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The active metabolite of prasugrel, R-138727, improves cerebral blood flow and reduces cerebral infarction and neurologic deficits in a non-human primate model of acute ischaemic stroke

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ABSTRACT

Previously, we showed preventive effects of prasugrel, a $P2Y_{12}$ antagonist, in a non-human primate model of thrombotic middle cerebral artery occlusion (MCAO); however, it remains unclear if $P2Y_{12}$ inhibition after MCAO reduces cerebral injury and dysfunction. Here we investigated the effects of R-138727, the major active metabolite of prasugrel, on *ex vivo* platelet aggregation at 5 min, 15 min, 60 min, and 24 h after administration to non-human primates ($n=3$). A single intravenous dose of R-138727 (0.03–0.3 mg/kg) resulted in significant and sustained dose-related effects on platelets for up to 24 h. R-138727 was administered 1 h after MCAO induction, and its effects on thrombosis, cerebral infarction, and neurological deficits were determined ($n=8$–10). R-138727 (0.3 mg/kg) significantly increased total patency rate of the MCA ($P=0.0211$). Although there was no effect on the patency rate before R-138727 dosing ($P=0.3975$), it increased 1 h after dosing ($P=0.0114$). R-138727 significantly reduced total ischaemic infarction volumes ($P=0.0147$), including those of basal ganglia ($P=0.0028$), white matter ($P=0.0393$), and haemorrhagic infarction ($P=0.0235$). Additionally, treatment with R-138727 reduced overall neurological deficits ($P=0.0019$), including the subcategories of consciousness ($P=0.0042$), sensory system ($P=0.0045$), motor system ($P=0.0079$) and musculoskeletal coordination ($P=0.0082$). These findings support the possible utility of $P2Y_{12}$ inhibition during early-onset MCAO to limit the progression and degree of cerebral ischaemia and infarction and also associated neurological deficits.

1. Introduction

Stroke is a leading cause of death and disability worldwide (Roger et al., 2011). Platelets play an important role in the pathophysiology of ischaemic stroke. Prasugrel is the third generation thienopyridine antiplatelet agent (Niitsu et al., 2005; Jakubowski et al., 2007; Dobesh, 2009) and its active metabolite, R-138727, effectively blocks the platelet $P2Y_{12}$ ADP receptor (Hasegawa et al., 2005). $P2Y_{12}$ receptor antagonists are proven therapeutics, and phase 3 trials of prasugrel monotherapy, which allows for more consistent inhibition of platelet aggregation (IPA) than clopidogrel, are in progress in Japan to assess efficacy and safety for treating ischaemic stroke (JapicCTI-111582[ja] and JapicCTI-121901[ja]).

Photochemically-induced middle cerebral artery occlusion (MCAO) is used as a model of thrombotic stroke in mice (Nagai et al., 2002), rats (Maeda et al., 2009), guinea pigs (Moriguchi et al., 2005), rabbits (Zhao et al., 2002), marmosets (Ikeda et al., 2013) and monkeys (Sawada et al., 2014). The photochemical response of rose bengal to irradiation with green light generates reactive oxygen species that injure the vascular endothelium, allowing for platelet adhesion with subsequent activation, and aggregation, and local thrombus formation (Matsuno et al., 1993). This model mimics, in part, the pathophysiological features of thrombotic stroke in humans.

Non-human primate brain structure and neurological function are very similar to that of humans. However, few published studies report using the MCAO model in non-human primates to evaluate the effects of antithrombotic and thrombolytic drugs (Sawada et al., 2014; Kaku et al., 1998; Maeda et al., 2005a; Furuichi et al., 2007). We reported the preventive effects, i.e., with pre-treatment,

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of a $P2Y_{12}$ antagonist, prasugrel, on non-cardioembolic stroke using a non-human primate model (Tomizawa et al., 2015). However, it remains unclear whether this model is useful for evaluating whether inhibiting $P2Y_{12}$ following MCAO is effective for treating cerebral infarction.

Several clinical trials demonstrate the clinical benefits of $P2Y_{12}$ antagonists such as the thienopyridine antiplatelet agents, ticlopidine and clopidogrel, for the secondary prevention of ischaemic stroke (Gent et al., 1989; CAPRIE Steering Committee, 1996; Fukuuchi et al., 2008; Sacco et al., 2008). When initiated early following ischaemic stroke or transient ischaemic attack caused by arterial atherosclerosis, dual antiplatelet therapy (DAPT) using clopidogrel and aspirin is most effective (Wang et al., 2013). Moreover, DAPT is associated with a significant benefit in the first few days following an ischaemic stroke, as shown by the dramatic divergence of event-curves for stroke-free survival (Liu et al., 2015). Furthermore, investigations are underway to evaluate the role of a monotherapy using ticagrelor, a more potent $P2Y_{12}$ antagonist than clopidogrel, to prevent acute stroke (Johnston et al., 2015). Thus, currently, antiplatelet therapy represents the major strategy for preventing recurrent non-cardioembolic stroke. However, the optimal regimen for therapy remains elusive and is a subject of controversy, particularly for patients with acute ischaemic stroke (Liu et al., 2015).

Therefore, here we used a non-human primate model of acute thrombotic stroke to determine whether $P2Y_{12}$ inhibition by R-138727, the active metabolite of prasugrel, after MCAO reduced cerebral infarction and associated neurologic deficits.

2. Materials and methods

2.1. Test agents and reagents

R-138727, the major active metabolite of prasugrel hydrochloride, was synthesized by Ube Industries, Ltd. (Yamaguchi, Japan) and dissolved in saline (Otsuka Pharmaceutical Factory, Ltd., Tokushima, Japan). ADP was purchased from Sakae Co., Ltd. (Gunma, Japan).

2.2. Experimental animals

All animal studies were approved by the appropriate Institutional Animal Care and Use Committees (Shin Nippon Biomedical Laboratories, Ltd., Hamamatsu Pharma Research, Inc., and Daiichi Sankyo Co., Ltd.), and they were conducted in compliance with the animal welfare guidelines of Shin Nippon Biomedical Laboratories, Ltd. and Drug Safety Research Laboratories, the guidelines for animal experimentation of Hamamatsu Pharma Research, Inc., and the *Guide for the Care and Use of Laboratory Animals* (National Research Council). The protocol was approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories (Permit Number: IACUC315-176) and the Institutional Animal Care and Use Committee of Hamamatsu Pharma (Permit Number: HPRIRB-106). All surgery was performed after monkeys were anaesthetized with ketamine hydrochloride and isoflurane, and all efforts were made to minimize suffering.

Male cynomolgus monkeys (5–8 years old) were supplied by Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan) and were housed under constant temperature, humidity, and a 12-h light/dark cycle. No treatment-related clinical signs or changes in body weights were observed during the study.

2.3. Measurement of ex vivo platelet aggregation

Male monkeys were divided into four groups ($n=3$ per group),

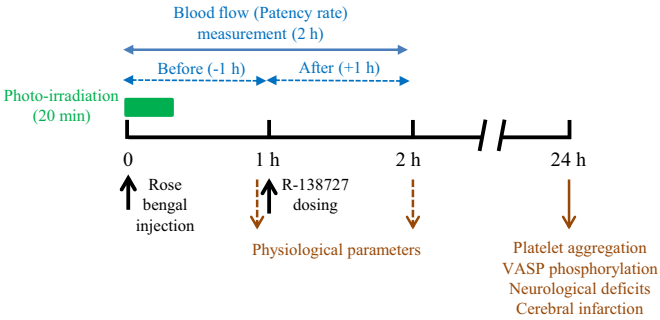

Fig. 1. Protocol for the study of thrombotic middle cerebral artery occlusion using a non-human primate model. VASP, vasodilator-stimulated phosphoprotein.

and each monkey received a single intravenous dose of R-138727 (0.03, 0.1, or 0.3 mg/kg) or vehicle (saline). Blood samples were collected before (baseline) and at 5 min, 15 min, 60 min, and 24 h after treatment. *Ex vivo* platelet aggregation was determined according to our previous report (Tomizawa et al., 2013). Briefly, platelet aggregation in platelet-rich plasma was monitored for 8 min after adding ADP (20 μM) and recorded as maximum platelet aggregation (MPA) using a 12-channel automated platelet aggregometer (MCM HEMA TRACER 313M, MC Medical, Inc., Tokyo, Japan). The extent of inhibition of platelet aggregation (IPA) at each time point in each animal was calculated as the percent of MPA at pre (before administration) according to the following equation: [(MPA at pre – MPA at each time point)/MPA at pre] × 100.

2.4. Photochemical induction of thrombotic MCAO

The protocol for thrombotic MCAO in non-human primates is shown in Fig. 1. Male cynomolgus monkeys were divided into two groups ($n=10$ for vehicle, $n=8$ for R-138727). Ketamine hydrochloride (10 mg/kg) and atropine sulphate (0.05 mg/kg) were intramuscularly administered. The monkeys were tracheostomized, immobilized with vecuronium bromide (0.08 mg/kg), and artificially ventilated. During surgery, monkeys were maintained on 0.8% isoflurane in a 7:3 mixture of N_2O:O_2 gas. The MCA was occluded using the photothrombotic method described by Maeda et al., (2005a). Intense green light (wavelength, 540 nm; 1,400,000 lx) from a xenon lamp (L-4887, Hamamatsu Photonics K.K., Shizuoka, Japan) was focused on the trunk of the MCA, and rose bengal (20 mg/kg, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was intravenously infused for 6 min. Photoirradiation was administered for 20 min. A pulse Doppler flow meter probe (PDV-20, Crystal Biotech, Northborough, MA, USA) was placed distally to the photoirradiation probe on the main trunk of the MCA, and the times to first occlusion and total occlusion were recorded. MCA patency was monitored for 120 min, and the patency rate was calculated as follows: [(120-total occlusion time)/120] × 100. R-138727 (0.3 mg/kg) or vehicle was intravenously administered once 1 h after initiating photoirradiation. After measuring MCA blood flow, the access site was closed, and after confirmation of spontaneous respiration, the monkeys were administered intramuscular neostigmine bromide (0.05 mg/kg) and atropine sulphate (0.05 mg/kg) and allowed to recover from anaesthesia. After regaining consciousness, the monkeys were administered intramuscular penicillin (100,000 U per body) and buprenorphine hydrochloride (0.02 mg per body).

2.5. Measurement of physiological parameters

A polyethylene catheter was inserted into the right femoral artery to measure mean arterial blood pressure (MABP), heart rate