

# Hepatic Microsomal Thiol Methyltransferase Is Involved in Stereoselective Methylation of Pharmacologically Active Metabolite of Prasugrel<sup>§</sup>

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## ABSTRACT

Prasugrel, a thienopyridine antiplatelet drug, is converted in animals and humans to the pharmacologically active metabolite R-138727 [(2Z)-{1-[(1RS)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-sulfanyl}piperidin-3-ylidene]ethanoic acid], which has two chiral centers, occurring as a mixture of four isomers. The RS and RR isomers are more active than the SS and SR isomers (RS > RR >> SR = SS). The pharmacologically active metabolite is further metabolized to an S-methylated metabolite that is the major identified inactive metabolite in humans. In rat, dog, and human liver microsomes supplemented with S-adenosyl methione, the SS and SR isomers of the active metabolite were extensively S-methylated while the RS and RR isomers were not. Addition of 2,3-dichloromethyl benzylamine (50  $\mu$ M) completely inhibited the S-methylation reaction, indicating that the microsomal and

cytosolic thiol methyltransferase but not the cytosolic thiopurine S-methyltransferase is involved in the methylation. The hepatic intrinsic clearance values for methylation of the RS, RR, SS, and SR isomers (ml/min/kg) were 0, 0, 40.4, and 37.6, respectively, in rat liver microsomes, 0, 0, 11.6, and 2.5, respectively, in dog liver microsomes, and 0, 0, 17.3, and 17.7, respectively, in human liver microsomes, indicating that the RS and RR isomers are not methylated in vitro and that the methylation of SS and SR isomers is high with rat > human > dog. This finding in vitro agreed well with the in vivo observation in rats and dogs, where the S-methylated SS and SR isomers were the major metabolites in the plasma whereas negligible amounts of S-methylated RS and RR isomers were detected after intravenous administration of the pharmacologically active metabolites.

## Introduction

Prasugrel is an approved thienopyridine antiplatelet agent for the reduction of thrombotic cardiovascular events in patients with acute coronary syndrome who are being managed by percutaneous coronary intervention (PCI) (Jakubowski et al., 2012). Prasugrel is a prodrug that requires the metabolic conversion to the pharmacologically active metabolite R-138727 [(2Z)-{1-[(1RS)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-sulfanyl}piperidin-3-ylidene]ethanoic acid] in vivo (Sugidachi et al., 2000). The pharmacologically active metabolite of prasugrel is a mixture of four stereoisomers, RS, RR, SS, and SR forms (the first letter indicates the configuration at the 4-position of the piperidyl group and

the second for that at the 1'-position of the benzyl group) (Fig. 1). The rank order of potency of these compounds in inhibiting platelet aggregation in vitro is: RS isomer > RR isomer >> SS isomer = SR isomer (Hasegawa et al., 2005). In humans, the pharmacologically active RS and RR isomers were detected in plasma at about 5-fold higher levels than the pharmacologically less active SS and SR isomers after dosing of prasugrel (Wickremsinhe et al., 2007). In rats, the RS and RR isomers were the major forms detected in plasma, and the SS and SR isomers were only detected at much lower concentrations in plasma (Kazui et al., 2008). In dogs, on the other hand, the levels of the RS and RR isomers were similar to those of the SS and SR isomers in plasma (Kazui et al., 2008). The S-methylated metabolite of the pharmacologically active metabolite was the major identified metabolite in humans and the second major metabolite in rats and dogs, indicating that the S-methylation is an important inactivation pathway of prasugrel (Asai et al., 2006; Farid et al., 2007a,b). The S-methylation reaction could be catalyzed by two enzymes: thiol S-methyltransferase (TMT) and thiopurine S-methyltransferase (TPMT). These two enzymes differ in their subcellular localization,

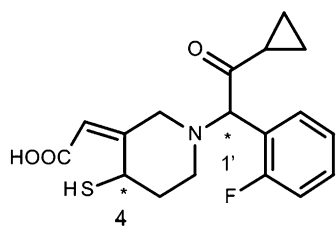
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**ABBREVIATIONS:** AUC<sub>0-inf</sub>, area under the plasma concentration-time curve extrapolated to infinity; CL<sub>int</sub>, intrinsic clearance; C<sub>max</sub>, maximum plasma concentration; DCMB, ( $\pm$ )-2,3-dichloro- $\alpha$ -methylbenzylamine hydrochloride; ESI, electrospray ionization; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; NADP,  $\beta$ -nicotinamide adenine dinucleotide phosphate sodium salt; ODS, octadecyl silane; R-106583, (2Z)-{1-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-(methylsulfanyl)piperidin-3-ylidene}ethanoic acid; R-121721, (2Z)-{1-[2-[(2,2,3,3-<sup>2</sup>H<sub>4</sub>)cyclopropyl]-1-(2-fluorophenyl)-2-oxoethyl]-4-[(<sup>2</sup>H<sub>3</sub>)methylsulfanyl]piperidin-3-ylidene}ethanoic acid; R-135766, (4-[[2-(4-bromophenyl)-2-oxoethyl]sulfanyl]-1-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-1,2,5,6-tetrahydropyridin-3-yl)acetic acid; R-138727, (2Z)-{1-[(1RS)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-sulfanyl}piperidin-3-ylidene]ethanoic acid; SAM, S-(5'-adenosyl)-L-methionine chloride; TMT, thiol-methyltransferase; TPMT, thiopurine S-methyltransferase; UPLC-UV, ultraperformance liquid chromatography coupled with ultraviolet detection; V<sub>max</sub>, maximal reaction rate.



Active metabolite of prasugrel

\*: denotes chiral center

Compounds	4-position of piperidyl group	1'-position of benzyl group
SS-form	S	S
SR-form	S	R
RR-form	R	R
RS-form	R	S

**Fig. 1.** Structures of four stereoisomers of the pharmacologically active metabolites of prasugrel. The first letter indicates the configuration at the 4-position of piperidyl group and the second for that at the 1'-position of benzyl group. \*Denotes chiral center.

substrate specificities, inhibitor sensitivities, and regulation. TPMT is a cytosolic enzyme and inhibited by *m*-anisic acid whereas TMT is a microsomal enzyme and inhibited by ( $\pm$ )-2,3-dichloro- $\alpha$ -methylbenzylamine hydrochloride (DCMB) (Lee and Kim, 1999). TPMT exhibits a genetic polymorphism (Weinshilboum, 1989), with 89% of whites and African Americans being extensive metabolizers, 11% intermediate metabolizers, and 0.33% poor metabolizers (Szumlanski et al., 1996; Hamdan-Khalil et al., 2003). Our study identified the S-methylating enzyme responsible for methylating the pharmacologically active metabolite of prasugrel and determined the stereoselectivity in this metabolic reaction using sub-cellular preparations of human and animal livers. Based on the assay data in vitro, we calculated the hepatic intrinsic clearance ( $CL_{in}$ ) values in vivo for the stereoselective S-methylation as the major elimination pathway of the pharmacologically active metabolite of prasugrel, and examined the relevance of these values to the in vivo profile of the S-methylated metabolite after intravenous administration of the pharmacologically active metabolite of prasugrel R-138727 to rats and dogs.

### Materials and Methods

The pharmacologically active metabolites of prasugrel (R-138727) containing four stereoisomers, the S-methylated form of the pharmacologically active metabolites containing four stereoisomers (R-106583 [(2Z)-{1-[2-(cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-(methylsulfanyl)piperidin-3-ylidene}ethanoic acid)] (Fig. 2), the deuterium-labeled S-methylated metabolite of the pharmacologically active metabolites of prasugrel containing four stereoisomers (R-121721 [(2Z)-1-(1-[2-[(2,2,3,3- $^2$ H<sub>4</sub>)cyclopropyl]-1-(2-fluorophenyl)-2-oxoethyl]-4-[( $^2$ H<sub>3</sub>)methylsulfanyl]piperidin-3-ylidene)ethanoic acid]), and the bromo-phenacyl derivative of the pharmacologically active metabolite (R-135766 [(4-[[2-(4-bromophenyl)-2-oxoethyl]sulfanyl]-1-[2-(cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-1,2,5,6-tetrahydropyridin-3-yl)acetic acid]) were obtained from Ube Industries, Ltd. (Ube, Japan) (Supplemental Figure 1). Each stereoisomer of the pharmacologically active metabolites of prasugrel was synthesized by Daiichi Sankyo Co., Ltd. R-135766 was used as the internal standard for an in vitro assay of the enantiomers of R-106583 by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). R-121721 was used as the internal standard for an in vivo assay of R-106583 and its enantiomer (i.e., RR/RS form and SR/SS form of R-106583) by LC-MS/MS. As the internal standard for the assay of the R-106583 by high-performance liquid chromatography (HPLC),  $\alpha$ -naphthoflavone was obtained from Sigma-Aldrich Corporation (St. Louis, MO). S-(5'-adenosyl)-L-methionine chloride (SAM), glucose-6-phosphate dehydrogenase from baker's yeast (G-6-PDH), D-glucose 6-phosphate disodium salt hydrate (G-6-P),  $\beta$ -nicotinamide adenine dinucleotide phosphate sodium salt (NADP), L-glutathione reduced (GSH), and DCMB were also purchased from Sigma-Aldrich Corporation. The derivatizing reagent for the assay of the pharmacologically active metabolites of prasugrel by LC-MS/MS (Farid et al., 2007b) *m*-methoxyphenacyl bromide (MPBr) and *m*-anisic acid were obtained from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were commercially available and of guaranteed reagent grade.

### Biological Samples

Pooled human liver microsomes (20 mg protein/ml), pooled male rat (Sprague-Dawley) liver microsomes (20 mg protein/ml), and pooled male dog

(beagle) liver microsomes (20 mg protein/ml) were purchased from BD Biosciences Company (Woburn, MA). Human liver microsomes (pooled from 10 donors) and human liver cytosol (pooled from 10 donors) for identification of the enzyme involved in the S-methylation of the pharmacologically active metabolites were purchased from the nonprofit Human & Animal Bridging Research Organization (Tokyo, Japan).

### Animals

Male Sprague-Dawley rats ( $n = 4$ ) were obtained from Charles River Japan (Yokohama, Japan) at 8 weeks of age. Four male beagle dogs were originally obtained from Nihon Nosan Corporation at the age of 6 months and were kept separately in stainless-steel cages in a controlled animal area. The controlled animal area was set at a room temperature of  $23 \pm 2^\circ\text{C}$  and  $55\% \pm 10\%$  relative humidity under a 12-hour cycle of light/dark artificial lighting (7:00 AM–7:00 PM). All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

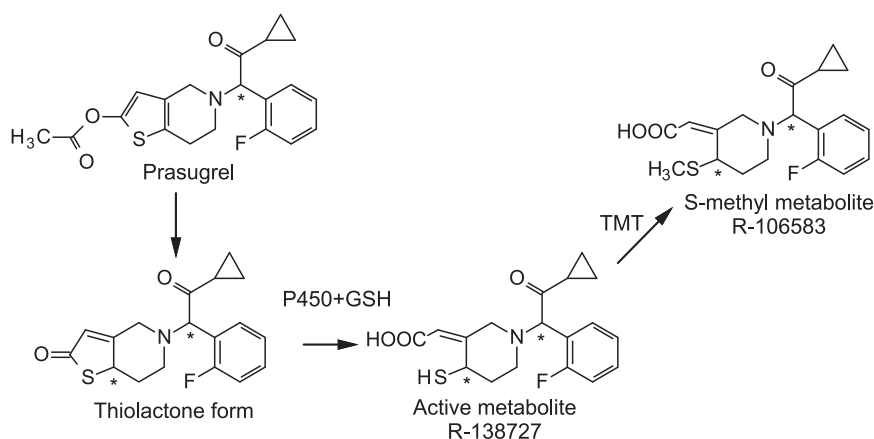
### S-Methylation of the Pharmacologically Active Metabolite of Prasugrel in Human Liver Microsomes and Cytosol

The assay for a TMT reaction was performed using pooled human liver microsomes. The incubation mixture contained 2 mg protein/ml of pooled human liver microsomes, 0.5 mM of SAM, and 50  $\mu\text{M}$  of the pharmacologically active metabolite of prasugrel (R-138727 racemic mixture) as the substrate in a final volume of 400  $\mu\text{l}$  of 100 mM potassium phosphate buffer (pH 7.9) containing 1 mM EDTA and 0.5% Triton X-100. The mixture without the substrate was preincubated at  $37^\circ\text{C}$  for 5 minutes, and the reaction was started by the addition of 4  $\mu\text{l}$  of the substrate solution in dimethylsulfoxide (DMSO). After incubation at  $37^\circ\text{C}$  for 0, 5, 10, 20, 30, and 45 minutes, a 50- $\mu\text{l}$  aliquot of the incubation mixture was collected and added to a mixture of 100  $\mu\text{l}$  of acetonitrile and 50  $\mu\text{l}$  of a solution of  $\alpha$ -naphthoflavone as the internal standard (5  $\mu\text{g}/\text{ml}$  in acetonitrile) to terminate the reaction. The mixture was centrifuged at 20,800g at  $4^\circ\text{C}$  for 3 minutes, and 2  $\mu\text{l}$  of the supernatant was injected into the ultraperformance liquid chromatography coupled with ultraviolet detection system (UPLC-UV; Waters Corporation) to determine the concentrations of the S-methylated metabolite, R-106583 (details of method to be explained herein).

The assay for a TPMT reaction was performed using pooled human liver cytosol. The incubation mixture contained 2.25 mg protein/ml of pooled human liver cytosol, 0.5 mM of SAM, and 50  $\mu\text{M}$  of R-138727 as the substrate in a final volume of 300  $\mu\text{l}$  of 400 mM potassium phosphate buffer (pH 6.2). The sample preparation and assay were performed in the same manner as described earlier for the assay for a TMT reaction in microsomes. Experiments were performed in triplicate.

### Inhibition of S-Methylation of R-138727, the Pharmacologically Active Metabolite of Prasugrel, in Human Liver Microsomes and Cytosol

Effects of DCMB as the TMT inhibitor and *m*-anisic acid as the TPMT inhibitor on S-methylation were evaluated. DCMB was added at a final concentration of 5, 50, or 500  $\mu\text{M}$  to the incubation medium consisting of 2 mg microsomal protein/ml of human liver, 0.5 mM SAM, and 100 mM potassium



**Fig. 2.** Metabolic pathway of prasugrel. Figure does not illustrate all metabolites of prasugrel that have been identified.

phosphate buffer (pH 7.9) containing 1 mM EDTA and 0.5% Triton X-100, and the mixture was preincubated at 37°C for 5 minutes. Similarly, DCMB was added at a final concentration of 5, 50, or 500  $\mu$ M to the incubation medium consisting of 2.25 mg protein/ml of human liver cytosol, 0.5 mM SAM, and 400 mM potassium phosphate buffer (pH 6.2), and the mixture was preincubated at 37°C for 5 minutes. *Meta*-anisic acid was added at a final concentration of 0.1, 1, and 10 mM to the incubation medium consisting of 2.25 mg protein/ml of human liver cytosol, 0.5 mM SAM, and 400 mM potassium phosphate buffer (pH 6.2), and the mixture was preincubated at 37°C for 5 minutes. Each reaction was initiated by the addition of 50  $\mu$ M of the active metabolite in DMSO and terminated after incubation at 37°C for 30 minutes and 45 minutes. A 50- $\mu$ l aliquot of the incubation mixture was collected and treated as described in the preceding section on S-methylation of the pharmacologically active metabolites of prasugrel in human liver microsomes and cytosol. The assay was conducted by the UPLC-UV method.

#### S-Methylation of Each Isomer of the Pharmacologically Active Metabolite of Prasugrel in Rat, Dog, and Human Liver Microsomes

The assays were performed using rat, dog, and human liver microsomes. The incubation mixture contained 2 mg liver microsomal protein/ml of each species, an NADPH-generating system (2.5 mM  $\beta$ -NADP, 22.5 mM D-glucose-6-phosphate, 10 mM magnesium chloride, and 0.5 units/ml of glucose-6-phosphate dehydrogenase), 0.5 mM of SAM, and 100  $\mu$ M of either of four stereoisomers of the pharmacologically active metabolite of prasugrel, SS, SR, RS, or RR form in DMSO solution, as the substrate in a final volume of 500 or 800  $\mu$ l of 100 mM potassium phosphate buffer (pH 7.9) containing 1 mM EDTA and 0.5% Triton X-100. A mixture without the substrate was preincubated at 37°C for 5 minutes, and the reaction was started by the addition of 5 or 8  $\mu$ l of a solution of the substrate. After incubation at 37°C for 0, 5, 15, 30, 60, 90, and 120 minutes, a 50- $\mu$ l aliquot of the incubation mixture was collected and added to a mixture of 100  $\mu$ l of acetonitrile and 50  $\mu$ l of a solution of  $\alpha$ -naphthoflavone as the internal standard (5  $\mu$ g/ml in acetonitrile) to terminate the reaction. The mixture was centrifuged at 20,800g at 4°C for 3 minutes, and 2  $\mu$ l of the supernatant was analyzed by the UPLC-UV method to determine the concentrations of the S-methylated metabolites. Experiments were performed in duplicate.

#### Enzyme Kinetic Parameters for S-Methylation of Each Stereoisomer of the Pharmacologically Active Metabolites of Prasugrel

The incubation mixture contained 2 mg protein/ml of the liver microsomes from rat, dog, or human liver, an NADPH-generating system, 0.5 mM SAM, 5 mM glutathione and 0.5, 1, 2, 5, 10, 50, 100, 200, or 400  $\mu$ M of one of four isomers of the pharmacologically active metabolite of prasugrel (RS, RR, SS, or SR form) in DMSO as the substrate in a final volume of 250  $\mu$ l of 100 mM potassium phosphate buffer (pH 7.9) containing 1 mM EDTA and 0.5% Triton X-100. A mixture without the substrate was preincubated at 37°C for 5 minutes, and the reaction was started by the addition of 2.5  $\mu$ l of a solution of the

substrate. After incubation at 37°C for 0 and 30 minutes, a 50- $\mu$ l aliquot of the incubation mixture was collected and added to a mixture of 100  $\mu$ l of acetonitrile and 50  $\mu$ l of a solution of R-135766 as the internal standard (2  $\mu$ M in acetonitrile) to terminate the reaction. The mixture was centrifuged at 20,800g at 4°C for 3 minutes, and 5  $\mu$ l of the supernatant was injected into an LC-MS/MS system to determine the concentrations of R-106583. This analytical method is referred to as the electrospray ionization (ESI) LC-MS/MS single-column method (details of the method explained herein). Experiments were performed in duplicate.

#### Plasma Concentrations of the S-Methylated Metabolites after Intravenous Administration of the Pharmacologically Active Metabolites of Prasugrel to Rats and Dogs

**Administration and Plasma Sample Preparations.** R-138727, a mixture of the four stereoisomers of the pharmacologically active metabolite of prasugrel, was dissolved in saline to prepare a 1 mg/ml solution and used for intravenous administration. Rats ( $n = 4$ ) and dogs ( $n = 4$ ) were fasted overnight and administered R-138727 intravenously at a dose of 1 mg/kg. At 0.033, 0.083, 0.25, 0.5, and 1 hour after the dose, approximately 0.4 ml of blood was collected from the jugular vein (rat) or the cephalic vein (dog) with a heparinized syringe. Each blood sample was transferred into a sampling tube containing 10  $\mu$ l of 500 mM *m*-methoxyphenacyl bromide (MPBr) in acetonitrile to derivatize the sulfhydryl-containing substances to chemically stable substances. The mixture was vortexed and allowed to stand at room temperature for about 10 minutes for the derivatization reaction. The blood samples were centrifuged at 21,600g for 3 minutes at 4°C to separate the plasma for the assay of each stereoisomer of the S-methylated metabolites.

#### Assay of S-Methylated Metabolites of the Pharmacologically Active Metabolite of Prasugrel (UPLC-UV Method, Nonstereoselective)

The S-methylated metabolite enantiomers produced from the pharmacologically active metabolite of prasugrel, in the rat, dog, and human liver microsomes were measured using a UPLC system (Acquity Ultra Performance system; Waters Corporation). After injection of the sample (2  $\mu$ l) to an ACQUITY UPLC BEH C<sub>18</sub> column (2.1  $\times$  50 mm, particle size of 1.7  $\mu$ m; Waters Corporation), the S-methylated metabolite was separated as a single peak via a gradient system with a flow rate of 0.5 ml/min. R-106583 (racemic mixture) was used as standard the S-methylated metabolites. The mobile phase consisted of a mixture of distilled water and trifluoroacetic acid (1000:0.05, v/v, solvent A) and a mixture of acetonitrile, distilled water, and trifluoroacetic acid (400:600:0.05, v/v/v, solvent B). The elution was started with 90% solvent A and 10% solvent B, and the proportion of solvent B was increased from 10% to 55% in 10 minutes, from 55% to 100% in 14 minutes, and returned to the initial condition in 19 minutes. Detection was performed at 220 nm for R-106583 and at 280 nm for  $\alpha$ -naphthoflavone, the internal standard, using a photodiode array detector (Waters Corporation).

**Assay of S-methylated Metabolite of the Pharmacologically Active Metabolite of Prasugrel (ESI-LC-MS/MS Single-Column Method, Nonstereoselective)**

For determination of the enzyme kinetic parameters of the S-methylation of the pharmacologically active metabolite of prasugrel, the S-methylated metabolite was measured by the ESI-LC-MS/MS single-column method, using R-106583 as the standard. The Quattro-LC MS/MS system (Micromass UK., Ltd., Manchester, United Kingdom) was used in the positive-ion detection mode with the ESI interface. The peak area of the  $m/z$  364→206 for R-106583 was measured against the peak areas of the  $m/z$  548→206 for the internal standard (R-135766). Separation by HPLC was conducted using an Alliance 2695 Separations Module (Waters Corporation) with an octadecyl silane column (Inertsil ODS-3, 2.1 mm × 150 mm, 5 μm; GL Sciences, Torrance, CA) at a flow rate of 0.2 ml/min with a mobile phase consisting of methanol, distilled water, and trifluoroacetic acid (TFA) [520:480:0.5 (v/v/v)].

**Assay of the Stereoisomers of the S-Methylated Metabolite in Plasma (ESI-LC-MS/MS Double-Column Method)**

For determination of the plasma concentrations of the stereoisomers of the S-methylated metabolite after intravenous administration of the pharmacologically active metabolite of prasugrel (R-138727) to rats and dogs, an ESI-LC-MS/MS system equipped with two columns was used. API5000 (Applied Biosystems/MDS SCIEX, Foster City, CA) was used in the positive-ion detection mode with the ESI interface. The peak area of the  $m/z$  364→206 for R-106583 was measured against the peak areas of the  $m/z$  371→153 for the internal standard (R-121721). Separation by UPLC was conducted using an

Acquity UPLC System (Waters Corporation) with an ODS column (Acquity UPLC BEH C<sub>18</sub>, 2.1 mm × 100 mm, 1.7 μm, Waters Corp.) and a chiral column (Chiralcel OJ-RH, 2 × 150 mm, 5 μm; Daicel Chemical Industries, Ltd., Osaka, Japan) at a flow rate of 0.25 ml/min with a mobile phase consisting of acetonitrile, distilled water, 1 M ammonium acetate, and trifluoroacetic acid (TFA) [275:715:10:0.5 (v/v/v)]. The two diastereomeric pairs (RR/RS and SS/SR) of R-106583 were separated chromatographically by the ESI-LC-MS/MS double-column method. Therefore, the ratios of the two diastereomeric pairs (RR/RS and SS/SR) of the S-methylated metabolite in plasma were determined using this assay.

The total concentration of the S-methylated metabolite in the plasma was determined using a slight modification of the ESI-LC-MS/MS single-column method: API5000 (Applied Biosystems/MDS SCIEX) in the positive-ion detection mode with the ESI interface and LC-20A UFLC system (Shimadzu Corporation, Kyoto, Japan) consisting of L-column ODS (2.1 × 150 mm, particle size of 5 μm; Chemicals Evaluation and Research Institute, Fukuoka, Japan) and a gradient system. Elution was started with 50% solvent A (distilled water and formic acid, 990:10 v/v) and 50% solvent B (acetonitrile), and the proportion of solvent B was increased linearly to 75% in 2 minutes, maintained at 75% for 6 minutes, and returned to the initial condition in 6.1 minutes.

**Data Handling**

The activity for producing the S-methylated metabolite ( $V$ , pmol/min/mg protein),  $V/S$  (μL/min/mg protein), and the inhibition ratio (%) were calculated according to the following equations.

$$V(\text{pmol/min/mg protein}) = \frac{\text{The concentration}(\mu\text{M})\text{ of the product} \times 1000}{\text{Incubation time}(\text{min}) \times \text{protein concentration}(\text{mg/mL})} \quad (1)$$

$$\frac{V}{S}(\mu\text{L/min/mg protein}) = \frac{\text{The mean } V(\text{pmol/min/mg protein})}{\text{The nominal concentration of each substrate}(\mu\text{M})} \quad (2)$$

$$\text{Inhibition ratio}(\%) = \frac{(\text{The mean } V \text{ without inhibitor} - \text{the mean } V \text{ with inhibitor}) \times 100}{\text{The mean } V \text{ without inhibitor}} \quad (3)$$

The values for  $V$ ,  $V/S$ , and the inhibition ratio were expressed to one decimal place.

The Michaelis-Menten constant ( $K_m$  in μM) and maximal reaction rate ( $V_{\max}$  in pmol/min/mg protein) were calculated from the Eadie-Hofstee plots of the reaction rate ( $V$ ) against  $V/S$  using Microsoft Office Excel 2003 (version SP2; Microsoft Corporation, Redmond, WA). The  $y$ -intercept and the slope obtained from the Eadie-Hofstee plots indicate  $V_{\max}$  and  $K_m$  values, respectively. The

intrinsic clearance ( $CL_{\text{int}}$  in ml/min/kg b.wt.) for the S-methylation in the animals was calculated according to eq. 4 and eq. 5. The microsomal protein (mg/g liver) of rats (44.8), humans (48.8), and dogs (77.9) and the weight of the liver (g) per body weight (kg) in rats (10 g/0.25 kg), humans (1800 g/70 kg), and dogs (320 g/10 kg) were from the literature reports (Davies and Morris, 1993; Iwatsubo et al., 1997).

$$CL_{\text{int, in vitro}}(\mu\text{L/min/mg protein}) = \frac{V_{\max}(\text{pmol/min/mg protein})}{K_m(\mu\text{M})} \quad (4)$$

$$CL_{\text{int}}(\text{mL/min/kg}) = \frac{CL_{\text{int, in vitro}} \times \text{the microsomal protein}(\text{mg/g liver}) \times \text{the weights of the liver were g/B.W.}(\text{kg})}{1000} \quad (5)$$

The calculated enzymatic kinetic parameters were expressed to two decimal places.

For calculating the plasma concentrations of each stereoisomer of the S-methylated metabolite produced after intravenous administration of the pharmacologically active metabolites of prasugrel to rats and dogs, we used Analyst 1.4.1 and Analyst 1.4.2 (Applied Biosystems/MDS SCIEX). The

plasma concentrations were expressed to three significant figures. The mean values and S.D. of the concentration and peak area ratio are calculated and expressed to three significant figures using Microsoft Office Excel 2003 (SP2; Microsoft Corporation). After dosing of R-138727, the enantiomer ratio of RS/RR or SS/SR isomers of R-106583 was calculated using the peak area ratios of RS/RR and SS/SR isomers by eq. 6.

$$\text{Enantiomer ratio of RS/RR or SS/SR isomers(\%)} = \frac{\text{Peak area ratio of RS/RR or SS/SR isomers}}{\text{Peak area ratio of (RS/RR isomers + SS/SR isomers)}} \times 100 \quad (6)$$

The plasma concentrations of R-106583 enantiomers (SS/SR isomers and RS/RR isomers) were calculated by eq. 7.

$$\text{Plasma concentrations of R-106583 enantiomers (RS/RR or SS/SR isomers)(ng/nL)} = \frac{\text{Plasma concentrations of R-10683(ng/mL)} \times \text{enantiomer ratio RS/RR or SS/SR isomers(\%)}}{100} \quad (7)$$

The pharmacokinetic parameters of R-106583 enantiomers were calculated using the computer program WinNonlin Professional (version 4.0.1; Pharsight Corporation, Mountain View, CA) based on the noncompartmental model.

## Results

### S-Methylation of the Pharmacologically Active Metabolites of Prasugrel, R-138727, in Human Liver Microsomes and Cytosol.

The S-methylation of the pharmacologically active metabolite of prasugrel was measured in human liver microsomes and cytosol. R-138727, the mixture of four stereoisomers of the pharmacologically active metabolites, was used as the substrate, and the production of the total amount of S-methylated isomers (R-106583) was determined by UPLC. As shown in Fig. 3, the S-methylating activity was  $56.7 \pm 5.0$  pmol/min/mg protein (mean  $\pm$  S.D.,  $n = 3$ ) in the microsomes and  $11.2 \pm 1.2$  pmol/min/mg protein (mean  $\pm$  S.D.,  $n = 3$ ) in the cytosol, indicating that the activity in human liver microsomes was higher than the activity in human liver cytosol.

**Inhibition of S-Methylation of the Pharmacologically Active Metabolite of Prasugrel.** Two S-methylating enzymes are known TMT in microsomes and TPMT in cytosols (Lee and Kim, 1999). Regarding TMT, low activity was observed in the cytosolic fraction also, which was likely due to a soluble isoform of TMT as reported previously because DCMB inhibited the cytosolic S-methylating activity (Glauser et al., 1992). The inhibitory effects of DCMB, the TMT inhibitor, on the S-methylating activity for the pharmacologically active metabolite of prasugrel in the human liver microsomes were determined as shown in Fig. 4. The addition of DCMB at a concentration of 50  $\mu$ M or 500  $\mu$ M completely inhibited the production of the S-methyl metabolite in the liver microsomes. Additionally, DCMB at a concentration of 5, 50, or 500  $\mu$ M completely inhibited the production of the S-methyl metabolite in the liver cytosol. On the other hand, *m*-anisic acid at a concentration of 0.1, 1, or 10 mM did not show any inhibitory effects on the production of the S-methyl metabolite in the liver cytosol (Fig. 4). These data indicate that TMT catalyzes the S-methylation of the

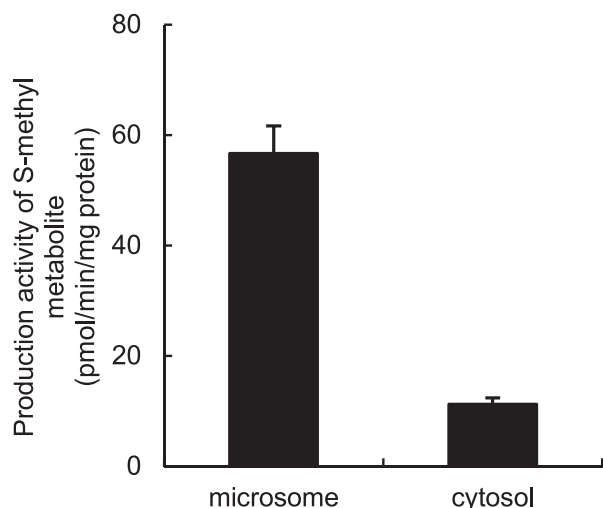
pharmacologically active metabolite of prasugrel in the liver microsomes and cytosol.

**Stereoselectivity and Species Difference in S-Methylation of the Pharmacologically Active Metabolite of Prasugrel In Vitro.** The activity for S-methylation of SS, SR, RR, and RS isomers of the pharmacologically active metabolite of prasugrel was determined separately using rat, dog, and human liver microsomes. The SS and SR forms were S-methylated in rat, dog, and human liver microsomes at varying activities, with the rat liver microsomes showing the highest activity, followed by the human and dog liver microsomes in that order (Fig. 5). Interestingly, neither the RS form nor the RR form were S-methylated in the liver microsomes of any of the three species.

**Enzyme Kinetics in S-Methylation of Each Enantiomer of the Pharmacologically Active Metabolite of Prasugrel.** We determined the enzyme kinetic parameters for the S-methylation of the SS and SR forms of the pharmacologically active metabolite of prasugrel in rat, dog, and human liver microsomes as shown in Table 1. In the case of the SS form, the  $K_m$  values for rat, dog, and human liver microsomes were 32.58, 155.24, and 26.29  $\mu$ M, respectively, and the  $V_{max}$  values were 734.80, 722.79, and 362.01 pmol/min/mg protein, respectively. In the case of the SR form, the  $K_m$  values for rat, dog, and human liver microsomes were 79.34, 208.04, and 17.26  $\mu$ M, respectively, and the  $V_{max}$  values were 1665.74, 205.30, and 243.50 pmol/min/mg protein, respectively.

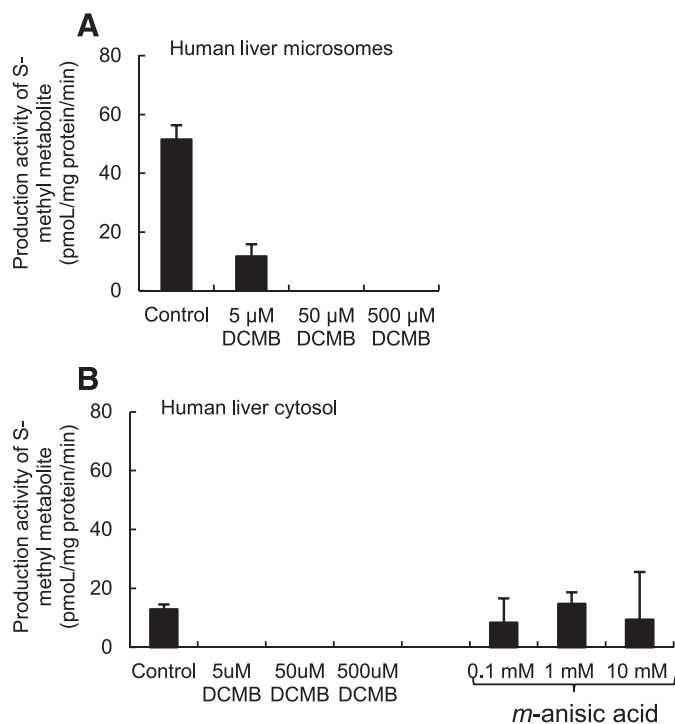
The  $CL_{int}$  values (ml/min/kg) for S-methylation of the SS and SR forms of the pharmacologically active metabolites of prasugrel in rat, dog, and human in vivo were calculated based on the in vitro data as shown in Table 1, and were 40.41, 11.62, and 17.28 ml/min/kg, respectively, for the SS form, and 37.61, 2.47, and 17.71 ml/min/kg, respectively, for the SR form. Therefore, the data indicate that rats show the highest  $CL_{int}$  in S-methylation of both enantiomers, followed by humans and dogs in that order.

**Plasma Concentrations of the S-Methylated Metabolites after Intravenous Administration of the Pharmacologically Active Metabolite to Rats and Dogs.** R-138727, the mixture of four isomers of the pharmacologically active metabolite of prasugrel was

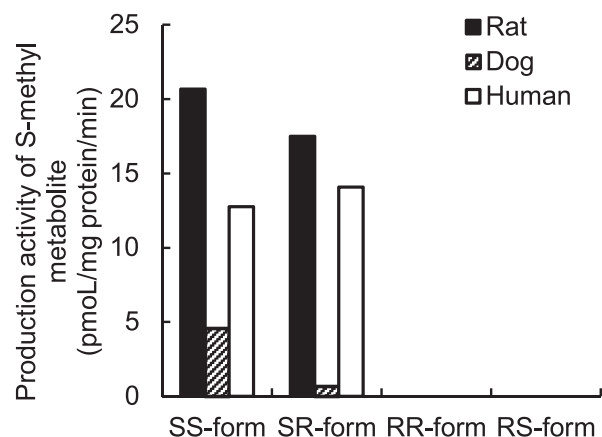


**Fig. 3.** S-methylation of the pharmacologically active metabolite of prasugrel in human liver microsomes and cytosol. The human liver microsomes or cytosol was incubated with 0.5 mM SAM and 50  $\mu$ M of the pharmacologically active metabolite of prasugrel at 37°C for 30 minutes or 45 minutes. Data are expressed as mean  $\pm$  S.D. of three experiments.

administered intravenously at a dose of 1 mg/kg to rats and dogs. The plasma concentrations of the S-methylated metabolites were measured as a mixture of the RS and RR isomers or as a mixture of SS and SR isomers by LC-MS/MS as shown in Fig. 6 (rat) and Fig. 7 (dog). We measured the combined concentration of the RS and RR isomers and the SS and SR isomers because the pair of the RS and RR isomers and the pair of SR and SS isomers were technically



**Fig. 4.** Inhibitory effects of DCMB and *m*-anisic acid on S-methylation of the pharmacologically active metabolite of prasugrel in human liver microsomes and cytosol. (A) S-methyl metabolite formation activity from the pharmacologically active metabolite with or without DCMB in pooled human liver microsomes. (B) S-methyl metabolite formation activity from the pharmacologically active metabolite with or without DCMB and *m*-anisic acid in pooled human liver cytosol. Data are expressed as mean  $\pm$  S.D. of three experiments.



**Fig. 5.** S-methylation of the single enantiomers of the pharmacologically active metabolite of prasugrel in rat, dog, and human liver microsomes. The rat, dog, and human liver microsomes were incubated with 0.5 mM SAM and 100  $\mu$ M the single enantiomer of the pharmacologically active metabolites of prasugrel at 37°C for 30 minutes. Data are expressed as the mean of duplicate experiments.

difficult to separate from each other using an ODS-column and a chiral column in tandem. The in vitro data demonstrated that the steric configuration at the 4-position of the piperidyl group was the crucial position for the S-methylation reaction (Fig. 5); therefore, the measurement of the RS and RR isomers together or the SS and SR isomers together was appropriate. Based on the plasma concentrations of the RS and RR isomers and the SS and SR isomers of S-methylated metabolite in rats and dogs, the pharmacokinetic parameters of S-methylated metabolites were calculated (Table 2).

As shown in Fig. 6 and Fig. 7, the amount of the SS and SR isomers of the S-methylated metabolite was greater than that of the RS and RR isomers of the S-methylated metabolite in both animal species. The maximum plasma concentration ( $C_{max}$ ) and area under the plasma concentration-time curve extrapolated to infinity ( $AUC_{0-inf}$ ) for the SS and SR isomers of S-methylated metabolites in rats were 614  $\pm$  124 ng/ml and 421  $\pm$  95.4 ng-h/ml, and the  $AUC_{0-inf}$  for the SS and SR isomers of S-methylated metabolites was about 8.5 times higher than that for the RS and RR isomers of S-methylated metabolite (Table 2). The  $C_{max}$  and  $AUC_{0-inf}$  for the SS and SR isomers of S-methylated metabolites in dogs were 156  $\pm$  21.2 ng/ml and 134  $\pm$  42.6 ng-h/ml, and the  $AUC_{0-inf}$  for the SS and SR isomers of S-methylated metabolite was about 76.6 times higher than that for the RS and RR isomers of S-methylated metabolite (Table 2). In comparison with the  $AUC_{0-inf}$  for the SS and SR isomers in rats and dogs, the  $AUC_{0-inf}$  of rats was about 3 times greater than that of dogs (Table 2).

## Discussion

Previous clinical studies demonstrated that the S-methylated metabolite of the pharmacologically active metabolite of prasugrel is the main metabolite identified in human plasma after oral administration of prasugrel (Asai et al., 2006; Farid et al., 2007a,b), indicating that the S-methylation is a major elimination pathway for the pharmacologically active metabolite. TMT and TPMT have been reported as the enzymes responsible for the S-methylation of xenobiotics (Glauser et al., 1992). In the present study, we identified the enzyme involved in the S-methylation of the pharmacologically active metabolites of prasugrel as TMT based on the localization of the activity being predominantly in the microsomal fraction and sensitive to the TMT inhibitor DCMB. Although the liver cytosol showed about one-fifth of the S-methylating activity in the microsomes, the cytosolic activity

TABLE 1

Apparent enzyme kinetic parameters and estimated  $CL_{int}$  values in S-methylation of SS and SR forms of the pharmacologically active metabolite of prasugrel

The assays were performed by using rat, dog, and human liver microsomes. Data are expressed as mean of duplicate experiments.  $CL_{int}$  in vitro and  $CL_{int}$  values were scaled to eq. 4 and eq. 5.

Pharmacologically Active Metabolite Substrate	$K_m$	$V_{max}$	$CL_{int}$ in vitro	$CL_{int}$
	$\mu M$	$pmol/min/mg$ protein	$\mu l/min/mg$ protein	$ml/min/kg$ b.wt.
SS form				
Rat	32.58	734.80	22.55	40.41
Dog	155.24	722.79	4.66	11.62
Human	26.29	362.01	13.77	17.28
SR form				
Rat	79.34	1665.74	20.99	37.61
Dog	208.04	205.30	0.99	2.47
Human	17.26	243.50	14.11	17.71

seems due to the cytosolic isoform of TMT (Glauser et al., 1992) as DCMB inhibited both the microsomal and cytosolic S-methylating activities. Additionally, *m*-anisic acid had little effect on the cytosolic S-methylating activities. Taken together, the S-methylation of the pharmacologically active metabolite was not catalyzed by TPMT, which was the enzyme showing a genetic polymorphism, but by TMT which was the enzyme showing no genetic polymorphism.

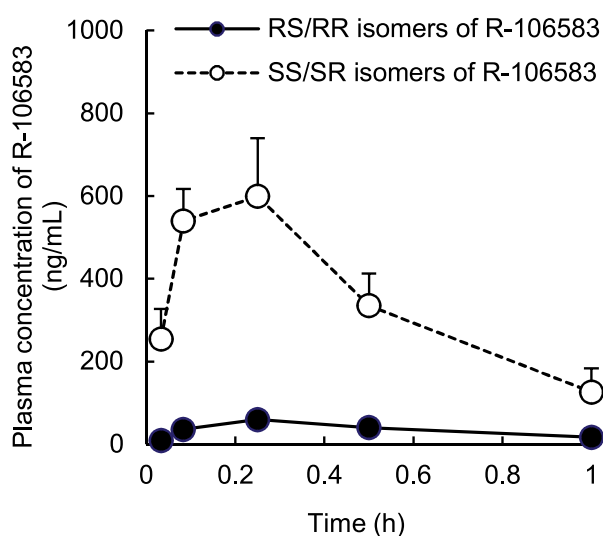
The RS and RR isomers of the pharmacologically active metabolite are more active in inhibiting platelet aggregation than the SS and SR isomers of the active metabolite (Hasegawa et al., 2005). Interestingly, the SR and SS isomers of the active metabolite were found to be extensively methylated in vitro whereas the RS and RR isomers of the active metabolite were not, demonstrating that TMT catalyzes the S-methylation reaction in a stereoselective manner (Fig. 5). It was reported that 2-mercaptopyrazine, diethyldithiocarbamate, and dihydrozipsidone, which is a metabolite of ziprasidone (an antipsychotic drug), were metabolized by TMT; however, stereoselectivity of TMT in liver microsomes (Glauser et al., 1993; Lee and Kim, 1999; Obach et al., 2012) was not reported.

This study is the first report describing stereoselectivity in S-methylation catalyzed by TMT. Specifically, only the SS form and SR form were S-methylated in the liver microsomes of all three species, demonstrating that the configuration of the 4-position of the piperidyl group is far more

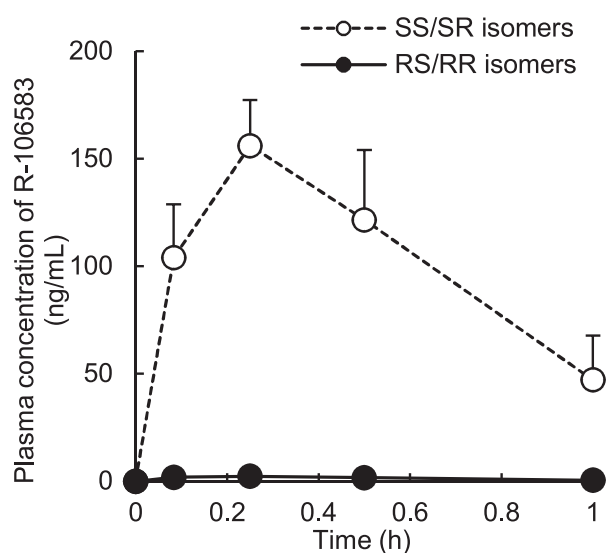
important than the configuration of the 1'-position of the benzyl group. The configuration of the chiral carbon adjacent to the thiol group seems to be the key for substrate recognition, but both of the two chiral carbon configurations appear to be important for platelet inhibition activity.

We also measured the plasma concentrations of the S-methylated metabolites in rats and dogs after intravenous administration of the pharmacologically active metabolite, and the results demonstrated that the S-methylated metabolite in vivo was almost exclusively the SS and SR isomers. Additionally, as shown in Table 1, the combined  $CL_{int}$  value obtained in vitro for the SS and SR isomers in rats (78.0 ml/min/kg) was about 5.5 times greater than that in dogs (14.1 ml/min/kg), and this difference approximated the 3.1-fold greater  $AUC_{0-inf}$  in rats over that in dogs (Table 2). Although in vitro the RS and RR isomers were not S-methylated, the S-methylated metabolite was detected in both rat and dog plasma at much lower amounts. At this time, it is unclear why the RS and RR isomers of the S-methylated metabolites were detected in both rat and dog plasma after intravenous administration of the pharmacologically active metabolite. Collectively, findings in vitro of stereoselective S-methylation were reflected in the observations in vivo in both rats and dogs.

Recently, the pharmacokinetics of enantiomers of clopidogrel active metabolites in patients with cardiovascular diseases were reported



**Fig. 6.** Plasma concentrations of the S-methylated metabolite (R-106583) enantiomers after intravenous administration of the racemic mixture of the pharmacologically active metabolite of prasugrel to rats at a dose of 1 mg/kg: RS/RR isomers (●) and SS/SR isomers (○). Data are expressed as mean  $\pm$  S.D. of four animals.



**Fig. 7.** Plasma concentrations of the S-methylated metabolite (R-106583) enantiomers after intravenous administration of the racemic mixture of the pharmacologically active metabolite of prasugrel to dogs at a dose of 1 mg/kg: RS/RR isomers (●) and SS/SR isomers (○). Data are expressed as mean  $\pm$  S.D. of four animals.

TABLE 2

Pharmacokinetic parameters of R-106583 enantiomers after intravenous administration of R-138727 to rats and dogs at a dose of 1 mg/kg

The pharmacokinetic parameters of R-106583 enantiomers were calculated using the computer program WinNonlin Professional (version 4.0.1, Pharsight Corporation) based on the noncompartmental model. Data are expressed as mean  $\pm$  S.D. of four animals.

Pharmacokinetic Parameters	Rat		Dog	
	RS+RR Isomers	SS+SR Isomers	RS+RR Isomers	SS+SR Isomers
$t_{1/2}$ (h)	0.446 $\pm$ 0.239	0.167 $\pm$ 0.0964	0.270 $\pm$ 0.112	0.424 $\pm$ 0.106
$C_{max}$ (ng/ml)	59.7 $\pm$ 2.15	614 $\pm$ 124	2.40 $\pm$ 1.52	156 $\pm$ 21.2
AUC <sub>0-1 h</sub> (ng·h/ml)	36.0 $\pm$ 3.81	351 $\pm$ 62.6	1.50 $\pm$ 1.04	103 $\pm$ 21.9
AUC <sub>0-inf</sub> (ng·h/ml)	49.3 $\pm$ 18.1	421 $\pm$ 95.4	1.75 $\pm$ 1.41	134 $\pm$ 42.6

(Karaźniewicz-Łada et al., 2014). Clopidogrel is another thienopyridine antiplatelet agent that has been widely used in the management of cardiovascular disease; the structure of the pharmacologically active metabolite of clopidogrel is somewhat analogous to that of prasugrel, and both compounds have the thiol group in the structure. Karaźniewicz-Łada et al. (2012) reported that the exposure to the more potent isomer (H4) of the clopidogrel active metabolite (Pereillo et al., 2002; Tuffal et al., 2011) was higher (1.5-fold and 2.2-fold) than that of the less potent isomer (H3) after oral administration of clopidogrel at doses of 75 mg and 300 mg, respectively.

For prasugrel, more potent RS and RR active metabolite isomers were detected in the plasma at about 5-fold higher levels than the less potent SS and SR isomers after dosing of prasugrel in humans (Wickremsinhe et al., 2007). Compared with clopidogrel, prasugrel showed a more drastic imbalance of isomer proportion with a more potent isomer at a higher level. Previously, it was reported that the pharmacologically active metabolite was produced in vitro from the thiolactone-metabolite of prasugrel by CYP3A4 or CYP3A5 in a stereoselective manner, and the production of the RR and RS isomers of the active metabolite was 1.6 times greater than that of the SS and SR isomers (Baker et al., 2008). Thus, the higher plasma concentrations of the RS and RR isomers than the SR and SS isomers of the active metabolite of prasugrel could be due to both the more stereoselective formation of the RS and RR isomers of the active metabolite from the thiolactone and the stereoselective S-methylation of the SR and SS isomers of the active metabolite.

To the best of our knowledge, there have been no reports on the stereoselectivity in clopidogrel S-methyl metabolite formation of H3 and H4 isomers. One factor in the difference in the plasma concentrations between clopidogrel and prasugrel active metabolites may be related to the selectivity in the formation of the S-methylated metabolites.

In conclusion, our results indicate that TMT is involved in the stereoselective S-methylation of the pharmacologically active metabolite of prasugrel, and this stereoselectivity in S-methylation will contribute to the higher plasma concentrations of RS and RR isomers of the pharmacologically active metabolite compared with the less pharmacologically active metabolites, SS and SR isomers, in animals and humans.

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#### Authorship Contributions

Participated in research design: Kazui, Kurihara.

Conducted experiments: Kazui, Hagihara.

Performed data analysis: Kazui, Hagihara, Kurihara.

Wrote or contributed to the writing of the manuscript: Kazui, Izumi, Ikeda, Kurihara.

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