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Full Length Article

Inhibitory effects of ASP6537, a selective cyclooxygenase-1 inhibitor, on thrombosis and neointima formation in rats

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ABSTRACT

Introduction: Percutaneous coronary interventions (PCIs), such as balloon angioplasty and stent placement, are effective in the treatment of coronary artery disease. PCI has drawbacks, however, including acute thrombosis after the procedure and restenosis of the vascular lumen due to abnormal neointimal hyperplasia. ASP6537 is a selective COX-1 inhibitor that has been investigated as a possible candidate for clinical development as an antiplatelet agent. In the present study, we evaluated the *in vivo* antithrombotic effect of ASP6537 and its effect on neointima formation after balloon angioplasty.
Material and methods: The antithrombotic effect of ASP6537 was examined using an arteriovenous shunt thrombosis model in rats while the effect of ASP6537 on neointima formation was evaluated in a rat carotid arterial balloon angioplasty model.
Results: In the thrombosis study, ASP6537 dose-dependently decreased the protein content of the thrombus, while no prolongation of template bleeding time was observed. In the neointima study, ASP6537 reduced neointima formation.
Conclusions: ASP6537 may be a promising agent for the prevention of acute thrombosis and restenosis after PCI in place of aspirin.

1. Introduction

Despite advances in the treatment of cardiovascular disease, ischemic heart disease is still the number one cause of death worldwide. A 2012 survey by the World Health Organization reported 7.4 million deaths were due to ischemic heart disease. Coronary heart disease (CHD) is characterized by the thrombotic occlusion of coronary arteries and subsequent ischemic damage in myocardial tissue. In the United States, 6.2% of the population suffer from CHD [1]. The major treatment strategy for CHD is revascularization of the occluded coronary artery. A growing number of patients are undergoing percutaneous coronary interventions (PCIs), such as balloon angioplasty and stent placement [2,3]. The major drawbacks of PCI are acute closure of the dilated coronary artery by thrombosis and restenosis of vascular lumen due to abnormal neointimal hyperplasia. Interactions between the implanted material and the blood and de-endothelialized vascular tissue during angioplasty induce platelet adhesion, activation and aggregation. This causes activation of the coagulation cascade, resulting in acute thrombosis [4]. The activation of platelets also induces leukocyte recruitment. Growth factors and cytokines released by activated platelets and leukocytes stimulate the migration and proliferation of vascular smooth muscle cells, which result in neointima formation and restenosis [5,6].Current guidelines [7–9] recommend dual antiplatelet therapy consisting of acetylsalicylic acid (aspirin) and a platelet $P2Y_{12}$ ADP receptor antagonist after PCI to prevent acute thrombosis. Aspirin is a non-selective cyclooxigenase (COX) inhibitor, and exerts its antithrombotic effects through inhibition of COX-1 mediated thromboxane A_2 (TXA_2) production.

ASP6537 is a highly selective COX-1 inhibitor synthesized by Astellas Pharma Inc. [10,11] that is under investigation as an antiplatelet agent for possible clinical development. We previously demonstrated that ASP6537 selectively inhibits TXA_2 production over prostaglandin (PG) I_2 [11]. The balance of the prothrombotic TXA_2 and antithrombotic PGI_2 has been reported to play central roles not only in thrombus formation but also neointimal hyperplasia [12–17]. Here, we investigated the inhibitory effect of ASP6537 on neointima formation in a rat model of balloon angioplasty to see if ASP6537 have potential to be effective in

Abbreviations: COX, cyclooxygenase; TXA_2, thromboxane A_2; I/M ratio, intima/media ratio.

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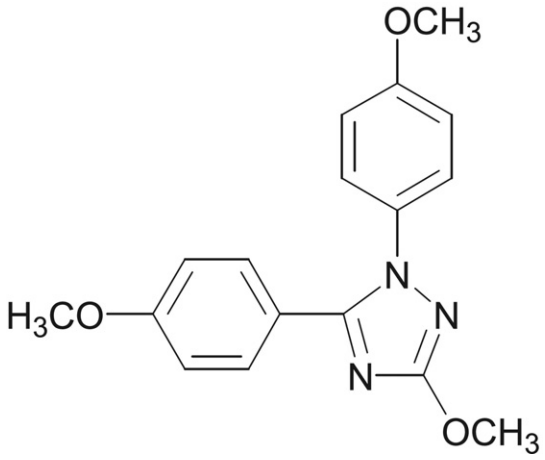

Fig. 1. Chemical structure of ASP6537.

restenosis after vascular injury. We also examined the *in vivo* antithrombotic effect of ASP6537 using arteriovenous shunt model in rats. All results were compared with aspirin.

2. Material and methods

2.1. Agents

ASP6537 (Batch ID. L12792447) was synthesized at the Chemical Laboratories of Astellas Pharma Inc. The chemical structure of ASP6537 is shown in Fig. 1. Aspirin (acetylsalicylic acid, lot no. 045K0101) and captopril (lot no: 056K1227) were purchased from Sigma-Aldrich Co. (St. Louis, MO). For the *in vitro* study, ASP6537 or aspirin was dissolved in dimethylsulfoxide and diluted with 50 mM Tris buffer (final dimethylsulfoxide concentration: 1%). For *in vivo* studies, ASP6537 or aspirin was suspended in 0.5% methylcellulose solution prior to use. Captopril was dissolved in 0.5% methylcellulose solution just prior to use.

The following platelet aggregation inducers were used: arachidonic acid (purchased from Biopool, Bray, Co. Wicklow, Ireland), type I collagen from equine tendon (Collagen reagent Horm®, Moriya Co., Tokyo, Japan), and adenosine 5′-diphosphate (ADP, MC Medical, Tokyo, Japan).

2.2. Animals

All animals were purchased from Clea Japan, Inc. (Tokyo, Japan) and were housed under conventional conditions with controlled temperature, humidity, and light (12-h light–dark cycle). Seven- and eight-week-old male Wistar rats were used in the platelet aggregation and arteriovenous shunt-bleeding models, respectively. The animals were fasted for 12–24 h before oral administration of test agents. For the rat model of balloon angioplasty, 18-week-old male Wistar rats were used. The animals were maintained on a normal diet and were allowed free access to chow and water throughout this study. All animal experiments were performed in accordance with the regulations of the Animal Ethics Committee of Astellas Pharma Inc.

2.3. Platelet aggregation assay

For the platelet aggregation assay using human platelets, 50 ml of blood was collected from each healthy human volunteer in plastic syringes containing 3.2% trisodium citrate solution (10% of the final volume). The subjects had not taken any medications for at least 1 week prior to blood collection. Platelet-rich plasma (PRP) was prepared by centrifuging at 160 $\times g$ for 10 min (Kubota 2420, Kubota Co., Tokyo, Japan). Platelet-poor plasma (PPP) was obtained by centrifuging the remaining platelet-rich plasma at 1800 $\times g$ for 10 min. Platelet counts were measured with an automatic cell counter (MEK-6258, Nihon Kohden, Tokyo, Japan). The platelet count of PRP was adjusted to 3×10^5/μL with PPP. In the cuvette, platelet-rich plasma was preincubated at 37 °C for 1 min after the addition of the test drug or vehicle. Platelet aggregation in the PRP was induced by adding arachidonic acid (0.5 to 1.0 mM), collagen (0.25 to 0.5 μg/ml) or ADP (5 μM). An aggregometer (MCM Hema Tracer 212, MC Medical) was used to measure the maximum extent of platelet aggregation by recording the increase in light transmission through a stirred suspension maintained at 37 °C for 5 min (arachidonic acid- and ADP-induced platelet aggregation) to 10 min (collagen-induced platelet aggregation).

For the *in vitro* platelet aggregation study in rats, 6 ml of blood was collected from the vena cava in a plastic syringe containing 3.2% trisodium citrate solution (10% of the final volume) under diethyl ether anesthesia. For the *ex vivo* study in rats, 1 h before blood sampling, the vehicle or ASP6537 (1–30 mg/kg) was administered through a gastric tube to conscious rats at a rate of 5 ml/kg body weight. Under diethyl ether anesthesia, 6 ml of blood was collected as described above. PRP adjusted to 3×10^5/μL was obtained as described in the human platelet aggregation assay. Rat platelet aggregation in the PRP was induced by adding 5–10 μg/ml of collagen. The maximum extent of platelet aggregation was recorded through a stirred suspension maintained at 37 °C for 10 min.

2.4. Arteriovenous shunt thrombosis model and bleeding time

The experimental protocol is shown in Fig. 2. ASP6537 or aspirin was orally administered 1 h before the induction of the thrombus. Rats were anesthetized with urethane (0.96 g/kg, ip). The neck was opened surgically, and segments of the left carotid artery and right jugular vein about 1 cm long were carefully extricated from the surrounding tissues. The vein and artery were then connected to a shunt containing a silk thread. This shunt was made of three polyethylene tubes bundled together tightly. The 10-cm long tube (SP67 [I.D. 0.97 mm, O.D. 1.27 mm]; Natsume, Tokyo, Japan) that formed the center section of the shunt housed the 9-cm length of silk thread (No. 1; Shin-Ei Ika Kogyo, Tokyo, Japan). Both ends of this tube were connected to 12-cm long tubes (PE-50 [I.D. 0.58 mm, O.D. 0.965 mm]; Becton Dickinson, NJ). Blood was allowed to circulate in this shunt for 15 min, and then the silk thread was withdrawn from the tube. After the uncoagulated blood was gently washed away with saline, the thrombus adhering to the thread was dissolved in 2 ml of 0.5 M NaOH for protein assay. The silk thread was removed from the NaOH after the thrombus was completely dissolved. The DC protein assay (Bio-Rad, Hercules, CA) was used to measure the thrombus protein content. The sole of the

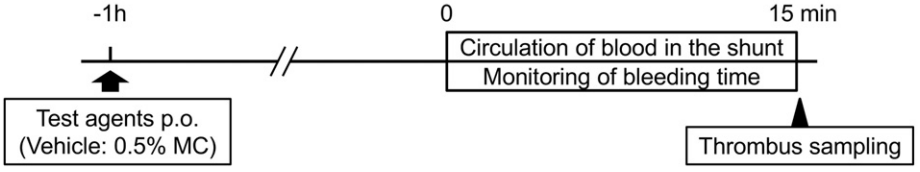

Fig. 2. Experimental protocol for the arteriovenous shunt model and bleeding time in rats. ASP6537 or aspirin were orally administered 1 h before thrombus induction. Blood was allowed to circulate in the shunt for 15 min. A cut was made on the bottom of the rat's foot using Surgicutt® at the same time as the blood started to circulate in the shunt. Bleeding time was simultaneously measured during the blood circulation in the shunt.