Title : Human intestinal Raf kinase inhibitor protein, RKIP, catalyzes prasugrel as a bioactivation hydrolase

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Running Title: New role of RKIP in the bioconversion of prodrug.

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Abbreviations: HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography/tandem mass spectrometry; RKIP, Raf1-kinase inhibitor protein; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; DMSO, dimethylsulfoxide; Tris, tris(hydroxymethyl)aminomethane, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GFP, green fluorescent protein; prasugrel, 2-acetoxy-5-(α-cyclopropylcarbonyl-2-fluorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-c]pyridine; hCE, human carboxylesterase; thiolactone metabolite, 2-[2-oxo-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl]-1-cyclopropyl-2-(2-fluorophenyl) ethanone; R-135766, (2Z)-(1-2-[(2,2,3,3-2H4)Cyclopropyl]-1-(2-fluorophenyl)-2-oxoethyl)-4-{[3H]methylsulfanyl}piperidin-3-ylidene)ethanoic acid.
Abstract

Prasugrel is a thienopyridine antiplatelet prodrug that undergoes rapid hydrolysis in vivo to a thiolactone metabolite by human carboxylesterase-2 (hCE2) during gastrointestinal absorption. The thiolactone metabolite is further converted to a pharmacologically active metabolite by cytochrome P450 isoforms. The aim of the current study was to elucidate hydrolases other than hCE2 involved in the bioactivation step of prasugrel in human intestine. Using size exclusion column chromatography of a human small intestinal S9 fraction, another peak besides hCE2 peak was observed to have prasugrel hydrolyzing activity and this protein was found to be ca. 20 kDa molecular weight. This prasugrel hydrolyzing protein was successfully purified from a monkey small intestinal cytosolic fraction by successive four-step column chromatography, and identified as Raf-1 kinase inhibitor protein (RKIP) by liquid chromatography mass spectrometry (LC-MS/MS). Secondly, we evaluated the enzymatic kinetic parameters for prasugrel hydrolysis using recombinant human RKIP and hCE2 and estimated the contributions of these two hydrolyzing enzymes to the prasugrel hydrolysis reaction in human intestine, which were approximately 40% for hRKIP and 60% for hCE2. Moreover, prasugrel hydrolysis was inhibited by anti-hRKIP antibody and carboxylesterase-specific chemical inhibitor (BNPP) by 30% and 60%, respectively. In conclusion, another protein capable of hydrolyzing prasugrel to its thiolactone metabolite was identified as RKIP and this protein may play a significant role with hCE2 in prasugrel bioactivation in human intestine. RKIP is known to have diverse functions in many intracellular signaling cascades, but this is the first report describing RKIP as a hydrolase involved in drug metabolism.
Introduction

Prasugrel (marketed as Effient® in the USA, and as Efient® in Europe, Japan and other countries) is a 3rd generation of thienopyridine antiplatelet prodrug (Angiolillo et al, 2008) indicated for the reduction of thrombotic cardiovascular events in patients with acute coronary syndrome who are being managed by percutaneous coronary intervention (PCI). Prasugrel was not detected in the plasma, urine or feces after oral administration of prasugrel to humans, because it is rapidly absorbed and extensively metabolized in human (Farid et al, 2007). Prasugrel undergoes rapid hydrolysis in vivo to a thiolactone metabolite, which is further converted to a pharmacologically active metabolite by cytochrome P450 isoforms (Figure 1). This rapid hydrolysis reaction of prasugrel may contribute to the rapid onset and the low individual variability of pharmacological effect of prasugrel compared with other thienopyridine antiplatelet agents such as clopidogrel (Tcheng et al, 2012).

We previously reported based on in vitro experiments that prasugrel was converted to a thiolactone metabolite by human carboxylesterase 2 (hCE2) and human carboxylesterase 1 (hCE1) (Williams et al, 2008). The hydrolysis of prasugrel was at least 25 times greater with hCE2 than hCE1. Prasugrel hydrolysis by hCE2 exhibited substrate inhibition at high substrate concentration, although this in vitro observation did not translate to in vivo relevance (Williams et al, 2008). A linear relationship has been shown between the prasugrel dose and the plasma exposure to thiolactone metabolite in human (Asai et al, 2006). Therefore, it was suggested that the formation of thiolactone metabolite from prasugrel was catalyzed by not only hCES but other unknown enzymes. The aim of this study was to identify the hydrolysis enzymes other than hCE2 involved in the hydrolysis of prasugrel in the intestine. Another aim was to determine the contribution of hCE2 and the newly identified enzyme to the hydrolysis of prasugrel to the thiolactone metabolite in human intestine by estimating enzyme kinetic parameters and enzyme inhibition.
Materials and Methods

Materials

Prasugrel, thiolactone metabolites (racemate) and R-135766 (internal standard for thiolactone assay) were synthesized by Ube Industries, Ltd. (Ube, Japan) (Supplementary Figure S1). Protease inhibitor cocktail (cOmplete mini: mixture of serine, cysteine and metaloprotease inhibitors) was purchased from Roche Applied Science (Mannheim, Germany). Bis p-nitrophenyl phosphate (BNPP) was purchased from Sigma-Aldrich Corporation (St. Louis, MO). Laemmli sample buffer, Flamingo fluorescent gel stain and 5-20% sodium dodecyl sulfate acrylamide gel were purchased from Bio-Rad Laboratories Ink. (Richmond, CA). Bovine serum albumin (BSA) was purchased from Thermo Scientific Pierce (Rockford, IL).

Biological samples

Pooled mixed gender human intestinal S9 was purchased from Xeno Tech, LLC (Lenexa, KS) and four individual human small intestinal S9 (HIS-063-S3: 16.6 mg protein/mL, HIS-067-S3: 12.6 mg protein/mL, HIS-084-S3: 19.9 mg protein/mL, HIS-111-S3: 5.7 mg protein/mL) were purchased from the non profit Human and Animal Bridging Research Organization (Chiba, Japan). Ethical approval was obtained from the ethics committee of Daiichi Sankyo Co., Ltd. FreeStyle 293F cells, cell culture media and 293fectin were purchased from Invitrogen (Carlsbad, CA). FLAG-M2 agarose was purchased from Sigma-Aldrich. The mouse anti-human Raf Kinase Inhibitor Protein (hRKIP) antibody was obtained from Invitrogen. For immuno-depletion, rabbit anti-hRKIP antibodies and the control rabbit IgG were produced by Immuno-Biological Laboratories Co., Ltd (Gunma, Japan). A rabbit polyclonal antibody to RKIP (RKIP (FL-187): sc-28837) was purchased from Santa Cruz Biotechnology, Inc. A rabbit monoclonal antibody to hCE2 was constructed in Immuno-Biological Laboratories Co., Ltd. Amersham ECL-anti-rabbit IgG, Horseradish Peroxidase-Linked Species-Specific Whole Antibody (ECL-anti-rabbit IgG-HRP Linked) and Amersham ECL Advance Western Blotting Detection Kit were purchased from GE Healthcare UK Limited. Peptide N-Glycosidase F (PNGase F, Lot 0360910) was obtained from New England Biolabs Japan, Inc.

Preparation of monkey small intestinal subcellular fractions

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. All
preparation below was conducted at 4°C. The Cynomolgus monkey (HAMURI Co., Ltd) was euthanized and the small intestine was removed. The small intestine was homogenized in 9 volumes of homogenization buffer (10 mM HEPES-NaOH (pH 7.0), 0.25 M sucrose and protease inhibitor cocktail) using a polytron. The homogenate was centrifuged at 9,000g for 30 min at 4°C and the supernatant was used as the S9 fraction. Additionally, the S9 fraction was ultracentrifuged at 105,000g for 1 hour at 4°C and the supernatant and precipitate fractions were used as the cytosolic and the microsome fraction, respectively. The subcellular fractions were stored at -80°C until use.

**Gel filtration of human small intestine S9 and monkey small intestine cytosol**

Two hundred µL of human small intestinal S9 fraction (10 mg/mL) was loaded onto a gel filtration column (Superdex 75, GE Healthcare, USA) and eluted with 20 mL 100 mM HEPES (pH 7.0) at 0.5 mL/min with fraction size of 0.5 mL. Similarly, 200 µL of monkey small intestinal cytosolic fraction (10 mg/mL) was separated by the same experimental condition.

**Purification of endogenous enzyme**

The cytosolic fraction equivalent to 5 g monkey small intestine was dialyzed against 20 mM sodium acetate (pH 6.0). The dialysate was loaded onto a HiPrep Heparin column (GE Healthcare; 20 mL) and eluted with a 20 mL linear gradient of 0 to 0.5 M NaCl. Each fraction was tested for prasugrel hydrolysis activity, and analyzed by immunoblotting with the monoclonal antibody to hCE2. The flow-through fractions (fraction No. 5-10) were pooled, and applied to a mono S 5/50 GL column (GE Healthcare; 1 mL). The bound proteins were eluted with a 30 mL linear gradient of 0 to 0.5 M NaCl. Each fraction was tested for prasugrel hydrolysis activity and the active fractions were pooled and dialyzed against 20 mM Tris-HCl (pH 9.0) buffer. The dialyzed sample was applied to a mono Q 5/50 GL column (GE Healthcare; 1 mL), and proteins were eluted with 30 mL linear gradient of 0 to 0.5 M NaCl. Each fraction was tested for the prasugrel hydrolysis activity, and a part of fractions was loaded on SDS-PAGE. The active fractions were dialyzed against 20 mM sodium acetate (pH 6.0), and applied to a mini S PC 3.2/3 column (GE Healthcare; 0.24 mL). The column was eluted with a 7.2 mL linear gradient of 0 to 0.35 M NaCl, and the presence of purified enzyme in eluted fractions was confirmed by prasugrel hydrolysis activity and SDS-PAGE.

**Prasugrel hydrolase assay for subcellular localization, gel filtration and endogenous...**
**enzyme purification**

The subcellular fraction at a final concentration of 1 mg protein/mL or 30 µL of fractions from endogenous enzyme purification was mixed with prasugrel DMSO solution at a final concentration of 6 mM in a final volume of 50 µL of 100 mM HEPES buffer (pH7.0). The mixture was incubated at 37°C for 15 min followed by adding 100 µL methanol to terminate the reaction. The samples was filtrated by an Ultrafree-MC 0.45 µm PVDF membrane filter unit (Millipore) and 2 µL of the filtrate was injected into LC-MS system to determine the concentrations of the thiolactone metabolite.

In case of fractions of gel filtration separation, 30 µL of each fraction was tested as described above with slight modification, i.e. fraction were tested with or without 1 mM BNPP, a specific inhibito of human carboxyl esterase (hCE) and incubation time was extended to 60 min.

**Electrophoresis**

The monoQ and miniS active fractions were resolved by 5-20% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the gel was stained with Flamingo (Bio-Rad Laboratories, In.) and scanned by molecular imager FX (Bio-Rad Laboratories, In.) system.

**Protein identification by LC-MS/MS**

For protein separation, the active fractions from the mini S PC 3.2/3 column were loaded on a 5-20% SDS-PAGE gel. After staining of the gel with the Flamingo staining, each gel piece was excised from the gel. The gel piece was subjected to in-gel reduction and alkylation, followed by trypsin digestion (Modified trypsin, Promega). The resulting peptides were extracted and sequenced with LC-MS/MS on a DiNa nano-flow liquid chromatography system (KYA tech) coupled to a LTQ-Orbitrap (Thermo Fisher Scientific). The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ MS/MS acquisitions. Orbitrap MS full scans were acquired in the Orbitrap analyzer in using lock mass recalibration in real time. Resolution in the Orbitrap MS acquisition was set to r = 15000. The tandem mass spectra of the six most intense peptide ions with charge states ≥2 were collected. Data from LC/MS/MS measurements were searched against the Swiss-Plot database using the Mascot algorithm (Matrix science) with the following parameters: trypsin specificity, two missed cleavage, cysteine carbamidomethylation (fixed), protein N-term acetylated, methionine oxidation and asparagine, glutamine...
deamidated (variable), and ESI-TRAP fragmentation. The maximum allowed mass deviation for MS and MS/MS scans was 5 ppm and 0.8 Da, respectively. The resulting files were summarized and rearranged by an in-house developed software iMAP2.

Expression and purification of recombinant proteins
The expression vector of hCE2 fused with carboxyl terminal FLAG tag was prepared as described (Ishizuka et al, 2013). The expression vector of the N-terminal FLAG-tagged enhanced GFP was kindly provided by Dr. Keisuke Fukuchi from Daiichi Sankyo Co., Ltd. Human RKIP was cloned from cDNA library of human 293F cells and the expression vector was constructed with N-terminal FLAG tag under CMV promoter as described (Kubota et al, 2015). GFP, RKIP and hCE2 expression vectors (30 μg) were transfected into 293F cells (3 × 10⁷ cells) using 293fectin according to the manufacturer's protocol, respectively. The transfected cells were cultured for 72 h. The cell culture was centrifuged and the collected cells were suspended for 5 min on ice in lysis buffer (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.1% NP-40). The cell extracts were centrifuged, and the supernatant was used as a cell lysate. Proteins were purified using their FLAG-tag by affinity chromatography. Five μL of anti-FLAG M2 agarose was added to 300 μL of the lysate. After 2 h incubation at 4°C, the resin was collected by centrifuge and washed three times with 500 μL of lysis buffer. Proteins bound to the gel were eluted with 100 μL of lysis buffer containing 0.1 mg/mL FLAG peptide. Each purified recombinant protein (i.e. hCE2, RKIP, GFP as control) was subjected to 5-20% SDS-PAGE and visualized by Flamingo staining, which confirming more than 90% purity (Supplementary Figure S2). Additionally, we evaluated prasugrel hydrolysis activity with each recombinant protein.

Determination of the enzymatic kinetic parameters for thiolactone formation from prasugrel
The assay was performed by using recombinant RKIP and hCE2. The incubation mixture contained 1 μg protein/mL recombinant RKIP or 0.25 μg protein/mL recombinant hCE2; and 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320 and 640 μM prasugrel in a final volume of 200 μL of 0.1N HEPES buffer (pH 7.0). A mixture without prasugrel was preincubated at 37°C for 5 min, and the reaction was started by the addition of 2 μL of a solution of prasugrel in DMSO. After incubation at 37°C for 2 min, 50 μL of the incubation mixture was collected and added to 100 μL of acetonitrile and 50 μL of a solution of R-135766 as the internal standard (2 μM in acetonitrile) to terminate the reaction. The mixture was centrifuged at 15,000 rpm at
4°C for 3 min, and 5 μL of the supernatant was injected into LC-MS/MS system to determine the concentration of the thiolactone metabolite.

**Western blot analysis**
Western blotting was performed to determine the amount of RKIP and hCE2 in four individual human small intestinal S9.

**Assay of the contents of RKIP in human small intestinal S9**
Recombinant RKIP (100 μg/mL) was diluted with 0.1N HEPES buffer (pH 7.0) and prepared at the concentration of 2.5, 5, 10, 15 and 20 μg/mL. Each human small intestinal S9 (n=4) was prepared at the concentration of 0.8 mg protein/mL in the same way. These prepared samples and GFP as control were diluted twice with Laemmlli sample buffer containing 5% mercaptoethanol and boiled for 5 min at 100°C. Ten μL of boiled samples was loaded onto commercially available 12.5% SDS-polyacrylamide gel (Ready Gel J, Bio-Rad Laboratories, Inc.), respectively. The electrophoresis was performed at 140 V for 80 min. The proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc.) using a blotter (Trans-blot, Bio-Rad Laboratories, Inc.) at 15 V for 40 min. The PVDF membrane was blocked with blocking buffer (Tris-buffered saline containing 0.05% Tween 20 (TTBS) and 2% skimmed milk) for 1 h at room temperature. After washing the PVDF membrane with TTBS, the PVDF membrane was incubated with the antibody to RKIP (dilution 1: 400) for 1 h at room temperature. After washing the PVDF membrane with TTBS for 30 min, the PVDF membrane was incubated with ECL anti-rabbit IgG-HRP-linked (dilution 1: 5000) for 1 h at room temperature. After washing with TTBS and water, the PVDF membrane was incubated with coloring reagent (Lumigen TMA-6, Western blotting kits) for 5 min and was analyzed using lumino-image analyzer (LAS-4000UVmini, Fujifilm Co., Ltd.).

**Assay of the contents of hCE2 in human small intestinal S9**
PNGase F-treated recombinant hCE2 (50 μg/mL) was diluted with Laemmli sample buffer containing 5% mercaptoethanol and prepared at the concentration of 1, 2.5, 5, 7.5 and 10 μg/mL. Each human small intestinal S9 (n=4) was prepared at the concentration of 1.6 mg protein/mL with 0.1N HEPES buffer (pH 7.0). The diluted human small intestinal S9 was treated with PNGase F. PNGase F-treated human small intestinal S9 samples were diluted twice with Laemmli sample buffer containing 5%
mercaptoethanol and boiled for 5 min at 100°C. Ten μL of boiled samples was loaded onto commercially available 7.5% SDS-polyacrylamide gel, respectively. The details of the Western blot analysis are described above.

In this Western blotting, the monoclonal antibody to hCE2 (dilution 1:5000) was used as the primary antibody and an ECL anti-rabbit IgG-HRP-linked antibody (dilution 1:20000) was used as the secondary antibody.

**Determination of RKIP and hCE2 contents using Lumino-image Analyzer**

The expression protein (ng/μg S9) of RKIP and hCE2 in human small intestinal S9 was determined using LAS-4000UVmini. The amount of chemiluminescence (AU) of each enzyme was computed using Multi Gauge version 3.0 software (Fujifilm Corp.). The calibration curve was constructed by linear least squares regression, plotting the AU against each recombinant concentration. The calibration curve range was from 12.5 ng/10 μL to 100 ng/10 μL for RKIP and from 10.0 ng/10 μL to 100 ng/10 μL for hCE2.

**Inhibition study**

Human small intestine S9 fractions (20 μg) were incubated with the anti-RKIP antibody, or the control rabbit antibody (20 μg) in TNN buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 1% NP-40) overnight at 4°C. The immune complexes were removed by incubation with 10 μL of protein G-Sepharose (GE healthcare). Depletion of RKIP was monitored by immunoblotting with anti-RKIP antibodies. The depleted S9 fractions (10 μL each) were mixed with Laemmli sample buffer containing 5% β-mercaptoethanol and subjected to 5-20% SDS-PAGE and transferred to a PVDF membrane using the iBlot dry blotting system (Invitrogen). The PVDF membrane was treated with TBS-T (20 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20) containing 10% Aqua block (Rockland) for 1 h at room temperature. The blot was sequentially incubated with anti-RKIP antibody (1:5000 dilution) or anti-β-actin antibody (1:5000 dilution) as primary antibody and anti-mouse IgG antibody conjugated with horseradish peroxidase as secondary antibodies (1:50000 dilution; GE healthcare). The membrane was visualized with the ECL plus or advance (GE healthcare) and developed using a NightOWL imaging system (Berthold technologies GmbH, Germany).

After that, the incubation mixture contained each depleted S9 fraction (i.e. the rabbit IgG-depleted S9 fraction, RKIP-depleted S9 fraction) (0.2 mg/mL) in a final volume of 100 μL of 50 mM HEPES buffer (pH 7.0) with or without 0.1 mM BNPP. A mixture without prasugrel was preincubated at 37°C for 2 min, and a reaction was started by the
adding of 40 μM prasugrel in a DMSO solution. The reaction mixtures were incubated at 37°C for 20 min. A 10 μL aliquot was removed and mixed with 90 μL of methanol/H₂O (1:1, v/v) to terminate the reaction. A 25 μL of this sample was diluted with 75 μL of methanol/ H₂O (1:1, v/v) and was injected into LC-MS/MS system to determine the concentration of thiolactone metabolite.

**Assay of thiolactone metabolites by LC-MS/MS System**

Thiolactone metabolites and R-135766 were analyzed using the LC-MS/MS system as described below. Separation by HPLC was conducted using an Alliance 2695 Separations Module HPLC system (Waters Corp.) with an ODS column (Inertsil ODS-3, 150 × 2.1 mm I.D., 5.0 μm, GL Science Inc.) at a flow rate of 0.2 mL/min with a mobile phase consisting of methanol, distilled water and trifluoroacetic acid (TFA) (570 / 430 / 0.5 (v/v/v) or 520 / 480 / 0.5 (v/v/v)). The column temperature was set at 40°C and the injection volume was 2 or 5 μL. The mass spectra were determined using a Quattro LC-MS/MS system (Micromass UK Ltd.) in positive ion detection mode at the electrospray ionization (ESI) interface. The precursor ions of the thiolactone [M+H]⁺ at m/z 332 and R-135766[M+H]⁺ at m/z 548 (for the internal standard) were obtained in the first quadrupole (Q1). After the collision-induced fragmentation in the second quadrupole (Q2), the product ion of the thiolactone metabolite [M+H]⁺ at m/z 149 and R-135766[M+H]⁺ at m/z 206 was monitored in the third quadrupole (Q3). The peak area ratio of each compound to the internal standard was computed using MassLynx version 4.0 SP4 (Waters Corp.) software.

**Enzyme Kinetic Analysis**

The reaction rate (V, pmol/min/μg protein) and V/the substrate concentration (S, μM) were calculated using Microsoft Office Excel 2003 (version SP2, Microsoft Corp.) according to Equation 1 and Equation 2.

\[
V \text{(pmol/min/μg protein)} = \frac{\text{generated concentration (μM) } \times 1000}{1 \text{ or } 0.25 \text{ μg protein/mL } \times \text{incubation time (min)}} \quad \ldots(1)
\]

\[
\frac{V}{S} = \frac{\text{the reaction rate (pmol/min/μg protein)}}{\text{substrate concentration (μM)}} \quad \ldots(2)
\]

Initially the obtained data were analyzed by an Eadie-Hofstee plot (x-axis: V/S, y-axis: V). Eadie-Hofstee plots were used to visually detect deviation from linearity. The
formation of the thiolactone metabolites from prasugrel by the recombinant hCE2 indicated a non-straight line in the Eadie-Hofstee plot, suggesting the involvement of the substrate inhibition kinetic properties. Therefore, the data were fitted to Equation 3 using WinNonlin Professional (version 4.0.1, Pharsight Corporation) in order to calculate the $K_m$ and $V_{max}$ values. $S$ ($\mu$M) is the substrate concentration, $K_m$ ($\mu$M) is the Michaelis-Menten constant, $V_{max}$ (pmol/min/\(\mu\)g protein) is the maximal reaction rate and $K_i$ is the inhibition constant for the substrate.

$$V = \frac{V_{max}}{1 + (K_m/S) + (S/K_i)} \quad \ldots(3)$$

On the other hand, $K_m$ and $V_{max}$ for the recombinant RKIP were calculated by using WinNonlin professional based on a pharmacodynamic compiled model (model No.101) since the best model was a Michaelis-Menten kinetics model. The contribution ratio (%) of RKIP and hCE2 that was responsible for the formation of the thiolactone metabolite in human small intestinal S9 was determined by using Equations 4 and 5.

$$\text{CL}_{int} \ (\mu\text{L/min/ng enzyme concentration}) = \frac{V_{max} \times \text{each enzyme concentration (ng/\(\mu\)g human small intestinal S9)}}{K_m} \quad \ldots(4)$$

The contribution ratio (%) = \frac{\text{CL}_{int} \ for \ each \ enzyme \times 100}{\Sigma \text{CL}_{int} \ for \ each \ enzyme} \quad \ldots(5)

Additionally, the remaining hydrolysis activity (%) in the inhibition study was determined by Equation 6 and 7.

The remaining hydrolysis activity (%) = \frac{\text{RKIP-depleted S9 with or without BNPP (nmol/mg/min) \times 100}}{\text{Control antibody treated S9 without BNPP (nmol/mg/min)}} \quad \ldots(6)

The remaining hydrolysis activity (%) = \frac{\text{Depleted S9 with BNPP (nmol/mg/min) \times 100}}{\text{Control antibody treated S9 without BNPP (nmol/mg/min)}} \quad \ldots(7)

The calculated $V$ and $V_{max}$ are expressed to an integral number. The calculated $K_m$ is expressed to three significant figures. The mean values, SD of the contribution ratio
and the remaining hydrolysis activity are also expressed to three significant figures. However, if the calculated values exceeded three digits, it was expressed to an integral number.
Results
Identification of several hydrolases in human and monkey small intestine
To define subcellular localization of the hydrolase involved in the prasugrel hydrolysis reaction, we determined the prasugrel hydrolysis activity using each subcellular fraction (i.e. homogenate, S9, microsome, cytosol) of monkey small intestine. The hydrolysis activity of prasugrel was localized mainly in the cytosolic fraction of monkey small intestine (Figure 2). Additionally, the gel filtration of human small intestinal S9 and monkey small intestinal cytosolic fraction was performed using a Superdex 75 column in order to confirm the characterization of expressing hydrolysis enzymes of prasugrel in human and monkey (Figure 3). Two hydrolysis activity peaks of prasugrel were detected in the human small intestinal S9 fractions. The first peak was observed at about 60 kDa molecular weight and the prasugrel hydrolase activity in this peak was inhibited by 70% with BNPP, a CES-specific chemical inhibitor. On the other hand, prasugrel hydrolase activity in the second peak was not inhibited with BNPP and its molecular weight was estimated to be about 20 kDa (Figure 3A). These results suggested that the first peak activity consists of CES2, and there was an unidentified hydrolase in the second peak. In the case of monkey small intestinal cytosolic fraction, a roughly similar result was obtained as human small intestinal S9 fraction (Figure 3B).

Purification of hydrolase
The monkey intestinal cytosolic fraction was primarily subjected to the HiPrep heparin column and two hydrolysis activity peaks of prasugrel were detected (Figure 4A). Each fraction of heparin chromatography was analyzed by western blotting using anti-CES2 antibody (Figure 4B). It was shown that CES2 was included in the bound fractions (Figure 4B). Taken together, it was shown that the unknown prasugrel hydrolase activity was separated from the CES2 catalyzed activity and eluted in flow-through fraction in the HiPrep heparin step. In the next step of mono-S fractionation of HiPrep flow-through sample, the unknown prasugrel hydrolysis activities were observed both in the flow-through and bound fraction (data not shown). The physical basis of this heterogeneity was not determined. The bound fraction, containing most of the activity, was used for the next step of mono Q chromatography. SDS-PAGE and Flamingo staining of the mono Q fractions revealed a band of 21 kDa associated with prasugrel hydrolase activity (Figure 5A). Compared to other fractions, fractions 6 and 7 contained higher prasugrel hydrolyzing activity and only 21 kDa protein content was higher in these two fractions while other protein band contents were
not. Because the mono Q active fraction contained several bands (Figure 5A), we applied the mono Q active fraction (bound fraction) next to the mini S column. As a result of successive four-step purification, our target unknown prasugrel hydrolyzing protein was purified. SDS-PAGE and Flamingo staining of the mini S active fractions revealed a single band of 21 kDa associated with prasugrel hydrolysis activity (Figure 5B). Faint 60 kDa bands were observed but did not correlate with the activity. Those bands would be well known to be human keratins, which were often contaminated in small amounts during experiments and visualized by high sensitivity staining such as fluorescent dye and silver staining.

**Protein identification by LC-MS/MS**

To identify the protein that was associated with prasugrel hydrolysis activity, the mini S active fractions were subjected to SDS-PAGE, and the band of 21 kDa was excised and subjected to in-gel trypsin digestion. The fragmented peptides were analyzed by LC-MS/MS, identified by Mascot. Twenty-one peptides were found to match with the amino acid coverage of 96% for monkey Raf-1 kinase inhibitory protein (RKIP) (Figure 6).

**Estimation of the enzymatic kinetic parameters for prasugrel hydrolysis by hRKIP and hCE2**

The V and V/S values of prasugrel hydrolysis for recombinant hRKIP and hCE2 were determined according to Equation 1 and Equation 2 and Eadie-Hofstee plots of these data are shown in Figure 7. The enzyme kinetic parameters, K_m and V_max for recombinant hCE2, were estimated according to Equation 3, since the formation of thiolactone from prasugrel indicated a substrate inhibition pattern in the Eadie-Hofstee plots. On the other hand, K_m and V_max values for recombinant RKIP were calculated by Michaelis-Menten model since it fit the data best, although we tried other models such as a substrate inhibition model. The CV (%) for the parameter estimates in the Michaelis-Menten model was the smallest of the models tested.

In the formation of thiolactone metabolite from prasugrel using recombinant RKIP, K_m and V_max were 49.9±7.96 μM and 14114±647 pmol/min/μg protein (Table 1). Similarly, in the case of using recombinant hCE2, K_m and V_max were 49.8±2.54 μM and 54839±1510 pmol/min/μg protein (Table 1). Thiolactone formation from prasugrel in GFP was almost never detected (data not shown).

**Estimation of the contribution of hRKIP and hCE2 involved in the prasugrel**
hydrolysis in human small intestinal S9
We determined the contents of hRKIP and hCE2 in individual human small intestinal S9 by western blotting method (Figure 8). As results, RKIP and hCE2 in individual human small intestinal S9 were 7.48 (ng/μg S9) - 15.6 (ng/μg S9) and 2.24 (ng/μg S9) - 7.91(ng/μg S9), respectively. From these data and enzyme kinetic parameters, it was estimated that the contribution ratio of RKIP and hCE2 involved in the hydrolysis reaction of prasugrel in human small intestinal S9 was 42.9±9.82% (Mean±SD) and 57.1±9.82%, respectively (Table 2).

Inhibition study of prasugrel hydrolysis in human small intestinal S9
RKIP was immuno-depleted from human small intestinal S9 fraction using the anti-RKIP antibody (Figure 9A). This immuno-depletion of RKIP clearly inhibited prasugrel hydrolysis activity by about 34.7%, while inhibition by BNPP was about 50.7% (Figure 9B, Table 3).
Discussion

Prasugrel and clopidogrel are both thienopyridine-type antiplatelet prodrugs and both need to be bioactivated via a thiolactone intermediate to their pharmacologically active metabolites (Farid et al, 2010). Prasugrel was converted more rapidly and more efficiently to the thiolactone metabolite compared with clopidogrel, since for prasugrel, the thiolactone formation is via rapid ester group hydrolysis during gastrointestinal absorption. Prasugrel itself is not detected in the blood circulation, but there is rapid appearance of the thiolactone metabolite. On the other hand, conversion of clopidogrel to its thiolactone metabolite is via hepatic cytochrome P450 oxidation including CYP2C19 which is known to have large interindividual variability. Furthermore, most of the clopidogrel undergoes hydrolysis of its ester group to form a carboxylic acid metabolite which cannot be converted to the active metabolite. (Hagihara et al, 2009, Kazui et al, 2010, Tang M et al, 2006). We previously reported that prasugrel was converted to the thiolactone metabolite primarily by hCE2 with a lesser contribution by hCE1 and that at high prasugrel concentrations in excess of 109 µM substrate inhibition was observed for hCE2 (Williams et al, 2008). The prasugrel-concentrations in the gastrointestinal tract at the doses of 2.5-, 10- and 75 mg are calculated to be 26.8, 107, and 803 µM, respectively, assuming that these doses of prasugrel are dissolved in a standard glass of water (250 mL). If the metabolic enzyme involved in the formation of thiolactone in the small intestine is only hCE2, the human exposure to thiolactone metabolite might saturate at more than 10 mg doses of prasugrel, however, the human plasma exposure to the thiolactone metabolite after oral administration of prasugrel at the doses of 2.5-, 10- and 75 mg were dose dependent (Asai et al, 2006). So that means the observed inhibition in vitro does not translate to in vivo relevance. Thus, we hypothesized another enzyme other than hCE2 might contribute to the prasugrel hydrolysis in the intestine. We also tried to determine the contributions of hCE2 and unknown enzyme to the prasugrel hydrolysis process.

As first step, we found that the prasugrel hydrolysis activity was localized in the cytosolic fraction of monkey small intestine, in addition to microsome fraction which contains most of CESs, suggesting another prasugrel hydrolase exists in the cytosol fraction. (Figure 2). Accordingly, we compared prasugrel hydrolysis activity profiles of human intestinal S9 fraction and monkey intestinal cytosol fraction in size-exclusion column chromatography, which resulted in almost the same profiles. In both matrices, first CES activity peak and second unknown activity peak were observed (Figure 3A, Figure 3B). Therefore, the further purification of the unknown enzyme was performed using monkey small intestinal cytosolic fraction since human source availability was
limited. As a result of successive four-step column chromatography purification, target enzyme protein was successfully purified. SDS-PAGE and Flamingo staining of the mini S active fractions revealed a single band of 21 kDa associated with prasugrel hydrolysis activity (Figure 5A, Figure 5B). The fragmented peptides of the 21 kDa band were analyzed by LC-MS/MS, identified by Mascot. As a result, twenty-one peptides were found to match with the amino acid coverage of 96% for Raf-1 kinase inhibitory protein (RKIP) (Figure 6). RKIP is a widely known protein as a member of the phosphatidylethanolamine-binding protein (PEBP) family. It is a small, evolutionarily conserved cytosolic protein that plays a pivotal modulatory role in several protein kinase signaling cascades (Bazzi et al. 1992, Corbit et al. 2003, Lorenz et al. 2003, Yeung et al. 2000, Yeung et al. 2001). Additionally, it is reported that RKIP exerts a significant impact on controlling the cell cycle and is also associated with centrosomes and kinetochores in cultured mammalian cells (AL-Mulla et al. 2013). Taken together, RKIP play several roles in regulating the process of cell growth. It is already known that a lot of hydrolysis enzymes such as carboxylesterase (CE), paraoxonase (PON), butyrylcholinesterase (BChE), acetylcholinesterase (AChE), carboxymethylenebutenolidase (CMBL) and albumin are involved in the bioconversion of ester-based prodrugs (Liedere et al. 2006, Ishizuka et al. 2013). However, it is not known that RKIP plays a role of the drug metabolizing enzyme (i.e. hydrolase). This study is, to the best of the author’s knowledge, the first report describing hydrolase activity of RKIP.

We determined the contributions of hRKIP and hCE2 for the prasugrel hydrolysis by estimating the enzymatic kinetic parameters of recombinant enzymes and by the inhibition study using human small intestinal S9. The experiment for estimating the enzymatic kinetic parameters show that both RKIP and hCE2 have similar $K_m$ values for the hydrolysis of prasugrel, suggesting they bind prasugrel with similar affinity (Table 1), but the $V_{max}$ value for hCE2 appears to be 4 times higher than that for RKIP (Table 1).

From these enzyme kinetic parameters with the enzyme contents determined by Western method, contribution ratio of RKIP and hCE2 involved in the prasugrel hydrolysis in the human small intestinal S9 was estimated to be 42.9±9.82% and 57.1±9.82%, respectively (Table 2). Additionally, the prasugrel hydrolysis in human small intestinal S9 was inhibited about 30-40% by anti-RKIP antibody, and further 40-50% by BNPP (Table 3, Figure 9B). The data from the inhibition study was consistent with the estimated contribution ratio from the enzyme kinetic parameters obtained using the recombinant enzymes. Therefore, we judged that the contribution of RKIP and hCE2
to prasugrel hydrolysis in human intestine was about 30%–40% and 60%, respectively.
In conclusion, hRKIP was identified as capable of hydrolyzing prasugrel to its
thiolactone metabolite and may play a significant role with hCE2 in prasugrel
bioactivation in the human intestine. RKIP is known to have diverse functions as a
master modulator of many intracellular signaling cascades. This is the first report
describing a new function of RKIP as hydrolase involved in drug metabolism.

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Authorship Contributions
Participated in research design: Kazui, and Kurihara.
Conducted experiments: Kazui, Ogura, Kubota and Hagihara
Performed data analysis: Kazui, Ogura, and Kubota
Wrote or contributed to the writing of the manuscript: Kazui, and Kurihara
DMD #66290

Reference


Ishizuka T, Yoshigae Y, Murayama N, Izumi T. (2013) Different hydrolases involved in bioactivation of prodrug-type angiotensin receptor blockers:

20
carboxymethylenebutenolidase and carboxylesterase 1. Drug Metab Dispos. 41:1888-1895.


Declaration of Conflicting Interests
Miho Kazui, Katsunobu Hagihara, and Atsushi Kurihara are employees of Daiichi Sankyo Co., Ltd. Yugi Ogura and Kazuishi Kubota are employees of Daiichi Sankyo RD Novare Co., Ltd.

Footnote
The study was sponsored by Daiichi-Sankyo Co., Ltd, Tokyo, Japan.
Figure Legends

Figure 1. Simplified metabolic pathway for prasugrel
Figure does not illustrate all metabolites of prasugrel that have been identified. Asterisk mark expresses an asymmetric center. Prasugrel is a racemate, and thiolactone and the active metabolite are the mixture of four stereoisomers.

Figure 2. Localization of prasugrel hydrolase in monkey small intestine
Comparison of hydrolysis activity of respective fraction. The small intestine subcellular fractions were incubated with 6 mM prasugrel for 15 min at 37°C.

Figure 3. Chromatography of human small intestinal S9 and monkey small intestinal cytosolic fraction on Superdex 75 gel filtration column
Two hundred microliters human small intestine S9 fraction (A), or monkey small intestine cytosolic fraction (B) was applied to a Superdex 75 gel filtration column. Each fraction was incubated with 6 mM prasugrel for 60 min at 37°C in the presence or absence of BNPP. The positions of the native molecular weight markers are indicated at the top.

Figure 4. Chromatography of monkey small intestinal cytosol fraction on HiPrep Heparin column and western blotting of each elution
Heparin fractions were assayed for prasugrel hydrolysis activity. Each fraction was incubated with 6 mM prasugrel at 37°C for 60 min. (A) Hydrolysis activity was found in fractions flow-through (1-12) and bound (19-28). (B) Each fraction was analyzed by immunoblotting with the indicated antibodies. IN; input.

Figure 5. Purification of prasugrel hydrolase
(A) Prasugrel activity on the mono Q column. Mono Q fractions were incubated with 6 mM prasugrel for 60 min at 37°C. Hydrolase activity was found in fractions 6-14. The mono Q fractions were also resolved by 5-20% SDS-PAGE, and the gel was stained with Flamingo and scanned by molecular imager FX. The band of 21 kDa (indicated by arrows) was associated with the activity. (B) The mini S active fractions were resolved by SDS-PAGE, and the gel was stained with Flamingo. Mini S fractions were incubated with 6 mM prasugrel for 60 min at 37°C.
Figure 6. Identified amino acid sequence as prasugrel hydrolase using monkey small intestinal cytosolic fraction

The active fractions from mini S were subjected to SDS-PAGE, and the band of 21 kDa was subjected to LC-MS/MS analysis. The peptides found to be identical to monkey RKIP are shown in bold (GenBank accession No. P48737). The identical peptides covered 96% of RKIP amino acid sequence.

Figure 7. Kinetic analysis of the thiolactone produced from prasugrel using recombinant RKIP and hCE2 by Eadie-Hofstee plots (A) and direct plots (B)

Figure 8. Expression of RKIP and hCE2 in human small intestinal S9 by Western blot analysis

(A) Expression of RKIP in human small intestinal S9. Lane 1, 2, 3, 4, 5 are recombinant RKIP at the concentrations of 12.5, 25, 50, 75 and 100 ng/10 μL, respectively. Lane 6, 7, 8 and 9 are individual human small intestinal S9.

(B) Expression of hCE2 in human small intestinal S9. Lane 1, 2, 3, 4, 5 are recombinant hCE2 at the concentrations of 10, 25, 50, 75 and 100 ng/10 μL, respectively. Lane 6, 7, 8 and 9 are individual human small intestinal S9.

Figure 9. Immunodepletion of RKIP from human small intestine S9 fraction

(A) Immunoblot analysis of the human small intestine S9 fractions depleted of RKIP. The anti-RKIP antibodies or non-specific rabbit IgGs were used to immunodeplete the RKIP. The RKIP-depleted S9 fraction was subjected to SDS-PAGE followed by immunoblotting analysis with the indicated antibodies. β-actin was used as an internal control. (B) The relative prasugrel hydrolysis activity of the RKIP-depleted S9 fraction. The depleted S9 fractions were incubated with 40 μM prasugrel for 20 min at 37°C in the presence or absence of 0.1 mM BNPP. The data is mean (n = 2).
Table 1 Enzymatic kinetic parameters of the thiolactone metabolite produced from prasugrel using recombinant RKIP and hCE2

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/µg protein)</th>
<th>$V_{max}/K_m$ (µL/min/µg protein)</th>
<th>Ki</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKIP</td>
<td>49.9 ± 7.96</td>
<td>14114 ± 647</td>
<td>283</td>
<td>N.D.</td>
</tr>
<tr>
<td>hCE2</td>
<td>49.8 ± 2.54</td>
<td>54839 ± 1510</td>
<td>1101</td>
<td>1380 ± 498</td>
</tr>
</tbody>
</table>

Enzymatic kinetic parameters were expressed as mean±SE of parameter estimate. N.D. is expressed as no data. The formation of thiolactone metabolite from prasugrel in the recombinant hCE2 indicated a non-straight line in the Eadie-Hofstee plot, suggesting the involvement of the substrate inhibition kinetic properties. Therefore, the data were fitted to Equation 3 in order to calculate the $K_m$ and $V_{max}$ values. $S$ (µM) is the substrate concentration, $K_m$ (µM) is the Michaelis-Menten constant, $V_{max}$ (pmol/min/µg protein) is the maximal reaction rate and $K_i$ is the inhibition constant for the substrate. $K_m$ and $V_{max}$ for the recombinant RKIP were calculated by using WinNonlin professional based on a pharmacodynamic compiled model (model No.101).
Table 2  Estimation of the contribution ratio of RKIP and hCE2 involved in the formation of the thiolactone metabolite from prasugrel in human small intestinal S9 by the enzymatic kinetic parameters

<table>
<thead>
<tr>
<th></th>
<th>$V_{max}/K_m$ (μL/min/μg protein)</th>
<th>Individual human small intestinal S9</th>
<th>Enzyme contents (ng/human small intestinal S9 μg)</th>
<th>CLint (μL/min/μg enzyme contents)</th>
<th>Contribution ratio (%)</th>
<th>Individual mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKIP</td>
<td>283</td>
<td>HIS-063-S3</td>
<td>14.9</td>
<td>4217</td>
<td>52.7</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>HIS-067-S3</td>
<td>12.8</td>
<td>3622</td>
<td>29.4</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>HIS-084-S3</td>
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<td>4415</td>
<td>43.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIS-111-S3</td>
<td>7.48</td>
<td>2117</td>
<td>46.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hCE2</td>
<td>1101</td>
<td>HIS-063-S3</td>
<td>3.44</td>
<td>3787</td>
<td>47.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HIS-067-S3</td>
<td>7.91</td>
<td>8709</td>
<td>70.6</td>
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<td></td>
<td></td>
<td>HIS-084-S3</td>
<td>5.24</td>
<td>5769</td>
<td>56.6</td>
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<tr>
<td></td>
<td></td>
<td>HIS-111-S3</td>
<td>2.24</td>
<td>2466</td>
<td>53.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The contents of RKIP and hCE2 in individual human small intestinal S9 (n = 4) were determined using recombinant RKIP and PNGase F-treated hCE2 by Western blotting method. The contribution ratio of RKIP and hCE2 involved in the hydrolysis reaction of prasugrel in human small intestinal S9 was estimated by using the obtained contents data and enzyme kinetic parameters.
Table 3  Immunodepletion of RKIP and effect of BNPP

<table>
<thead>
<tr>
<th>Individual human small intestinal S9</th>
<th>Production of thiolactone form (nmol/mg protein/min)</th>
<th>Remaining hydrolysis activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (BNPP (-))</td>
<td>Anti-RKIP treatment (BNPP (+))</td>
</tr>
<tr>
<td>HIS-063-S3</td>
<td>54.5</td>
<td>33.4</td>
</tr>
<tr>
<td>HIS-111-S3</td>
<td>50.5</td>
<td>18.8</td>
</tr>
</tbody>
</table>

The relative prasugrel hydrolase activity of RKIP depleted human small intestine S9. The depleted S9 fractions were incubated with 40 μM prasugrel for 20 min at 37°C in the presence of or absence of 0.1 mM BNPP.
Prasugrel: racemate
Thiolactone, active metabolite: mixture of four stereoisomers

*: Asymmetric center
Prasugrel: racemate
Thiolactone, active metabolite: mixture of four stereoisomers
Figure 2

Prasugrel hydrolysis activity [μg/mL]

Homogenate  S9  Microsome  Cytosol
Figure 3

A

Prasugrel hydrolysis activity

Fr.8 Fr.9 Fr.10 Fr.11 Fr.12 Fr.13 Fr.14 Fr.15 Fr.16 Fr.17 Fr.18 Fr.19 Fr.20 Fr.21 Fr.22 Fr.23 Fr.24 Fr.25 Fr.26 Fr.27 Fr.28 Fr.29 Fr.30 Fr.31 Fr.32 Fr.33 Fr.34 Fr.35 Fr.36 Fr.37

BNPP +
BNPP -

CES2
Human small intestine S9 fraction

B

Prasugrel hydrolysis activity

Fr.8 Fr.9 Fr.10 Fr.11 Fr.12 Fr.13 Fr.14 Fr.15 Fr.16 Fr.17 Fr.18 Fr.19 Fr.20 Fr.21 Fr.22 Fr.23 Fr.24 Fr.25 Fr.26 Fr.27 Fr.28 Fr.29 Fr.30 Fr.31 Fr.32 Fr.33 Fr.34 Fr.35 Fr.36 Fr.37

BNPP +
BNPP -

CES2
Monkey small intestine cytosolic fraction

A

(kDa)

(kDa)
Figure 4

A: HiPrep Heparin chromatography

Prasugrel hydrolysis activity [μg/mL]

Fr.1 & 2
Fr.3 & 4
Fr.5 & 6
Fr.7 & 8
Fr.9 & 10
Fr.11 & 12
Fr.13 & 14
Fr.15 & 16
Fr.17 & 18
Fr.19 & 20
Fr.21 & 22
Fr.23 & 24
Fr.25 & 26
Fr.27 & 28
Fr.29 & 30
Fr.31 & 32
Fr.33 & 34
Fr.35 & 36
Fr.37 & 38
Fr.39 & 40

Flow-through fraction

Bound fraction

conductivity [mS] (-----)

0  50  100  150  200  250  300

0  80

B: Western blotting

anti-CES2
Figure 5

**A**

Prasugrel hydrolysis activity [μg/mL] vs. fraction numbers (Fr.) 5 to 15.

**B**

Prasugrel hydrolysis activity [μg/mL] vs. fraction numbers (Fr.) 5 to 15 for mini S.

(kDa)
Figure 6

1 MPVDLSK WSG PLSQEVEDEQ PQHPLHVTYA GAALDELGKV LTPTQVKNP
51 TSISWDGLDS GKLTYTLVLTDPDAPSRKDPKYREWHHFLVVNMKGNDISSG
101 TVLSDYVGSG PPKGTGLHRY VWLVYEQARP LKCDEPILSN RSGDHRGKFK
151 VASFRKKYEL GAPVAGACYQ AEWDDYVPKL YEQLSGK
Figure 8

(A)

(B)
Figure 9

(A) HIS-063-S3  HIS-111-S3

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>Δ RKIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIS-063-S3</td>
<td>anti-RKIP</td>
<td>anti-β-actin</td>
</tr>
<tr>
<td>HIS-111-S3</td>
<td>anti-RKIP</td>
<td>anti-β-actin</td>
</tr>
</tbody>
</table>

(B) The remaining hydrolysis activity (%)

<table>
<thead>
<tr>
<th></th>
<th>BNPP (-)</th>
<th>BNPP (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIS-063-S3 ctrl</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>HIS-063-S3 RKIP</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>HIS-111-S3 ctrl</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>HIS-111-S3 RKIP</td>
<td>60</td>
<td>40</td>
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