin-induced platelet aggregation assay was adapted from a method described previously (Look *et al*, 1997). Platelet-rich plasma (PRP) was prepared from samples of whole blood collected from prescreened, healthy human subjects known to give a positive response in the heparin-induced platelet aggregation assay. PRP, patient serum, and study drug or saline control were mixed and immediately assayed for platelet aggregation over a 20-min period in a PAP-4 Platelet Aggregometer (Bio/Data, Horsham, PA, USA). Results were reported as percentage platelet aggregation, with 100% platelet aggregation defined as the light transmission of platelet-poor plasma (PPP).

Flow cytometric analysis of platelet activation. Whole blood was collected from prescreened, healthy human subjects known to give a positive response in the flow cytometry assay, and immediately added to a mixture of patient serum and study drug or saline control, and incubated at 37°C for 15 min, with stirring at 600 rpm. Samples were then fixed in paraformaldehyde and labelled with CD61 fluorescein isothiocyanate (FITC; a marker of glycoprotein IIIa on platelets; Becton-Dickinson, San Jose, CA, USA) and CD62P phycoerythrin (PE; a marker of P-selectin on activated platelets; Becton-Dickinson). Samples were analysed using the Epics XL Flow Cytometer (Beckman-Coulter, Miami, FL, USA). Ten thousand CD61 FITC-positive events, gated for particle size relevant to platelets, were measured and classified according to particle size (single platelets, platelet aggregates and platelet microparticles). Platelet P-selectin expression was determined by measuring CD62P PE-positive events (% CD62P PE-positive single platelet events and mean fluorescence intensity).

PF4 release assay

Resting platelets. Whole blood collected from healthy human subjects was incubated with study drug or saline control (37°C, 30 min; stirring at 600 rpm), and centrifuged to obtain PPP. The release of PF4 from platelets was quantified by ELISA (Diagnostics Stago, Parsippany, NJ, USA) according to the manufacturer's instructions.

Activated platelets. Study drug or saline control was added to whole blood collected from healthy human subjects. Samples were then incubated with recombinant human tissue factor (TF; a gift from Dr Yale Nemerson, Mt. Sinai Hospital, NY, USA) diluted with calcium chloride (1:1) (37°C, 30 min; stirring at 600 rpm). PPP was obtained by centrifugation and assayed for PF4 by ELISA (Diagnostics Stago), according to manufacturer's instructions. This experiment was also performed using PRP instead of whole blood.

PF4 interaction assay

Purified human PF4 (10 μg/ml; Hyphen BioMed, Neuville-sur-Oise, France) was added to study drug or saline control in Tris buffer pH 8·4. The mixture was incubated at 37°C for 30 min, while stirring at 600 rpm. To determine the ability of PF4 to bind and neutralize study drugs, drug activity was measured before and after incubation with PF4. Antithrombin activity (for heparin and argatroban) and anti-FXa activity (for rivaroxaban, enoxaparin and fondaparinux) were measured using the Automated Coagulation Laboratory instrument (Beckman-Coulter). For heparin and argatroban, samples were incubated with pooled normal human plasma, Tris buffer pH 8·4, and human thrombin (Enzyme Research Labs, South Bend, IN, USA) at 37°C for 1 min. Optical density (OD) readings were recorded at 405 nm for 30 s immediately after the addition of a chromogenic substrate specific for thrombin (Spectrozyme® TH, American Diagnostica, Stamford, CT, USA). For rivaroxaban, enoxaparin and fondaparinux, samples were incubated with pooled normal human plasma, Tris buffer pH 8·4, and bovine FXa (Enzyme Research Labs) at 37°C for 2 min. OD readings were recorded at 405 nm for 30 s immediately after the addition of a chromogenic substrate specific for FXa (Spectrozyme® Xa, American Diagnostica). The activity of each study drug was quantified by comparing OD readings with a pre-prepared calibration curve.

Statistical analysis

Data were analysed using the Friedman-repeated-measures analysis of variance (ANOVA) by ranks or a one-way repeated ANOVA, followed by the Student Newman–Keuls test, as appropriate; $P < 0{\cdot}05$ was considered statistically significant.

Results

Cross-reactivity with HIT antibodies

^{14}C-serotonin release assay. Rivaroxaban ($n = 79$) did not cause any platelet activation in the presence of HIT antibodies, as demonstrated by the lack of a concentration-dependent increase in % serotonin release (Fig 1). Two other non-heparin anticoagulants, fondaparinux ($n = 10$) and argatroban ($n = 10$), also had a negative response in the ^{14}C-serotonin release assay. Study drugs were tested over a wide concentration range that included therapeutic concentrations. Results at all concentrations remained at or below those of the saline control and, therefore, were classified as a negative response. In contrast, and as expected, strong concentration-dependent platelet activation was observed with therapeutic doses of both UFH ($n = 8$) and enoxaparin

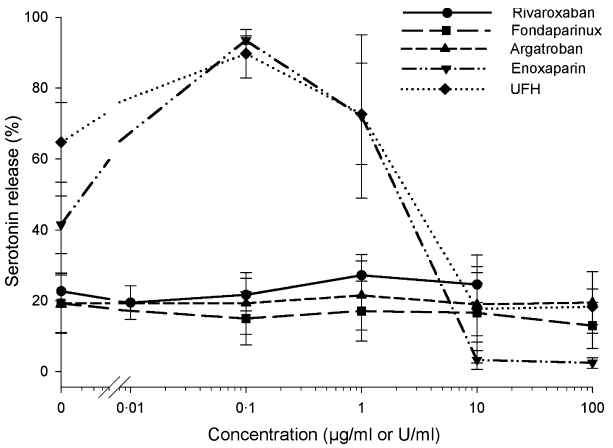

Fig 1. Effect of rivaroxaban (n = 79), fondaparinux (n = 10), argatroban (n = 10), enoxaparin (n = 8) and unfractionated heparin (UFH; n = 8) on platelet activation in the presence of heparin-induced thrombocytopenia antibodies, measured using the ^{14}C-serotonin release assay. Values are shown as means ± SD. Units of concentration are μg/ml for rivaroxaban, fondaparinux and argatroban, and U/ml for enoxaparin and UFH.

(n = 8) in the presence of HIT antibodies. Platelet activation was not observed at supra-therapeutic concentrations of heparin (10 and 100 U/ml), as is typical of a heparin-dependent platelet activation response.

Heparin-induced platelet aggregation assay. Rivaroxaban (n = 12), in contrast to UFH (n = 12), did not cause platelet aggregation in the presence of HIT antibodies over a wide concentration range (Fig 2). In a second study, where drugs were evaluated at 1·0 μg/ml (rivaroxaban and fondaparinux) or 0·1 U/ml (UFH and enoxaparin) using the platelet aggregation assay for HIT (Fig 3), rivaroxaban (n = 18)

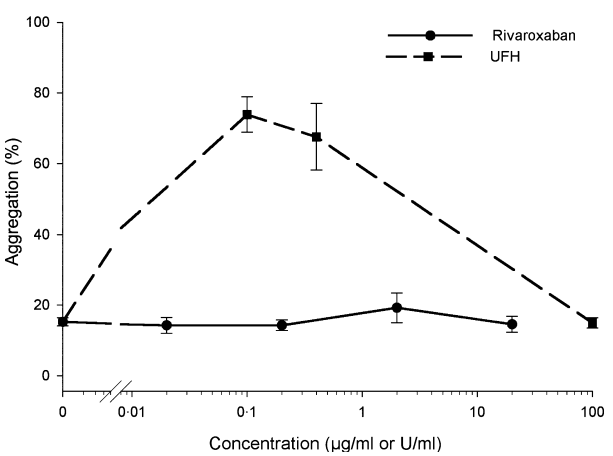

Fig 2. Effect of rivaroxaban (n = 12) and unfractionated heparin (UFH; n = 12) on platelet aggregation in the presence of heparin-induced thrombocytopenia antibodies, as measured using the heparin-induced platelet aggregation assay. Values are shown as means ± SD. Units of concentration are μg/ml for rivaroxaban and U/ml for UFH.

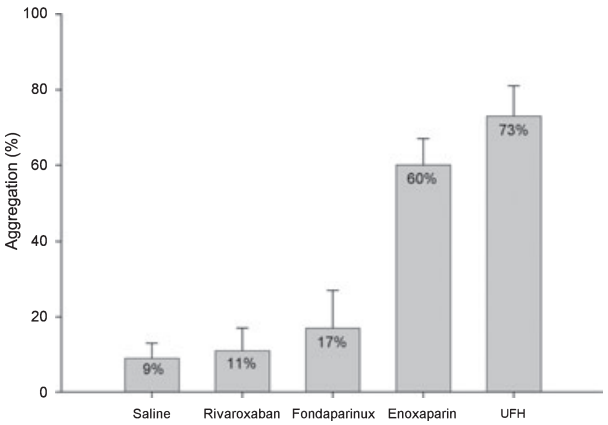

Fig 3. Effect of rivaroxaban, fondaparinux, enoxaparin and unfractionated heparin (UFH) on platelet activation (defined as >20% aggregation) in the presence of heparin-induced thrombocytopenia antibodies (n = 18), as measured using the heparin-induced platelet aggregation assay. Concentrations used were 1 μg/ml rivaroxaban and fondaparinux, and 0·1 U/ml UFH and enoxaparin. Values are shown as means ± SD.

consistently gave a negative response, whereas fondaparinux (n = 18) caused a weak positive aggregation response (platelet aggregation 30%) in one of 18 HIT patient sera (~5%). In contrast, enoxaparin (n = 18) and UFH (n = 18) induced strong platelet activation in 70% and 100% of HIT sera samples, respectively (Fig 3).

Flow cytometric analysis of platelet activation. Rivaroxaban (n = 21) did not cause any platelet activation in the presence of HIT antibodies, as demonstrated by the absence of platelet microparticle formation and platelet P-selectin up-regulation (Fig 4). There was no significant change in the parameters of

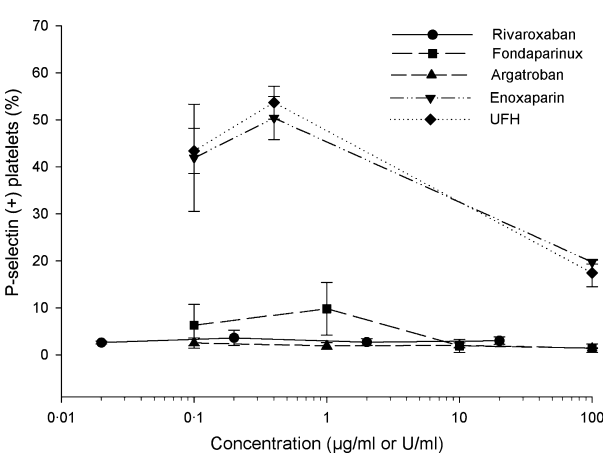

Fig 4. Effect of rivaroxaban (n = 21), fondaparinux (n = 5), argatroban (n = 5), enoxaparin (n = 4) and unfractionated heparin (UFH; n = 21) on platelet P-selectin expression (% positive platelets) in the presence of heparin-induced thrombocytopenia antibodies, as measured by flow cytometry. Units of concentration are μg/ml for rivaroxaban, fondaparinux and argatroban, and U/ml for enoxaparin and UFH. Values are shown as means ± SD.

platelet activation compared with saline control over a wide concentration range. Conversely, a positive concentration-dependent response was obtained with UFH ($n = 21$) and enoxaparin ($n = 4$) for both microparticle formation (data not shown) and P-selectin. Profiles for fondaparinux ($n = 5$) and argatroban ($n = 5$) were similar to that of rivaroxaban.

PF4 release

Resting platelets. Quiescent platelets were activated by UFH (5 μg/ml, approximately 0·5 U/ml), resulting in the release of PF4, as demonstrated in a simple whole blood system (Fig 5A). Rivaroxaban 1 μg/ml ($n = 5$) and fondaparinux 1 μg/ml ($n = 5$) did not cause release of PF4 from resting platelets, whereas enoxaparin 5 μg/ml ($n = 5$) caused platelet activation, indicated by a release of PF4 similar to that with UFH. Similar results were obtained when whole blood was replaced with PRP (data not shown).

Activated platelets. Modifying the whole blood system with the addition of TF to activate the platelets produced higher levels of PF4 release (Fig 5B). Rivaroxaban, fondaparinux and enoxaparin did not promote further release of PF4 from TF-activated platelets in whole blood, compared with the saline control (Fig 5B). Only rivaroxaban reduced PF4 release to a level below that of the saline control (Fig 5B). UFH promoted PF4 release from TF-activated platelets in whole blood, compared with the control. Similar results were obtained when whole blood was replaced with PRP (data not shown).

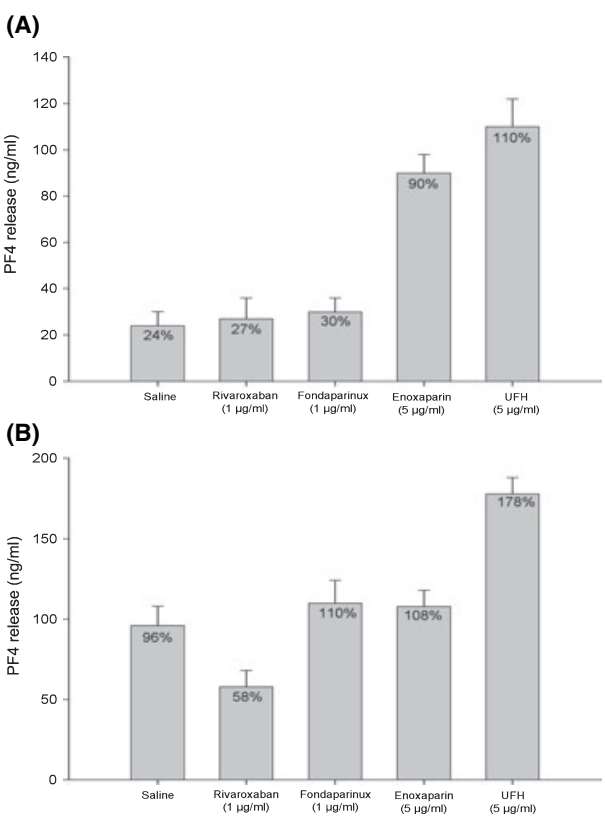

Fig 5. Effect of rivaroxaban, fondaparinux, enoxaparin and unfractionated heparin (UFH) on platelet factor 4 (PF4) release in whole blood ($n = 5$ per drug): (A) assay performed with resting platelets; and (B) assay performed with tissue-factor-activated platelets.

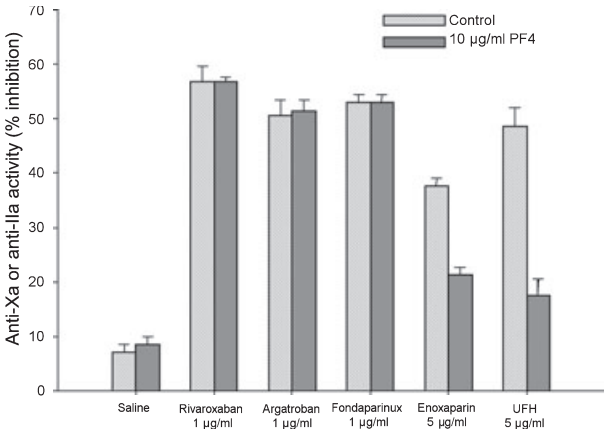

Fig 6. The effect of platelet factor 4 (PF4) on the anticoagulant activity (anti-Factor Xa or antithrombin) of rivaroxaban, fondaparinux, argatroban, enoxaparin and unfractionated heparin (UFH). Controls are anticoagulant activity without PF4. Values are shown as means ± SD.

PF4 interaction

Rivaroxaban 1 μg/ml does not interact with PF4, as demonstrated by its unchanged anti-FXa activity before (control) and after incubation with purified PF4 (Fig 6). Similarly, the anti-FXa activity of fondaparinux (1 μg/ml) and the antithrombin activity of argatroban (1 μg/ml) were unchanged after incubation with PF4 (Fig 6). In contrast, the antithrombin activity of UFH (5 μg/ml) and the anti-FXa activity of enoxaparin (5 μg/ml) were decreased by 43% and 23%, respectively, compared with the control.

Discussion

This study clearly shows that the oral, direct Factor Xa inhibitor rivaroxaban does not cross-react with HIT antibodies. Rivaroxaban did not cause platelet activation or aggregation with any of the HIT sera evaluated in three different assays of platelet function (^{14}C-serotonin release assay, heparin-induced platelet aggregation assay, and flow cytometry). These consistent findings suggest that rivaroxaban does not cross-react with HIT antibodies, which is as expected, given the non-heparin structure of the drug.

Therefore, rivaroxaban is unlikely to exacerbate the hypercoagulable state in patients with HIT, and may consequently have the potential to be used for the prevention and treatment of thrombosis in these patients. Furthermore, results with rivaroxaban in all three assays of platelet activation or aggregation were similar to those with the DTI argatroban, a drug currently recommended for use in patients with HIT.

This provides supportive evidence of the potential for rivaroxaban to be used as an alternative anticoagulant in patients with HIT.

Consistent results in the three different assays of platelet function used in this study allow a high level of confidence to be placed on HIT antibody cross-reactivity data. The ^{14}C-serotonin release assay and the heparin-induced platelet aggregation assay are used widely for the clinical diagnosis of HIT. Each detects platelet activation by a different mechanism. Thus, the combination of the two tests provides a comprehensive account of HIT antibody interaction with platelets. Flow cytometry provides additional platelet activation information: its use permits the highly specific and sensitive analysis of a range of parameters characteristic of platelet activation that can occur with or without the final aggregation step (Lee *et al*, 1996). The positive results obtained with heparins and the negative results obtained with the DTI argatroban validate the choice of assays.

Further support for the potential of rivaroxaban as an alternative anticoagulant in patients with HIT is provided by results of the PF4 release and binding assays. Rivaroxaban did not stimulate the release of PF4 from resting platelets, suggesting that the drug would not promote the platelet activation process associated with HIT by enhancing the available PF4 pool. In addition to this, rivaroxaban also suppressed the release of PF4 from platelets activated with TF (an important platelet agonist, which is generated during the acute phase of HIT), which, in the clinical setting, may further reduce the potential for the pathology of HIT to continue in affected patients. TF promotes the generation of thrombin, which activates platelets, which cause PF4 release. This is suppressed by rivaroxaban but not by the other drugs tested. Although fondaparinux did not promote the release of PF4 from resting or activated platelets, it did not reduce the release of PF4 from activated platelets to a level below that of the control (unlike rivaroxaban). In contrast to rivaroxaban and fondaparinux, UFH stimulated PF4 release from both resting and activated platelets, and enoxaparin stimulated PF4 release from resting platelets.

Unlike UFH and enoxaparin, rivaroxaban did not bind to PF4, as shown in the PF4 interaction assay, and, therefore, is unlikely to induce the conformational changes in PF4 necessary for cross-reaction with HIT antibodies. Not only does this suggest that rivaroxaban could be used in patients with HIT, but also that rivaroxaban would not initiate HIT antibody production if used instead of heparins as the initial anticoagulant. Fondaparinux and the DTI argatroban also did not interact with PF4. This was as expected, given that fondaparinux and the DTIs are structurally dissimilar to UFH. Heparins bind to PF4, causing a structural alteration in the PF4 molecule that exposes neoepitopes. The PF4–heparin complex is an immunological target to which antibodies are generated (Amiral *et al*, 2000; Newman & Chong, 2000; Kelton, 2005). If rivaroxaban does not interact with PF4, the immunogenic target for HIT will not be generated.

In conclusion, this study clearly demonstrated that rivaroxaban does not cross-react with HIT antibodies, mobilize PF4 from platelets, or interact with PF4. In addition, the study showed that rivaroxaban blocks the release of PF4 from activated platelets. Therefore, rivaroxaban may be considered as a new option for the management of patients with HIT. Further investigation in the clinical setting is required to confirm the potential of rivaroxaban as an alternative anticoagulant for use in patients with HIT.

Rivaroxaban may offer certain advantages over both of the currently recommended DTIs and fondaparinux in the management of patients with HIT because of its oral route of administration, wide therapeutic window, synthetic (non-recombinant) small molecule nature, no need of a co-factor to express anticoagulant activity and lack of effect on the regulatory functions of existing thrombin. Because it is an oral drug, rivaroxaban could also provide convenient, long-term secondary prevention after an acute episode of HIT-associated thrombosis. It would be the first oral drug to be used in the hypercoagulable state represented by acute HIT. Furthermore, patients initially receiving rivaroxaban (instead of heparins) would be unlikely to develop HIT.

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