Different hydrolases involved in bioactivation of prodrug-type angiotensin receptor blockers: carboxymethylenebutenolidase and carboxylesterase 1

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Running Title:
Bioactivation of ARB prodrugs by CMBL and CES1

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Abbreviations: ARB, angiotensin receptor blocker; AM, azilsartan medoxomil; BNPP, bis-p-nitrophenylphosphate; CC, candesartan cilexetil; CES, carboxylesterase; CMBL, carboxymethylenebutenolidase homolog; $K_m$, Michaelis constant; LC-MS/MS, liquid chromatography with tandem mass spectrometry; OM, olmesartan medoxomil; S9,
9,000×g supernatant; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; $V_{\text{max}}$, maximum velocity
Abstract

Olmesartan medoxomil (OM) is a prodrug-type angiotensin II type 1 receptor blocker (ARB). We recently identified carboxymethylenebutenolidase homolog (CMBL) as the responsible enzyme for OM bioactivation in humans. In the present study, we compared the bioactivating properties of OM with those of other prodrug-type ARBs, candesartan cilexetil (CC) and azilsartan medoxomil (AM), by focusing on interspecies differences and tissue specificity. In in-vitro experiments with pooled tissue subcellular fractions of mice, rats, monkeys, dogs, and humans, substantial OM-hydrolase activities were observed in cytosols of the liver, intestine, and kidney in all the species tested except for dog intestine showing negligible activity, whereas lung cytosols showed relatively low activities compared with the other tissues. AM-hydrolase activities were well correlated with the OM-hydrolase activities. In contrast, liver microsomes exhibited the highest CC-hydrolase activity among various tissue subcellular fractions in all the species tested. As a result of Western blot analysis with the tissue subcellular fractions, the band intensities stained with anti-human CMBL and carboxylesterase 1 (CES1) antibodies well reflected OM- and AM-hydrolase activities and CC-hydrolase activity, respectively, in animals and humans. Recombinant human CMBL and CES1 showed significant AM- and CC-hydrolase activities, respectively, whereas CC hydrolysis was hardly catalyzed with recombinant carboxylesterase 2 (CES2). In conclusion, OM is bioactivated mainly via intestinal and additionally hepatic CMBL not only in humans but also in mice, rats, and monkeys, while CC is via hepatic CES1 rather than intestinal enzymes including CES2. AM is a substrate for CMBL.
Introduction

Historically, many ester-based prodrugs have been developed with the aim of overcoming a number of barriers to drug-like properties (Liederer and Borchardt, 2006; Huttunen et al., 2011). Esterases, which are involved in bioactivation of ester-based prodrugs and inactivation for clinical drugs as well, are typically represented by carboxylesterases (CES) (Satoh and Hosokawa, 2006; Hosokawa, 2008), cholinesterases (Taylor, 1991; Taylor and Radic, 1994) and paraoxonases (Draganov and La Du, 2004). In addition, recent works revealed several valuable aspects of other esterases responsible for drug hydrolysis; valacyclovirase as a bioactivating hydrolase for the amino-acid ester prodrugs valacyclovir and valcyclovir (Kim et al., 2003; Lai et al., 2008), ary lacetamide deacetylase as a hydrolase of clinical drugs such as flutamide, phenacetine, and rifamycins, and being associated with their organ toxicities (Watanabe et al., 2009; Watanabe et al., 2010; Nakajima et al., 2011), α/β hydrolase domain containing 10 (ABHD10) which functions as the detoxification enzyme of mycophenolic acid acyl-glucuronide (Iwamura et al., 2012).

Although esterases are attractive targets for prodrug bioactivating enzymes due to their wide distribution in the blood, liver, intestine and many other biological fluids and tissues (Berry et al., 2009; Fukami and Yokoi, 2012), interspecies differences in tissue-specific esterase expressions at times cause challenges to extrapolate animal data to humans. For instance, many examples have shown higher hydrolase activity in rodent species than humans, while there is minimal hydrolase activity in dog small intestine (Liederer and Borchardt, 2006; Satoh and Hosokawa, 2010). These observations suggest the importance of selecting appropriate animal species that most closely represent humans in drug development processes based on comprehensive
understanding of the species differences.

Angiotensin receptor blockers (ARBs) that directly inhibit the binding of angiotensin II to angiotensin II type 1 receptor provide an effective pharmacologic strategy in the management of hypertension, cardiovascular disease, and renal disease (De Gasparo et al., 2000). Among eight market-available ARBs, candesartan cilexetil (CC), olmesartan medoxomil (OM), and azilsartan medoxomil (AM) are ester-based prodrugs which require hydrolysis to be converted into their respective active metabolites. As shown in Fig. 1, the prodrug moieties of OM and AM are identical, where the medoxomil moiety links to the carboxylic acid of the drug via an ester bond (Laeis et al., 2001; Kawaguchi et al., 2013), while the CC is a cyclohexyloxy carbonyloxy ethyl ester of the carboxylic acid of candesartan (Nishikawa et al., 1997). We recently reported that a novel human hydrolase, carboxymethylenebutenolidase homolog (CMBL), is responsible for the OM bioactivation (Ishizuka et al., 2010; Ishizuka et al., 2012), however, responsible enzymes for CC- and AM-bioactivations have not been clearly identified thus far.

This study was designed to investigate the difference in the bioactivating properties of the prodrug-type ARBs by comparing CC- and AM-hydrolase activities with that of OM using human and animal tissue subcellular fractions and recombinant proteins of candidate human hydrolases. Also, this work provides new insights into interspecies differences in tissue-specific expressions and activities of the novel hydrolase CMBL between humans and other animal species.
Materials and Methods

Materials.

Olmesartan medoxomil (OM), olmesartan, candesartan, and RNH-6272 were synthesized in Daiichi Sankyo (Tokyo, Japan). RNH-6272 is a structural analog of olmesartan and was used as an internal standard for determination of prodrug active metabolites (olmesartan, candesartan, and azilsartan). Candesartan cilexetil (CC) was synthesized at Chemtech Lab. Inc. (Tokyo, Japan). Azilsartan medoxomil (AM) and azilsartan were purchased from Cosmo Bio (Tokyo, Japan) and Sigma-Aldrich Japan (Tokyo, Japan), respectively. Bis-p-nitrophenylphosphate (BNPP) was purchased from Tokyo Chemical Industry (Tokyo, Japan). All the other chemicals and reagents were of the highest grade available and were obtained from standard commercial sources.

Tissue subcellular fractions and plasma.

Pooled male CD1 mouse, male Sprague Dawley rat, male cynomolgus monkey, male beagle dog, and mixed gender human tissue (liver, intestine, kidney, and lung) cytosolic and microsomal fractions were used in the study. Mouse intestinal, human kidney, rat, monkey, dog, and human lung cytosolic fractions were separated from commercially available 9,000xg supernatant (S9) fractions of respective tissues (XenoTech, LLC., Lenexa, KS) as supernatant by ultracentrifugation at 105,000xg at 4°C for 1 h. Mouse lung microsomal fraction was also prepared from a mouse lung S9 fraction (XenoTech, LLC.). The pellet obtained by ultracentrifugation of the S9 fraction at 105,000xg at 4°C for 1 h was washed and re-pelleted with 10 mM potassium phosphate buffer (pH 7.4) containing 1.15% KCl by ultracentrifugation with the same conditions as above. The obtained pellet was re-suspended with 10 mM potassium phosphate buffer (pH 7.4) containing 30% glycerol to use as the microsomal fraction.
The other cytosolic and microsomal fractions were purchased from XenoTech, LLC. Pooled male CD1 mouse, male Sprague Dawley rat, male cynomolgus monkey, male beagle dog, and male human plasma were used in the study. Blood was collected from 3 or more individuals and centrifuged to separate plasma.

Recombinant human carboxylesterase.

To construct a destination vector containing a FLAG epitope tag, the synthesized oligonucleotides encoding FLAG tag (amino acid sequence: DYKDDDDK) and reading frame A (Invitrogen, Carlsbad, CA) were inserted into pcDNA3.1(+) (Invitrogen) as a NheI/HindIII and EcoRV fragment, respectively. The resulting vector pcDNA3.1-CFLAG-GW was verified by sequencing. pDONR-human CES1 and pDONR-human CES2 entry clones, which contain human CES1 and CES2 genes without their native stop codon, respectively (produced by Invitrogen), were recombined with the destination vector pcDNA3.1-CFLAG-GW using LR clonase II (Invitrogen) to generate the expression vectors pcDNA3.1-CFLAG-CES1 and pcDNA3.1-CFLAG-CES2, which encodes human CES1 and CES2, respectively, with a FLAG tag at the c-terminus. The recombinant proteins were produced in mammalian FreeStyleTM 293-F Cells (Invitrogen). Gene transfected cells were cultured for 7 days in FreeStyleTM 293 Expression Medium (Invitrogen). The conditioned media from the cells overexpressing human CES1 and CES2 were dialyzed against 20 mM Tris-HCl buffer (pH 8.0) and filtered with a polyethersulfone membrane filter (0.45 µm, Thermo Fisher Scientific, Rockford, IL). The overexpressed FLAG-tagged protein in the filtrate was purified with a two-step purification process: anion chromatography with HiTrap Q-XL (GE Healthcare) followed by anti-FLAG M2 affinity gel (Sigma Aldrich). The eluates were collected, desalted with PD midi Trap G-25 (GE Healthcare), and then
the resultant protein was stored frozen at -80°C until use.

**Hydrolase Activity Measurement in tissue subcellular fractions.**

The hydrolase activities for the three prodrug-type ARBs, OM, CC, and AM in animal and human tissue subcellular fractions were measured with 10 μM of each prodrug as a substrate (final solvent concentration: 2.5% acetonitrile) in duplicate. After 5-min pre-incubation, the reaction was initiated by adding the substrate. OM and AM were incubated in 100 mM HEPES buffer (pH 7.4, incubation volume of 0.2 ml) with 100 μg cytosolic or microsomal protein/ml, and CC was incubated in 100 mM Tris-HCl buffer (pH 7.5, incubation volume of 0.2 ml) at 37 °C for 1, 2, and 10 min with 10 to 100 μg cytosolic or microsomal protein/ml. 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 the respective active metabolite concentrations and 0.25% formic acid for preventing the non-enzymatic degradation of the prodrugs. The mixture, with the volume of ca. 200 μL, was filtered using a Captiva 96-well filter plate.

For olmesartan measurement, the Captiva filtrate was 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) and an API3200 (Applied Biosystems/MDS SCIEX) as previously reported (Ishizuka et al., 2013). For candesartan and azilsartan measurements, the filtrate was directly analyzed by LC-MS/MS consisting of a Prominence CBM-20A system (Shimadzu) and an API4000 (Applied Biosystems/MDS SCIEX). The two metabolites were separated with a reversed-phase ODS column (Shim-pack XR-ODS, 2.0 mm ID×300 mm, Shimadzu). The mobile phase total flow was set to 0.75 ml/min with
binary gradient elution, using solvent A (5% acetonitrile, 0.2% formic acid, 100 mM ammonium acetate) and B (95% acetonitrile, 0.2% formic acid, 100 mM ammonium acetate). The gradient started with 45% B for 0.5 min and was increased to 100% B for 0.5 min. Elution was continued for 1 min at 100% B, and then the total flow was increased to 5 ml/min to wash the column. Candesartan and azilsartan were determined by monitoring the ion transition of \( m/z \) 441 to \( m/z \) 263 and \( m/z \) 457 to \( m/z \) 233, respectively; with multiple reaction monitoring in the positive electrospray ionization mode.

The enzymatic activity was expressed as a metabolite formation rate (nmol/min/mg protein) based on the production of respective active metabolite for the reaction by each enzyme source, from which the buffer control as non-enzymatic hydrolysis was subtracted. The incubation time and cytosolic/microsomal protein concentration were selected to be in the linear range for the metabolite formation.

**Hydrolysis by Recombinant human CES1 and CES2.**

The OM-, CC- and AM-hydrolase activities by recombinant human CES1 and CES2 were measured with 10 \( \mu \)M of the prodrugs as substrates (final solvent concentration: 2.5% acetonitrile) with or without 1 mM BNPP (final solvent concentration: 1% dimethyl sulfoxide) in triplicate. After 5-min pre-incubation, the reaction was initiated by adding the substrate. The prodrugs were incubated in 50 mM Tris-HCl buffer (pH 7.5, incubation volume of 0.2 ml) containing 5% (v/v) conditioned media from CES1- or CES2-overexpressed cells as an enzyme source at 37 °C for 2 min. The enzymatic activity was expressed as a metabolite formation rate per reaction (nmol/min/reaction) based on the production of respective metabolites.

**Hydrolysis by Recombinant human CMBL.**
The OM-, CC- and AM-hydrolase activities by recombinant human CMBL were measured with 10 μM of the prodrugs as substrates (final solvent concentration: 2.5% acetonitrile) in triplicate. After 5-min pre-incubation, the reaction was initiated by adding the substrate. The prodrugs were incubated in 100 mM HEPES buffer (pH 7.4, incubation volume of 0.2 ml) at 37 °C for 5 min with 100 μg of the human CMBL-transfected mammalian cell lysate constructed in-house (Ishizuka et al., 2010) as an enzyme source. The enzymatic activity was expressed as a metabolite formation rate (v₀: nmol/min/mg protein) based on the production of azilsartan, from which the vector control was subtracted as non-enzymatic hydrolysis.

In addition, the kinetic analysis for AM hydrolysis by recombinant human CMBL was carried out with a substrate range of 0.781 to 100 μM. The kinetic constant (Kₘ) and maximum velocity (Vₘₐₓ) for AM hydrolysis were estimated using WinNonlin Professional (version 1.4.1, Pharsight, CA) by a non-linear least-square regression analysis fitting to the Michaelis-Menten equation, v₀ = Vₘₐₓ × [S]/(Kₘ + [S]), where v₀ and [S] represent initial velocity and substrate concentration, respectively.

**Western Blot Analysis of CMBL and CES1 Expression.**

The following tissue subcellular fractions (liver, intestine, kidney, and lung cytosol and microsomes) and diluted plasma were subjected to Western blot analysis. Mouse, rat and dog; 2 μg protein for each subcellular fraction and 10 μl of 100-fold diluted plasma, monkey and human; 0.5 μg protein for each subcellular fraction and 5 μl of 200-fold diluted plasma. As positive controls, 0.5 μg of human CMBL-overexpressed mammalian Freestyle 293-F cell lysate, 10 ng of purified recombinant protein of human CES1 were also analyzed. The sample proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% sodium dodecyl
sulfate-polyacrylamide gel (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 CMBL and CES1 proteins in animals and humans were stained with affinity-purified rabbit polyclonal IgG against a human CMBL peptide (Immuno-Biological Laboratories, 1:5000 dilution) and affinity-purified rabbit polyclonal IgG against a human CES1 peptide (ab52941; abcam, 1:1000 dilution) as primary antibodies, respectively, and ECL anti-rabbit IgG HRP-linked, from donkey (Amersham Biosciences, 1:10000 dilution) as a secondary antibody. These immunoblots were visualized by chemiluminescence 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).

**Protein Assay.**

Protein concentration was determined by the Bradford method using bovine serum albumin as the reference standard.
Results

**Prodrug hydrolysis in tissue subcellular fractions.**

The OM-hydrolase activities with mouse, rat, monkey, dog, and human tissue cytosolic and microsomal fractions are shown in Fig. 2. The activity was generally higher in cytosols than in respective microsomes. Liver, intestine, and kidney cytosols exhibited substantial OM-hydrolase activities in all the species tested except for dog intestine, whereas lung cytosols showed relatively low activities. In rats, liver and kidney microsomes showed comparable activities to those of respective cytosols. In AM hydrolysis, similar patterns of the species- and tissue-specific activity to OM hydrolysis were observed in both cytosols and microsomes (data not shown). In contrast to OM hydrolysis, the CC-hydrolase activity was generally higher in microsomes than in respective cytosols as shown in Fig. 3. In all the species tested, liver microsomes exhibited the highest CC-hydrolase activity among various tissue subcellular fractions. The CC hydrolysis was mostly liver specific in humans, whereas significant activities were observed in the kidney and lung along with liver in the other species. Minimal activity of CC hydrolysis was observed in the intestine in all the species tested.

Using the above activity data, the CC-hydrolase and AM-hydrolase activities were plotted against the OM-hydrolase activity. Twenty data points for each cytosolic and microsomal fractions prepared from 4 different tissues (liver, intestinal, kidney, and lung) of 5 species (mouse, rat, monkey, dog, and human) were used. As shown in Fig. 4, no correlation was observed between the CC- and OM-hydrolase activities in both cytosolic and microsomal fractions, whereas the AM-hydrolase activities were well correlated with the OM-hydrolase activities in both cytosolic and microsomal fractions.
with correlation coefficients of 0.929 and 0.832, respectively.

**Hydrolysis by recombinant human CES1 and CES2.**

To confirm the substrate specificities of the recombinant human CES1 and CES2 in conditioned media from the overexpressing cells, the hydrolase activities of p-nitrophenyl acetate (PNPA), clopidogrel, and CPT-11, which are a general esterase substrate, CES1 specific and CES2 specific substrate, respectively, were measured. The CES1 and CES2 conditioned media showed comparable hydrolytic activity toward PNPA, and that CES1 and CES2 specifically catalyzed the clopidogrel hydrolysis forming clopidogrel carboxylic acid and the CPT-11 hydrolysis forming SN-38, respectively (Supplemental Fig. S1).

As shown in Fig. 5, CC was rapidly de-esterified with the recombinant CES1 and produced its active metabolite candesartan at a rate of 514 pmol/min/reaction, whereas the reaction was hardly catalyzed with the recombinant CES2 (24.2 pmol/min/reaction). Both activities were completely abolished by the addition of 1 mM BNPP, a carboxylesterase inhibitor. Although the recombinant CES1 showed slight OM- and AM- hydrolase activities being higher than that of the mock control, the activities were much lower than that for CC hydrolysis. The OM- and AM-hydrolase activities by the recombinant CES2 were comparable with the mock control regardless with or without BNPP, suggesting that only non-enzymatic hydrolysis was slightly observed.

**Hydrolysis by recombinant human CMBL.**

AM, which is another medoxomil-ester prodrug-type ARB, was substantially hydrolyzed by the recombinant human CMBL as well as OM (Fig. 6A), and exhibited simple Michaelis-Menten kinetics (Fig. 6B) with $K_m$ and $V_{max}$ values of 14.8 +/- 1.3 µM and 5.40 +/- 0.16 nmol/min/mg protein (the mean +/- standard error of triplicate
determinations). CC was not hydrolyzed by the recombinant CMBL (Fig. 6A).

**Western Blot Analysis of CMBL and CES1 Expression.**

The immunoblots stained with polyclonal anti-human CMBL and anti-human CES1 antibodies were shown in Fig. 7. In the human liver, intestine, and kidney cytosols, the anti-human CMBL antibody intensely stained a single band at approximately 25 kDa, which was almost consistent with the theoretical mass 28 kDa, whereas it was weakly stained in human lung cytosols. Compared to the cytosols, the weaker CMBL expression was observed in the respective microsomes. Regarding the other animal species as well, liver, intestine, and kidney cytosols demonstrated strongly stained bands with approximately 25 kDa molecular mass except for dog intestine, whereas lung cytosols showed weak protein bands. The band intensity was generally lower in microsomes than in respective cytosols in the animal species as seen in humans. Collectively, these data indicate that the anti-human CMBL antibody recognized the CMBL orthologs in the animal species tested. No significant protein was detected in plasma samples of all the species tested including humans.

The anti-human CES1 antibody used in the present study was reported to be CES1 specific without cross-reactivity with CES2 proteins by probing against cynomolgus monkey, dog, and human CES1 and CES2 enzymes (Williams et al., 2011). As previously reported (Satoh and Hosokawa, 2010; Williams et al., 2011), CES1 proteins were highly expressed in the liver in the three species, and were significantly expressed in monkey intestine and kidney, dog kidney, lung, and plasma, and human lung as a single band with a molecular mass approximately at 60 kDa. Regarding mouse and rat samples, single or multiple bands were detected in all the tissues and plasma.

The protein band intensities of CMBL in Western blot analysis mostly
corresponded with the distribution of OM- and AM-hydrolase activities, and those of CES1 mostly corresponded with the distribution of CC-hydrolase activities in the eight different tissue subcellular fractions tested. The analysis plots for OM- and CC-hydrolase activities toward CMBL and CES1 protein expressions in humans were presented in Fig. 8 as exemplary cases. As shown in Table 1, the correlation coefficients between CMBL protein expression levels and OM- or AM-hydrolase activities were over 0.8 with one exception, where that between rat CMBL protein and OM-hydrolase activity was 0.477. The correlation coefficients between CES1 protein expression levels and CC-hydrolase activities were also over 0.8 without exception.
Discussion

In the present study, we comparatively showed the difference between the bioactivating properties of prodrugs OM and CC which belong to the same class of antihypertensive, ARB. We previously reported that the bioactivation of OM predominantly proceeds via intestinal CMBL in humans and that hepatic CMBL and plasma paraoxonase 1 are additionally involved (Ishizuka et al., 2010; Ishizuka et al., 2012). On the contrary, present in vitro studies demonstrated that the contribution of intestinal enzymes to the CC bioactivation is relatively small and that hepatic CES1 is mainly involved. Although the responsible hydrolases and bioactivation sites were presented to be different between these two prodrugs, they were both reported to be rapidly and completely converted to their active metabolites through the first pass metabolism before they reach the systemic circulation and showed an increase of bioavailability as respective active metabolites (AstraZeneca, 2000; Laeis et al., 2001), meaning that OM and CC are both good exemplary cases of absorbability improvement by esterification of the carboxylic acid group.

Also, we presented for the first time species- and tissue-specific OM hydrolysis catalyzed via CMBL, which was recently identified as an OM-bioactivating hydrolases and had not received much attention until then (Ishizuka et al., 2010), using various tissue subcellular fractions of animals and humans. The kidney and lung were selected in addition to the liver and intestine, which were already demonstrated in humans as bioactivating organs for the prodrug OM (Ishizuka et al., 2010). Human kidney cytosol showed significant OM hydrolase activity which is comparable to those of liver and intestinal cytosol, whereas lung cytosol showed meaningfully lower activity. This tissue distribution pattern of the activity, namely high in the liver, intestine, and kidney
and low in the lung, is consistent with the CMBL protein expression pattern
demonstrated by Western blotting in the present study and also with the mRNA
expression pattern previously reported (Ishizuka et al., 2010). The predominant
expression of CMBL in the liver, intestine, and kidney implies that CMBL has primarily
functioned in detoxification of xenobiotic compounds through the evolutional process of
the widely distributed protein CMBL from bacteria to humans. Bacterial CMBL, also
called dienelactone hydrolase, has been reported as a catabolic enzyme enabling to
utilize chloroaromatic compounds (Schmidt and Knackmuss, 1980; Ngai et al., 1987),
which are generally toxic to organisms.

Mice, rats, and monkeys, which are all commonly used experimental animal
species, exhibited similar tissue distribution patterns to humans in the OM-hydrolase
activity and CMBL protein expression. Based on the results, these animal species
would provide dependable information to predict human bioactivation of ester
compounds hydrolyzed by CMBL in the preclinical drug discovery and development
settings. Although relatively high activities in monkey liver and kidney, and low
activity in rat intestine appeared compared to those in humans, the in vitro study results
would be helpful when considering the quantitative difference of prodrug bioactivation
between species. As a notable exception of the tissue distribution of CMBL, in the
dog intestine slight OM-hydrolase activity or negligible CMBL protein expression was
observed while intestinal cytosols in humans and the other animal species exhibited
significant activities and clear protein expressions. The absence of dog intestinal
CMBL was observed despite highly conserved amino acid sequences with 87%
homology between humans and dogs (GenBank Accession Numbers NP_620164.1 and
XP_535793.1, respectively). This is a common feature to well investigated esterases
such as CES, paraoxonases, and cholinesterases (Williams et al., 2011), and shows
interesting functional and evolutional implications. Low capacity to detoxify esters in
dog intestine potentially suggests the minor importance of the ability in carnivores,
which would have infrequent exposure to toxic substances in plants.

Moreover, we also demonstrated that CMBL is a bioactivating enzyme for recently
launched prodrug-type ARB, AM, whose prodrug moiety is identical to that of OM.
Kawaguchi et al. recently reported a likely estimation that enzymes involved in the
hydrolysis of AM would be the same as OM by considering the identical chemical
structures of the prodrug moiety between the two ARBs and in vitro metabolic study
results using liver and intestinal S9 fractions and plasma (Kawaguchi et al., 2013). As
shown in the results of our in vitro study using recombinant human CMBL, obvious
AM-hydrolase activity clearly demonstrated the involvement of CMBL in the reaction.
Furthermore, the clear positive correlation between OM- and AM-hydrolase activities
suggests the significant contribution of CMBL, not only in humans but also other tested
animal species, to the hepatic and intestinal AM bioactivation. Only limited
information on the substrate specificity of CMBL is available at present: it specifically
catalyzes ring-opening reaction of cyclic esters such as faropenem medoxomil and
lenampicillin (Ishizuka et al., 2010) as well as OM and AM. What seems to be lacking,
as a further interest, is to define the endogenous substrates and to elucidate the
physiological functions of CMBL proteins.

Also, we presented that human CES1 rather than CES2 catalyzed the bioactivation
of CC using recombinant proteins. It has often been reported that CC is converted to
candesartan by carboxylesterases via intestinal and hepatic first-pass metabolism (Miwa
et al., 1998; Easthope and Jarvis, 2002), however, which isozymes of CES1 or CES2 are
a major contributor remained unknown until now. Although Laizure et al. supposed that CC is a CES2 substrate because of the CC’s large alcohol group of the prodrug moiety which CES2 prefers as substrates (Casey Laizure et al., 2013), in the present study, the recombinant CES2 exhibited only low CC-hydrolase activity in contrast to rapid hydrolysis by the recombinant CES1. Moreover, the intestinal subcellular fractions showed minimal CC-hydrolase activity in all the species tested including humans, indicating negligible involvement of CES2 which have been reported to be expressed in the intestine in mice, rats, monkeys, and humans (Taketani et al., 2007; Satoh and Hosokawa, 2010). On the contrary to CC hydrolysis, the recombinant human CES1 showed slight OM- and AM-hydrolase activities, suggesting that human CES1 may partially catalyze OM- and AM-hydrolysis in the liver. However, these activities were abundantly observed in cytosolic fraction of the liver rather than the microsomal fraction which human CES1 is mostly distributed (Fig. 2 for OM hydrolysis). This fact indicates that the CES1 contribution in the liver to OM and AM hydrolysis is limited. CES2 showed only mock-control level OM- and AM-hydrolase activities. Three prodrug-type ARBs, namely OM, CC, and AM, similarly require hydrolytic bioactivation to exert their antihypertensive effect, however, needless to say, the responsible hydrolases depend on their chemical structures of prodrug moiety; OM and AM are substrates for CMBL while CC is a substrate for CES1.

In conclusion, we comparatively demonstrated the difference in the bioactivating properties of prodrug-type ARBs; OM is bioactivated mainly via intestinal and additionally hepatic CMBL, therefore the newly identified CMBL substrate AM is likely to be similarly bioactivated to OM, while CC is via hepatic CES1 rather than intestinal enzymes. Also, we presented species differences in hydrolytic activity and
protein expression of the uninvestigated hydrolase CMBL in four major organs, namely liver, intestine, kidney, and lung, by comparison between humans and other animal species.
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Authorship Contributions.

Participated in research design: Ishizuka, Yoshigae, and Murayama.
Conducted experiments: Ishizuka.
Performed data analysis: Ishizuka.
Wrote or contributed to the writing of the manuscript: Ishizuka, Yoshigae, Murayama, and Izumi.
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olmesartan medoxomil limits the risk of clinically relevant drug interaction.


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Footnotes

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Figure Legends

Figure 1  Bioactivation of the prodrug-type ARBs
Hydrolytic reactions of olmesartan medoxomil (A), candesartan cilexetil (B), and azilsartan medoxomil (C) are shown.

Figure 2  OM-hydrolase activities with animal and human tissue subcellular fractions
Prodrug OM was incubated with animal and human tissue cytosolic (A) and microsomal (B) fractions, and formed active metabolite olmesartan was measured by LC-MS/MS. Data represent the means of duplicate determinations.

Figure 3  CC-hydrolase activities with animal and human tissue subcellular fractions
Prodrug CC was incubated with animal and human tissue cytosolic (A) and microsomal (B) fractions, and formed active metabolite candesartan was measured by LC-MS/MS. Data represent the means of duplicate determinations.

Figure 4  Correlation analysis of hydrolase activities for prodrug-type ARBs with animal and human tissue subcellular fractions
CC-hydrolase (A and B) and AM-hydrolase (C and D) activities are plotted against OM-hydrolase activity. Twenty data points for each cytosolic (A and C) and microsomal (B and D) fractions prepared from 4 different tissues (liver, intestinal, kidney, and lung) of 5 species (mouse, rat, monkey, dog, and human) were used. The prodrugs were incubated with animal and human tissue cytosolic and microsomal fractions and their respectively formed active metabolites were measured by
LC-MS/MS. Data represent the means of duplicate determinations.

Figure 5  Hydrolysis of prodrug-type ARBs by recombinant human CES1 and CES2. Prodrugs OM, CC, and AM were incubated with 5% (v/v) conditioned media from human CES1- or CES2-overexpressed cell culture as an enzyme source under the conditions with or without 1 mM BNPP, and formed active metabolites, olmesartan, candesartan, and azilsartan, were respectively measured by LC-MS/MS. Data represent the means ± SD of triplicate determinations.

Figure 6  Hydrolysis of prodrug-type ARBs by recombinant human CMBL. A: OM-, CC-, and AM-hydrolase activities by recombinant human CMBL. Prodrugs OM, CC, and AM were incubated with recombinant CMBL-overexpressed cell lysate as an enzyme source, and formed active metabolites, olmesartan, candesartan, and azilsartan, were respectively measured by LC-MS/MS. Data represent the means ± SD of triplicate determinations. B: Kinetic analysis of AM hydrolysis. The solid line is the best fit by non-linear least-squares regression to the Michaelis-Menten equation. Data represent the means ± SD of triplicate determinations. The respective Eadie-Hofstee plot is presented in the inset.

Figure 7  Western blot analysis of CMBL and CES1 expressions in animals and humans. The following tissue subcellular fractions (liver, intestine, kidney, and lung cytosol and microsomes) and diluted plasma were subjected to Western blot analysis: Mouse, rat, and dog; 2 μg protein for each subcellular fraction and 10 μl of 100-fold diluted plasma,
monkey and human; 0.5 μg protein for each subcellular fraction and 5 μl of 200-fold
diluted plasma. As positive controls, 0.5 μg of human CMBL-overexpressed
mammalian Freestyle 293-F cell lysate, and 10 ng of purified recombinant protein of
human CES1 were also analyzed. The proteins were separated by SDS-PAGE,
transferred electrophoretically onto a polyvinylidene difluoride membrane, and stained
by polyclonal anti-human CMBL and anti-human CES1 antibodies. The signal
intensities of the protein bands in the subcellular fractions at mobility with closed
triangles were semi-quantified. White bars and open triangles represent molecular
mass marker proteins.

Figure 8  Correlation between protein expression levels of hydrolases and
prodrug-hydrolase activities in humans.

Data of eight different human tissue subcellular fractions (liver, intestine, kidney, and
lung cytosolic and microsomal fractions) were employed to show the correlations
between the protein expressions and activities. A, CMBL vs. OM-hydrolase activity; B,
CMBL vs. CC-hydrolase activity; C, CES1 vs. OM-hydrolase activity; D, CES1 vs.
CC-hydrolase activity.
Table 1  Correlation coefficients between protein expression levels of hydrolases and prodrug-hydrolase activities.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>OM-hydrolase activity</th>
<th>CC-hydrolase activity</th>
<th>AM-hydrolase activity</th>
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</thead>
<tbody>
<tr>
<td>CMBL</td>
<td>Mouse</td>
<td>0.986</td>
<td>-0.236</td>
<td>0.864</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>0.477</td>
<td>-0.221</td>
<td>0.942</td>
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<tr>
<td></td>
<td>Monkey</td>
<td>0.988</td>
<td>0.012</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td>Dog</td>
<td>0.995</td>
<td>-0.019</td>
<td>0.998</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>0.842</td>
<td>-0.200</td>
<td>0.913</td>
</tr>
<tr>
<td>CES1</td>
<td>Mouse</td>
<td>-0.221</td>
<td>0.970</td>
<td>0.171</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>0.443</td>
<td>0.827</td>
<td>0.021</td>
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<td></td>
<td>Monkey</td>
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<td>0.985</td>
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<td></td>
<td>Dog</td>
<td>0.048</td>
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<td>0.005</td>
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<tr>
<td></td>
<td>Human</td>
<td>-0.167</td>
<td>0.959</td>
<td>-0.097</td>
</tr>
</tbody>
</table>

Data of protein expressions and activities of eight different subcellular fractions (liver, intestine, kidney, and lung cytosolic and microsomal fractions) were employed for each species. The protein levels were semi-quantified as signal intensities of the immunoreactive bands at the mobility with closed triangles in Fig. 7.
Figure 1

A

\[
\text{Hydrolysis}
\]

\[
\text{Olmesartan medoxomil [ester prodrug]}
\]

\[
\text{Olmesartan [active metabolite]}
\]

B

\[
\text{Hydrolysis}
\]

\[
\text{Candesartan cilexetil [ester prodrug]}
\]

\[
\text{Candesartan [active metabolite]}
\]

C

\[
\text{Hydrolysis}
\]

\[
\text{Azilsartan medoxomil [ester prodrug]}
\]

\[
\text{Azilsartan [active metabolite]}
\]
**Figure 2**

A

![Bar chart showing OM-hydrolase activity in different tissues of various species.]

B

![Bar chart showing OM-hydrolase activity in different tissues of various species.]

- Liver
- Intestine
- Kidney
- Lung
Figure 4

A

B

C

D

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**Figure 5**

Metabolite formation (pmol/min/reaction)

- CES1
- CES2
- Mock

- BNPP (-)
- BNPP (+)

OM, CC, AM
Figure 6

A

B

OM  CC  AM

Substrate

v₀ (pmol/min/mg protein)

Substrate (µM)

v₀ (nmol/min/mg protein)

v₀/S
**Figure 8**

(A) OM-hydrolase activity (nmol/min/mg protein) vs. CMBL protein expression (relative band intensity). 

R = 0.842

(B) CC-hydrolase activity (nmol/min/mg protein) vs. CMBL protein expression (relative band intensity). 

R = -0.200

(C) OM-hydrolase activity (nmol/min/mg protein) vs. CES1 protein expression (relative band intensity). 

R = -0.167

(D) CC-hydrolase activity (nmol/min/mg protein) vs. CES1 protein expression (relative band intensity). 

R = 0.959
Different hydrolases involved in bioactivation of prodrug-type angiotensin receptor blockers: carboxymethylenebutenolidase and carboxylesterase 1

Tomoko Ishizuka, Yasushi Yoshigae, Nobuyuki Murayama, and Takashi Izumi.

Drug Metabolism and Disposition

Supplemental Figure S1

PNPA, BNPP (-)

PNPA, BNPP (+)

Clopidogrel, BNPP (-)

Clopidogrel, BNPP (+)

CPT-11, BNPP (-)

CPT-11, BNPP (+)
Supplemental Figure S1  Hydrolysis of p-nitrophenyl acetate (PNPA), clopidogrel, and CPT-11 by recombinant human CES1 and CES2.

PNPA (1 mM), clopidogrel, and CPT-11 (10 μM each) were incubated in 50 mM Tris-HCl buffer (pH 7.5) with 5% (v/v) conditioned media from human CES1- or CES2-overexpressed cell culture as an enzyme source under the conditions with or without 1 mM bis-p-nitrophenylphosphate (BNPP). PNPA-hydrolase activity at 30 °C was determined by monitoring the absorbance at 405 nm. Clopidogrel- and CPT-11-hydrolase activities at 37 °C were determined by measuring formed metabolites, clopidogrel carboxylic acid and SN-38, respectively, by LC-MS/MS. Data represent the means ± SD of triplicate determinations.