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Synergistic effect of a factor Xa inhibitor, TAK-442, and antiplatelet agents on whole blood coagulation and arterial thrombosis in rats

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ABSTRACT

Introduction: Activated platelets facilitate blood coagulation by providing factor V and a procoagulant surface for prothrombinase. Here, we investigated the potential synergy of a potent factor Xa/prothrombinase inhibitor, TAK-442, plus aspirin or clopidogrel in preventing arterial thrombosis and whole blood coagulation.
Methods: Thrombus formation was initiated by $FeCl_3$-induced rat carotid injury. Bleeding time was evaluated with the rat tail transection model. Whole blood coagulation was assessed by thromboelastographic examination (TEG) for which blood obtained from control, aspirin-, or clopidogrel-treated rats was transferred to a TEG analyzer containing, collagen or adenosine diphosphate (ADP), and TAK-442 or vehicle.
Results: TAK-442 (3 mg/kg, po), aspirin (100 mg/kg, po) or clopidogrel (3 mg/kg, po) alone had no significant effect on thrombus formation, whereas the combination of TAK-442 with aspirin and clopidogrel remarkably prolonged the time to thrombus formation without additional significant prolongation of bleeding time. TEG demonstrated that the onset of collagen-induced blood coagulation were slightly longer in aspirin-treated rats than control; however, when the blood from aspirin-treated rats was subsequently treated *in vitro* with 100 nM TAK-442, the onset of clotting was significantly prolonged. In contrast, only marginal prolongation was observed with TAK-442 treatment of blood from control animals. The onset time of ADP-induced blood coagulation was slightly longer in clopidogrel-treated rats compared with control, and it was further extended by TAK-442 treatment.
Conclusion: These results demonstrate that blood coagulation can be markedly delayed by the addition of TAK-442 to antiplatelets treatment which could contribute to synergistic antithrombotic efficacy in these settings.

Introduction

Atherosclerotic plaque rupture stimulates platelet activation and blood coagulation leading to thrombus formation [1]. The resultant intracoronary artery thrombosis is the major cause of acute coronary syndromes, such as myocardial infarction and unstable angina. Antiplatelet and anticoagulating agents therefore form the foundation of pharmacological strategies to reduce the risks of these clinical events. Aspirin, a cyclooxygenase inhibitor, is the mainstay of long-term antiplatelet therapy in patients with acute coronary syndrome, reducing the relative risk of myocardial infarction, stroke, or vascular death by about 25% [2–6]. Clopidogrel, an adenosine diphosphate (ADP) receptor ($P2Y_{12}$) blocker, improves the efficacy of aspirin for the prevention of ischemic complications [7,8]. Long-term anticoagulation with warfarin, an oral vitamin K antagonist, or with ximelagatran, a direct thrombin inhibitor, provides additional benefit for patients receiving aspirin therapy, further reducing their risk of cardiovascular events [9–11].

Regardless of the benefit of the combination, aspirin plus warfarin or aspirin plus ximelagatran, in preventing arterial thrombosis, the combination therapy increases the risk of bleeding complications [9–12]. Because of its critical role in the blood coagulation cascade, recent investigations have focused on factor Xa (FXa) as a potential target for new oral anticogaulation therapies. Several reports demonstrated that oral FXa inhibitors had potent antithrombotic effects on various types of experimental arterial thrombosis, with lower bleeding potential than warfarin or thrombin inhibitors [13–15]. Wong et al. reported that minimum or moderate effective doses of direct FXa inhibitors, DPC-423, razaxaban and apixaban, enhanced the antithrombotic effect of aspirin and clopidogrel without further increasing bleeding time in a rabbit model of arterial thrombosis [15–17]. More recently, it was reported that direct FXa inhibitors, rivaroxaban and apixaban,

Abbreviations: FXa, factor Xa; TEG, thromboelastographic/thromboelastograph; ADP, adenosine diphosphate; PT, prothrombin time; APTT, activated partial thromboplastin time; TTO, time to occlusive thrombus formation; PRP, platelet rich plasma; PPP, platelet poor plasma.

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tended to improve the efficacies of antiplatelet therapies, aspirin alone or aspirin plus clopidogrel, with a dose-related increase in bleeding in phase II studies for acute coronary syndrome [18,19]. On the basis of these observations, phase III studies of antiplatelet therapies plus low-dose rivaroxaban or low-dose apixaban are currently undergoing.

In the whole blood coagulation process, activated platelets provide factor V and a procoagulant surface on which the prothrombinase complex [FXa-factor Va-phospholipids-calcium] assembles, thereby accelerating thrombin generation by 5-6 orders of magnitude [20]. Because direct FXa inhibitors inhibit prothrombinase activity [21–23], we speculated that whole blood coagulation may be effectively blocked by the combination of an antiplatelet agent and a low dose of FXa inhibitor, perhaps resulting in synergistic inhibition of arterial thrombosis. TAK-442 is an orally active direct and competitive FXa inhibitor, similar to rivaroxaban and apixaban, and is currently under phase II clinical evaluations in patients with venous and arterial thrombosis [24,25]. TAK-442 potently inhibits prothrombinase activity, with an IC_{50} value of 51 nM [Konishi N, et al. submitted for publication]. In the present study, we evaluated anti-arterial thrombotic effects of TAK-442 alone, and investigated possible synergistic effects of TAK-442 plus aspirin or clopidogrel on arterial thrombus formation and on whole blood clot formation.

Materials and Methods

Agents

TAK-442 and ximelagatran were synthesized by Takeda Pharmaceutical Company, Ltd. (Osaka, Japan). Clopidogrel bisulfate was extracted from PLAVIX® (Sanofi-Aventis, NJ, USA) at Takeda Pharmaceutical Company, Ltd. Aspirin (acetylsalicylic acid) and fondaparinux (Arixtra®) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Sanofi-Aventis (NJ, USA), respectively. The following chemicals and reagents were used: $FeCl_3 \cdot 6H_2O$ (Wako Pure Chemical Industries), ADP (Wako Pure Chemical Industries), collagen (collagen reagent Horm®, Nycomed Pharma GmbH, Unterschleiβheim, Germany). STA Neoplastin plus (Roche Diagnostics, Tokyo, Japan) and Pathromtin SL (Dade Behring, Tokyo, Japan) were used as prothrombin time (PT) and activated partial thromboplastin time (APTT) reagents, respectively. All other reagents were purchased commercially at the highest grade available. Drugs were suspended in 0.5% methyl-cellulose solution for oral administration, and were given after the animals had fasted for more than 12 hr.

Animals

All experiments were conducted in accordance with Animal Care and Use Committee regulations of Takeda Pharmaceutical Company Ltd. Seven-week-old male Sprague-Dawley rats (CLEA Japan, Tokyo, Japan) were anaesthetized using 5-sec-butyl-5-ethyl-2-thiobarbituric acid (Inactin) for the arterial thrombosis model studies or with sodium pentobarbital for bleeding time measurements, *ex vivo* assays, and thromboelastographic examinations.

Arterial thrombosis model in rats

An approximately 10 mm length of the right carotid artery was surgically exposed and parafilm placed under the vessel to isolate it from surrounding tissue. Carotid blood flow was then measured with a flow probe (diameter: 0.6 mm) linked to an electromagnetic flowmeter (MDL-1401, Skalar, Berda, Netherlands). Thrombus formation was induced by applying a piece of filter paper (3 × 3 mm) saturated with 50% $FeCl_3$ solution to chemically damage the vascular endothelium. The filter paper was placed on the carotid artery in a position distal to the flow probe for 10 min. When carotid blood flow decreased to zero, the time to occlusive thrombus formation (TTO) was noted. If the artery remained patent 60 min after the $FeCl_3$ application, the TTO was expressed as 60 min. In our preliminary *ex vivo* experiments, TAK-442, aspirin, ximelagatran, and fondaparinux showed maximal effects at 30, 30, 60, and 60 min after administration, respectively (data not shown). All the drugs, except for fondaparinux, were therefore orally administered at 30 min (TAK-442 or aspirin) or 60 min (ximelagatran) before the $FeCl_3$-saturated filter paper was applied. Fondaparinux was administered subcutaneously 60 min before inducing thrombus formation. Clopidogrel was orally administered 120 min before inducing thrombus formation according to the report by Hérault JP et al. [26].

Tail transection model for measurement of bleeding time in rats

Each rat tail was transected at a site 3 mm proximal to the tip. Blood was blotted every 30 sec with filter paper until either bleeding had stopped or 1800 sec had elapsed. If bleeding had not yet stopped during this interval, the bleeding time was recorded as 1800 sec. Drugs were orally administered 30 min (TAK-442 or aspirin) or 120 min (clopidogrel) before starting the measurement of bleeding time.

Platelet aggregation

Five mL of blood (3 mL for platelet aggregation assay and 2 mL for clotting time measurement) was drawn from the abdominal aorta in a plastic syringe containing 3.8% sodium citrate (1:9 citrate/blood, v/v). Platelet rich plasma (PRP) was obtained by centrifugation (2700 × g) for 7 sec at 20 °C and platelet poor plasma (PPP) was prepared by centrifugation (2700 × g) for 10 min at 20 °C. The platelet number in PRP was adjusted to approximately 1×10^6/μL using an automatic blood cell counter (K-4500, Sysmex, Kobe, Japan). Platelet aggregation was induced by addition of 20 μL of aggregation agent to 220 μL PRP that had been pre-warmed for 2 minutes at 37 °C in a cuvette stirred at 1000 rpm. The maximum percentage of platelet aggregation was determined by measuring the change in light transmittance using an aggregometer (NBS Hematracer 801, MC Medical, Tokyo, Japan). Drugs were orally administered 30 min (TAK-442 or aspirin) or 120 min (clopidogrel) before obtaining blood samples. Collagen and ADP, the most common inducers of platelet aggregation, have been shown to be useful for the evaluation of the antiplatelet effects of aspirin and clopidogrel, respectively [27,28]. Therefore, collagen (final concentration: 7.5 μg/mL) or ADP (final concentration: 3 μM) was used in this experiment.

Clotting time

PPP was obtained by centrifugation (9300 × g) for 10 min at 4 °C and stored at -80 °C until used for coagulation assays. Clotting time (PT and APTT) was measured with an automatic blood coagulometer (STA compact, Diagnostica Stago, Asnières, France) using clinical assay kits described above. In the mono-treatment study, 2 mL of blood was drawn from the abdominal aorta in a plastic syringe containing 3.8% sodium citrate (1:9 citrate/blood, v/v). PPP was prepared by centrifugation (9300 × g) for 10 min at 4 °C. Drugs were administered 30 min (TAK-442) or 60 min (ximelagatran and fondaparinux).

Thromboelastographic examination

Blood was obtained from the abdominal aorta of vehicle-, aspirin-, or clopidogrel-treated animals in a plastic syringe containing 3.8% sodium citrate (1:9 citrate/blood, v/v). Drugs were orally administered 30 min (aspirin) or 120 min (clopidogrel) before obtaining blood samples. Whole blood coagulation was monitored with a