ORIGINAL ARTICLE

Identification of activating enzymes of a novel FBPase inhibitor prodrug, CS-917

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Abstract

CS-917 (MB06322) is a selective small compound inhibitor of fructose 1,6-bis-phosphatase (FBPase), which is expected to be a novel drug for the treatment of type 2 diabetes by inhibiting gluconeogenesis. CS-917 is a bisamidate prodrug and activation of CS-917 requires a two-step enzyme catalyzed reaction. The first-step enzyme, esterase, catalyzes the conversion of CS-917 into the intermediate form (R-134450) and the second-step enzyme, phosphoramidase, catalyzes the conversion of R-134450 into the active form (R-125338). In this study, we biochemically purified the CS-917 esterase activity in monkey small intestine and liver. We identified cathepsin A (CTSA) and elastase 3B (ELA3B) as CS-917 esterases in the small intestine by mass spectrometry, whereas we found CTSA and carboxylesterase 1 (CES1) in monkey liver. We also purified R-134450 phosphoramidase activity in monkey liver and identified sphingomyelin phosphodiesterase, acid-like 3A (SMPADL3A), as an R-134450 phosphoramidase, which has not been reported to have any enzyme activity. Recombinant human CTSA, ELA3B, and CES1 showed CS-917 esterase activity and recombinant human SMPDL3A showed R-134450 phosphoramidase activity, which confirmed the identification of those enzymes. Identification of metabolic enzymes responsible for the activation process is the requisite first step to understanding the activation process, pharmacodynamics and pharmacokinetics of CS-917 at the molecular level. This is the first identification of a phosphoramidase other than histidine triad nucleotide-binding protein (HINT) family enzymes and SMPDL3A might generally contribute to activation of the other bisamidate prodrugs.

Abbreviations

APTX, ataxia; ASML3a, acid sphingomyelinase-like phosphodiesterase 3a; BNPP, bis(4-nitrophenyl) phosphate; BSA, bovine serum albumin; CES, carboxylesterase; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid; CMBL, carboxymethylenebutenolidase homolog; CTSA, cathepsin A; DBCCR1, deleted in the bladder cancer chromosome region 1; DM, dodecyl-β-maltoside; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; ELA, elastase; FBPase, fructose 1,6-bisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIC, hydrophobic interaction chromatography; HINT, histidine triad nucleotide-binding protein; HIT, histidine triad; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LXRs, liver X receptors; MES, 2-(N-morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS-

PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SMPDL3A, sphingomyelin phosphodiesteraseacid-like; TEAA, triethylamine acetate.

Introduction

CS-917 (MB06322) is a selective small molecule inhibitor of fructose 1,6-bisphosphate (FBPase) (Erion et al. 2005; van Poelje et al. 2007). Inhibition of FBPase will lead to the suppression of gluconeogenesis (Yoshida et al. 2008), and thus, CS-917 was expected to be a drug for type 2 diabetes with a novel mode of action.
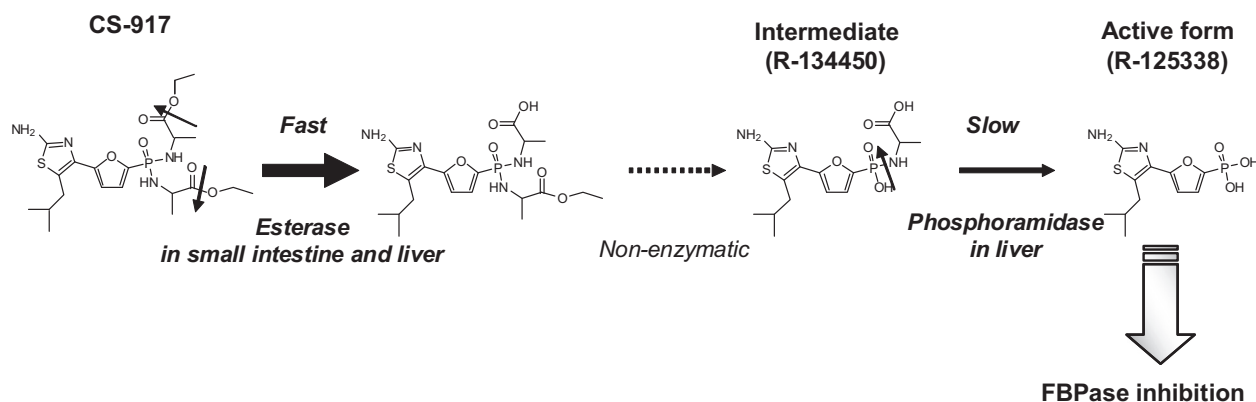
CS-917 is a bisamidate prodrug of R-125338, which inhibits FBPase in the human liver in a low nano-molar range (IC50: 16 nmol/L) (Erion et al. 2005). R-125338 was designed to target the adenosine monophosphate (AMP)-binding site of FBPase using a structure-based drug design (Erion et al. 2007). Although R-125338 showed good FBPase inhibition, its oral bioavailability was not sufficient. To improve the oral bioavailability of R-125338, CS-917 was designed as a bisamidate prodrug of R-125338, which gave us sufficient bioavailability and FBPase inhibition in vivo (Dang et al. 2007).

After oral administration, CS-917 is converted into the active form, R-125338, by a two-step enzyme-catalyzed reaction (Fig. 1) in rat hepatocytes (Dang et al. 2007) and primary human hepatocytes (Erion et al. 2005). In the first step, CS-917 is hydrolyzed by an esterase and the resultant monoester is spontaneously hydrolyzed to form a monoamidate intermediate (R-134450), mainly in the first-pass metabolism in the small intestine and liver. Then in the second step, the P–N bond of the intermediate is hydrolyzed by a phosphoramidase to generate the active form, R-125338, mainly in the liver. Investigation of the conversion rate using rat, monkey, and human liver homogenates demonstrated that the first esterase-catalyzed step was fast and that the second phosphoramidase-catalyzed step was slow and rate-limiting, analogous to other phosphoramidase prodrugs (Saboulard et al. 1999; Beltran et al. 2001).

Clinical studies showed a good maximum plasma concentration and total exposure of R-125338 indicating efficient conversion in humans as expected, although bioavailability in humans cannot be calculated due to the lack of pharmacokinetic data of intravenous administration of R-125338 (Walker et al. 2006). A large variability in the plasma concentration of CS-917 and R-125338 was also observed. Since CS-917 esterase activity in human plasma was relatively weak compared to that in the small intestine and liver (data not shown), the plasma concentration of CS-917 would be determined by the degree of CS-917 hydrolysis in the first-pass metabolism and phosphoramidase of the intermediate R-134450 could contribute to R-125338 variation.

Phosphoramidate prodrugs have been exploited for the delivery of nucleoside monophosphate antiviral and anti-cancer agents (Abraham et al. 1996; Saboulard et al. 1999; Freel Meyers and Borch 2000; Freel Meyers et al. 2000; Beltran et al. 2001; Egron et al. 2003), and molecular characterization of the activation process of nucleotide bisamidate prodrugs has been described recently. Birkus et al. (2007) identified cathepsin A (CTSA) as the first hydrolysis esterase by biochemical purification for GS-7340 and GS-9131, anti-retroviral nucleotide

Figure 1. Two-step activation of bisamidate prodrug CS-917 to the active form, R-125338. CS-917 is activated into the active form (R-125338) by a two-step enzyme-catalyzed reaction after oral administration. In the first step, CS-917 is hydrolyzed by an esterase and the resultant monoester is spontaneously hydrolyzed to form a monoamidate intermediate (R-134450) in the small intestine and the liver. In the second step, the P–N bond of the R-134450 is hydrolyzed by a phosphoramidase to generate R-125338 in the liver. The first step is observed to be fast and the second step is observed to be slow and rate-limiting.

phosphoramidate prodrugs. In the study of GS-9191, a prodrug for human papillomavirus treatment, they also found CTSA for the first esterase and contribution of both the unidentified enzyme and acid-driven hydrolysis for the second P–N bond cleavage (Birkus et al. 2011). Murakami et al. demonstrated carboxylesterase 1 (CES1) and CTSA involvement for the first ester hydrolysis and histidine triad nucleotide-binding protein 1 (HINT1) for the second phosphoramidase hydrolysis for PSI-7851, an anti-hepatitis C virus nucleotide prodrug (Murakami et al. 2010). They also reported CES1, CES2, and CTSA for the first hydrolysis and HINT1 for the second hydrolysis for the related drug, GS-6620 (Murakami et al. 2014). These previous studies showed that different enzymes were involved from compound to compound.

To understand the pharmacodynamics/pharmacokinetics of CS-917, including the variability of the plasma concentration of R-125338, identification of CS-917 esterase and R-134450 phosphoramidase at a molecular level was necessary. The structure of CS-917 is different from that of those nucleotide bisamidate prodrugs for anti-viral treatment, thus there is a possibility of unexpected enzymes which contribute to the activation process, although these studies provided candidate enzymes. Here, we describe successful identifications of both CS-917 esterase in the small intestine and liver and R-134450 phosphoramidase in the liver by biochemical purification to identify unexpected enzymes.

Materials and Methods

Materials

CS-917, R-134450, and R-125338 were provided by Drug Metabolism and Pharmacokinetics Research Laboratories, Sankyo Co. Ltd., and cynomolgus monkey livers and small intestines were provided by Pharmacodynamics Research Laboratories and Medicinal Safety Research Laboratories, Sankyo Co., Ltd. All experimental procedures were performed in accordance with the in-house guideline of the Institutional Animal Care and Use Committee of Sankyo Co., Ltd.

Purification of small intestine CS-917 esterase

All purification procedures were conducted at 4°C and the small intestines from 5 monkeys (monkey #1 to #5) were separately investigated. Each cynomolgus monkey small intestine was minced and homogenized in nine volumes of 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0, containing 0.25 mol/L sucrose and a protease inhibitor cocktail (complete

tablet; Roche Applied Science, Indianapolis, IN, USA) using a polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenate was filtrated by a few sheets of gauze, the filtrate was sonicated by a Bioruptor (COSMO BIO, Tokyo, Japan), and the suspension was centrifuged at 25,000g for 1 h. To the supernatant, ammonium sulfate was added to 20% saturation, which was then stirred for 3 h and centrifuged at 18,000g for 30 min. To the collected supernatant, ammonium sulfate was further added to 70% saturation, which was then stirred for 12 h and centrifuged at 18,000g for 30 min. The collected 20–70% saturated ammonium sulfate precipitate was stored at −80°C until use.

Ammonium sulfate precipitate equivalent to 12 g of monkey small intestines was dissolved in 80 mL of 20 mmol/L HEPES, pH 7.0, containing 1 mol/L ammonium sulfate, and dialyzed against 2 L of the same buffer. The dialyzed sample was centrifuged at 15,000g for 10 min, and the supernatant was filtrated and loaded onto a hydrophobic interaction chromatography (HIC) column (HiPrep Butyl 4 FF; GE Healthcare, Pittsburgh, PA, USA). The bound proteins were eluted with a linear gradient from 1 to 0 mol/L ammonium sulfate in 20 mmol/L HEPES, pH 7.0. Each fraction was tested for CS-917 esterase activity. The CS-917 esterase activity formed two peaks (the first active peak and the second active peak) in the case of the small intestine from monkey #1.

For the first active peak purification, the active fractions (20 mL) were dialyzed against 2 L of HC buffer (20 mmol/L HEPES, pH 7.0, containing 0.1% [w/v] 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid [CHAPS]). The dialyzed sample was loaded onto a dye-affinity column (HiTrap Blue HP 1 mL; GE Healthcare) and the bound proteins were eluted with a linear gradient of 0–2 mol/L NaCl in HC buffer. The active fractions (2 mL) were dialyzed against 1 L of HC buffer. The dialyzed sample was loaded onto an anion exchange column (Mini Q PC 3.2/3; GE Healthcare), and the bound proteins were eluted with a linear gradient of 0–350 mmol/L NaCl in HC buffer. A portion of the active fraction (50 μL) from the Mini Q column was loaded onto a gel filtration column (Superdex 200 PC 3.2/30; GE Healthcare) equilibrated with 20 mmol/L HEPES, pH 7.0, containing 150 mmol/L NaCl and 0.1% CHAPS.

For the second active peak purification, the active fractions (20 mL) were dialyzed against 10 mmol/L sodium phosphate, pH 6.8, containing 0.1% CHAPS. The dialyzed sample was loaded onto a hydroxylapatite column (CHT2-I; Bio-Rad Laboratories, Hercules, CA, USA) and the bound proteins were eluted with linear gradient of

10–500 mmol/L sodium phosphate, pH 6.8, containing 0.1% CHAPS. The active fractions (2 mL) were dialyzed against 1 L of HC buffer. The dialyzed sample was loaded onto an anion exchange column (Mini Q PC 3.2/3) and the bound proteins were eluted with a linear gradient of 0–350 mmol/L NaCl in HC buffer. A portion of the active fraction (50 μL) from the Mini Q column was loaded onto a gel filtration column (Superdex 200 PC 3.2/30; GE Healthcare) equilibrated with 20 mmol/L HEPES, pH 7.0, containing 150 mmol/L NaCl and 0.1% CHAPS.

Ten microliters of the fractions around the active fractions were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (5–20% gel; Bio-Rad Laboratories) under reducing conditions, and the gel was stained with a fluorescent dye (Flamingo gel stain; Bio-Rad Laboratories). The band intensity on the stained gel was quantified using a fluorescent scanner (Molecular Imager FX; Bio-Rad Laboratories).

Purification of liver CS-917 esterase

All purification procedures were conducted at 4°C. A monkey liver homogenate was prepared as described above, as a small intestine homogenate. Twenty to seventy percent saturated ammonium sulfate precipitation of the cytosolic fraction was collected and stored at −80°C until use. Ammonium sulfate precipitate equivalent to 11 g of monkey liver was dissolved in 80 mL of 20 mmol/L HEPES, pH 7.0, containing 1 mol/L ammonium sulfate, and dialyzed against 2 L of the same buffer. The dialyzed sample was centrifuged at 15,000g for 10 min, and the supernatant was filtrated and loaded onto an HIC column (HiPrep Butyl 4 FF; GE Healthcare). The bound proteins were eluted with a linear gradient from 1 to 0 mol/L ammonium sulfate in 20 mmol/L HEPES, pH 7.0. Each fraction was tested for CS-917 esterase activity. The CS-917 esterase activity formed two peaks (the first active peak and the second active peak).

Each active peak was separately purified further using the same purification procedures. The active fractions (20 mL) were dialyzed against 20 mmol/L HEPES, pH 7.0. The dialyzed sample was loaded onto a dye-affinity column (HiTrap Blue HP 1 mL; GE Healthcare), and the bound proteins were eluted with a linear gradient of 0–1 mol/L NaCl in 20 mmol/L HEPES, pH 7.0. The active fractions (4 mL) were dialyzed against 2 L of 20 mmol/L HEPES, pH 7.0. The dialyzed sample was loaded onto an anion exchange column (Mono Q 5/50 GL; GE Healthcare), and the bound proteins were eluted with a linear gradient of 0–500 mmol/L NaCl in 20 mmol/L HEPES, pH 7.0. Ten microliters of the fractions around the active fractions was loaded onto SDS-

PAGE gel. The gel was stained with the fluorescent dye and analyzed by the fluorescent scanner as described above.

Purification of R-134450 phosphoramidase from the liver

All purification procedures were conducted at 4°C. A monkey liver homogenate was prepared as described above. Twenty to seventy percent saturated ammonium sulfate precipitation of the cytosolic fraction was collected and stored at −80°C until use. Ammonium sulfate precipitate equivalent to 30 g of monkey liver was dissolved in 50 mL of CEX buffer (20 mmol/L CH$_3$COONa, pH 4.0, containing 0.1% CHAPS, 5 mmol/L MgCl$_2$ and 1 mmol/L dithiothreitol [DTT]) and dialyzed against 2 L of CEX buffer. The dialyzed sample was centrifuged at 3000g for 5 min and the supernatant was filtrated and loaded onto a cation exchange column (HiPrep SP 16/10 XL; GE Healthcare). The bound proteins were eluted with a linear gradient of 0–1 mol/L NaCl in CEX buffer. The active fractions (20 mL) were dialyzed against CEX buffer, and loaded onto another cation exchange column (Mono S HR 5/5; GE Healthcare). The bound proteins were eluted with a linear gradient of 0–300 mmol/L NaCl in CEX buffer. The active fractions (3 mL) were dialyzed against AEX buffer (20 mmol/L Tris, pH 10.0, containing 0.1% CHAPS, 5 mmol/L MgCl$_2$, and loaded onto an anion exchange column (Mini Q PC 3.2/3; GE Healthcare). The bound proteins were eluted with a linear gradient of 0–300 mmol/L NaCl in AEX buffer. A portion of the active fraction (50 μL) from the Mini Q column was loaded onto a gel filtration column (Superdex 75 PC 3.2/30; GE Healthcare) equilibrated with 20 mmol/L HEPES, pH 7.0, containing 150 mmol/L NaCl, 0.1% CHAPS, 5 mmol/L MgCl$_2$, and 1 mmol/L DTT. Ten microliters of the fractions around the active fractions were loaded onto SDS-PAGE gel. The gel was stained with the fluorescent dye, and analyzed by the fluorescent scanner as described above.

Protein identification and quantitation by mass spectrometry

To identify proteins from the gel, the bands on the gel were excised, digested in gel, and subjected to liquid chromatography equipped with tandem mass spectrometry (LC-MS/MS) as described previously (Ishizuka et al. 2010). In the case of identification from the solution for shotgun proteomics, the proteins were digested in solution, and subjected to LC-MS/MS as described previously (Kubota et al. 2009). The MS/MS spectra were searched against the GenBank nonredundant protein database

compiled by the National Center for Biotechnology Information using the Mascot program (Matrix Sciences, London, UK). A label-free spectral counting method, emPAI (Ishihama et al. 2005), was used to quantify identified proteins in shotgun proteomics.

Molecular cloning and expression vector construction

Human genes of CES1, CES2, elastase 2A (ELA2A), ELA2B, ELA3A, ELA3B, HINT3, aprataxin (APTX) and sphingomyelin phosphodiesterase, and acid-like (SMPDL3A) were amplified by a polymerase chain reaction (PCR) from human brains, livers, pancreas, skeletal muscle, prostates, or their mixed cDNA library. HINT1 and HINT3 were amplified from a commercially available cDNA clone (Ultimate clone; Invitrogen, Tokyo, Japan). The resulting PCR products were purified and cloned into a pDONR221 entry vector (Invitrogen) by a Gateway BP reaction. The sequences were confirmed by DNA sequencing. In the case of proteins with secretion signal sequences (CES1, CES2, ELA2A, ELA2B, ELA3A, ELA3B, and SMPDL3A), an entry clone was recombined with a pFLAG-CMV-5.1GW destination vector, in which the gene was fused with a C-terminal FLAG-tag by a Gateway LR reaction. In other cases (HINT1, HINT2, HINT3, APTX), each entry clone was recombined with pFLAG-CMV-2GW in which the gene was fused with an N-terminal FLAG-tag. The construction of the expression vectors was confirmed by the insert length and DNA sequencing. The expression vector of the N-terminal FLAG-tagged enhanced green fluorescent protein (EGFP) was kindly provided by Dr. Keisuke Fukuchi from Daiichi Sankyo Co., Ltd.

Expression and purification of recombinant proteins

Each expression vector (30 μg) was transfected into human embryonic kidney 293-F cells (3×10^7 cells; Invitrogen), using 293-fectin according to the manufacturer's protocol. The transfected cells were cultured for 72 h. The cell culture was centrifuged and the supernatant was filtered and used as a conditioned medium. The collected cells were suspended for 5 min on ice in Cellytic-M (Sigma-Aldrich, St. Louis, MO, USA) containing 1 mmol/L DTT. The cell extracts were centrifuged and the supernatant was used as a cell lysate.

Proteins were purified using their FLAG-tag by affinity chromatography. In the case of purification from a conditioned medium (ELA2A, ELA2B, ELA3A, ELA3B, and SMPDL3A), the conditioned medium was adjusted to have a 0.1% dodecyl-β-maltoside (DM), and 20–25 μL of anti-FLAG M2 agarose (Sigma-Aldrich) was added. In the case of purification from a lysate (CES1, CES2, EGFP, HINT1, HINT2, HINT3, and APTX), 5 μL of anti-FLAG M2 agarose was added to the lysate. After 12 h incubation at 4°C, the resin was collected by centrifuge and washed five times with 500 μL of wash buffer (20 mmol/L Tris-Cl, pH 7.5, containing 500 mmol/L NaCl, 1% DM) and once with 200 μL (for the conditioned medium samples) or 100 μL (for the lysate samples) of wash buffer containing 0.1 μg/mL FLAG peptide (Sigma-Aldrich). Proteins bound to the gel were eluted with 200 μL (for the conditioned medium samples) or 100 μL (for the lysate samples) of wash buffer containing 100 μg/mL FLAG peptide.

Confirmation of protein expression and quantitation of the purified proteins were accomplished by Western blot analysis. Proteins were loaded onto SDS-PAGE gel (5–20% gradient) and then electro-transferred onto a polyvinylidene difluoride membrane (Millipore, Temecula, CA, USA). After blocking with an ECL blocking agent (GE Healthcare), the membrane was incubated with an anti-FLAG M2 antibody conjugated with horse radish peroxidase (Sigma-Aldrich) followed by an ECL Western blotting detection system (GE Healthcare). The band intensity on the X-ray film was scanned by an optical scanner (GT-9700F, EPSON) and quantified using C-terminal FLAG-bacterial alkaline phosphatase (Sigma-Aldrich) as a standard protein.

Elastase purified from human leukocytes was purchased from Sigma-Aldrich and we confirmed by mass spectrometry that this elastase was human ELA2 without any contamination by other elastases or esterases. The total protein concentration of the sample was regarded as the concentration of the activated ELA2.

Activation of recombinant enzymes

In the case of the recombinant elastases (ELA2A, ELA2B, ELA3A and ELA3B), 1 ng/μL trypsin was added after purification and incubated at 37°C for 15 min. The activated elastases were tested for CS-917 esterase activity without inactivation of trypsin because trypsin alone did not show any CS-917 esterase activity (data not shown) and most trypsin inhibitors are known to inhibit elastases as well. A part of the activated elastases was loaded onto SDS-PAGE gel and the gel was stained by a fluorescent dye (Flamingo gel stain; Bio-Rad Laboratories). The band intensity corresponding to the predicted molecular weight was quantified using a fluorescent scanner (Molecular Imager FX; Bio-Rad Laboratories).

Human recombinant CTSA and cathepsin L were purchased from R&D Systems (Minneapolis, MN, USA). To activate cathepsin L, it was incubated on ice for 15 min in 50 mmol/L 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.0, containing 5 mmol/L DTT. To activate CTSA,

1 μg/mL cathepsin L was added to 10 μg/mL CTSA in 25 mmol/L MES, pH 6.0, containing 5 mmol/L DTT and incubated at 37°C for 30 min. After the activation of CTSA, cathepsin L was inactivated by adding 10 μmol/L E-64 (Sigma-Aldrich).

CS-917 esterase assay

The sample was incubated at 37°C for 1 h with 300 μg/mL CS-917 in 50 μL of 100 mmol/L HEPES, pH 7.0, containing 500 mmol/L NaCl and 200 μg/mL bovine serum albumin (BSA). Regarding investigation of the CES specific inhibitor, bis(4-nitrophenyl) phosphate (BNPP; Sigma-Aldrich), BNPP dissolved with dimethyl sulfoxide at 50 mmol/L was added to the sample before the addition of CS-917, and after 5 min preincubation at 37°C, CS-917 was added to the reaction mixture. The concentration of BNPP in the reaction mixture was 1 mmol/L in the case where there was no explicit description. To stop the reaction, the sample was mixed with 100 μL of methanol, and 10 μL of the filtrate (0.45 μm; Millipore) was subjected to a reversed-phase chromatography column (ZORBAX SB C18, 2.1 mm ID × 0 mm length, 1.8 μm; Agilent Technologies, Santa Clara, CA, USA), which was conditioned at 40°C and pre-equilibrated with 20 mmol/L triethylamine acetate (TEAA), pH 7.0, containing 5% acetonitrile. The compounds were eluted with a linear gradient of 5–95% acetonitrile in 20 mmol/L TEAA, pH 7.0, over 2.5 min at a flow rate of 0.5 mL/min. Absorbance at 303 nm was monitored, and CS-917 esterase activity was determined by measuring the peak area of the produced R-134450 using a known amount of a synthesized R-134450 as the standards. One unit of CS-917 esterase activity was defined as the activity required for 1 μg/mL production of R-134450 under these experimental conditions.

R-134450 phosphoramidase assay

To the 35 μL of the sample was added 5 μL of 1 mol/L HEPES, pH 7.0, 5 μL of 2 mg/mL BSA and 5 μL of 10 mmol/L R-134450 dissolved in 5% dimethyl sulfoxide, and this was incubated at 37°C for 1 h. After the reaction, the sample was mixed with 100 μL of methanol to inactivate the enzyme and 10 μL of the filtrate (0.45 μm) was subjected to a reversed-phase chromatography column (XTerra MS C18 5 μm, 4.6 mm ID × 150 mm length; Waters, Milford, MA, USA) with a guard column of the same material (3.9 mm ID × 20 mm length, Waters), then analyzed with isocratic elution in 10 mmol/L TEAA, pH 7.0, containing 16% acetonitrile. Absorbance at 260 nm was monitored, and R-134450 phosphoramidase activity was determined by measuring the peak area of produced R-125338, using a known amount of synthesized

R-125338 as the standard. One unit/mL R-134450 phosphoramidase activity was defined as the concentration required for 1 μmol/L production of R-125338 under these experimental conditions.

Protein assay

The total protein concentration was determined by a modified Bradford assay (Coomassie Plus Protein Assay; Pierce, Tokyo, Japan), using BSA as a standard protein.

Compliance with design and statistical analysis requirements

All experiments were independently conducted more than once to confirm reproducibility except Figure 4, where we show all of the data from individual animals. A representation of those independent experiments is shown in each figure and table, and the number of independent experiments is described in each figure and table legend. Therefore, replicates in the Figures were 'used only to test the precision of $n = 1$' in this study.

Results

Purification and identification of ELA3B and CTSA as CS-917 esterases in monkey small intestines

To identify the enzyme responsible for CS-917 esterase activity in the small intestine, we used cynomolgus monkeys as a purification source because of the abundant sample availability and their similarity to humans. The monkey small intestine was homogenized and separated to nuclear, mitochondrial, microsomal, and cytosolic fractions by centrifugation and the activity was found to be mostly localized in the cytosolic and soluble fraction (Fig. S1). The observed molecular weight of the activity was 60–70 kDa determined by gel filtration chromatography, and interestingly, the activity from one out of the five monkeys (monkey #1) was bound to an HIC column and formed two almost equivalent peaks (Fig. 2A).

The active peaks purified with the HIC column were further characterized. The first active peak was bound to a blue dye affinity column at pH 7 and was also weakly bound to a heparin affinity column. On the other hand, the second peak was not bound to either the blue dye or the heparin affinity columns, but was bound to a hydroxylapatite column. Based on their characters, we purified CS-917 esterase activity from the small intestine from monkey #1. We used the HIC column for the first column and the first and the second active peaks were purified separately afterwards. As a result of successive

five-step purification, the first and the second active peaks were purified more than 4000-fold (Table 1) and 8000-fold (Table 2), respectively. In the fourth (Fig. S2A and B) and final (Fig. 2B and C) purification step of the first active peak, we found bands of 30, 17, and 15 kDa on SDS-PAGE gels which correlated well with the CS-917 esterase activity. Therefore, we analyzed the proteins in these three bands by mass spectrometry and identified monkey elastase 3B (NCBI gene identifier: 62510667, human homologous gene symbol: ELA3B) from all three bands. Similarly, in the fourth (Fig. S2C and D) and final (Fig. 2D and E) purification step of the second active peak, we found bands of 26 and 17 kDa on SDS-PAGE gels which correlated well with the enzyme activity, and we identified the protein in these bands as monkey cathepsin A (NCBI gene identifier: 109091751, human homologous gene symbol: CTSA) by mass spectrometry.

Confirmation of CS-917 esterase activity of candidate genes in the small intestine

To confirm that CTSA and ELA3B have CS-917 esterase activity and to determine whether other possible candidate genes have CS-917 esterase activity, we compared the specific activity of the candidate genes for CS-917 esterase. We cloned human homologous genes because our final goal is to identify CS-917 esterase in humans. Considering the differences between species, we regarded other human pancreatic elastases (ELA2A, ELA2B and ELA3A) as candidates for CS-917 esterase in addition to ELA3B. We produced the recombinant proteins in human cells, purified them using FLAG-tag fused with their carboxyl-terminal, activated them if necessary, and tested their specific activity. In addition to pancreatic elastases, we also tested ELA2. Fortunately, active ELA2 purified from human leukocytes was commercially available. With regard to CTSA, we obtained commercially available human CTSA and activated them with cathepsin L as described in Materials and Methods.

As shown in Figure 3, CTSA demonstrated the highest specific activity as CS-917 esterase while ELA2 and ELA3B exhibited moderate specific activity, and ELA3A showed weak specific activity. ELA2A and ELA2B did not hydrolase CS-917. In parallel, we tested ELA2A and ELA2B's elastase activity using synthetic peptide substrates. The results revealed ELA2A had elastase activity but ELA2B did not (Fig. S3), indicating that our ELA2A was certainly active but did not have CS-917 esterase activity. With regard to ELA2B, a recent report showed ELA2B was devoid of proteolytic activity (Szepessy and Sahin Toth 2006). Thus, our ELA2B was supposed to be inactive due to its intrinsic character.

Phenylmethylsulfonyl fluoride (PMSF) is an inhibitor for a broad range of serine proteases. Since both ELA3B and CTSA are serine proteases, we tested its inhibitory effect on the elastases and CTSA. CS-917 esterase activity of the elastases was almost completely inhibited with 1 mmol/L PMSF, whereas CTSA was relatively insensitive to PMSF (Fig. S4). Next, we investigated the PMSF effect on CS-917 esterase activity separated with an HIC column in monkey small intestine homogenates. Interestingly, we found that all 4 homogenates prepared from monkeys (monkey #2 to #5) other than monkey #1 formed a single active peak after HIC column separation and that the activity was relatively insensitive to 1 mmol/L PMSF (Fig. 4). These observations strongly suggested that CTSA was the major CS-917 esterase in monkey small intestine homogenates and that monkey #1 was exceptional for unknown reasons, probably insufficient washing of the inside of the small intestine.

Purification and identification of CES1 and CTSA as CS-917 esterases in monkey liver

The CS-917 esterase activity of human liver S9 was partially removed by CTSA immunodepletion, whereas that of the human small intestine was almost completely removed (Fig. S5). These results strongly suggested that there was an enzyme(s) which had CS-917 esterase activity other than CTSA in the human liver.

CES is well known as an activating enzyme for many prodrugs, including bisamidate prodrugs and is supposed to be a good candidate for an additional CS-917 esterase in the liver (Imai 2006; Liederer and Borchardt 2006; Satoh and Hosokawa 2006; Murakami et al. 2010). To test this hypothesis, we investigated the effects of a CES-specific inhibitor, BNPP, on human liver S9 and observed that the high concentration of BNPP (10 mmol/L) did not show an inhibitory effect on human liver S9, but it did show significant inhibition on dialyzed human liver S9 (Fig. S6). These data indicated that BNPP could not inhibit CS-917 esterase activity in a crude sample for some reason. Therefore, we separated 20–70% ammonium sulfate precipitate of monkey liver homogenates by HIC, and tested the BNPP effect on its fractions. Considering the possibility of the purification of liver CS-917 esterase, we used cynomolgus monkey liver for the same reasons we used the monkey small intestine. As shown in Figure 5A, the CS-917 esterase activity formed two active peaks and the first active peak was strongly inhibited by BNPP, which suggested that the first active peak was contributed by CES and that the second active peak was contributed by CTSA.

Among the two major CES in humans, CES1 is highly expressed in the liver but is hardly expressed in the small intestine and contributes 80–95% CES activity in the human liver (Imai 2006), indicating CES1 is the enzyme

(A)

(B)

(C)

(D)

(E)
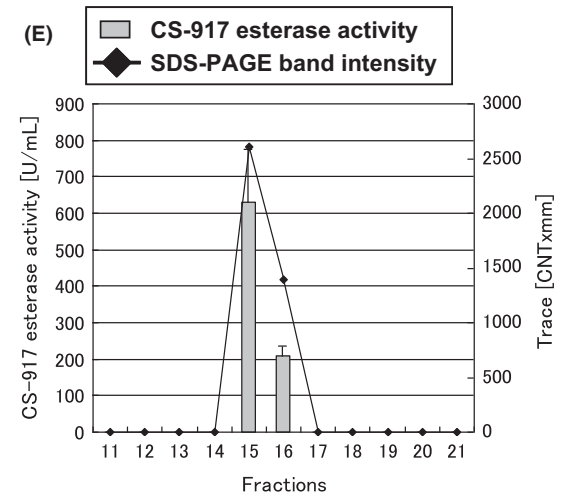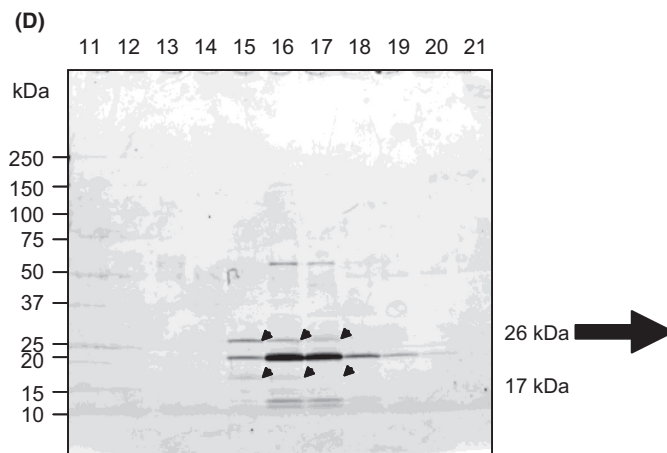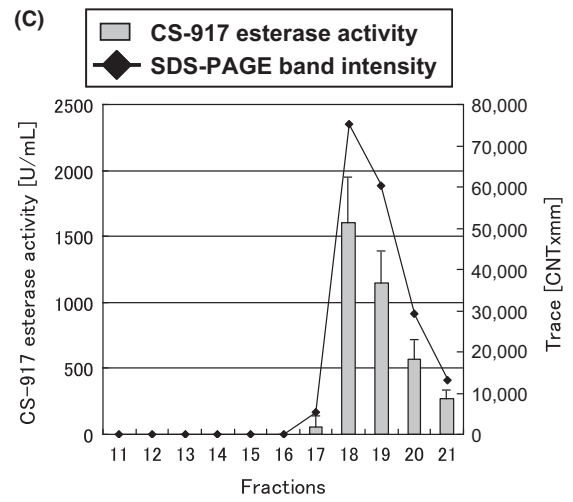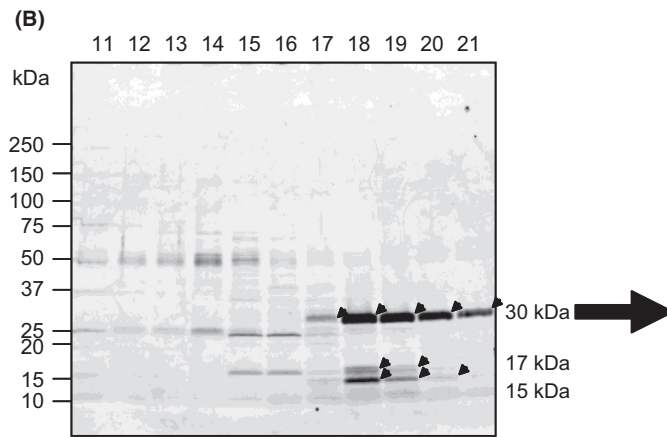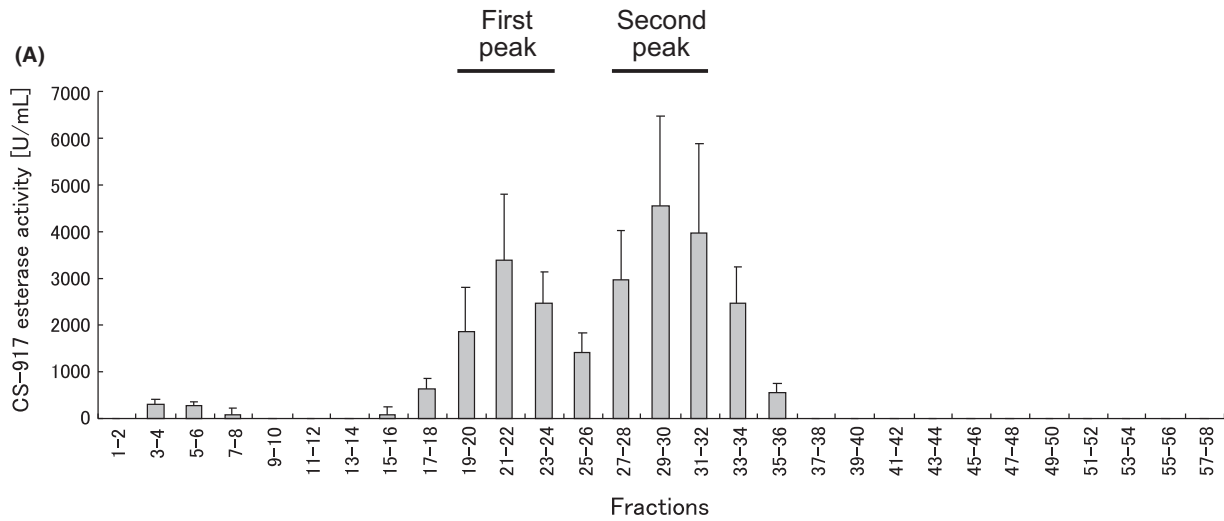
Figure 2. Purification and identification of CS-917 esterase activity from the small intestine of monkey #1. (A) The CS-917 esterase activity precipitated by ammonium sulfate from monkey #1 was subjected to a hydrophobic interaction chromatography (HIC) column. The activity was bound to a column and formed two active peaks by a linear gradient elution of 1–0 mol/L ammonium sulfate. CS-917 esterase activity of each active peak in the HIC was separately purified by successive chromatography. (B) Fractions of the final purification step of the first active peak were subjected to SDS-PAGE and the gel was stained by a fluorescent dye. Bands of 30, 17, and 15 kDa correlating well with the enzyme activity are indicated by arrowheads. (C) The enzyme activity of the fractions (solid line) and the intensity of arrowed bands of 30 kDa in (B) are shown as bars, respectively. (D) Fractions of the final purification step of the second active peak were subjected to SDS-PAGE and the gel was stained by a fluorescent dye. Bands of 26 and 17 kDa correlating well with the enzyme activity are indicated by arrowheads. (E) The enzyme activity of the fractions (solid line) and the intensity of the arrowed bands of 26 kDa in (D) are shown as bars, respectively. Three independent experiments were conducted, and the average of technical duplicates from a representative single experiment is shown with error bars representing the higher value. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 1. Purification of the first active peak in hydrophobic interaction chromatography.

Step	Protein concentration (μg/mL)	Activity[1] (U/mL)	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Step fold change	Overall fold change	Step recovery (%)	Overall recovery (%)
Monkey small intestine homogenate	14,000	330	430	6000	140,000	23	–	1	–	100
1. Ammonium sulfate precipitation	20,000	17,000	80	1600	1,400,000	85	37	37	970	970
2. Hydrophobic interaction (HiPrep Butyl)	390	1700	20	7.8	35,000	4500	5.2	190	2.6	25
3. Dye affinity (HiTrap Blue)	130	5700	2.0	0.26	11,000	44,000	9.8	1900	33	8.2
4. Anion exchange (Mini Q)	58	4600	0.24	0.014	1100	79,000	1.8	3400	10	0.8
5. Gel filtration (Superdex 75)	15	1600	0.10	0.0015	160	110,000	1.4	4700	14	0.11

Three independent experiments were conducted, and a representative purification table from a single experiment is shown.
[1]One unit of CS-917 esterase activity is defined as the activity required of 1 μg/mL R-134450 production in our assay condition described in Materials and Methods.

**Table 2.** Purification of the second active peak in hydrophobic interaction chromatography.

Step	Protein concentration (μg/mL)	Activity[1] (U/mL)	Volume (mL)	Total protein (mg)	Total activity p(U)	Specific activity (U/mg)	Step fold change	Overall fold change	Step recovery (%)	Overall recovery (%)
Monkey small intestine homogenate	14,000	327	430	6000	140,000	23	–	1	–	100
1. Ammonium sulfate precipitation	20,000	17,000	80	1600	1,400,000	850	37	37	967	967
2. Hydrophobic interaction (HiPrep Butyl)	160	2700	20	3.2	53,000	17,000	20	700	3.9	38
3. Hydroxylapatite (CHT2-I)	88	2800	2.0	0.18	5700	32,000	1.9	1400	11	4.1
4. Anion exchange (Mini Q)	34	3700	0.24	0.008	900	110,000	3.4	4700	16	0.6
5. Gel filtration (Superdex 75)	3.4	630	0.10	0.0003	63	190,000	1.7	8000	7	0.04

Three independent experiments were conducted, and a representative purification table from a single experiment is shown.
[1]One unit of CS-917 esterase activity is defined as the activity required of 1 μg/mL R-134450 production in our assay condition described in Materials and Methods.

responsible for the BNPP sensitive CS-917 esterase activity. To examine this, we partially purified CS-917 esterase activity and analyzed it by mass spectrometry. We separated the activity from the ammonium sulfate precipitate monkey liver using an HIC column. Afterward, the first and the second active peaks were purified separately by two successive columns, a dye-affinity column and an anion exchange column. CS-917 esterase activities separated by the final anion exchange chromatography are shown in Figure 5B (the first active peak) and D (the second active peak). The fractions which showed CS-917 esterase activity were tested to determine whether their
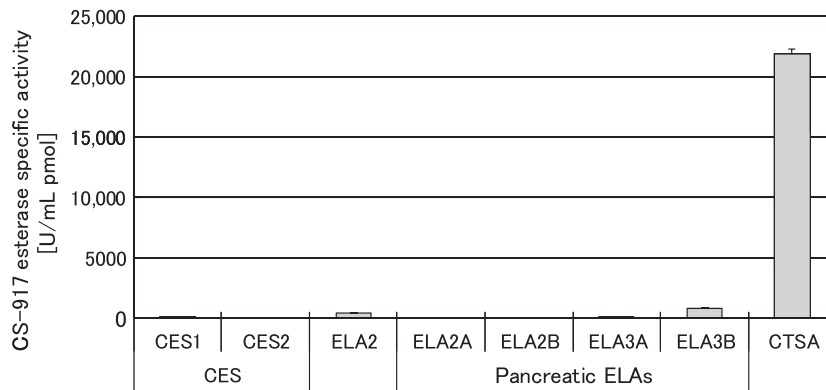
Figure 3. Specific CS-917 esterase activity of candidate genes. Specific activities of human candidate genes as CS-917 esterase were compared. Carboxylesterases (CES1 and CES2) were cloned, expressed in human cells, and purified from the lysate. Activated ELA2 purified from human leukocytes was commercially available. Pancreatic elastases (ELA2A, ELA2B, ELA3A, and ELA3B) were cloned, expressed in human cells, purified from the conditioned medium, and activated with trypsin. Purified CTSA expressed in *E. coli.* was obtained from a commercial source, and activated with cathepsin L. Two to five independent experiments were conducted (2 for ELA2, 3 for CES1, CES2, ELA2A, and ELA3B, and 5 for ELA3A, ELA3B, and CTSA), and the average of technical duplicates from a representative single experiment is shown with error bars representing the higher value. CTSA, cathepsin A; ELA, elastase.
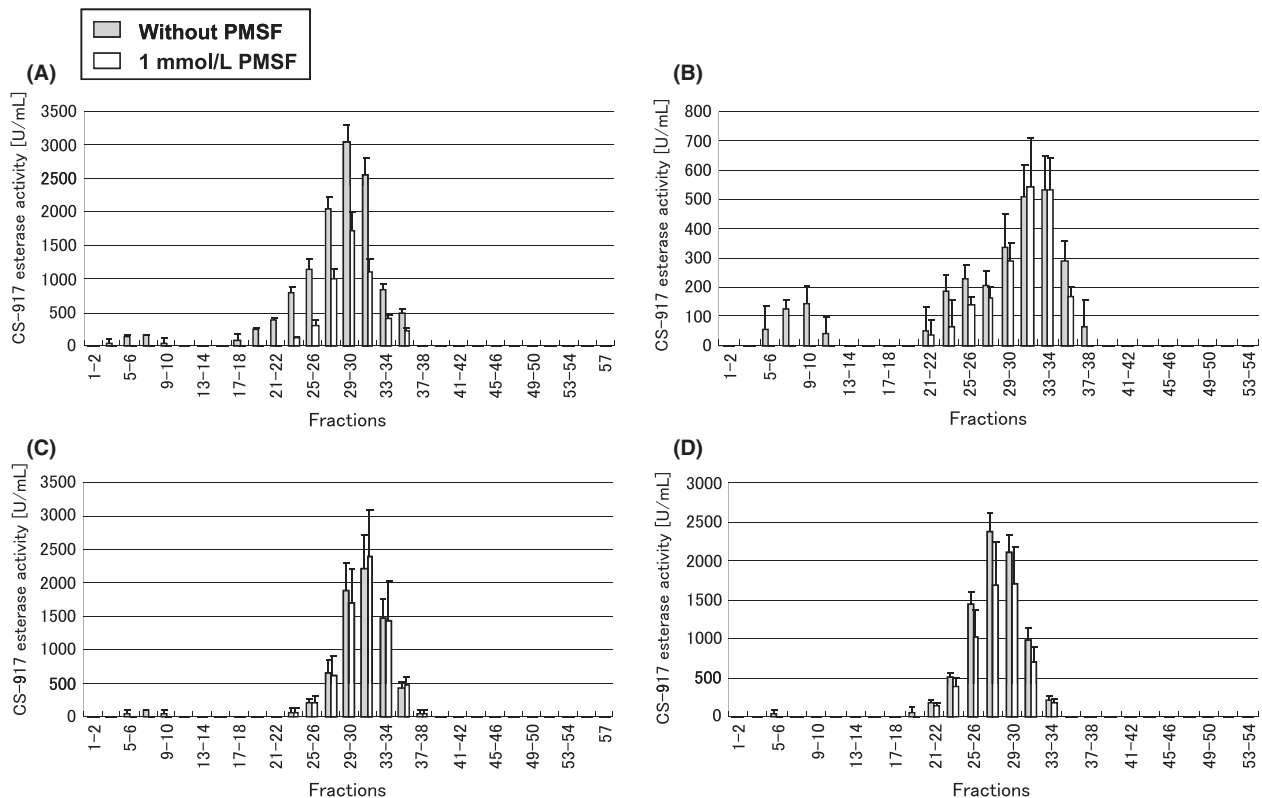
Figure 4. CS-917 esterase activity in the small intestine from different monkeys. The CS-917 esterase activity precipitated by ammonium sulfate from monkeys other than monkey #1 was subjected to a hydrophobic interaction chromatography column. In all of the cases, the activity formed a single active peak by a linear gradient elution of 1–0 mol/L ammonium sulfate. The activity was tested in the presence or absence of 1 mmol/L PMSF, a protease inhibitor for serine proteases including elastases. (A) Monkey #2, (B) monkey #3, (C) monkey #4 and (D) monkey #5. The data are shown as the average of technical duplicates from a single experiment with error bars representing the higher value. PMSF, phenylmethylsulfonyl fluoride.
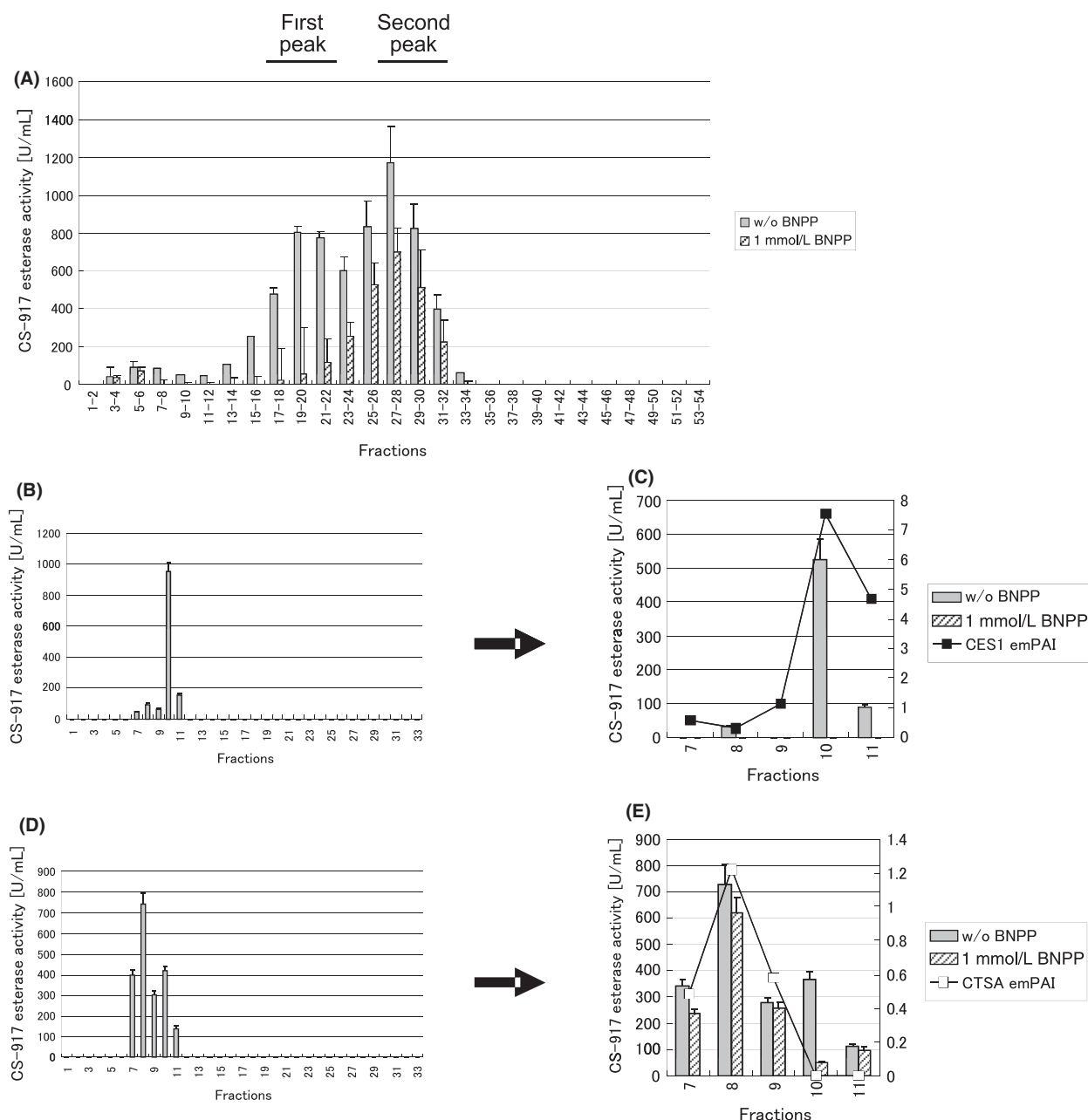
Figure 5. Purification and identification of CS-917 esterase activity from the monkey liver. (A) The CS-917 esterase activity precipitated by ammonium sulfate from monkey liver was subjected to a hydrophobic interaction (HIC). The activity was tested in the absence (gray bars) or presence (hatched bars) of 1 mmol/L BNPP, a CES specific inhibitor. CS-917 esterase activity of each active peak in the HIC was separately purified by successive chromatography. The CS-917 esterase activities were separated by the final anion exchange column purified from the first (B) or the second (D) active peak. The BNPP inhibitory effect was further tested on the active fractions from the first (C) or the second (E) active peak (gray and hatched bars). Quantitation of CES1 (C) and CTSA (E) by mass spectrometry using emPAI (closed and open box lines) correlated well with the enzyme activity. Four independent experiments were conducted, and the average of technical duplicates from a representative single experiment is shown with error bars representing the higher value. CTSA, cathepsin A; BNPP, bis(4-nitrophenyl) phosphate.

activities were inhibited by BNPP. Expectedly, the fractions from the first active peak were well inhibited by BNPP, indicating CES1 as a responsible enzyme (Fig. 5C), whereas the fractions from the second active peak were not inhibited by BNPP indicating another enzyme was involved (Fig. 5E). Then we attempted to identify proteins in the active fractions by shotgun proteomics, where total proteins are digested as a mixture and the digested

peptides are analyzed by LC-MS/MS (Hunt et al. 1992; Dongre et al. 1997; McCormack et al. 1997; Kubota et al. 2009). As expected, CES1 was identified in the fractions from the first active peak, and CTSA was identified in those from the second active peak. We quantified the relative abundance of CES1 and CTSA using a label-free spectral count method and found that the CES1 amount correlated well with CS-917 esterase activity purified from the first active peak and that the CTSA amount correlated well with the activity purified from the second active peak in the presence of 1 mmol/L BNPP (Fig. 5C and E). These results suggested that CES1 was the enzyme which was inhibited by BNPP and that CTSA and CES1 were the major CS-917 esterases in monkey liver.

Confirmation of CS-917 esterase activity of CES1

We prepared recombinant human CES1 and CES2 to confirm that CES1 has CS-917 esterase activity. Although contribution of CES2 is 5–15% in the human liver, CES2 is known to be the major CES in the small intestine (Imai 2006). CES2 was not identified as CS-917 esterase in the small intestine, therefore CES2 was not supposed to have CS-917 esterase activity, but we included CES2 as a good negative control. Both recombinant CES1 and CES2 showed CES activity on a typical CES substrate, *p*-nitrophenylacetate, and the only CES1 demonstrated CS-917 esterase activity as expected (Fig. 3).

Purification and identification of SMPDL3A as the intermediate (R-134450) phosphoramidase in monkey liver

To identify the second and rate-limiting enzyme of CS-917 activation, we attempted to purify the intermediate

(R-134450) phosphoramidase from monkey liver. We chose the liver as a purification source because the liver is the target organ of the FBPase inhibitor. As a result of preliminary experiments, R-134450 phosphoramidase activity was localized in the cytosolic fraction (Fig. S7) and the observed molecular weight was 65–83 kDa by gel filtration chromatography (Fig. S8).

We purified R-134450 phosphoramidase activity from monkey liver by successive five-step purification after ammonium sulfate precipitation. The enzyme activity was concentrated more than 9000-fold (Table 3). The enzyme activity formed a single peak in all chromatography steps, which indicated the enzyme was single. After the third purification step, we found that bands of 50 kDa on SDS-PAGE gels were correlating well with the enzyme activity (Fig. 6). We identified the protein of these 50 kDa bands by mass spectrometry as sphingomyelin phosphodiesterase, acid-like 3A (Human homologous gene symbol: SMPDL3A).

## Confirmation and comparison of candidate genes for R-134450 phosphoramidase

As far as we know, only two genes were reported as phosphoramidases in the literature (Bieganowski et al. 2002; Seidle et al. 2005). These two genes, HINT1 and APTX, belong to the same HINT enzyme family, and this HINT enzyme family consists of four genes (HINT1, HINT2, HINT3, and APTX) in humans. HINT1 could hydrolyze the phosphoramide bond of AMP-lysine (Bieganowski et al. 2002) and moreover the bisamidate nucleotide prodrug PSI-7851 (Murakami et al. 2010). In addition, APTX was also found to have the same AMP-lysine phosphoramidase activity (Seidle et al. 2005). Therefore, we included these four HINT family genes as candidate genes for R-134450 in addition to SMPDL3A, although none of

Table 3. Purification of R-134450 phosphoramidase from the monkey liver.

Step	Protein concentration (μg/mL)	Activity[1] (U/mL)	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Step fold change	Overall fold change	Step recovery (%)	Overall recovery (%)
Monkey liver homogenate	7000	71	200	1400	14,000	10	1.0	1.0	–	100
1. Ammonium sulfate precipitation	1400	320	50	72	16,000	224	22	22	110	110
2. Cation exchange (HiPrep SP XL)	13	680	30	0.40	20,000	53,000	240	5300	130	140
3. Cation exchange (Mono S)	18	300	3.0	0.054	890	16,000	0.31	1600	4.4	6.2
4. Anion exchange (Mini Q)	8.3	160	0.24	0.0020	37	19,000	1.2	1900	4.2	0.26
5. Gel filtration (Superdex 75)	2.1	190	0.10	0.00021	19	92,000	4.9	9100	52	0.14

Three independent experiments were conducted, and a representative purification table from a single experiment is shown.
[1]One unit of R-134450 phosphoramidase activity is defined as the activity required of 1 μmol/L R-125338 production in our assay condition described in Materials and Methods.
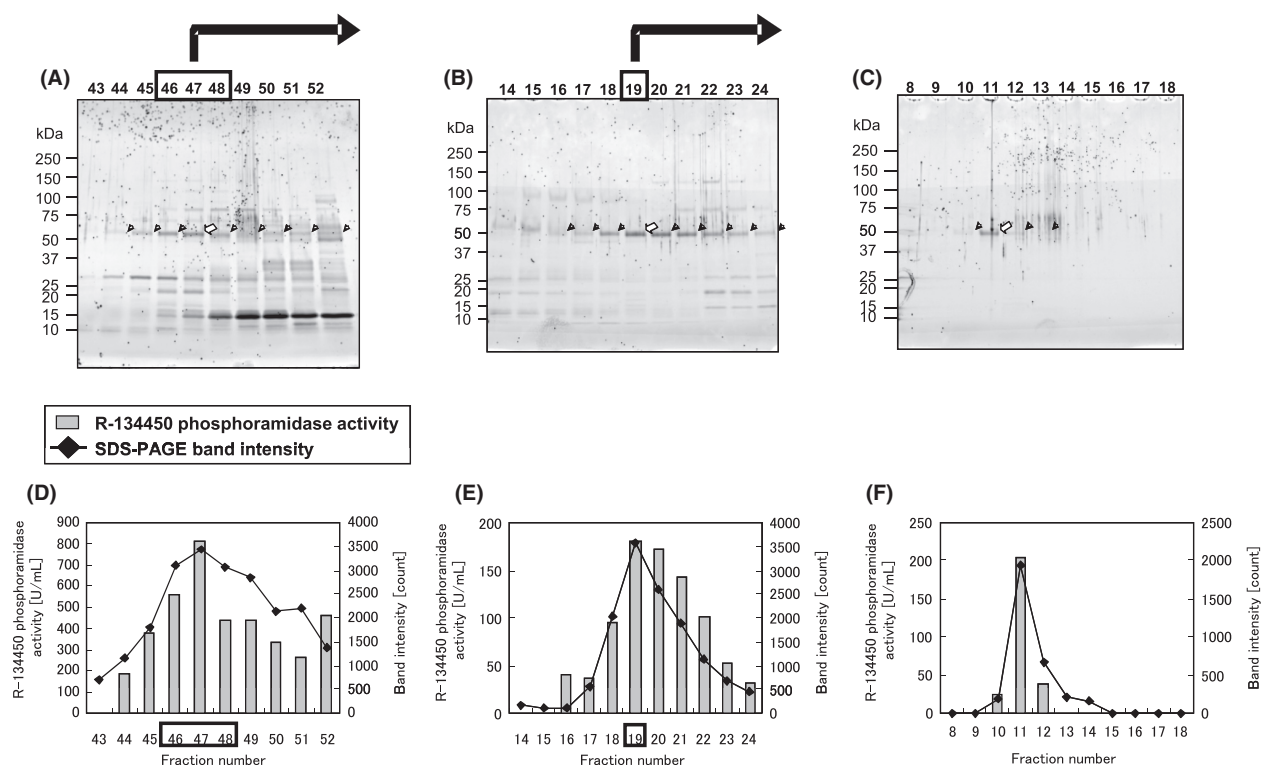
Figure 6. Purification and identification or R-134450 phosphoramidase activity from monkey liver. R-134450 phosphoramidase activity was purified from monkey liver by ammonium sulfate precipitation and successive five-step chromatography. Fractions of the third (A), fourth (B) and final (C) purification step were subjected to SDS-PAGE and the gel was stained by a fluorescent dye. Bands of 50 kDa correlating well with the enzyme activity are indicated by arrowheads. The bands indicated by open arrowheads were cut and analyzed by mass spectrometry. Enzyme activity of the fractions (solid line) and the intensities of the arrowed bands in the above panel (bars) are shown in the third (D), fourth (E) and final (F) purification step. Boxed fractions were subjected to the next purification step. Three independent experiments were conducted, and the average of technical duplicates from a representative single experiment is shown with error bars representing the higher value. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

them was identified in the purification of the enzyme activity from monkey liver.

We cloned the human genes of SMPDL3A, HINT1, HINT2, HINT3, and APTX, produced recombinant proteins in human cells, purified them using FLAG-tag fused with the protein, and tested their R-134450 phosphoramidase activity. As shown in Figure 7, SMPDL3A showed remarkable R-134450 phosphoramidase activity while HINT1, HINT2, and APTX had much weaker specific activity than SMPDL3A.

Discussion

In this study, we molecularly identified the enzymes that can catalyze the activation process of a bisamidate prodrug, CS-917. As activating enzymes in the first step, we purified and identified CTSA and ELA3B from the monkey small intestine. We also found ELA2 had relatively weak CS-917 esterase activity. Based on the fact that homogenates from only one specific monkey showed high

CS-917 esterase activity derived from ELA3B (Figs. 2, 4) and the immunodepletion experiment (Fig. S5), CTSA was probably the major responsible enzyme for CS-917 esterase activity in the small intestine. In addition, we have revealed that CES1 could contribute as CS-917 esterase as well as CTSA in the liver.

Orally administrated CS-917 would be partially hydrolyzed by pancreatic elastases, mainly ELA3B, in the lumen of the small intestine and would be further hydrolyzed by CTSA in the small intestine. CS-917 that escaped from those enzymes in the small intestine would be hydrolyzed by CTSA and CES1 in the liver, which is the target organ of CS-917. CS-917 hydrolyzed before reaching the liver would hardly contribute to the drug action of CS-917, because R-134450 is hydrophilic and has a low transition between organs due to its low membrane permeability. CS-917 esterase activity in human plasma was very weak compared to that in the small intestine or liver (data not shown), which indicated ELA2 was a minor factor in CS-917 metabolism. Taken together, ELA3B and CTSA, not
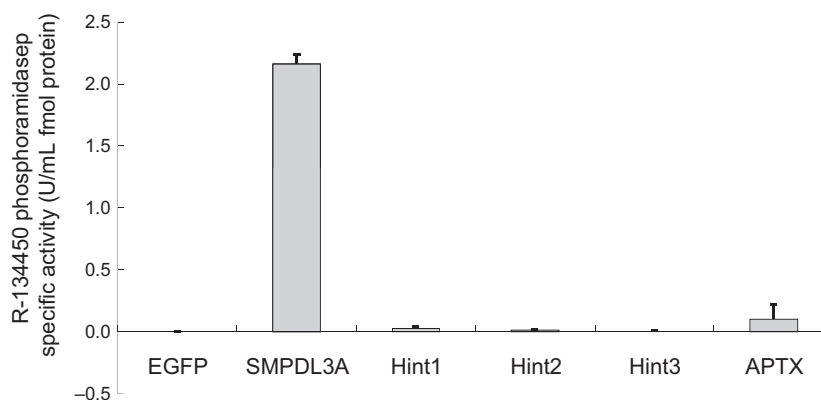
Figure 7. Specific R-134450 phosphoramidase activity of candidate genes. Candidate genes (SMPDL3A, HINT1, HINT2, HINT3, and APTX) and the negative control (EGFP) were expressed in the human cell line, the recombinant proteins were purified using fused FLAG-tag, and the R-134450 phosphoramidase activity was tested. The purified proteins were quantified by Western blotting, and the specific activity was calculated. Four to seven independent experiments were conducted (4 for HINT1, HINT2, HINT3, and APTX, and 7 for EGFP and SMPDL3A). The results are expressed as the means ± SD (three technical replicates from a single experiment). EGFP, enhanced green fluorescent protein.

ELA2, should play an important role in determining the CS-917 amount in the liver, and CTSA and CES1 would be an important factor for R-125338 concentration in the liver.

Elastases belong to the serine protease family. In humans, six elastase genes (elastase 1 (ELA1), ELA2, ELA2A, ELA2B, ELA3A, and ELA3B) are known. ELA1 is expressed in skin keratinocytes (Kawashima et al. 1992; Talas et al. 2000). ELA2, also known as neutrophil elastase or leukocyte elastase, is expressed in bone marrow myelocytic precursor cells, mostly promyelocytes (Fouret et al. 1989; Lee and Downey 2001). ELA2A, ELA2B, ELA3A, and ELA3B are known as pancreatic elastases, which are expressed and stored as an inactive zymogen in the pancreas, secreted into the intestine, activated by trypsin and participate in food digestion (Hartley and Shotton 1971; Shen et al. 1987; Tani et al. 1988; Fouret et al. 1989). Although the elastase was defined as a protease which degrades elastin, ELA3A and ELA3B are known to have a slight elastolytic activity but do have protease activity with preferential cleavage after alanine (Bode et al. 1989). The partial structure around the hydrolyzed ester of CS-917 was alanine ethyl ester, which supported ELA3B as the CS-917 esterase. It was reported that mature ELA3A/ELA3B had a molecular weight of 30 kDa and highly concentrated ELA3A/ELA3B showed rapid autolysis (Mallory and Travis 1975), which suggested that the 17 and 15 kDa identified as ELA3B by mass spectrometry would be its autodigested products (Fig. 2B). All of the data were consistent with ELA3B as one of the CS-917 esterases in the small intestine.

CTSA, also known as a lysosomal protective protein or lysosomal carboxypeptidase A, is a serine protease expressed ubiquitously (Hiraiwa 1999). This enzyme has three distinctive enzymatic activities: (1) peptidase activity optimal at pH 5.0 and (2) esterase and (3) deamidase activity optimal at pH 7.0 (Jackman et al. 1990). Besides its catalytic activities, it plays an important role in stabilizing lysosomal β-galactosidase and neuraminidase by its association. Interestingly, this protective function is independent of its catalytic activities (Galjart et al. 1991). CTSA is synthesized as a precursor of 54 kDa which is proteolytically processed into a mature two-chain form of 32 and 20 kDa polypeptides linked by disulfide bridges. This agrees well with the observations that the 26 and 17 kDa bands under a reducing condition (Fig. 2D) correlated well with CS-917 esterase activity on SDS-PAGE gels and were all identified as CTSA by mass spectrometry.

SMPDL3A, also referred to as acid sphingomyelinase-like phosphodiesterase 3a (ASML3a), is a protein whose biological or enzymatic function is mostly unknown. One study using a yeast 2-hybrid system reported that SMPDL3A interacts with the tumor suppressor gene deleted in the bladder cancer chromosome region 1 (DBCCR1) (Wright et al. 2002), and another study demonstrated SMPDL3A expression is regulated by liver X receptors (LXRs) (Noto et al. 2012). We found that the protein has one predicted phosphohydrolase domain (COG1409, NCBI), where the phosphoramidase reaction is similar to the phosphohydrolase reaction. mRNA expression of human SMPDL3A was high in the skin, small intestine, colon, rectum, liver, kidney, and prostate but low in other tissues, including blood vessels, which was consistent with the observation of the R-134450 phosphoramidase activity profile (data not shown). All experimental data supported that SMPDL3A was the enzyme responsible for R-134450 phosphoramidase activity.

We have identified SMPDL3A as an R-134450 phosphoramidase, which is thought to be a rate-limiting enzyme for CS-917 activation (Erion et al. 2005; Dang et al. 2007). We deduced that SMPDL3A, not HINT1, is the major R-134450 phosphoramidase in vivo because of the reasons below: (1) The enzyme activity formed a single peak in all purification steps, which suggested the enzyme in the monkey liver was unique; (2) Only SMPDL3A showed relatively strong specific activity compared to HINT family proteins; (3) We tested the R-134450 phosphoramidase activity of two isoforms of SMPDL3B, the most homologous protein to SMPDL3A, but they did not show any R-134450 phosphoramidase activity (data not shown) because homologous drug metabolism enzymes often have similar enzyme activity. To determine whether SMPDL3A is the major enzyme responsible for R-134450 phosphoramidase activity, further studies are needed. For example, a neutralizing antibody against SMPDL3A can be used for such experiments. Moreover, investigation of the relationship between SMPDL3A and pharmacokinetic and pharmacodynamic analysis of CS-917 will reveal the importance of SMPDL3A in CS-917 activation.

In previous literatures, contribution of CTSA for the first ester hydrolysis of nucleotide bisamidate prodrugs was reported for GS-7340 and GS-9131 (Birkus et al. 2007), PSI-7851 (Murakami et al. 2010), GS-9191 (Birkus et al. 2011) and GS-6620 (Murakami et al. 2014), whereas CES1 was partially involved, such as with PSI-7851 (Murakami et al. 2010) and GS-6620 (Murakami et al. 2014). Neutrophil elastase (ELA2) was also described to have the esterase activity for PSI-7851 (Murakami et al. 2010) and we identified ELA3B as CS-917 esterase. Therefore, the first step reaction of CS-917 was found to be similar to those nucleotide prodrugs. On the other hand, HINT1 (Murakami et al. 2010, 2014) or an unidentified enzyme (Saboulard et al. 1999; Birkus et al. 2011) or acid-driven hydrolysis (Birkus et al. 2011) was described for the second hydrolysis of the P–N bond for prodrugs, which was different from SMPDL3A in CS-917. Thus, SMPDL3A would be the first identified phosphoramidase other than the HINT family enzymes. Saboulard et al. (1999) reported that anti-HIV prodrug phosphoramidase from rat liver was enriched in a cytosolic fraction and that its observed molecular weight was 50–100 kDa. These data agree well with the characteristics of SMPDL3A, but not with HINT1 or APTX. Thus, SMPDL3A might be the unreported phosphoramidase for the anti-HIV prodrug.

Identification of an enzyme based on biochemical purification is sometimes regarded as a classic and conservative approach. In this study, we purified ELA3B and CTSA from monkey small intestine (Fig. 2, Tables 1, 2) and SMPDL3A from monkey liver (Fig. 6 and Table 3).

Technical limitations of this study were to use monkey tissue as starting materials for purification, and the potential to bias activities observed toward a more stable enzyme by a biochemical purification approach. However, many enzymes were discovered through this approach, especially the discovery of unforeseen enzymes, and once we identify the candidates, we could validate the candidates by the following experiments using human samples. Human recombinant protein experiments (Figs. 3, 7) in this study are the initial step, and correlation analysis between inter-individual enzyme activity and candidate proteins, a selective inhibitor experiment and gene knock-out animal experiment would follow for the purpose. In the purification scheme, recovery of enzyme activities at the early purification steps showed more than 100%. This is not an uncommon observation as we reported previously (Yonesu et al. 2011) and as written by others (Ryan 1996), indicating a presence of inhibitors of the enzyme reaction in crude samples. With regard to identification strategy, CES1 and CTSA can be expected as a CS-917 esterase, ELA3B could be speculated based on the activity of ELA2 for PSI-7851 (Murakami et al. 2010), however identification of SMPDL3A as an R-134450 phosphoramidase was completely unexpected because SMPDL3A was very far from the HINT family proteins which have only been reported as having phosphoramidase activity. Recent advancement of proteomics technology has made the requirements to identify candidate proteins much lower than before. We did not have to purify candidate proteins to homogeneity. Instead, in this study, we identified candidate proteins from faint bands of a nanogram level which correlated with enzyme activity (Figs. 2, 6) or even without SDS-PAGE (Fig. 5) by using a quantitation method by mass spectrometry. Recently, we reported the carboxymethylene butenolidase homolog (CMBL) as a hydrolase of a new antihypertensive drug, olmesartan medoxomil, by the same strategy (Ishizuka et al. 2010), where the identified protein was also completely unexpected. The classical biochemical approach with cutting-edge proteomics technology could contribute to identify enzymes beyond our limited imagination.

In conclusion, we identified the enzymes involved in both two-step activation processes of the novel phosphoramidate prodrug, CS-917, at the molecular level. All of the observations presented here were obtained through in vitro experiments, thus the next challenge would be to determine the contribution of enzymes identified in this study in vivo, as other enzymes or even nonenzymatic lysosomal hydrolysis could be involved. Nevertheless, this study would be an important, initial, and necessary step for a better understanding of the activation mechanism, pharmacodynamics, and pharmacokinetics of this new drug candidate and possibly other phosphoramidate pro-

drugs. Novel identification of new phosphoramidase, which could be responsible for the activation process of other bisamidate drugs, would contribute to research and development of this class of prodrugs in the future. In addition, this study would be another good example showing that our biochemical approach with proteomics technology is an efficient strategy to identify unexpected metabolic enzymes, which are supposed to be necessary in the personalized medicine era.

Acknowledgement

Authors Contribution

Kubota, Inaba, Ichikawa, Takahashi, Izumi, and Shinagawa participated in research design. Kubota, Nakano, Watanabe, Sakurai, and Fukushima conducted experiments. Kubota, Nakano, Watanabe, Sakurai, and Fukushima performed data analysis. Kubota, Inaba, Izumi, and Shinagawa wrote or contributed to the writing of the manuscript.

Disclosures

All of the authors are employee of Daiichi Sankyo group who co-developed CS-917.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Organelle fractionation of CS-917 esterase activity in monkey small intestine. The monkey small intestine was minced and homogenized in 9 volumes of 10 mmol/L HEPES, pH 7.0, including 0.25 mol/L sucrose (homogenate buffer). The homogenate was centrifuged at 600*g* for 10 min and the pellet was resuspended in the same volume of the homogenate with homogenate buffer (nucleus). The supernatant was centrifuged at 8000*g* for 10 min and the pellet was resuspended in the same volume of the supernatant with homogenate buffer (mitochondria). The supernatant was centrifuged at 105,000*g* for 60 min and the pellet was resuspended in the same volume of the supernatant with homogenate buffer (microsome). The supernatant was collected as cytosol. In addition, a part of the homogenate was sonicated and centrifuged at 105,000*g* for 60 min. The supernatant was collected as soluble fraction and the pellet was resuspended in the same volume of the homogenate with homogenate buffer (insoluble fraction). The data are shown as the average of duplicates with error bars representing the minimum and maximum values.

Figure S2. Purification and identification of CS-917 esterase activity from the small intestine of monkey #1. (A) Fractions of the fourth purification step of the first active peak were subjected to SDS-PAGE and the gel was stained by a florescent dye. Bands of 30 kDa, 17 and 15 kDa correlating well with the enzyme activity are indicated by arrowheads. (B) The enzyme activity of the fractions (solid line) and the intensity of the arrowed bands of 30 kDa in (A) are shown as bars, respectively. (C) Fractions of the fourth purification step of the second active peak were subjected to SDS-PAGE and the gel was stained by a florescent dye. Bands of 26 and 17 kDa correlating well with the enzyme activity are indicated by arrowheads. (D) The enzyme activity of the fractions (solid line) and the intensity of the arrowed bands of 26 kDa in (C) are shown as bars, respectively. The data are shown as the average of duplicates with error bars representing higher value.

Figure S3. Esterase activity of ELA2A and ELA2B. Recombinant human ELA2A and ELA2B were purified and subjected to esterase assay using Suc-AAPL-PNA as substrate with or without prior activation by trypsin. Substrate (300 μg/mL) was incubated with enzymes or buffer in 100 mmol/L HEPES, pH 7.0, 200 μg/mL BSA, and 500 mmol/L NaCl for 1 h and OD 405 nm was measured.

Figure S4. PMSF inhibition on CS-917 esterase activity of recombinant human CTSA. Recombinant human CTSA activated and pre-incubated with PMSF at 37°C for 5 min. PMSF treated CTSA was subjected to CS-917 esterase assay. CS-917 esterase activity was normalized the activity without PMSF treatment. The data are shown as the average of duplicates with error bars representing the minimum and maximum values.

Figure S5. Immunodepletion of CTSA in human jejunal and liver S9. Human jejunal S9, human liver S9 or human recombinant CTSA (1 μg/mL) was mixed with anti-CTSA antibody (MAB1049; R&D systems) with the indicated concentration and protein G beads at 4°C for 2 h. The mixture was centrifuged and the supernatant was subjected to CS-917 esterase activity. CS-917 esterase activity was normalized by the activity without immuno-precipitation (No-IP). The data are shown as the average of duplicates with error bars representing the minimum and maximum values.

Figure S6. BNPP inhibition on CS-917 esterase activity in human liver S9. Human liver S9 with or without dialysis was pre-incubated with DMSO (negative control) or BNPP dissolved in DMSO with indicated concentration at 37°C for 5 min, and was subjected to CS-917 esterase assay. CS-917 esterase activity was normalized by the activity of DMSO-treated. The data are shown as the average of duplicates with error bars representing higher value.

Figure S7. Organelle fractionation of R-134450 phosphoramidase activity in monkey liver. The monkey liver was minced and homogenized in 9 volumes of 10 mmol/L HEPES, pH 7.0, including 0.25 mol/L sucrose (homogenate buffer). The homogenate was centrifuged at 600*g* for 10 min and the pellet was resuspended in the same volume of the homogenate with homogenate buffer (nucleus). The supernatant was centrifuged at 8000*g* for 10 min and the pellet was resuspended in the same volume of the supernatant with homogenate buffer (mitochondria). The supernatant was centrifuged at 105,000*g* for 60 min and the pellet was resuspended in the same volume of the supernatant with homogenate buffer (microsome). The supernatant was collected as cytosol. In addition, a part of the homogenate was sonicated and centrifuged at 105,000*g* for 60 min. The supernatant was collected as soluble fraction and the pellet was resuspended in the same volume of the homogenate with homogenate buffer (insoluble fraction). The data are shown as the average of duplicates with error bars representing the minimum and maximum values.

Figure S8. Gel filtration chromatography (GFC) fractionation of R-134450 phosphoramidase activity in monkey

liver. Twenty to seventy percent saturated ammonium sulfate precipitation of the cytosolic fraction of monkey liver was dissolved in 50 mmol/L HEPES, pH 7.0, 150 mmol/L NaCl, 0.1% CHAPS, 5 mmol/L $MgCl_2$ and 1 mmol/L DTT, and subjected to gel filtration column (Superdex 75 10/300 GL; GE Healthcare). A part of fraction was sampled and assessed by R-134450 phosphoramidase assay. Estimated molecular weight based on gel filtration standard was indicated by arrows with molecular weight. The data are shown as the average of duplicates with error bars representing higher value.