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Interindividual variability of carboxymethylenebutenolidase homolog, a novel olmesartan medoxomil hydrolase, in human liver and intestine

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Running Title:

Individual variation in CMBL in human liver and intestine

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Abbreviations: OM, olmesartan medoxomil; CMBL, carboxymethylenebutenolidase homolog; CES, carboxylesterases; PON, paraoxonases; SNP, single nucleotide polymorphism; CYP, cytochrome P450; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; K_m,

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Michaelis constant; V_{max}, maximum velocity, F_g; intestinal first-pass availability

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Abstract

Olmesartan medoxomil (OM) is a prodrug-type angiotensin II type 1 receptor antagonist. OM is rapidly converted into its active metabolite olmesartan by multiple hydrolases in humans, and we recently identified carboxymethylenebutenolidase homolog (CMBL) as one of the OM bioactivating hydrolases. In the present study, we further investigated the interindividual variability of mRNA and protein expression of CMBL and OM-hydrolase activity using 40 individual human liver and 30 intestinal specimens. In the intestinal samples, OM-hydrolase activity strongly correlated with the CMBL protein expression, clearly indicating that CMBL is a major contributor to the prodrug bioactivation in human intestine. The protein and activity were highly distributed in the proximal region (duodenum and jejunum), and decreased to the distal region of the intestine. Although there was high interindividual variability (16-fold) in both the protein and activity in the intestinal segments from the duodenum to colon, the interindividual variability in the duodenum and jejunum was relatively small (3.0- and 2.4-fold, respectively). In the liver samples, the interindividual variability in the protein and activity was 4.1- and 6.8-fold, respectively. No sex differences in the protein and activity were shown in the human liver or intestine. A genetically engineered Y155C mutant of CMBL, which was caused by a single nucleotide polymorphism rs35489000, showed significantly lower OM-hydrolase activity than the wildtype protein although no minor allele was genotyped in the 40 individual liver specimens.

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Introduction

Esterases, which are typically represented by carboxylesterases (CES), cholinesterases, and paraoxonases (PON), have been well investigated in particular as key enzymes responsible for the bioactivation of ester-based prodrugs (Satoh, 2005; Liederer and Borchardt, 2006; Fukami and Yokoi, 2012). The ester-prodrug strategy has been used frequently to overcome problematic drug-like properties, such as chemical and enzymatic instability, low aqueous solubility, low passive intestinal absorption, and consequently to improve the oral bioavailability of drugs (Beaumont et al., 2003; Ettmayer et al., 2004). Although these esterases are widely distributed in the blood, liver, intestine and many other biological fluids and tissues (Testa and Mayer, 2003), in most cases, intestinal and hepatic esterases serve as the major enzymes in bioactivation of oral prodrugs during the first pass through the gut and liver after absorption.

Variability in the activity of drug metabolizing enzymes may determine the pharmacokinetics of the drug entities, and also affect their therapeutic efficacy and safety when drug responses are closely related to drug exposure. In the case of prodrugs, insufficient or excessive conversion of the prodrugs into their active forms may directly lead to a lack of therapeutic response or unexpected adverse effects. In addition to physiological variables such as age, gender, and diseases, genetic polymorphism in the genes encoding the enzymes is one of the main factors contributing to the interindividual variability (Liederer and Borchardt, 2006). For example, Zhu *et al.* reported that two single nucleotide polymorphisms (SNPs) in the *CES1* gene altered pharmacokinetics and enhanced pharmacodynamic effects of CES1 substrate methylphenidate (Zhu et al., 2008). Furthermore, SNPs with amino acid

substitutions of R34W and V142M in the *CES2* gene have been reported to cause a lack of the enzyme activity and reduce conversion of CPT-11 to its active metabolite (Kubo et al., 2005).

Olmesartan medoxomil (OM) is a prodrug-type angiotensin receptor blocker which is prescribed worldwide as monotherapy and in combination with a thiazide diuretic and/or a calcium channel blocker (Scott and McCormack, 2008); (Chrysant, 2008); (Rump and Sellin, 2010); (Deeks, 2011). As shown in Fig. 1, prodrug OM was rapidly absorbed and converted to its active metabolite olmesartan (von Bergmann et al., 2001); (Scott and McCormack, 2008). Following oral administration of radiolabeled OM in healthy volunteers, no components other than the active metabolite olmesartan were detected in plasma, feces, or, with the exception of trace amount of polar material, in urine either (Laeis et al., 2001), suggesting the prodrug is completely converted to its pharmacologically active form in the gastrointestinal mucosa, portal blood and liver before it reaches the systemic circulation. Olmesartan is not further metabolized and is excreted both into urine and feces (Schwocho and Masonson, 2001); (Laeis et al., 2001). OM has a low potential for pharmacokinetic drug-drug interactions via cytochrome P450 (CYP) enzymes or interindividual variability due to variation of CYP activities since OM does not undergo metabolism via CYPs (Scott and McCormack, 2008). We recently identified human carboxymethylenebutenolidase homolog (CMBL, EC 3.1.1.45) as a bioactivating enzyme for OM in the liver and intestine (Ishizuka et al., 2010), demonstrating its function as hydrolase in humans for the first time. CMBL is a novel hydrolase and its biological functions and enzyme characteristics mostly remain uninvestigated in contrast to the well-characterized prodrug bioactivating esterases like CES and PON. Therefore, in the present study, we quantitatively characterized

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CMBL's mRNA and protein expressions, and interindividual variation of its enzymatic activity with a large number of individual human tissue samples, 40 liver and 30 intestinal specimens. Furthermore, to clarify the CMBL's contribution to OM bioactivation in human liver and intestine, we performed correlation analyses between the protein level and OM-hydrolase activity using those individual sample sets. Also, we investigated the effects of two non-synonymous SNPs of human CMBL on OM-hydrolase activity using site-directed mutants.

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Materials and Methods

Materials.

Olmesartan medoxomil (OM), olmesartan, and RNH-6272, a structural analog of olmesartan used as the internal standard for olmesartan determination, were synthesized in Daiichi Sankyo (Tokyo, Japan). Pooled human liver and intestinal cytosolic fractions were purchased from XenoTech LLC (Lenexa, KS).

Individual Human Liver and Intestine.

Forty individual human liver (14 females and 26 males; median age 63 years, range 16 - 95 years) and 30 intestinal (10 females and 20 males; median age 68 years, range 44 - 84 years) specimens were collected and used to isolate DNA and RNA, and to prepare cytosolic fraction at Daiichi Sankyo Europe GmbH (Munich, Germany). Human liver and intestinal tissue sections were obtained from Caucasian patients undergoing partial hepatectomy and gastrointestinal surgery for cancer, respectively, and normal portions of the tissue sections were used in the study. Informed consent was obtained from all patients and approved by the local ethical committees of the University of Regensburg, Germany and the Ludwig-Maximilian-University (Munich, Germany). Each individual liver and intestinal specimen was made anonymous and assigned to an individual code by the charitable state-controlled foundation, Human Tissue and Cell Research foundation (Regensburg, Germany; (Thasler et al., 2003)). The records of liver and intestinal sample donors are shown in Supplemental Table S1 and S2, respectively.

Preparation of hepatic and intestinal cytosol and microsomes.

Liver and intestinal cytosolic fractions were prepared as described previously (Paine et al., 1997) with some modifications. Briefly, frozen liver tissue or intestinal

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mucosa was thawed by directly adding a 4-fold volume (v/w) of homogenizing buffer (50 mM Tris/HCl, 154 mM potassium chloride, 250 mM sucrose, and 1 mM EDTA; pH 7.4) In the case of intestinal tissue, the homogenizing buffer was enriched with protease inhibitor cocktail (Complete EDTA-free, Roche Applied Science, Indianapolis, IN). After thawing, the tissue was homogenized using an Ultra Turrax homogenizer (IKA Labortechnik, Staufen, Germany) with a motor-driven pestle (18 K, 18 mm diameter) at 13,500 rpm for 10 sec, three times. The homogenate was centrifuged at 677 *g* for 10 min, subsequently at 6,940 *g* for 10 min and finally at 10,844 *g* for 10 min. The obtained supernatant was filtered through gauze and centrifuged at 111,000 *g* for 60 min. The supernatant (cytosol) was collected and stored at -80°C until use. The protein concentration was measured using the Lowry method (Lowry et al., 1951) with bovine serum albumin as the reference standard.

Expression Analysis of Human CMBL Transcript.

The total cellular RNA was isolated from tissue samples submerged in RNALater solution (Qiagen, Hilden, Germany) using an RNeasy Mini kit (Qiagen) in accordance with the manufacturer's instructions. After determination of the quantity and quality of isolated RNA using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, NanoDrop Technologies, Wilmington, DE), cDNA was prepared from the total cellular RNA using an Omniscript RT Kit (Qiagen) with oligo-dT primers, according to the manufacturer's instructions. Quantitative real-time PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) with the reaction mixture containing the cDNA, TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (both from Applied Biosystems). A TaqMan gene expression assay for human CMBL (Hs00540853_m1) and Pre-Developed TaqMan Assay Reagent human GAPDH, as the

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internal reference for normalization of mRNA expression levels, were purchased from Applied Biosystems.

Semi Quantitative Western Blot Analysis of Human CMBL.

The following protein samples were subjected to Western blot analysis of human CMBL: 500 ng of 40 human individual liver and 30 intestinal cytosolic fractions, 500 and 250 ng of human pooled liver and intestinal cytosolic fractions, respectively, and 250 ng of human CMBL-overexpressed mammalian Freestyle 293-F cell lysate as a positive reference. The protein samples were separated by SDS-PAGE using 12.5% or 12% sodium dodecyl sulfate-polyacrylamide gel (Ready Gels J 12.5% or Mini-PROTEAN TGX precast gel 12%, Bio-Rad) and were transferred electrophoretically onto a polyvinylidene difluoride membrane (Immun-Blot PVDF membrane, 0.2 μm, Bio-Rad). The native CMBL proteins expressed in the human tissue preparations were detected with affinity-purified rabbit polyclonal IgG against a human CMBL peptide (Immuno-Biological Laboratories, Takasaki, Japan, 1:5000 dilution) as a primary antibody and an anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences, 1:10000 dilution) as a secondary antibody. These immunoblots were visualized by enhanced chemiluminescence (ECL) with an ECL Advance Western Blotting Detection Kit (Amersham Biosciences). The immuno-reactive signals were detected by a lumino-image analyzer (LAS-4000UV mini, Fujifilm) and the signal intensities were semi-quantified by Science Lab 2005 Multi Gauge software (ver. 3.0, Fujifilm). Each band intensity of individual liver and intestinal samples was normalized to those of pooled liver and intestinal cytosols, respectively.

Hydrolase Activity Measurement.

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The OM-hydrolase activity in human liver or intestinal cytosolic fractions (0.05 mg protein/ml) was measured in 100 mM HEPES buffer (pH 7.4, incubation volume of 0.25 ml) with OM as a substrate. After 5-min pre-incubation at 37 ℃, the reaction was initiated by adding the substrate (final substrate concentration: 10 μM of OM except as otherwise noted, final solvent concentration: 2.5% acetonitrile). After 5-min incubation at 37 °C, the reaction was terminated by adding a 4-fold volume of ice-cold 87.5% acetonitrile containing RNH-6272 as an internal standard for the determination of olmesartan concentration and 0.25% formic acid for preventing the non-enzymatic degradation of OM. The mixture, with the volume of ca. 200 μL, was filtered using a Captiva 96-well filter plate, mixed with 200 μL of 50% methanol containing 1% formic acid, and was analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) consisting of a Prominence LC-20A system (Shimadzu Corp.) and an API3200 (Applied Biosystems/MDS SCIEX). Olmesartan was separated with a reversed-phase C_{18} column (Atlantis T3, S-5 μm, 2.1 mm ID×150 mm, Waters) and a mobile phase of 64% methanol containing 0.2% formic acid at a flow rate of 0.2 ml/min, and was determined by monitoring the ion transition of *m/z* 447 to *m/z* 207 with multiple reaction monitoring in the positive electrospray ionization mode. The lower limit of quantitation was set at 20 nM. The enzymatic activity was expressed as a metabolite formation rate (nmol/min/mg protein) based on the production of olmesartan for the reaction by each enzyme source, from which that in the buffer control was subtracted as non-enzymatic hydrolysis.

Expression of Human CMBL in E. coli and Mutant Generation.

The plasmid vector containing the open reading frame of the full-length human CMBL (Ishizuka et al., 2010) was used as the template for generating mutants. A

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QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to create two single mutants in human CMBL (Y155C or A150T) in this study. The forward and reverse primers used for the mutagenesis were shown as follows: 5'-GTGTCCGTCTGTGGCATTGTCAAGGATTCTGAAGAC-3' and 5'-GACAATGCCACAGACGGACACCCCTGCCCTG-3' for the mutant Y155C, and 5'-CTCAGAATTCAGGACAGGGGTGTCCGTCTATGGC-3' and 5'-GGACACCCCTGTCCTGAATTCTGAGTATTTCATCATCAAATG-3' for the mutant A150T. To confirm the desired mutation and verify the absence of unintended mutations, the constructs were sequenced. Each plasmid for the native CMBL and the mutants was transformed into E. coli BL21(DE3) competent cells (Novagen, Madison, WI), which were then grown in MagicMedia E. coli expression medium (Invitrogen) containing ampicillin (100 μg/ml). The wildtype protein and two mutants were expressed as N-terminal 6×His-tagged proteins and purified using a column packed with His-select Nickel affinity gel (Sigma-Aldrich). From the eluted recombinant proteins, the tag was cleaved by biotinylated thrombin (Novagen, Madison, WI) treatment. The affinity-purified proteins with or without the thrombin treatment were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with GelCode Blue (Pierce, Rockford, IL) staining. The resulting protein solutions were stored at 4 ℃ until use.

Kinetic analysis.

The enzyme kinetics for OM hydrolysis by the recombinant CMBL proteins was evaluated at substrate concentrations [S] ranging from 7.81 to 1000 μM. The buffer control was incubated in parallel as non-enzymatic hydrolysis. The enzymatic activity was measured as stated above. Kinetic parameters, namely Michaelis constant (K_m)

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and maximum velocity (V_{max}), were estimated from the data of the substrate concentrations [S] and initial velocity (v) using WinNonlin Professional (version 5.2.1, Pharsight, Sunnyvale, CA) by a non-linear least-square regression analysis fitted to the Michaelis-Menten equation, $v = V_{max} \times [S]/(K_m + [S])$.

Statistical method.

Unpaired, two-tailed Student t-test was performed with Microsoft Excel 2010 for statistical analyses.

Ethical approval.

This study was performed under a protocol approved by the Institutional Human Ethical Committee of Daiichi Sankyo. All experimental procedures for human tissue collection and sample preparation at Daiichi Sankyo Europe GmbH were performed according to the guidelines of the Human Tissue and Cell Research foundation (Thasler et al., 2003).

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Results

Correlation Analysis of CMBL mRNA, Protein and Activity in the Intestine.

The intestinal sample set contained all intestinal segments from duodenum to sigmoid colon. Around 11-fold difference in the CMBL mRNA levels were observed among all the tested 20 individual samples as shown in Fig. 2. Although the CMBL protein and OM-hydrolase activity were detected in all the tested 30 individual samples, both of these showed greater variability, ~16-fold, than the mRNA levels. Correlations between the CMBL mRNA and protein expressions (n=20), and between the protein expression and OM-hydrolase activity (n=30) in human intestine are shown in Fig. 2. A weak positive correlation was observed between the mRNA and protein expression levels (r = 0.669). Remarkably, OM-hydrolase activity strongly correlated with the CMBL protein expression resulting in a correlation coefficient of 0.958.

Distribution of OM-hydrolase Activity in Intestinal Segments.

We analyzed the distribution of the CMBL protein expression and OM-hydrolase activity in different intestinal segments, namely duodenum (n = 11), jejunum (n = 2), ileum (n = 3), ascending colon (n = 5), transverse colon (n = 3), descending colon (n = 2), and sigmoid colon (n = 4). The OM-hydrolase activity was highly distributed in the proximal region of the intestine, and less distributed in the distal region and so was the CMBL protein level (Fig. 3). The mean value of the activity in each intestinal region was duodenum > jejunum > ileum > colon (ascending colon to sigmoid colon). The OM-hydrolase activities among 13 individual duodenal and jejunal specimens, showing abundant activities, were relatively consistent and came within the range from 0.814 to 1.97 nmol/min/mg protein, whereas the lowest value of a transverse colon specimen was 0.126 nmol/min/mg protein. Also, the activities in the 13 individual

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duodenum and jejunal specimens were well correlated with the CMBL protein expression levels at a correlation coefficient of 0.968 (data not shown).

Correlation Analysis of CMBL mRNA, Protein and Activity in the Liver.

The CMBL mRNA was detected in all the liver samples except only one out of 34 individuals. The interindividual variation in mRNA levels in the liver samples was much greater (136-fold difference) than that in intestinal samples (11-fold difference). In contrast to the great variation in the mRNA expression levels, the interindividual variation in the protein levels was only 4.1-fold. Correlation analysis (n=34) in Fig. 4A showed that CMBL protein levels followed a saturation curve against the mRNA levels. All 40 individual samples showed substantial OM-hydrolase activity, and a 6.8-fold difference in the activity was observed. The OM-hydrolase activity showed a weak positive correlation with the CMBL protein expression (Fig. 4B, n=40, r=0.619). Low OM-hydrolase activities in two individual samples compared to the others were attributed to their low mRNA and protein expression levels of CMBL.

Sex Differences in OM-hydrolase Activity in Human Liver and Intestine.

The sex differences in the CMBL protein expression and OM-hydrolase activity in human liver (26 males and 14 females) and intestine (8 males and 5 females) were analyzed. For analysis of intestinal samples, the data of the duodenum and jejunum in which OM-hydrolase activity was highly distributed were employed. In both the liver and intestine, no statistically significant sex differences in the protein level and activity were observed (Fig. 5). *P*-values in the analysis of liver and intestinal samples were 0.24 and 0.21 in the protein level and 0.07 and 0.27 in the activity, respectively.

Mutant Generation of CMBL and OM-hydrolase Activity.

The mutant proteins of human CMBL, Y155C and A150T, were successfully

created by site-directed mutagenesis in order to evaluate the effects of two non-synonymous CMBL SNPs rs35489000 and rs34487157 (Supplemental Table S3), respectively, which were previously reported in the NCBI dbSNP (build 130; http://www.ncbi.nlm.gov/SNP), on OM-hydrolase activities. All of the recombinant proteins were purified using their affinity tag, and the tag was cleaved from the proteins by thrombin treatment. SDS-PAGE analysis indicated the high levels of purity and complete tag cleavage, and gel filtration analysis demonstrated that both mutants behaved in the same manner as the wildtype protein (data not shown).

In the kinetic analysis, OM was hydrolyzed and converted to pharmacologically active olmesartan by the mutant proteins as well as the wildtype protein in a simple Michaelis-Menten kinetics manner (Fig. 6). As summarized in Table 1, the mutant Y155C showed slightly higher K_m and lower V_{max}, and A150T showed slightly higher V_{max}. The intrinsic metabolic clearance calculated as the V_{max}/K_m for the hydrolysis by the mutant Y155C was approximately half of that by the wildtype (13.1 and 29.5 ml/min/mg protein, respectively), whereas the mutant A150T showed an almost consistent value (35.4 ml/min/mg protein) as the wildtype.

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Discussion

We recently identified CMBL as one of the OM bioactivating hydrolases (Ishizuka et al., 2010), and further investigated the interindividual variability of mRNA and protein expression of CMBL and OM-hydrolase activity using 40 individual human liver and 30 intestinal specimens in the present study.

The clear positive correlation between OM-hydrolase activity and CMBL protein expression among 30 individual human intestinal cytosolic fractions demonstrated that CMBL is a major contributor to the OM bioactivation in the *in vitro* preparations. In our previous work, we demonstrated that the recombinant CMBL produced in mammalian cells exhibited OM-hydrolase activity with the K_m value well agreed with those in human liver and intestinal cytosols, and that chemical inhibition pattern in the recombinant CMBL was quite consistent with those in human liver and intestinal cytosols; where strong inhibition by *p*-chloromercuribenzoate, a free thiol modifier, and partial inhibition by *bis*-*p*-nitrophenylphosphate, a carboxylesterase inhibitor, were observed (Ishizuka et al., 2010), suggesting a substantial contribution of the enzyme to the OM hydrolysis in these *in vitro* preparations. However, the contribution of CMBL was yet to be demonstrated since specific chemical inhibitors or neutralizing antibodies against this novel enzyme are not available. In the present study, the correlation analysis using a large number of human individual specimens revealed that CMBL must be the key enzyme responsible for OM bioactivation in human intestinal cytosol by showing a significant correlation between CMBL protein and the target activity, in addition to the our previously published data on enzyme kinetics and chemical inhibition stated above. The intestine is considered to be the first site of exposure of orally administered prodrug OM to be converted to its active moiety olmesartan by

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metabolic bioactivation. According to the computer simulated intestinal first-pass availability (F_g) of the prodrug OM (Ishizuka et al., 2012), the majority of the prodrug is converted to active olmesartan in the intestinal epithelial cells. In fact, active olmesartan is reported to be the only species in human blood circulation (Laeis et al., 2001). Taken together, where the dominant involvement of CMBL in the intestinal bioactivation of OM and the extensive conversion to olmesartan that may occur in the intestine, it is convincing that the intestinal CMBL is the key enzyme in bioactivation of prodrug OM.

The distribution of OM-hydrolase activity, correspondingly with CMBL protein expression, was not uniform along the length of the intestine. The activity was significantly higher in the proximal region (duodenum > jejunum > ileum) than in the distal region (colon) with some extent of variation within each region. Likewise, the distribution of most CYP enzymes representing the most important class in phase-I drug metabolism is not uniform along the length of the small intestine and is generally higher in the proximal regions of the small intestine (Zhang et al., 1999; Thelen and Dressman, 2009). As for other known hydrolases, there is a relative paucity of knowledge of enzyme distribution in human enterocytes despite the potential for intestinal hydrolases to play major roles in oral prodrug bioactivation in the drug absorption process. However, Imai and Ohura recently reported that intestinal mRNA expression of carboxylesterases belonging to the CES2 gene family (hCE2) and their activities are nearly constant along the jejunum and ileum (Imai and Ohura, 2010), while Schwer and colleagues reported higher mRNA expression of hCE2 in the jejunum than the ileum (Schwer et al., 1997). In the present study, the CMBL protein and OM-hydrolase activity were considerably varied both with approximately 16-fold interindividual

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differences in the sample set from duodenum to colon. However, by focusing on the data of 11 duodenum and 2 jejunum specimens which exhibited abundant CMBL distribution, the activity and protein content did not largely vary with only 3.0- and 2.4-fold difference. Our computer simulation with the previously reported method (Ishizuka et al., 2012) estimated the Fg value of the prodrug OM to be only 20%, even with the lowest activity (0.814 ng/min/mg protein) among 13 cytosolic fractions of the duodenum and jejunum in the present *in-vitro* study. Notably, a clear positive correlation was observed between OM-hydrolase activity and the CMBL protein expression level among the 13 samples, as well as all 30 samples from the duodenum to colon (data not shown). In addition, our *in situ* closed gastrointestinal loop experiment in rats presented high regional absorption rates of radiolabeled OM from the duodenal and jejunal loops compared to those of ileum and stomach (data not shown), suggesting efficient bioactivation of OM due to the agreement of the primary sites of OM absorption and bioactivation in the intestine.

From the analysis using a sample set of 40 individual human liver specimens, the variability seen in CMBL mRNA expression levels was much higher than those observed for the protein level and activity (136-, 4.1- and 6.8-fold difference, respectively). Looking at carboxylesterases, Hosokawa *et al*. reported more than 8-fold interindividual difference in hCES1 protein levels among 12 human liver microsomal preparations (Hosokawa et al., 1995) and Xu *et al*. reported a 3-fold difference for hCES2 among 13 human liver microsomal preparations (Xu et al., 2002). In addition, in both hCES1 and hCES2, age-dependent mRNA expression (adult > child > fetus) in the liver was reported (Yang et al., 2009), therefore further research is needed for CMBL as well to demonstrate developmental expression. In the correlation

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analysis among the liver samples, OM-hydrolase activity exhibited a poor correlation with CMBL protein content in contrast to the analysis for the intestine. One possible reason for the poor correlation in liver cytosols is a significant contribution of different enzymes including another OM-hydrolase PON1 (Ishizuka et al., 2012), which is localized in the liver microsomal fraction (Leviev et al., 1997); (Marsillach et al., 2007) as well as in plasma. Possible contribution of the CMBL protein to OM hydrolysis might be obscured by the contamination of PON1 from the liver microsomal fraction to cytosolic fraction during their separation process.

Moreover, the effects of two non-synonymous SNPs rs35489000 and rs34487157 (NCBI dbSNP build 130, Supplemental Table S3), which result in the two single amino acid substitutions of Y155C and A150T, respectively, were investigated. According to our previous work (Ishizuka et al., 2010), the prediction of a three-dimensional structure of CMBL protein showed that 155Tyr was located near the active center of the protein and could interact with its ligands. In accordance with the prediction, the mutant Y155C by the site-directed mutagenesis led a certain decrease of the OM-hydrolase activity while the mutant A150T did not. In the present study, two outliers out of 40 individual livers demonstrated considerably low mRNA and protein expression levels of CMBL and consequently low OM-hydrolase activities. To elucidate whether the low activities arose from some genetic variations of CMBL, we genotyped the two non-synonymous SNPs above and 6 other SNPs chosen from NCBI dbSNP build 130 (rs10067744, rs1287736, rs1287735, rs3995688, rs687670, and rs10072686, Supplemental Table S3) using DNA extracts from liver specimens of 40 individual Caucasians. As a result, no mutations of the two non-synonymous SNPs were detected among these, which are consistent with the minor allele frequencies in the Caucasian

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population reported in dbSNP (Supplemental Table S3). Besides this, we found no relationship between the CMBL mRNA/protein/activity and the genetic variations of the other 6 SNPs listed above, although variants were detected for all the SNPs with well-matched minor allele frequencies previously reported to dbSNP (Supplemental Table S3). Given the high metabolic clearance of intestinal CMBL estimated using human intestinal cytosol (Ishizuka et al., 2010), it is unlikely that the mutation leads to a substantial *in vivo* decrease of the first-pass conversion rate of the prodrug OM into its active metabolite in human intestine. Also, plasma and hepatic esterase PON1 (Ishizuka et al., 2012), as well as hepatic CMBL, are considered to play a supplemental role to complete the *in vivo* prodrug bioactivation. These multiple bioactivating enzymes in multiple sites allow us to disregard the genetic polymorphism in each bioactivating enzyme which may cause a varied production of the pharmacologically active metabolite.

In the present study, no statistically significant sex-related differences were observed in the liver and intestinal CMBL protein levels and OM-hydrolase activities. In the previous population pharmacokinetic analysis of olmesartan following oral administration of its prodrug OM in healthy volunteers and hypertensive patients (Yoshihara et al., 2005), being female was reported to be one of the effective covariates on a lower apparent oral clearance. However, the observed change was slight and not considerable (13.5% lower in women than men) to adjust the dosage.

In conclusion, the correlation analysis using a number of individual human specimens clearly showed that CMBL is the key enzyme for OM bioactivation in the intestine, where the prodrug OM is practically and predominantly converted into its active metabolite olmesartan after oral administration. The interindividual variations

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in the OM-hydrolase activity and CMBL protein in the duodenum and jejunum, where the activity and protein were highly distributed, were relatively low with only 3.0- and 2.4-fold differences, respectively. In the liver, the variations in the activity and protein were 4.1- and 6.8-fold differences, respectively.

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Authorship Contributions.

Participated in research design: Ishizuka, Yoshigae, and Kurihara.

Conducted experiments: Ishizuka, Rozehnal, Fischer, and Kato.

Performed data analysis: Ishizuka, Rozehnal, Fischer, Kato, and Endo.

Wrote or contributed to the writing of the manuscript: Ishizuka, Rozehnal, Yoshigae, Kurihara, and Izumi.

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Footnotes

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Figure Legends

Figure 1 Bioactivation of olmesartan medoxomil.

Hydrolysis liberates its active metabolite olmesartan and generates a diketone. Another possible product, RNH-8097, was not detected in the *in vitro* reaction mixture.

Figure 2 Correlation analysis of CMBL mRNA, protein and activity in human intestine.

Correlations between the CMBL mRNA and protein expressions (*A*, n=20) and between the protein expression and OM-hydrolase activity (*B*, n=30) are shown. The CMBL mRNA level was determined by quantitative real-time RT-PCR and normalized to those of GAPDH mRNA in single determinations. The CMBL protein expression level was measured by semi quantitative Western blot analysis in single determinations. The OM-hydrolase activity was expressed as an olmesartan formation rate, from which that in the HEPES buffer was subtracted as non-enzymatic hydrolysis, and data represent the means of duplicate determinations.

Figure 3 Distribution of the CMBL protein expression and the OM-hydrolase activity in human intestinal segments.

Thirty individual intestinal cytosolic fractions were prepared from seven different intestinal segments (duodenum; n=11, jejunum; n=2, ileum; n=3, ascending colon; n=5, transverse colon; n=3, descending colon; n=2, sigmoid colon; n=4). The CMBL protein expression level (*A*) was measured by semi quantitative Western blot analysis in single determinations. The OM-hydrolase activity (*B*) was expressed as an olmesartan

formation rate, where the HEPES buffer was subtracted as non-enzymatic hydrolysis, and data represent the means of duplicate determinations. Closed circles and gray bars represent the individual and average values of each intestinal segment, respectively.

Figure 4 Correlation analysis of CMBL mRNA, protein and activity in human liver.
Correlations between the CMBL mRNA and protein expressions (*A*, n=34) and between the protein expression and OM-hydrolase activity (*B*, n=40) are shown. The CMBL mRNA level was determined by quantitative real-time RT-PCR and normalized to those of GAPDH mRNA in single determinations. The CMBL protein expression level was measured by semi quantitative Western blot analysis in single determinations. The OM-hydrolase activity was expressed as an olmesartan formation rate, from which that in the HEPES buffer was subtracted as non-enzymatic hydrolysis, and data represent the means of duplicate determinations.

Figure 5 Sex differences in the CMBL protein expression and OM-hydrolase activity in human liver and intestine.
Data for 40 liver samples (male; n=26, female; n=14) and 11 intestinal samples (all duodenum, male; n=7, female; n=4) were analyzed and represent the means ± SD. The CMBL protein expression level (*A*) was measured by semi quantitative Western blot analysis in single determinations. The OM-hydrolase activity (*B*) was expressed as an olmesartan formation rate, from which that in the HEPES buffer was subtracted as non-enzymatic hydrolysis, and data represent the means of duplicate determinations.

Figure 6 Enzyme kinetics for OM-hydrolase activities by wildtype and mutant

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proteins of human CMBL.

Enzyme kinetics of the wildtype protein of human CMBL and the site-directed single mutants each with an amino acid substitution (Y155C or A150T) led by the non-synonymous SNPs (rs35489000 and rs34487157, respectively) was investigated. The initial velocity (OM-hydrolase activity) was expressed as an olmesartan formation rate by each recombinant CMBL protein, from which that in the HEPES buffer was subtracted as non-enzymatic hydrolysis. Panels A and B show the direct plot and the Eadie-Hofstee plot, respectively. Data represent the means ± SD (*A*) and means (*B*) of triplicate determinations. The *solid line* is the best fit by non-linear least-squares regression to the Michaelis-Menten equation. S: substrate concentration, v_0: initial velocity.

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Table 1 Kinetic parameters for OM-hydrolase activities by wildtype and mutant proteins of human CMBL

Recombinant CMBL	K_m	V_{max}	V_{max}/K_m
	μM	*nmol/min/mg protein*	*ml/min/mg protein*
Wildtype	119	3510	29.5
Y155C	156	2040	13.1
A150T	114	4030	35.4

Data generated in triplicate determinations were fitted to the single enzyme Michaelis-Menten model by nonlinear least-squares regression.

Fig. 1

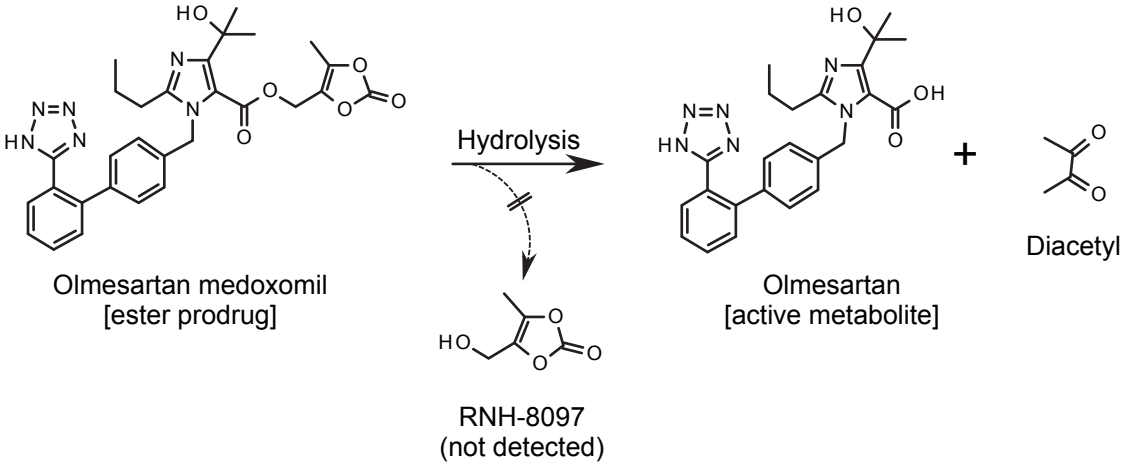

HO
N
N=N
HN
N
O
O
O
O
O
Hydrolysis
OH
+
Diacetyl
Olmesartan medoxomil
[ester prodrug]
Olmesartan
[active metabolite]
RNH-8097
(not detected)

Fig. 2

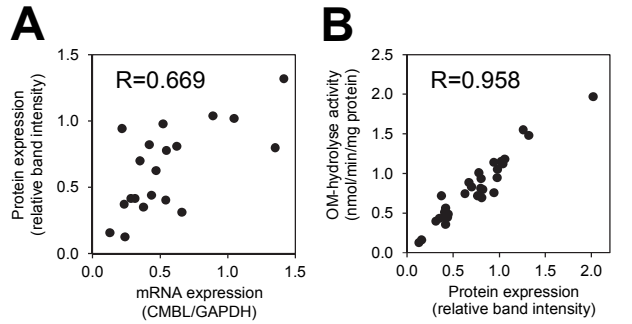
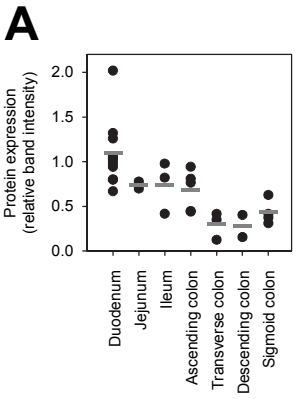

A
R=0.669
Protein expression
(relative band intensity)
mRNA expression
(CMBL/GAPDH)
0.0
0.5
1.0
1.5
B
R=0.958
OM-hydrolyse activity
(nmol/min/mg protein)
Protein expression
(relative band intensity)
0.0
0.5
1.0
1.5
2.0
2.5

Fig. 3


A
Protein expression
(relative band intensity)
2.0
1.5
1.0
0.5
0.0
Duodenum
Jejunum
Ileum
Ascending colon
Transverse colon
Descending colon
Sigmoid colon

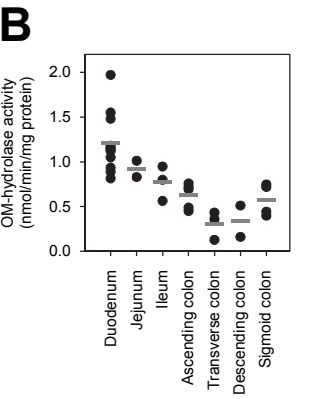
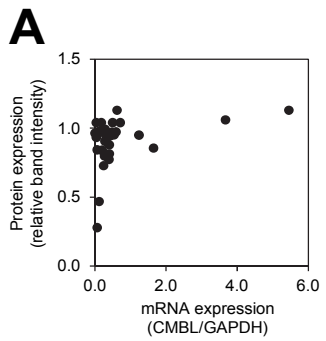

B
OM-hydrolase activity
(nmol/min/mg protein)
2.0
1.5
1.0
0.5
0.0
Duodenum
Jejunum
Ileum
Ascending colon
Transverse colon
Descending colon
Sigmoid colon

Fig. 4


A
Protein expression
(relative band intensity)
1.5
1.0
0.5
0.0
0.0
2.0
4.0
6.0
mRNA expression
(CMBL/GAPDH)

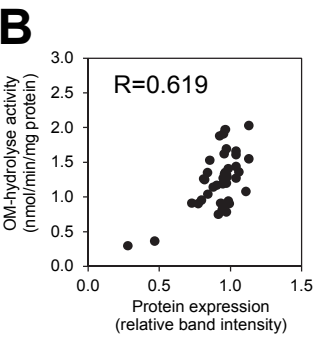
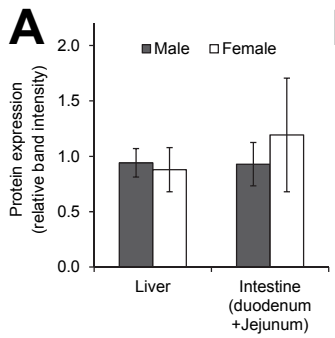

B
OM-hydrolyse activity
(nmol/min/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
R=0.619
0.0
0.5
1.0
1.5
Protein expression
(relative band intensity)

Fig. 5


A
Male
Female
Protein expression
(relative band intensity)
2.0
1.5
1.0
0.5
0.0
Liver
Intestine
(duodenum
+Jejunum)

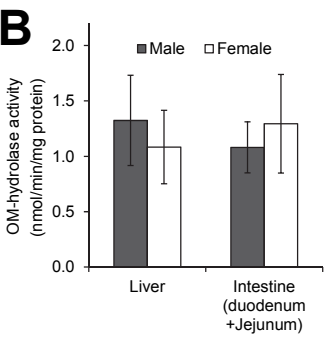
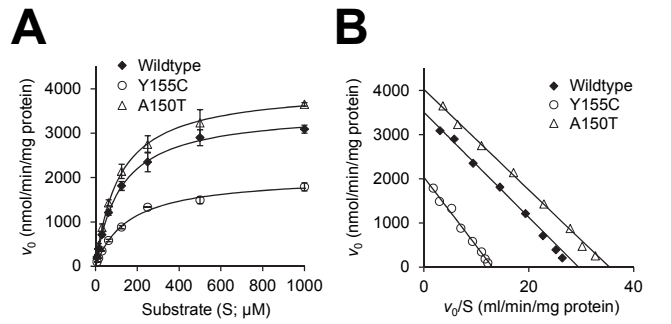

B
Male
Female
OM-hydrolase activity
(nmol/min/mg protein)
2.0
1.5
1.0
0.5
0.0
Liver
Intestine
(duodenum
+Jejunum)

Fig. 6


A
Wildtype
Y155C
A150T
4000
3000
2000
1000
0
v_0 (nmol/min/mg protein)
0
200
400
600
800
1000
Substrate (S; μM)
B
Wildtype
Y155C
A150T
4000
3000
2000
1000
0
v_0 (nmol/min/mg protein)
0
20
40
v_0/S (ml/min/mg protein)

Interindividual variability of carboxymethylenebutenolidase homolog, a novel olmesartan medoxomil hydrolase, in human liver and intestine

Tomoko Ishizuka, Veronika Rozehnal, Thomas Fischer, Ayako Kato, Seiko Endo, Yasushi Yoshigae, Atsushi Kurihara, and Takashi Izumi

Drug Metabolism and Disposition

Supplemental Table S1 The records of liver sample donors

No.	Sex	Age	BMI	Diagnosis
1	male	60	29	-
2	male	72	23	colon cancer
3	male	57	27	rectum carcinoma, liver metastasis segm V+VII
4	male	48	25	-
5	female	70	20	-
6	male	64	22	sigma carcinoma
7	female	57	24	sigma carcinoma
8	female	64	28	sigma carcinoma
9	male	77	26	chronic cholestasis
10	male	74	22	rectum carcinoma
11	female	62	24	gastrointestinal struma tumors
12	male	53	25	colorectal carcinoma, liver metastasis
13	female	68	28	adenocarcinoma, liver and lung failure
14	female	37	37	liver adenoma
15	male	72	31	hepatocellular carcinoma
16	female	47	19	colon carcinoma
17	male	73	29	-
18	female	41	20	sigma carcinoma, liver metastasis
19	male	54	23	neuroendocrine carcinoma
20	male	16	22	hepatocellular carcinoma
21	male	49	25	hepatocellular carcinoma
22	female	71	32	cholangiocellular carcinoma
23	male	95	unknown	rectum carcinoma, liver failure
24	female	61	26	sigma carcinoma, liver failure
25	male	68	30	sigma carcinoma, liver failure
26	male	73	28	rectum carcinoma
27	male	72	24	cholangiocellular carcinoma, lung metastasis
28	male	31	25	hemangiom
29	male	72	25	colon carcinoma
30	male	81	21	colon carcinoma, liver metastasis segm V
31	male	31	23	hepatic atrophy
32	female	69	33	cholangiocellular carcinoma
33	male	74	25	hepatocellular carcinoma segm II
34	female	62	31	rectum carcinoma, liver failure segm II+III
35	male	36	21	liver metastasis segm VIII
36	male	59	30	sigma carcinoma
37	male	69	26	liver metastasis segm VI
38	female	50	29	-
39	female	62	19	cholangiocellular carcinoma
40	male	66	43	colorectal liver failure

Interindividual variability of carboxymethylenebutenolidase homolog, a novel olmesartan medoxomil hydrolase, in human liver and intestine

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Drug Metabolism and Disposition

Supplemental Table S2 The records of intestinal sample donors

No.	Sex	Age	BMI	Diagnosis	Intestinal segment
1	female	69	18	suspicion of pancreatic cancer	duodenum
2	male	63	26	pancreatic cancer	duodenum
3	female	76	26	pancreatic cancer	duodenum
4	male	47	24	pancreatic cancer	duodenum
5	male	56	26	pancreatic cancer	duodenum
6	male	72	26	pancreatic cancer	duodenum
7	male	46	22	pancreatic cancer	duodenum
8	male	69	28	pancreatic cancer	duodenum
9	female	82	24	pancreatic cancer	duodenum
10	female	63	17	pancreatic cancer	duodenum
11	male	80	30	pancreatic cancer	duodenum
12	male	62	23	metastatic liver cancer	jejunum
13	female	44	35	leiomyosarcoma	jejunum
14	male	63	24	metastatic liver cancer	ileum
15	male	81	unknown	colon cancer	ileum
16	male	70	35	right hemicolectomy	ileum
17	male	78	24	colon cancer	ascending colon
18	female	56	unknown	colon cancer	ascending colon
19	male	70	35	right hemicolectomy	ascending colon
20	female	73	23	colon cancer	ascending colon
21	male	65	32	colon cancer	ascending colon
22	female	82	22	colon cancer	transverse colon
23	male	78	29	colon cancer	transverse colon
24	female	67	24	sigmoid colon cancer	transverse colon
25	male	59	21	retroperitoneal sarcoma	descending colon
26	male	66	25	rectal cancer	descending colon
27	male	57	25	rectal cancer	sigmoid colon
28	female	77	25	rectal cancer	sigmoid colon
29	male	55	23	rectal cancer	sigmoid colon
30	male	84	29	sigmoid colon cancer	sigmoid colon

Interindividual variability of carboxymethylenebutenolidase homolog, a novel olmesartan medoxomil hydrolase, in human liver and intestine

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Drug Metabolism and Disposition

Supplemental Table S3 Genotyped single nucleotide polymorphisms in the human CMBL gene

dbSNP rs#	Gene structure	Allele	Amino acid substitution	Minor allele frequency* (build 130; HapMap-CEU)	TaqMan SNP genotyping assay
rs35489000	exon4	A/G	C155Y	G=0	C__25770201_10
rs34487157	exon4	A/G	A150T	A=0	C__25770225_10
rs10067744	exon5	A/G	I157I	A=0.305	C___2839206_10
rs1287736	3'UTR	A/T	-	A=0.108	C___8798909_20
rs1287735	3'UTR	A/G	-	A=0.168	C___8798908_10
rs3995688	3'UTR	G/T	-	G=0.277	C__27324906_10
rs6876709	intron	C/G	-	C=0.314	C___2951754_10
rs10072686	5' near gene	A/G	-	G=0.305	C__30343761_10

Polymorphisms were genotyped using the TaqMan SNP genotyping assays above (Applied Biosystems, Foster City, CA) on ABI Prism 7900HT Fast Real-Time PCR system (Applied Biosystems) according to the manufacturer's instructions. UTR; untranslated region. *No update until the latest release (build 137).