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Darexaban: Anticoagulant effects in mice and human plasma *in vitro*, antithrombotic effects in thrombosis and bleeding models in mice and effects of anti-inhibitor coagulant complex and recombinant factor VIIa

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ABSTRACT

Here, we investigated the anticoagulant effects of darexaban in mice and human plasma *in vitro*, effects of darexaban in thrombosis and bleeding models in mice, and reversal effects of anti-inhibitor coagulant complex (ACC) and recombinant factor VIIa (rFVIIa) on anticoagulant effects of darexaban. In mice, darexaban inhibited FXa activity in plasma with an ED_{50} value of 24.8 mg/kg. Both darexaban and warfarin prolonged prothrombin time (PT) at 3 mg/kg and 0.3 mg/kg/day, respectively. PT and activated partial thromboplastin time (aPTT) prolonged by darexaban were dose-dependently reversed by intravenously-administered rFVIIa, significantly so at 1 mg/kg. In a pulmonary thromboembolism (PE) mouse model, both darexaban and warfarin dose-dependently reduced the mortality rate, significantly so at 10 mg/kg and 3 mg/kg/day, respectively. In a $FeCl_3$-induced venous thrombosis (VT) mouse model, darexaban (0.3-10 mg/kg) dose-dependently decreased the thrombus protein content, significantly so at doses of 3 mg/kg or higher. In a tail-transection mouse model, darexaban had no significant effect on the amount of blood loss at doses up to 10 mg/kg, while warfarin showed a dose-dependent increase in blood loss, significantly so from 1 mg/kg/day. Darexaban and its metabolite darexaban glucuronide significantly prolonged PT and aPTT in human plasma *in vitro*, and while rFVIIa concentration-dependently reversed the prolonged PT in this plasma, ACC dose-dependently reversed both PT and aPTT changes prolonged by darexaban. Taken together, these results suggest that darexaban has a potential to be an oral anticoagulant with a better safety profile than warfarin, and that rFVIIa and ACC may be useful as antidotes to darexaban in cases of overdose.

Introduction

Anticoagulation is an extremely common form of medical intervention, increasingly used for primary or secondary prevention of the thromboembolic complications of vascular disease. Although low-molecular-weight heparins and pentasaccharide fondaparinux are standard therapies for the immediate prophylaxis of deep vein thrombosis, they can be administered only parenterally [1], making them inconvenient for long-term use. Vitamin K antagonists, such as warfarin, have been the mainstay of long-term anticoagulant therapy. However, these drugs have a slow onset of action and require frequent monitoring due to their narrow therapeutic window and their multiple drug and food interactions [2].

After more than 50 years of thrombosis treatment and prophylaxis using heparin and vitamin K antagonists, a new generation of oral, direct anticoagulants that may overcome the above shortcomings is now available [3]. Darexaban (YM150) is an oral direct factor Xa (FXa) inhibitor [4] that is rapidly and extensively metabolized into its glucuronide conjugate (YM-222714) post-administration. Both darexaban and darexaban glucuronide competitively and selectively inhibited human FXa, and they also inhibited the prothrombin activation induced by prothrombinase complex or whole blood clot with similar potency to free FXa [4]. Unlike other FXa inhibitors, darexaban and darexaban glucuronide were very sensitive in the prothrombin time (PT) test over a wide range of FXa inhibition in human plasma [5]. Studies using human platelets showed darexaban glucuronide had no effect on platelet activation and aggregation [6]. In venous and A–V shunt thrombosis models in rats, darexaban strongly suppressed thrombus formation without affecting bleeding time [4]. In rabbit atherothrombosis model, plasma FXa activity and PT correlated with the antithrombotic effects of darexaban [7]. Effects of darexaban as a clinically effective antithrombotic

Abbreviations: FXa, factor Xa; PE, pulmonary thromboembolism; VT, venous thrombosis; PT, prothrombin time; aPTT, activated partial thromboplastin time; rFVIIa, recombinant factor VIIa; PCC, prothrombin complex concentrate; ACC, anti-inhibitor coagulant complex; MC, methylcellulose; RVV, Russell's viper venom factor X activator; SD, standard deviation; CI, confidence interval.

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agent have previously been demonstrated in the prevention of VTE in patients undergoing major orthopedic surgery in clinical studies [8–10]. Clinical studies of darexaban in patients with atrial fibrillation and acute coronary syndrome have also been conducted [11].

Hemorrhaging is a potential adverse effect of antithrombotic therapies, particularly when an overdose is inadvertently administered. In such cases, antidotes may be required to reverse the effects of anticoagulants. However, no recognized antidotes are able to reverse the effects of new anticoagulants that directly inhibit either FXa or thrombin. Recent paper has demonstrated that the anticoagulant effects of rivaroxaban and edoxaban, other direct FXa inhibitors, are reversed by prothrombin complex concentrate (PCC) and recombinant factor VIIa (rFVIIa) in rabbits and rats, respectively [12,13]. It is also reported that the anticoagulant effects of rivaroxaban is reversed by PCC in healthy volunteers, while reversal effect of PCC on anticoagulant effects of dabigatran is controversial [14,15]. Therefore, commercially available hemostatic agents may have potential to reverse the effects of direct FXa inhibitors, however, it has to be confirmed for each agent. Just recently, some new approaches are also reported. Plasma derived or recombinant FXa, modified to lack catalytic and membrane binding activities, have the potential to act as universal antidotes for reversal of anticoagulation of all FXa inhibitors, both small molecule and antithrombin dependent [16]. A fully humanized monoclonal antibody fragment (Fab) against direct thrombin inhibitor dabigatran reversed dabigatran-induced anticoagulation and bleeding effects [17,18].

Here, using mouse models we compared antithrombotic effects and effects on bleeding between darexaban and warfarin. Further, to explore potential antidotes for darexaban, effects of hemostatic agents on the anticoagulant activity of darexaban were also examined.

Materials and Methods

Animals

Male ICR mice (SLC; Shizuoka, Japan or CLEA Japan Inc., Tokyo, Japan) were used in this study. Animals were sacrificed by exsanguination at the end of experiments. Animal facilities, animal care and study programs were in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Astellas Pharma Inc.

Agents

Darexaban and darexaban glucuronide were manufactured by Astellas Pharma Inc. (Tokyo, Japan). For the *in vivo* study, darexaban was suspended in 0.5% methylcellulose (MC) or dissolved in DMSO/Cremophor® EL/H_2O [2:1:7]. Warfarin was purchased from Sigma (St. Louis, MO, USA). Cremophor® EL, Iron (III) chloride hexahydrate, Russell's viper venom factor X activator (RVV), Hirudin, *Hirudo medicinalis*, recombinant, and S-2765TM were purchased from Nacalai Tesque (Kyoto, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), American Diagnostica Inc. (Stamford, CT, USA), Calbiochem (La Jolla, CA, USA), and Chromogenix Instrumentation Laboratory SpA (Milano, Italy), respectively. Recombinant factor VIIa (rFVIIa, NovoSeven®) was purchased from Novo Nordisk Pharma (Tokyo, Japan) or Novo Norsidk A/S (Bagsvaerd, Denmark). An anti-inhibitor coagulant complex (Feiba®) was purchased from Baxter AG (Vienna, Austria). PT and aPTT reagents were purchased from Instrumentation Laboratory (Lexington, MA, USA) or Ortho-Clinical Diagnostics K.K. (Tokyo, Japan).

Inhibition of FXa Activity in Mice

Either vehicle or darexaban (0.3, 1, 3, 10, 30, 100, and 300 mg/kg) was orally administered to fasted mice (n = 5/dose group). Blood samples were collected from the inferior vena cava of each mouse under anesthesia with urethane (1 g/kg, ip) at about 30 min after drug administration and centrifuged to isolate platelet-poor plasma. The chromogenic assay for FXa activity in plasma was performed according to the method of Perzborn et al. [19] with minor modifications. Plasma diluted 2-fold with buffer consisting of 50 mmol/L Tris-HCl and 200 mmol/L NaCl (pH 8.4) was used. The diluted plasma (22.5 μL) was mixed with 2.5 μL hirudin (2,000 U/mL) and 25 μL S-2765 (1.75 mmol/L). The reactions were initiated by the addition of 25 μL RVV (10 mAU/mL with 50 mmol/L $CaCl_2$), and the color was measured every 10 min for 30 min at 405 and 490 nm using a SpectraMax 340PC (Molecular Devices, Sunnyvale, CA, USA). FXa activity was defined as the increment of absorbance (Δ405-490 nm) in the first 10 min.

Anticoagulant Activity in Mice Ex Vivo

Darexaban (0.3-10 mg/kg) was orally administered to fasted mice (n = 6/group). Thirty minutes after administration, 1 mL of citrated-blood samples were collected from the inferior vena cava of mice anesthetized with ether. Similarly, warfarin (0.3-3 mg/kg) dissolved in a 0.5% MC solution was orally administered to non-fasted male mice for 3 days, and blood was collected 2 h after the last dosing (n = 6/group). Plasma was immediately prepared by centrifugation. The anticoagulant activity of each test substance was measured with a coagulometer (KC-10A; Amelung; Lehbrinksweg, FRG). If the coagulation time for a test substance was 10-times the control coagulation time, measurement was stopped and recorded as 10-times the control value.

Effects of Recombinant Factor VIIa on Coagulation Time in Mice While Using Darexaban for Anticoagulation

Either darexaban (100 mg/kg) or 0.5% MC was orally administered to fasted mice (n = 6/group). As an antidote, rFVIIa (0.01-1 mg/kg) or saline (5 mL/kg) was intravenously injected 25 min after drug administration. Five minutes after antidote injection, a blood sample (1 mL) was collected from the inferior vena cava of each ether-anesthetized mouse, and the PT and aPTT were measured.

Pulmonary Thromboembolism (PE) Model in Mice

Mice were injected with 15 mg/kg (10 mL/kg) thromboplastin (Ortho-Clinical Diagnostics K.K.; Tokyo, Japan) via the tail vein (n = 15/group). Survival for 10 min after thromboplastin injection was recorded as the index of the antithrombotic effect exerted by each agent. Darexaban (0.3-10 mg/kg) was orally administered to fasted mice 30 min before the injection of thromboplastin. Warfarin (0.3-3 mg/kg) was orally administered to non-fasted mice for 3 days, and the last administration was performed 2 h before the injection of thromboplastin.

$FeCl_3$-induced Venous Thrombosis (VT) Model in Mice

Either vehicle or darexaban (0.3-10 mg/kg) was orally administered to fasted mice (n = 8/group). A thrombus was induced 30 min after administration under anesthesia with urethane (0.05 g/mouse, intraperitoneal). Vena cava thrombosis was induced following the method devised by Wang et al. [20]. Briefly, the vena cava was exposed via a midline abdominal incision, and the venous surface was cleared using blunt dissection between the renal and iliolumber veins. A piece of filter paper (2 mm × 4 mm) presaturated with $FeCl_3$ solution (10% in water) was placed on the vena cava for 3 min and then removed. Thirty minutes after the initial filter paper application, the vena cava containing the thrombus was dissected free, and a section of the vessel was cut out. The clot was dissected from the vessel in saline. The protein contents of the thrombus were then measured.