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The influence of $P2Y_{12}$ receptor deficiency on the platelet inhibitory activities of prasugrel in a mouse model: Evidence for specific inhibition of $P2Y_{12}$ receptors by prasugrel

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ABSTRACT

Prasugrel is a novel orally active thienopyridine with faster, higher and more reliable inhibition of platelet aggregation than clopidogrel reflecting its metabolism *in vivo* to an active metabolite with selective $P2Y_{12}$ antagonistic activity. Several lines of evidence support the contention that prasugrel provides selective $P2Y_{12}$ receptor antagonistic activity. To date, however, direct evidence of $P2Y_{12}$ specific action by prasugrel *in vivo* is limited. In the present study, effects of prasugrel on *ex vivo* platelet aggregation were examined in wild type (WT) and $P2Y_{12}^{-/-}$ mice. In WT mice, prasugrel showed platelet inhibition that was 8.2 times more potent than clopidogrel. In $P2Y_{12}^{-/-}$ mice, ADP induced platelet aggregation was minimal, and its extent was similar to that in prasugrel-treated WT mice. In addition, no further inhibition of platelet aggregation was observed after administration of prasugrel to $P2Y_{12}^{-/-}$ mice. Furthermore, prasugrel-treated WT mice showed similar aggregation patterns using collagen- and murine PAR-4 agonist peptide to those of $P2Y_{12}^{-/-}$ mice treated with vehicle or prasugrel. Overall, these results clearly provide additional *in vivo* evidence that prasugrel has selective $P2Y_{12}$ antagonistic activity.

1. Introduction

Thrombus formation at a site of arterial injury is initiated by the adhesion of platelets to the damaged arterial wall, with several agonists such as adenosine 5′-diphosphate (ADP), 5-hydroxytryptamine and thromboxane A_2, released from the activated platelets, promoting additional platelet activation and aggregation [1]. ADP is one of the more important agonists inducing and accelerating platelet activation and those effects are mediated by at least two subtypes of G-protein coupled ADP receptors existing on the platelet membrane, namely $P2Y_1$ and $P2Y_{12}$ [2,3]. Both $P2Y_1$ and $P2Y_{12}$ ADP receptors are reported to be essential for full platelet activation and aggregation [4,5]. Furthermore, several studies have shown that $P2Y_{12}$ receptors are an effective therapeutic target for antithrombotic agents [6]. The $P2Y_{12}$ receptor was cloned by three groups in 2001 [6–8]. In addition, numerous investigators have elucidated the role of $P2Y_{12}$ in mediating platelet aggregation and thrombus formation using selective $P2Y_{12}$ antagonists such as AR-C69931MX, ticlopidine and clopidogrel

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[9–12]. In parallel with these investigations, study of $P2Y_{12}$-deficient (knockout, KO) mice has been pursued by a number of groups, further clarifying the role of $P2Y_{12}$ in thrombus formation [13,14].

Prasugrel (CS-747, LY640315), now under phase 3 evaluation [15], is a novel thienopyridine antiplatelet prodrug with fast onset, high potency and irreversible action in experimental animals [16,17] and humans [18,19]. This activity is believed to be mediated via $P2Y_{12}$ receptor antagonism by its active metabolite [20,21]. Notably, there is evidence both in healthy subjects and stable cardiac patients that the pharmacodynamic response variability noted with clopidogrel is less after a loading dose of 60 mg prasugrel compared to 300 mg clopidogrel [22,23], supporting the potential clinical utility of prasugrel. To date, *in vitro* and *ex vivo* studies of prasugrel have been persued, however, *in vivo* evidence for selective $P2Y_{12}$ inhibition by prasugrel has not been fully described. In addition, the antiplatelet activity of prasugrel in mice has not been explored.

In the present study, we examined the antiplatelet activity of prasugrel in both WT and $P2Y_{12}$-deficient mice, and report additional *in vivo* evidence that prasugrel specifically inhibits $P2Y_{12}$ receptors.

2. Materials and methods

2.1. Chemicals

Prasugrel hydrochloride ($C_{20}H_{20}FNO_3S{\cdot}HCl$, M.W. 409.90) and clopidogrel hydrogen sulfate ($C_{16}H_{16}ClNO_2S{\cdot}H_2SO_4$, M.W. 419.90) were supplied by Ube Industries, Ltd. (Ube, Yamaguchi, Japan) and stored at −20 °C. Both test compounds were suspended in 5% (w/v) gum arabic (Sigma–Aldrich Co. St. Louis, MO, USA) solution. The vehicle (5% gum arabic solution) or suspensions of prasugrel or clopidogrel were orally administered to mice in a volume of 10 mL/kg. Adenosine 5′-diphosphate (ADP) sodium salt was obtained from Sigma–Aldrich Co. Murine protease activated receptor-4 (mPAR-4) agonist peptide (GYPGKF) was obtained from Sigma Genosys Japan Co. (Ishikari, Hokkaido, Japan). Collagen was obtained from Nycomed Pharma GmbH Co. (Unterschleiβheim, Bayern, Germany). Sodium pentobarbital (Nembutal™) was obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan).

2.2. Experimental animals

$P2Y_{12}$ receptor-deficient ($P2Y_{12}^{-/-}$) mice were generated according to the report by Foster et al. [13] in the Medicinal Safety Research Laboratories of Sankyo Co. Ltd. To confirm the $P2Y_{12}$-deficient genotype, tail DNA from the putative $P2Y_{12}^{-/-}$ mice was screened for targeted recombination by a PCR strategy, then by Southern blots of restriction enzyme-digested DNA (Fig. 1). The SphI fragment detected by the indicated probe is reduced from 7.6 to 5.5 kb due to a new SphI site in the targeted locus, indicating knockout of $P2Y_{12}$ gene [13]. Age- and gender-matched C57BL/6J × 129S1 control mice which do not differ genetically from $P2Y_{12}^{-/-}$ mice, except at the targeted locus, were used as wild-type (WT) mice. $P2Y_{12}^{-/-}$ mice and their offspring were maintained at Charles River Japan, Inc., and used for the present study at 11–18 weeks of age. The WT mice were also maintained on a mixed C57BL/6J × 129S1 genetic background at Charles River Japan, Inc., and used for the present study at 11–33 weeks of age. The mice were housed in animal quarters set at a constant temperature of 23–24 °C, humidity of 48–68% and a light/dark cycle of 12 h light. They were maintained with free access to water and food (FR-2, Funabashi Farm Co. Ltd., Funabashi, Chiba, Japan) after transporting them to the animal facility of Sankyo Co. Ltd., Tokyo.

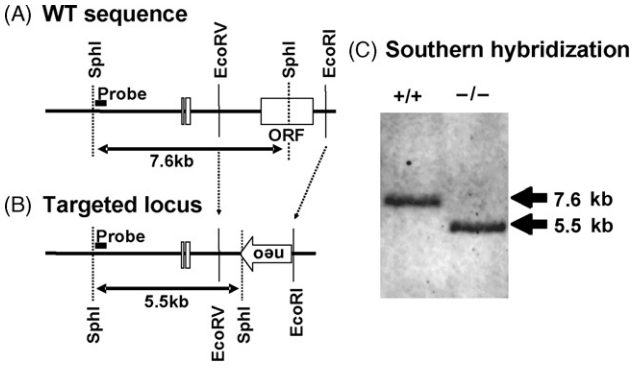

Fig. 1 – (A) Wild type $P2Y_{12}$ gene locus: the exonic sequence is shown by three open rectangles (ORF). (B) Targeted locus: the *neo* gene is inserted. (C) Southern hybridization of SphI-digested genomic DNA from a WT (+/+) and a KO (−/−) mouse. The SphI fragment detected by the indicated probe is reduced from 7.6 to 5.5 kb due to a new SphI site in the targeted locus.

2.3. Platelet preparation

We performed two separate series of experiments, for Fig. 2 and Figs. 3–6, using individual platelet-rich plasma (PRP) and

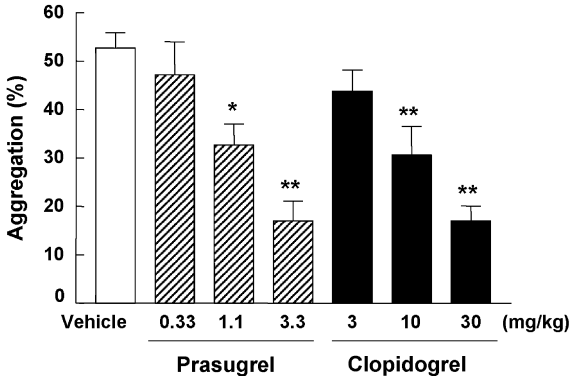

Fig. 2 – *Ex vivo* effects of single oral administration of prasugrel and clopidogrel on ADP induced platelet aggregation in mice. Platelet aggregation induced by ADP (3 μM) was measured 4 h after the dosing of prasugrel (0.33, 1.1 and 3.3 mg/kg) and clopidogrel (3, 10 and 30 mg/kg). Results are expressed as the mean ± S.E. (n = 5). Comparison between the test compound-treated groups and the vehicle-treated control group was carried out using the Dunnett test; *P < 0.05, **P < 0.01 vs. vehicle-treated control group.