Arylacetamide deacetylase is responsible for activation of prasugrel in human and dog

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**Abbreviations:** AADAC, arylacetamide deacetylase; CES, carboxylesterase; CYP, cytochrome P450; DFP, diisopropyl fluorophosphate; DIM, dog intestinal microsomes; DLM, dog liver microsomes; DMSO, dimethyl sulfoxide; HIM, human intestinal microsomes; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple-reaction monitoring; PMSF, phenylmethylsulfonyl fluoride; RAF, relative activity factor
Abstract

Prasugrel, a thienopyridine anti-platelet agent, is pharmacologically activated by hydrolysis and hydroxylation. It is efficiently hydrolyzed in the intestine after oral administration, and the enzyme responsible for the hydrolysis in human was demonstrated to be carboxylesterase (CES) 2. Prasugrel hydrolase activity is detected in dog intestine where CES enzymes are absent, so this prompted us to investigate the involvement of enzyme(s) other than CES. Human arylacetamide deacetylase (AADAC) is highly expressed in the small intestine, catalyzing the hydrolysis of several clinical drugs containing small acyl moieties. In the present study, we investigated whether AADAC catalyzes prasugrel hydrolysis. Recombinant human AADAC was shown to catalyze prasugrel hydrolysis with a $CL_{int}$ value of $50.0 \pm 1.2 \text{ mL/min/mg protein}$ with a similar $K_m$ value to human intestinal and liver microsomes, whereas the $CL_{int}$ values of human CES1 and CES2 were $4.6 \pm 0.1 \text{ mL/min/mg protein}$ and $6.6 \pm 0.3 \text{ mL/min/mg protein}$, respectively. Inhibition studies using various chemical inhibitors and the relative activity factor approach suggested that the contribution of AADAC to prasugrel hydrolysis in human intestine is comparable to that of CES2. In dog intestine, the expression of AADAC, but not CES1 and CES2, was confirmed by measuring the marker hydrolase activities of each human esterase. The similar $K_m$ values and inhibition profiles between recombinant dog AADAC and small intestinal microsomes suggest that AADAC is a major enzyme responsible for prasugrel hydrolysis in dog intestine. Collectively, we found that AADAC largely contributes to prasugrel hydrolysis in both human and dog intestine.
Introduction

Prasugrel, a thienopyridine anti-platelet agent, is used for patients with ischemic cardiac events who are undergoing percutaneous coronary intervention. It is a prodrug that is activated by hydrolysis and hydroxylation in the body. Previous reports demonstrated that after oral administration to human, prasugrel is efficiently hydrolyzed to prasugrel thiolactone by carboxylesterase (CES) 2 in the intestine, followed by oxidization to an active metabolite by cytochrome P450 (CYP) 3A4 and 2B6 in the intestine and liver (Rehmel et al., 2006; Farid et al., 2007; Williams et al., 2008) (Fig. 1). A previous report has demonstrated a benefit of prasugrel, such that its platelet inhibition effects showed faster onset and less inter-individual variability than that of clopidogrel (Payne et al., 2007), because ~85% of the absorbed clopidogrel is rapidly hydrolyzed to an inactive metabolite by CES1 (Hagihara et al., 2009).

CES enzymes are involved in the hydrolysis of various types of drugs and xenobiotics (Fukami and Yokoi, 2012). Although CES enzymes are divided into five families in mammals, the CES families involved in drug metabolism are mainly CES1 and CES2 (Holmes et al., 2010; Imai et al., 2006). CES1 is mainly expressed in the liver, and CES2 is highly expressed in the intestine and liver (Imai, 2006). In the case of human CES1 and CES2, substrate specificities are generally defined depending on the size of the acyl and alcohol/amine moieties in the compounds (Fukami and Yokoi, 2012). CES1 prefers substrates containing large acyl and small alcohol/amine moieties, whereas CES2 prefers substrates containing large alcohol/amine and small acyl moieties. The chemical structure of prasugrel fits the characteristics of CES2 substrates.

In the processes of drug development, prediction of drug disposition in humans using experimental animals is usually adopted to estimate pharmacological effects and toxicity of drug candidates. However, species differences in drug-metabolizing enzymes sometimes make the extrapolation to human difficult. In dog, both CES1 and CES2 are expressed in the liver, but not in the intestine (Taketani et al., 2007). Although substrates of CES enzymes are predicted to be unlikely hydrolyzed in dog intestine, a previous study reported that the dog
intestinal S9 fraction showed prasugrel hydrolase activity comparable to the human intestinal S9 fraction (Hagihara et al., 2011). This result made us hypothesize that enzyme(s) other than CES2 are involved in prasugrel hydrolysis.

Arylacetamide deacetylase (AADAC), which is expressed in the gastrointestinal tract and liver, is involved in the metabolism of various drugs. Until now, we have shown that human AADAC catalyzes the hydrolysis of flutamide, phenacetin, rifamycins, and indiplon (Watanabe et al., 2009 and 2010; Nakajima et al., 2011; Shimizu et al., 2014a). Our recent studies uncovered that human AADAC prefers compounds containing a quite small acyl moiety (Fukami et al., 2015). Based on the chemical structure of prasugrel (Fig. 1), we surmised that AADAC catalyzes prasugrel hydrolysis.

Identification of enzyme(s) involved in the metabolism of a drug is required to estimate the inter-individual variability of drug efficacy and drug-drug interactions. In the present study, we investigated whether human AADAC can catalyze prasugrel hydrolysis and whether the efficient hydrolysis of prasugrel in dog intestine, where CES enzymes are not expressed, might be explained by AADAC. As results, we found that both human and dog AADAC can catalyze prasugrel hydrolysis, and AADAC is an enzyme responsible for high efficient hydrolysis of prasugrel in dog intestine.
Materials and Methods

Chemicals and Reagents. Prasugrel hydrochloride was purchased from Toronto Research Chemicals (Ontario, Canada). Phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP) and eserine sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Telmisartan was obtained from LKT laboratories (St. Paul, MN). Human intestinal (pooled HIM, n = 7) and liver microsomes (pooled HLM, n = 50) were purchased from Corning (Corning, NY). Dog intestinal (pooled DIM, n = 3, male) and liver microsomes (pooled DLM, n = 8, male) were obtained from Xenotech, LLC (Lenexa, KS). All primers were commercially synthesized at Greiner Japan (Tokyo, Japan) or Sigma-Aldrich (St. Louis, MO). Other chemicals were of the highest commercially available grade.

Recombinant Esterases Expressed in Sf21 Cells. Sf21 cell homogenates expressing human AADAC, CES1, and CES2 were previously prepared (Watanabe et al., 2010; Fukami et al., 2010). An expression system of dog AADAC was constructed by using a Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer’s protocol. Dog AADAC cDNA was obtained by reverse transcription-polymerase chain reaction using a dog liver RNA sample (UNITECH, Chiba, Japan) with AADAC EcoR I (5’-GGGAATTCGTGTTGCCAAGTCAAGAGAG-3’) and AADAC Xho I (5’-CCCCTCGAGCCCCTTCCTCAGATTTTTAC-3’) primers. The nucleotide sequences (referred to accession no. XM_534309) were confirmed by DNA sequence analysis (FASMAC, Kanagawa, Japan). The cDNA was transferred into the pFastBac1 vector and digested with EcoR I and Xho I. The pFastBac1 vector containing AADAC cDNA was transformed into DH10Bac-competent cells, followed by transposition of the inserts into bacmid DNA. Non-recombinant bacmid DNA (mock) was also prepared by the same procedures. Other steps including transfection of bacmid DNA into Spodoptera frugiperda Sf21 cells (Invitrogen) and preparation of cell homogenates were performed according to the method described previously (Iwamura et al., 2012). Protein concentrations were determined
according to the method of Bradford (1976) using γ-globulin as the standard.

**Prasugrel Hydrolase Activity.** Prasugrel hydrolase activities were determined as follows: a typical incubation mixture (final volume of 0.2 mL) contained 100 mM potassium phosphate buffer (pH 7.4) and enzyme sources (HLM: 0.01 mg/mL, HIM: 0.01 mg/mL, DLM: 0.005 mg/mL, DIM: 0.03 mg/mL, Sf21 cell homogenates expressing human AADAC: 0.01 mg/mL, CES1: 0.01 mg/mL, CES2: 0.01 mg/mL, and dog AADAC: 0.005 mg/mL). After a 2 min pre-incubation at 37°C, the reactions were initiated by the addition of prasugrel (final concentration was 5 µM). Prasugrel hydrochloride was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the incubation mixture was 2%. After a 1 min incubation, the reactions were terminated by addition of 100 µl of ice-cold acetonitrile. In a preliminary study, we confirmed that the formation rates of prasugrel thiolactone from prasugrel were linear with respect to protein concentration (< 0.02 mg/mL HIM, 0.015 mg/mL HLM, 0.04 mg/mL DIM, and 0.006 mg/mL DLM) and incubation time < 1.5 min. A 50 µL portion of the supernatant obtained by centrifugation at 12,000 g for 5 min was subjected to high-performance liquid chromatography (HPLC). The HPLC analysis was performed using an L-2130 pump (Hitachi, Tokyo, Japan), an L-2200 autosampler (Hitachi), an L-2400 UV detector (Hitachi), and a D-2500 Chromato-Integrator (Hitachi) equipped with an Inertsil-ODS3 column (5-μm particle size, 4.6 mm i.d.×250 mm; GL Science, Tokyo, Japan). The eluent was monitored at 254 nm using a UV detector equipped with noise-base clean Uni-3 (Union, Gunma, Japan), which can reduce the noise by integrating the output and increase the signal 3-fold by differentiating the output and 5-fold by further amplification with an internal amplifier, resulting in a maximum 15-fold amplification of the signal. The mobile phase was 70% acetonitrile containing 5 mM potassium phosphate buffer (pH 7.4). The flow rate was 0.6 ml/min. The column temperature was 35°C. Because prasugrel is non-enzymatically hydrolyzed to some extent, the content of prasugrel thiolactone in the mixture incubated without the enzyme sources was subtracted from that with the enzyme sources. The quantification of prasugrel thiolactone was performed as described below.
Identification and Quantification of Prasugrel Thiolactone. Because the authentic standard of prasugrel thiolactone is not commercially available, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed to determine whether the concerned peak represents prasugrel thiolactone. The LC equipment comprised an HP1100 system with a binary pump, an automatic sampler, and a column oven (Agilent Technologies, Santa Clara, CA) equipped with ZORBAX (3.5 μm particle size, 2.1 mm i.d. × 50 mm; Agilent Technologies). The column temperature was 25°C. The mobile phase was 55% methanol with 0.1% formic acid. The flow rate was 0.2 ml/min. The LC was connected to a PE Sciex API2000 tandem mass spectrometer (ABSciex, Framingham, MA), which was operated in the positive electrospray ionization mode. In the multiple-reaction monitoring (MRM) mode, the ion transitions of \( m/z \) 332.1 > 109.0 for prasugrel thiolactone and \( m/z \) 374.1 > 206.8 for prasugrel were monitored. Turbo gas was maintained at 550°C. Nitrogen was used as the nebulizing, turbo, and curtain gas at 60, 85, and 40 psi, respectively. The collision energy was 25 V. The analytical data were processed using the Analyst software (version 1.5; Applied Biosystems, Foster City, CA). After injection of samples incubated with prasugrel and HLM to LC-MS/MS, the fraction of prasugrel thiolactone confirmed with MRM (Fig. 2A) was collected, concentrated, and subjected to HPLC as described above (Fig. 2B). An HPLC peak detected with a sample incubated with HLM and 10 μM prasugrel for 15 min showed the same retention time to that collected from LC-MS/MS (Figs. 2B - E), indicating that prasugrel was efficiently hydrolyzed to prasugrel thiolactone.

The formed prasugrel thiolactone was quantified by HPLC analysis based on the decreased amount of prasugrel, according to a method described in our previous study for indiplon hydrolysis (Shimizu et al., 2014a). Briefly, prasugrel at concentrations of 1 and 10 μM was incubated with HLMs (0.1 mg/mL). After a 15-min incubation, prasugrel was completely converted to prasugrel thiolactone (Figs. 2D and E). The amounts of prasugrel were assigned for the formed prasugrel thiolactone. After determining the peak height per known content of prasugrel thiolactone, the value was used to calculate hydrolase activity of
Kinetic Analyses of Prasugrel Hydrolysis in Tissue Microsomes and Recombinant Esterases. Kinetic analyses of prasugrel hydrolysis were performed at ranges of 1 - 80 µM of prasugrel concentrations. The parameters were estimated from the fitted curves using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for non-linear regression analyses.

Inhibition Studies of Prasugrel Hydrolase Activity. To confirm the involvement of AADAC in prasugrel hydrolysis in human and dog, inhibition studies with HIM, HLM, DIM, and recombinant dog AADAC were performed using representative esterase inhibitors including DFP, PMSF, eserine, and telmisartan. Final concentrations of the inhibitors were 100 µM (DFP, PMSF) or 10 µM (eserine, telmisartan). DFP, a type of organophosphate, is known to potently inhibit CES and AADAC at 100 µM (Tabata et al., 2004; Watanabe et al., 2009). PMSF is a general inhibitor of serine esterases and potently inhibits CES enzymes at 100 µM (Fujiyama et al., 2010), but does not inhibit AADAC (Watanabe et al., 2009; Shimizu et al., 2014b). Eserine potently inhibits CES2 and AADAC at 10 µM (Kobayashi et al., 2012a). Telmisartan specifically inhibits CES2 at 10 µM (Shimizu et al., 2014b). DFP, PMSF, and telmisartan were dissolved in DMSO so that the final concentration of DMSO in the incubation mixture was 2%. Eserine was dissolved in distilled water. The experimental procedures and conditions were the same as above. It was confirmed that 2% DMSO did not inhibit prasugrel hydrolase activity, and the control activity was determined in the presence of 2% DMSO.

Contributions of AADAC, CES1, and CES2 to Prasugrel Hydrolase Activity in HIM and HLM. The percentage contributions of AADAC, CES1, and CES2 to the prasugrel hydrolase activity in HIM and HLM were estimated by applying the relative activity factor (RAF) as the ratio of activity values, as described previously (Watanabe et al., 2010). In this study, the RAF...
values for AADAC (RAF_{AADAC, HIM} or RAF_{AADAC, HLM}) were determined as the ratios of the indiplon hydrolase activities in HIM and HLM to the value by recombinant human AADAC.

The RAF values for CES1 (RAF_{CES1, HIM} or RAF_{CES1, HLM}) were determined as the ratios of the fenofibrate hydrolase activities in HIM and HLM to the value by recombinant human CES1.

The RAF values for CES2 (RAF_{CES2, HIM} or RAF_{CES2, HLM}) were determined as the ratios of the procaine hydrolase activities in HIM and HLM to the value by recombinant human CES2.

Using the RAF values, the predicted prasugrel hydrolase activities by AADAC (V_{AADAC, HIM} or V_{AADAC, HLM}), CES1 (V_{CES1, HIM} or V_{CES1, HLM}), and CES2 (V_{CES2, HIM} or V_{CES2, HLM}) in HIM or HLM were calculated as follows:

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\begin{align*}
(1) \quad V_{AADAC, HIM} &= V_{rec-AADAC} \times RAF_{AADAC, HIM} \\
(2) \quad V_{AADAC, HLM} &= V_{rec-AADAC} \times RAF_{AADAC, HLM} \\
(3) \quad V_{CES1, HIM} &= V_{rec-CES1} \times RAF_{CES1, HIM} \\
(4) \quad V_{CES1, HLM} &= V_{rec-CES1} \times RAF_{CES1, HLM} \\
(5) \quad V_{CES2, HIM} &= V_{rec-CES2} \times RAF_{CES2, HIM} \\
(6) \quad V_{CES2, HLM} &= V_{rec-CES2} \times RAF_{CES2, HLM}
\end{align*}
\]

where \( V_{rec-AADAC}, V_{rec-CES1}, \) and \( V_{rec-CES2} \) are the prasugrel hydrolase activities by recombinant AADAC, CES1, and CES2, respectively. The contributions of AADAC, CES1, and CES2 to the prasugrel hydrolase activities in HIM or HLM were calculated using the following equations:

\[
\begin{align*}
(7) \quad \text{Contribution of AADAC in HIM} (\%) &= \left( \frac{V_{AADAC, HIM}}{V_{HIM}} \right) \times 100 \\
(8) \quad \text{Contribution of AADAC in HLM} (\%) &= \left( \frac{V_{AADAC, HLM}}{V_{HLM}} \right) \times 100 \\
(9) \quad \text{Contribution of CES1 in HIM} (\%) &= \left( \frac{V_{CES1, HIM}}{V_{HIM}} \right) \times 100 \\
(10) \quad \text{Contribution of CES1 in HLM} (\%) &= \left( \frac{V_{CES1, HLM}}{V_{HLM}} \right) \times 100 \\
(11) \quad \text{Contribution of CES2 in HIM} (\%) &= \left( \frac{V_{CES2, HIM}}{V_{HIM}} \right) \times 100 \\
(12) \quad \text{Contribution of CES2 in HLM} (\%) &= \left( \frac{V_{CES2, HLM}}{V_{HLM}} \right) \times 100
\end{align*}
\]

where the \( V_{HIM} \) and \( V_{HLM} \) values are the observed prasugrel hydrolase activities in HIM and HLM, respectively.
Indiplon, Phenacetin, Fenofibrate, and Procaine Hydrolase Activity. Indiplon, phenacetin, fenofibrate, and procaine hydrolase activities were measured according to our previous studies (Shimizu et al., 2014a; Watanabe et al., 2010; Fukami et al., 2015). Their substrate concentrations were 1 mM, 5 mM, 25 µM, and 5 mM, respectively.
Results

Kinetic Analyses of prasugrel hydrolase activity by recombinant human esterases, HIM, and HLM. Prasugrel hydrolysis was reported to be mainly catalyzed by CES2 in human intestine (Williams et al., 2008). To investigate whether CES2 is the sole enzyme involved in this hydrolysis, we performed kinetic analyses of prasugrel hydrolase activity using recombinant human AADAC, CES1, and CES2. The activity was detected by all recombinant human esterases, and data for activities followed Michaelis-Menten equations (Fig. 3A). The $K_m$, $V_{max}$, and $CL_{int}$ values for prasugrel hydrolysis by recombinant human AADAC were $4.6 \pm 0.2 \mu M$, $228.4 \pm 13.6 \text{ nmol/min/mg protein}$, and $50.0 \pm 1.2 \text{ mL/min/mg protein}$, respectively. In human CES1 and CES2, $K_m$ values were higher and $V_{max}$ values were lower than those in human AADAC (Table 1). Thus, human AADAC showed the highest catalytic efficiency among three recombinant esterases, although we cannot directly compare the $V_{max}$ values because expression levels may be different among expression systems. This is the first study to report the kinetics of prasugrel hydrolysis in HLM and HIM. The $K_m$, $V_{max}$, and $CL_{int}$ values in HIM were $5.3 \pm 0.2 \mu M$, $560.6 \pm 20.7 \text{ nmol/min/mg protein}$, and $106.4 \pm 1.4 \text{ mL/min/mg protein}$, respectively, and those in HLM were $4.6 \pm 0.5 \mu M$, $382.0 \pm 27.9 \text{ nmol/min/mg protein}$, and $83.9 \pm 3.1 \text{ mL/min/mg protein}$, respectively (Fig. 3B, Table 1). Because the $K_m$ values in HIM and HLM were similar to that of recombinant human AADAC, it was suggested that AADAC would be one of the responsible enzymes for prasugrel hydrolysis in human intestine and liver.

Effects of Chemical Inhibitors on Prasugrel Hydrolase Activities in HIM and HLM. To investigate the contribution of each esterase to prasugrel hydrolysis in human intestine and liver, the effects of various chemical inhibitors on prasugrel hydrolase activities in HIM and HLM were determined (Fig. 4). The prasugrel hydrolase activities in HIM and HLM were efficiently inhibited by DFP and eserine, but were slightly inhibited by PMSF. Although telmisartan slightly inhibited the activity in HIM, it did not inhibit the activity in HLM. These
results suggest that AADAC is involved in prasugrel hydrolysis in human intestine and liver.

**Contribution of AADAC, CES1, and CES2 to Prasugrel Hydrolase Activity in HIM and HLM.** To estimate the contribution of AADAC, CES1, and CES2 to prasugrel hydrolase activity in HIM and HLM, RAF values for each esterase in HIM and HLM were calculated (Table 2). We previously demonstrated that indiplon, fenofibrate, and procaine were specifically hydrolyzed by AADAC, CES1, and CES2, respectively (Shimizu et al., 2014a; Fukami et al., 2015). Therefore, we selected these compounds as the specific substrates of each esterase. The values of indiplon hydrolase activity at 1 mM in HIM and HLM were 221.0 and 338.2 pmol/min/mg protein, respectively. Using the activity of recombinant AADAC (265.0 pmol/min/mg protein), RAF_{AADAC,HIM} and RAF_{AADAC,HLM} values were calculated as 0.83 and 1.27, respectively. The value of fenofibrate hydrolase activity at 25 µM in HLM was 421.4 nmol/min/mg protein, whereas the activity was not detected in HIM. Using the activity of recombinant CES1 (84.0 nmol/min/mg protein), the RAF_{CES1,HLM} value was calculated as 5.01. The values of procaine hydrolase activity at 5 mM in HIM and HLM were 11.1 and 2.3 nmol/min/mg protein, respectively. Using the activity of recombinant CES2 (4.3 nmol/min/mg protein), RAF_{CES2,HIM} and RAF_{CES2,HLM} values were calculated as 2.60 and 0.54, respectively. The contribution ratio of each esterase in HIM and HLM was estimated according to the equations described in the Materials and Methods, resulting in the values of AADAC, CES1, and CES2 in HIM being 50.6%, 0%, and 46.4%, respectively, and those in HLM were 57.3%, 31.6%, and 7.1%, respectively. The cumulative contribution of the three enzymes are illustrated in Fig. 5. The total contributions of AADAC, CES1, and CES2 in HIM and HLM were 97.0% and 96.0%, respectively. It was shown that AADAC is one of the enzymes highly contributing to prasugrel hydrolysis.

**Hydrolase Activity of Phenacetin, Fenofibrate and Procaine in Dog Tissue.** We surmised that the hydrolysis of prasugrel in dog intestine might be catalyzed by AADAC. Because it was unknown whether AADAC is expressed in dog intestine, we sought to measure the
hydrolase activity of indiplon in DLM and DIM as a marker activity of AADAC. However, recombinant dog AADAC hardly showed indiplon hydrolysis activity at 1 mM substrate concentration (data not shown). As a second marker substrate of AADAC, we selected phenacetin because recombinant dog AADAC showed high activity toward phenacetin hydrolysis (5.4 ± 0.0 nmol/min/mg protein at a 5 mM substrate concentration). The hydrolase activity at 5 mM phenacetin was detected in DIM (0.3 ± 0.1 nmol/min/mg protein), although the value was much lower than that in DLM (4.0 ± 0.0 nmol/min/mg protein) (Fig. 6A). On the other hand, the hydrolase activities of fenofibrate and procaine, which were human CES1 and CES2 marker substrates, respectively, were not detected in DIM (Figs. 6B and C), although these activities were highly detected in DLM (153.9 ± 1.5 nmol/min/mg protein and 3.4 ± 0.2 nmol/min/mg protein, respectively). We confirmed that recombinant dog AADAC did not show fenofibrate and procaine hydrolase activity (data not shown). These results indicate that AADAC is functionally expressed in dog intestine.

Kinetic Analyses of Prasugrel Hydrolase Activity by Recombinant Dog AADAC and DIM. To investigate whether dog AADAC can catalyze prasugrel hydrolysis, kinetic analysis of prasugrel hydrolase activity by DIM and recombinant dog AADAC was performed (Fig. 7A and Table 3). Data for the activity in DIM followed Michaelis-Menten equations, and the $K_m$, $V_{max}$, and $CL_{int}$ values were 5.3 ± 0.6 μM, 29.8 ± 2.0 nmol/min/mg protein, and 5.6 ± 0.3 mL/min/mg protein, respectively (Table 3). The activity was also detected by recombinant dog AADAC, and data for the activity followed Michaelis-Menten equations. The $K_m$, $V_{max}$, and $CL_{int}$ values were 1.5 ± 0.1 μM, 67.4 ± 6.5 nmol/min/mg protein, and 45.9 ± 3.1 mL/min/mg protein, respectively (Table 3). Thus, recombinant dog AADAC showed a similar $K_m$ value to DIM, suggesting that AADAC is the major enzyme involved in prasugrel hydrolysis in dog intestine.

Effects of Chemical Inhibitors on Prasugrel Hydrolase Activity by DIM and Recombinant Dog AADAC. To support the major contribution of AADAC to prasugrel
hydrolysis in dog intestine, the inhibition profiles of some inhibitors were compared between DIM and recombinant dog AADAC (Fig. 7B). The hydrolase activity in DIM was potently inhibited by DFP and eserine, but was not inhibited by PMSF and telmisartan. The prasugrel hydrolase activity by recombinant dog AADAC was also potently inhibited by DFP and eserine, but was not inhibited by PMSF and telmisartan. This inhibitory characteristic was similar to that in human tissues (Fig. 4). Collectively, these results support that AADAC is major contributor to prasugrel hydrolysis in dog intestine.
Discussion

Prasugrel is efficiently hydrolyzed to prasugrel thiolactone in the intestine after oral administration, followed by oxidation to an active metabolite by CYP3A4 and CYP2B6 in the intestine and liver (Rehmel et al., 2006; Farid et al., 2007; Williams et al., 2008). It has been believed that prasugrel hydrolysis was catalyzed by CES2 in humans (Williams et al., 2008). High prasugrel hydrolase activity was observed in dog intestine (Hagihara et al., 2011), even though CES2 protein is not expressed in dog intestine (Taketani et al., 2007). This fact implies a possibility that enzyme(s) other than CES enzymes would catalyze prasugrel hydrolysis. Because we have recently found that AADAC prefers compounds containing small acyl moieties, we expected that AADAC would be involved in the hydrolysis of prasugrel, which has a small acyl moiety.

First, we performed kinetic analyses of prasugrel hydrolase activity by recombinant human AADAC, CES1, and CES2 (Fig. 3A), showing that the highest catalytic potency was detected in human AADAC (Table 1). The $K_m$ value obtained by recombinant human AADAC was similar to those in HIM and HLM (Fig. 3B and Table 1). HIM and HLM were selected for this study because the intestine is a main organ for prasugrel hydrolysis, and the liver is an organ that metabolizes a majority of drugs. The results suggested that AADAC is involved in prasugrel hydrolysis in human intestine and liver. The high $CL_{int}$ value in HIM suggests that a large portion of prasugrel was hydrolyzed in the intestine after oral administration, as previously reported (Hagihara et al., 2011). The $K_m$ value by recombinant CES1 in this study (16.7 ± 1.9 µM) was similar to that reported in a previous study using purified CES1 (9.3 ± 0.8 µM) (Williams et al., 2008). Data for the prasugrel hydrolysis by recombinant human CES2 also fit the Michaelis-Menten equation, with a $K_m$ value of 17.1 ± 1.8 µM in our study (Fig. 3A, Table 1). However, Williams et al. (2008) reported that data for human CES2 fit a Hill equation at prasugrel concentrations < 40.5 µM and an inhibition plot at concentrations > 40.5 µM. This inconsistency may be due to the difference in the enzyme sources (homogenates of Sf21 cells expressing human CES2 versus purified human CES2).
We performed inhibition studies using several inhibitors to estimate the contribution of AADAC to prasugrel hydrolysis in HIM and HLM (Fig. 4). The activities in both HIM and HLM were strongly inhibited by DFP and eserine, which are known to inhibit AADAC activity. In addition, the inhibition profiles were similar between HIM and HLM. These results suggest that AADAC highly contributes to prasugrel hydrolysis in HIM and HLM. Telmisartan, a specific inhibitor of human CES2, showed the inhibitory effect on only HIM, although the effect was slight. The results suggest that the contribution of CES2 to prasugrel hydrolysis in HIM would be higher than that in HLM.

To further quantitatively elucidate the contribution of each esterase in HIM, we utilized the RAF approach. A concept of RAF was proposed by Crespi (1995) to extrapolate the data of recombinant CYP enzymes to the activity in human liver. For the calculation of the RAF, the $V$ value was used. RAF using the $V$ value requires an appropriate substrate concentration to obtain a reasonable prediction (Crespi and Miller, 1999). In this study, we selected concentrations approximately 5-fold higher than the $K_m$ value for marker substrates to calculate the RAF values. As a result, it was estimated that AADAC showed similar or higher contribution ratios in the intestine compared to CES2 (Fig. 5 and Table 2). Although the hydrolysis in human liver appears to hardly contribute to pharmacological activation of prasugrel in the body owing to the high clearance in human intestine, the contribution ratio of each esterase in HLM was also estimated by the RAF approach. CES1 is predominantly expressed in human liver (Imai, 2006) and highly contributes to the hydrolysis of many compounds. Nevertheless, AADAC showed the highest contribution to prasugrel hydrolysis in the liver. Considering the results of the inhibition study (Fig. 4) and the RAF approach (Fig. 5) together, AADAC plays a strong role in prasugrel hydrolysis in human.

Next, we investigated whether prasugrel hydrolysis in dog intestine might be catalyzed by dog AADAC. Because it had been unknown that AADAC is expressed in dog intestine, the mRNA expression levels of dog AADAC in the intestine and liver were initially measured with real-time reverse-transcription polymerase chain reaction. The mRNA expression was detected in both the liver and intestine, and the level in the liver was 2.7-fold higher than that
in the intestine (data not shown). However, because protein expression is not necessarily correlated with mRNA expression, the expression of AADAC protein in DIM and DLM was attempted to be evaluated by immunoblot analysis. However, a commercially available polyclonal antibody against human AADAC did not react with dog AADAC, even though it reacted with rat and mice AADAC (data not shown). Accordingly, we sought to alternatively investigate dog AADAC expression by measuring the hydrolase activity of a specific substrate of human AADAC. Because recombinant dog AADAC could not efficiently hydrolyze indiplon, phenacetin was used as a marker substrate of AADAC for the dog study (Fig. 6A). Such species differences are not surprising because we have previously reported that there are species differences in substrate specificity among human, rat, and mouse AADAC (Kobayashi et al., 2012b).

The phenacetin hydrolase activity was detected in both DIM and DLM, although the activity in DIM was only 8% of that in DLM. Therefore, it would be permissible to claim that AADAC was functionally expressed in both dog intestine and liver, although the expression level in the intestine was relatively low. The expressions of CES1 and CES2 in DIM and DLM were also investigated by measuring fenofibrate and procaine hydrolysis, and it was found that both enzymes are expressed in DLM, but not in DIM (Figs. 6B and C). This result was consistent with a previous report showing no expression of CES1 and CES2 in dog intestine (Taketani et al., 2007). Collectively, it was demonstrated that AADAC, but not CES enzymes, is expressed in dog intestine.

The $K_m$ values of recombinant dog AADAC and DIM for prasugrel hydrolysis showed similar values (Table 3). In addition, inhibition profiles of recombinant dog AADAC and DIM were similar (Fig. 7B). The activity in DIM ($V_{\text{max}}$, 29.8 ± 2.0 nmol/min/mg) was much lower than that in DLM ($V_{\text{max}}$, 1326.2 ± 107.2 nmol/min/mg) (data not shown), and the tendency was similar to the finding for phenacetin hydrolysis (Fig. 6A). These results suggest that AADAC majorly contributes to prasugrel hydrolysis in dog intestine. The inhibition potencies of DFP and eserine to prasugrel hydrolysis by dog AADAC appeared to be slightly lower than those by human AADAC. It was reported that the $IC_{50}$ value of loperamide was
0.562 µM for purified human CES2, whereas that for purified dog CES2 was 93.6 µM (Williams et al., 2011). Thus, the differences in the inhibitory potencies of DFP and eserine are due to species differences.

The CL\text{int} value of prasugrel hydrolase activity in dog intestine was 20-fold lower than that in human intestine (Tables 1 and 3). The difference may be explained by the difference in the expression levels of CES2 and AADAC between human and dog intestine. CES2 is highly expressed in human intestine, but not in dog intestine. Because phenacetin hydrolase activity in DIM (322.5 ± 65.1 pmol/min/mg protein at 5 mM phenacetin) was lower than that in HIM (1927.5 ± 67.9 pmol/min/mg protein, data not shown), it was surmised that AADAC expression in dog intestine would be much lower than that in human intestine. Nevertheless, it has been reported that prasugrel is extensively hydrolyzed in the small intestine after oral doses in dog (Hagihara et al, 2011). This would be due to the high absolute value of CL\text{int} of prasugrel hydrolysis in DIM (5.6 ± 0.3 mL/min/mg protein).

In our recent study, we investigated the inhibitory potencies of 542 compounds against human AADAC using a recombinant enzyme, and found that several compounds or drugs, such as benz bromarone and vinblastine, potently inhibited AADAC activity (Shimizu et al., 2014b). However, there are no clinical reports for drug-drug interaction in which the involvement of AADAC is suspected, probably because such combination would be rare in clinical. As for prasugrel, drug-drug interaction caused by metabolic inhibition has not been reported. One of the feasible reasons would be that the clearance of prasugrel hydrolysis in both intestine and liver is much high. If drugs that are specifically hydrolyzed by AADAC with low Km values are found or developed, we would have to pay attention to the possibility of drug-drug interaction.

In conclusion, we found that prasugrel hydrolysis is catalyzed not only by CES enzymes but also AADAC in human. AADAC majorly contributes to prasugrel hydrolysis in dog intestine, where CES1 and CES2 are not expressed. It should be appreciated that AADAC is an important esterase to be considered in drug development.
AUTHORSHIP CONTRIBUTION

Participated in research design: Kurokawa, Fukami and Nakajima.

Conducted experiment: Kurokawa, Fukami, and Yoshida.

Contributed new reagent or analytic tools: Kurokawa and Fukami.

Performed data analysis: Kurokawa and Fukami.

Wrote or contributed to the writing of the manuscript: Kurokawa, Fukami and Nakajima.
References


metabolite formation from thienopyridine antiplatelet agents, prasugrel and clopidogrel.

*Drug Metab Dispos* **37**:2145-2152.


Footnotes

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Figure Legends

Fig. 1. Metabolic pathways of prasugrel and responsible enzymes in human. A previous study demonstrated that prasugrel is hydrolyzed by CES2. Because prasugrel is hydrolyzed in dog intestine where CES2 is not expressed, the involvement of enzyme(s) other than CES2 is presumed.

Fig. 2. LC-MS/MS and HPLC chromatograms of prasugrel and prasugrel thiolactone. (A) In the MRM mode, the ion transitions of \(m/z\) 374.1 > 206.8 for prasugrel and \(m/z\) 332.1 > 109.0 for prasugrel thiolactone were monitored. Prasugrel (100 µM) was incubated with HLM for 60 min, and the incubation mixtures were subjected to LC-MS/MS. (B) The fraction of prasugrel thiolactone confirmed with MRM was injected to HPLC. The eluent was monitored at 254 nm. (C – E) (C) HLM (0.1 mg/mL), (D) prasugrel (10 µM), and (E) HLM and prasugrel were incubated at 37°C for 15 min and then analyzed with HPLC. Peak 1, prasugrel thiolactone; Peak 2, prasugrel.

Fig. 3. Kinetic analyses of prasugrel hydrolase activity by recombinant human esterases and human tissue microsomes. (A) Recombinant human AADAC, CES1, or CES2 (0.01 mg/mL), and (B) HIM or HLM (0.01 mg/mL) were incubated with prasugrel for 1 min. Prasugrel hydrolase activity was measured by HPLC. Each point represents the mean ± SD of triplicate determinations.

Fig. 4. Inhibitory effects of chemical inhibitors on prasugrel hydrolase activity. HIM or HLM (0.01 mg/mL) were incubated with 5 µM prasugrel for 1 min. The control activity values by HIM and HLM were 267.5 ± 12.5 nmol/min/mg protein and 248.0 ± 13.8 nmol/min/mg protein, respectively. Each column represents the mean ± SD of triplicate determinations.
Fig. 5. Contribution of each esterase to prasugrel hydrolysis in HIM and HLM. Extrapolation was determined by RAF using HIM, HLM, and recombinant human enzymes. Prasugrel concentration used in this study was 5 µM.

Fig. 6. Hydrolase activities of phenacetin, fenofibrate, and procaine in DIM and DLM. DIM or DLM ((A) 0.4 mg/mL, (B) 0.025 mg/mL, and (C) 0.5 mg/mL) were incubated with (A) 5 mM phenacetin specific for AADAC, (B) 5 µM fenofibrate specific for CES1, and (C) 1 mM procaine specific for CES2, respectively. Each column represents the mean ± SD of triplicate determinations. N.D.: Not detected.

Fig. 7. (A) Kinetic analyses of prasugrel hydrolase activity by DIM and recombinant dog AADAC. DIM (0.03 mg/mL) or recombinant dog AADAC (0.005 mg/mL) were incubated with prasugrel for 1 min. Prasugrel hydrolase activity was measured by HPLC. Each point represents the mean ± SD of triplicate determinations. (B) Inhibitory profile of chemical inhibitors against prasugrel hydrolase activity. DIM (0.03 mg/mL) or recombinant dog AADAC (0.005 mg/mL) were incubated with 5 µM prasugrel for 1 min. The control activity values by DIM and recombinant dog AADAC were 9.55 ± 0.14 and 4.23 ± 0.13 nmol/min/mg protein, respectively. Each column represents the mean ± SD of triplicate determinations.
TABLE 1
Kinetic parameters of prasugrel hydrolysis by recombinant esterases and human tissue microsomes.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Km (µM)</th>
<th>Vmax (nmol/min/mg protein)</th>
<th>CLint (mL/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AADAC</td>
<td>4.6 ± 0.2</td>
<td>228.4 ± 13.6</td>
<td>50.0 ± 1.2</td>
</tr>
<tr>
<td>CES1</td>
<td>16.7 ± 1.9</td>
<td>76.5 ± 6.9</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>CES2</td>
<td>17.1 ± 1.8</td>
<td>111.7 ± 6.8</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td>HIM</td>
<td>5.3 ± 0.2</td>
<td>560.6 ± 20.7</td>
<td>106.4 ± 1.4</td>
</tr>
<tr>
<td>HLM</td>
<td>4.6 ± 0.5</td>
<td>382.0 ± 27.9</td>
<td>83.9 ± 3.1</td>
</tr>
</tbody>
</table>
TABLE 2
RAF values calculated from the marker activity and the contributions of AADAC, CES1, and CES2 to prasugrel hydrolysis in HIM and HLM.

<table>
<thead>
<tr>
<th>Hydrolase activity</th>
<th>RAF</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AADAC</td>
<td>CES1</td>
</tr>
<tr>
<td>Indiplon Fenofibrate Procaine Prasugrel</td>
<td>pmol/min/mg protein</td>
<td>nmol/min/mg protein</td>
</tr>
<tr>
<td>AADAC</td>
<td>265.0</td>
<td>136.7</td>
</tr>
<tr>
<td>CES1</td>
<td>84.0</td>
<td>19.2</td>
</tr>
<tr>
<td>CES2</td>
<td>4.3</td>
<td>40.2</td>
</tr>
<tr>
<td>HIM</td>
<td>221.0</td>
<td>0</td>
</tr>
<tr>
<td>HLM</td>
<td>338.3</td>
<td>421.4</td>
</tr>
</tbody>
</table>
### TABLE 3

Kinetic parameters of prasugrel hydrolysis by DIM and dog AADAC.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$CL_{int}$ (mL/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIM</td>
<td>5.3 ± 0.6</td>
<td>29.8 ± 2.0</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>AADAC</td>
<td>1.5 ± 0.1</td>
<td>67.4 ± 6.5</td>
<td>45.9 ± 3.1</td>
</tr>
</tbody>
</table>
Fig. 1

Prasugrel → Prasugrel thiolactone → Active metabolite

- CES2
- CYP3A4
- CYP2B6
**Fig. 2**

A

![Graph showing retention time vs. intensity (cps)](image)

- **Prasugrel**
  - $m/z$ 374.1 $\rightarrow$ 206.8

- **Prasugrel thiolactone**
  - $m/z$ 332.1 $\rightarrow$ 109.0

B

- **Standard**
  - 1

C

- **Blank**
  - (No prasugrel)

D

- **Blank**
  - (No enzyme)

E

- **Sample**
  - 1
Fig. 3

(A) Prasugrel hydrolyase activity (nmol/min/mg protein) against Prasugrel concentration (μM) for AADAC, CES2, and CES1.

(B) Prasugrel hydrolyase activity (nmol/min/mg protein) against Prasugrel concentration (μM) for HIM and HLM.
Fig. 6

Panel A: Phenacetin hydrolyase activity (nmol/min/mg protein)
- DIM: 0.5 nmol/min/mg protein
- DLM: 4.0 nmol/min/mg protein

Panel B: Fenofibrate hydrolyase activity (nmol/min/mg protein)
- DIM: N.D.
- DLM: 160 nmol/min/mg protein

Panel C: Procaine hydrolyase activity (nmol/min/mg protein)
- DIM: N.D.
- DLM: 3.5 nmol/min/mg protein
Fig. 7

A

Prasugrel hydrolyase activity (nmol/min/mg protein)

Prasugrel concentration (μM)

AADAC

DIM

B

Remaining activity (%)

DFP  Eserine  PMSF  Termisartan

DIM  AADAC