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Difference in substrate specificity of carboxylesterase and arylacetamide deacetylase between dogs and humans

Tomohiro Yoshida[a], Tatsuki Fukami[a,*], Takaya Kurokawa[a], Saki Gotoh[a], Akifumi Oda[b], Miki Nakajima[a]

[a] Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
[b] Biophysical Chemistry, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan

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ABSTRACT

Carboxylesterase (CES) and arylacetamide deacetylase (AADAC) are the major enzymes responsible for the hydrolysis of various clinical drugs. Our recent study demonstrated that the identity of the responsible hydrolase can be roughly surmised based on the chemical structures of compounds in humans. Dogs are used for preclinical studies in drug development, but the substrate specificities of dog CES and AADAC remain to be clarified. The purpose of this study is to characterize their substrate specificities. We prepared recombinant dog CES1, CES2, and AADAC. *p*-Nitrophenyl acetate, a general substrate for esterases, was hydrolyzed by dog CES1 and AADAC, while it was not hydrolyzed by CES2. CES2 protein was not substantially detected in the recombinant system or in the dog liver and intestinal microsomes by Western blot using anti-human CES2 antibodies. *In silico* analyses demonstrated slight differences in the three-dimensional structures of dog CES2 and human CES2, indicating that dog CES2 might be unstable or inactive. By evaluating the hydrolase activities of 22 compounds, which are known to be substrates of human CES and/or AADAC, we found that the activities of dog recombinant CES1 and AADAC as well as dog tissue preparations for nearly all compounds were lower than those of human enzymes. The dog enzymes that were responsible for the hydrolysis of most compounds corresponded to the human enzymes, but the following differences were observed: oseltamivir, irinotecan, and rifampicin were not hydrolyzed in the dog liver or by any of the recombinant esterases and procaine, a human CES2 substrate, was hydrolyzed by dog CES1. In conclusion, the present study could provide new finding to facilitate our understanding of species differences in drug hydrolysis, which can facilitate drug development and drug safety evaluation.

1. Introduction

Drug metabolism refers to the biochemical transformation of compounds into more polar chemical forms. Although cytochrome P450-mediated metabolism is the most common route of drug metabolism (over 50%), non-P450 enzymes also play a significant role in the metabolism of recently developed drugs (20–35%) (Cerny, 2016). Among non-P450 enzymes, esterases significantly contribute to the metabolism of clinical drugs (approximately 35%) (Cerny, 2016). Esterases are involved in pharmacological bioactivation of prodrugs containing ester, amide, and thioester bonds, or inactivation of drugs. Carboxylesterase (CES) enzymes, CES1 and CES2, are well known to catalyze the hydrolysis of various xenobiotics (Satoh and Hosokawa, 2006; Hosokawa, 2008). CES1 is mainly expressed in the human liver, and it hydrolyzes drugs, such as clopidogrel, imidapril, oseltamivir, and fenofibrate (Imai et al., 2006; Fukami et al., 2015). CES2 is expressed in the human liver and intestine, and it hydrolyzes drugs such as irinotecan and procaine (Xu et al., 2002; Fukami et al., 2015). We have demonstrated that arylacetamide deacetylase (AADAC) is also involved in the hydrolysis of various drugs, such as flutamide, phenacetin, rifamycins, indiplon, prasugrel, and ketoconazole (Watanabe et al., 2009 and 2010; Nakajima et al., 2011; Shimizu et al., 2014; Fukami et al., 2016; Kurokawa et al., 2016). AADAC is expressed in the human liver and gastrointestinal tract, which is where CES enzymes are also expressed (Watanabe et al., 2009). A previous study revealed that CES1 prefers compounds with a large acyl moiety and a small alcohol or amine

Abbreviations: AADAC, arylacetamide deacetylase; CES, carboxylesterase; CPGA, 2-cyclohexyl-2-phenylglycolic acid; DIM, dog intestinal microsomes; DLM, dog liver microsomes; DMSO, dimethyl sulfoxide; EndoH, endoglycosidase H; HIM, human intestinal microsomes; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; MD, molecular dynamics; PNGase-F, peptide: *N*-glycosidase F; PNPA, *p*-nitrophenylacetate; RMSD, root mean square deviation; RMSFs, root mean square fluctuation

* Corresponding author.
E-mail address: tatsuki@p.kanazawa-u.ac.jp (T. Fukami).

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moiety, while CES2 prefers those with a large alcohol or amine moiety and a small acyl moiety (Imai et al., 2006). Our recent study revealed that AADAC prefers substrates with smaller acyl moieties than CES2 (Fukami et al., 2015). Based on their rough characteristics, we can surmise the human hydrolase responsible for the hydrolysis of the compounds of concern, although experimental confirmation is needed.

In the processes of drug development, extrapolation of animal data to humans in predicting drug disposition is used to estimate the pharmacological effects and toxicity of drug candidates. In preclinical studies, mouse, rats, rabbits, guinea pigs, and monkeys are used in for toxicological and toxicokinetics studies. Dogs are also used for pharmacokinetics studies. Species differences in drug-metabolizing enzymes often make it difficult to extrapolate animal data to humans. Therefore, an evaluation of species differences in CES and AADAC is important for drug development. CES and AADAC are conserved in animal species, including mice, rats, dogs, and monkeys (Imai et al., 2006; Taketani et al., 2007; Kobayashi et al., 2011; Williams et al., 2011; Uno et al., 2015), although mice and rats have multiple Ces1 and Ces2 isoforms (Ces1a-Ces1h and Ces2a-Ces2h for mice, and Ces1a-Ces1f and Ces2a-Ces2j for rats) (Holmes et al., 2010). It has been reported that dog CES1 and CES2 are expressed in the liver, but they are not expressed in the intestine (Taketani et al., 2007). We recently found that AADAC is expressed in dog liver and intestine by measuring the hydrolase activity of phenacetin, a specific substrate of human AADAC, although the expression level in the intestine is much lower than that in the liver (Kurokawa et al., 2016). Substrate specificities of CES and AADAC in humans and rodents have been characterized (Luan et al., 1997; Kobayashi et al., 2011; Ozaki et al., 2013; Kurokawa et al., 2015). For example, pranlukast is hydrolyzed by Ces1d and Ces1f (trivial names: carboxylesterase pI 6.0 and pI 6.2, respectively) in rats, whereas it is not hydrolyzed in humans (Luan et al., 1997; Takai et al., 1997). In addition, we demonstrated that diltiazem is hydrolyzed by Ces2a in the rat liver, whereas this is not hydrolyzed in human liver (Kurokawa et al., 2015). Rifamycins including rifampicin, rifabutin, and rifapentine are hydrolyzed by AADAC in human liver, but not in mice and rat liver (Kobayashi et al., 2012). However, substrate specificities of dog CES and AADAC remain to be clarified. In this study, we established recombinant systems for dog CES and AADAC, and we measured their hydrolase activities for 22 compounds, which are known substrates for human CES and/or AADAC, to evaluate species differences in the substrate specificities of esterases between dogs and humans.

2. Materials and methods

2.1. Chemicals and reagents

p-Aminobenzoic acid, 2-cyclohexyl-2-phenylglycolic acid (CPGA), 3,4-dichloroaniline, fluorescein, fluorescein diacetate, flutamide, imidapril hydrochloride, lidocaine, mycophenolic acid, *p*-nitrophenol, 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1), phenacetin, prilocaine, propanil, temocapril hydrochloride, *o*-toluidine, and 2,6-xylidine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Clofibrate, clofibric acid, fenofibrate, oxybutynin chloride, procaine hydrochloride, dapsone, *p*-phenetidine, and *p*-nitrophenylacetate (PNPA) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Acetyldapsone, clopidogrel hydrogen sulfate, clopidogrel carboxylic acid, 25-desacetylrifabutin, 25-desacetylrifampicin, 25-desacetylrifapentine, fenofibric acid, imidaprilat, irinotecan hydrochloride trihydrate, rifabutin, rifampicin, rifapentine, SN-38, and temocaprilat were purchased from Toronto Research Chemicals (Toronto, Canada). Mycophenolate mofetil was from Cayman Chemical Company (Ann Arbor, MI). Oseltamivir phosphate and oseltamivir carboxylate were from LKT Laboratories (St. Paul, MN) and Medchemexpress (Princeton, NJ), respectively. Indiplon was from Carbosynthe (Berkshire, UK). Human liver (pooled HLM, n = 50) and intestinal microsomes (pooled HIM, n = 7) were purchased from Corning (Corning, NY). Dog liver (pooled DLM, n = 8, male) and intestinal (pooled DIM, n = 3, male) microsomes were from Xenotech, LLC (Lenexa, KS). *Spodoptera frugiperda* Sf21 cells were obtained from Invitrogen (Carlsbad, CA). All primers were commercially synthesized at Rikaken (Nagoya, Japan) or Hokkaido System Science (Sapporo, Japan). Other chemicals were of the highest commercially available grade.

2.2. Recombinant esterases expressed in Sf21 cells

Sf21 cell homogenates expressing dog AADAC and mock control were previously prepared (Watanabe et al., 2010; Kurokawa et al., 2016). Expression systems of dog CES1 and CES2 were prepared using a Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer's protocol. Dog CES1 and CES2 cDNA were obtained *via* a reverse transcription-polymerase chain reaction using dog liver RNA sample (UNITECH, Chiba, Japan) with primer sets of CES1 *Eco*R I (GGGAATTCCACGATGTGGCTCTTCGATC) and CES1 *Sac* I (CCCGAGCTCCTCTGTAGAATAGCCCTGTC), or CES2 *Xho* I (GGGCTCGAGCCAGTCCAAACTCCGAACAC) and CES2 *Kpn* I (CCGGTACCCAGCTCTGTGTGCTTCTTCTCC). The underlined nucleotides represent the recognition sites of restriction enzymes. The nucleotide sequences (accession no. NM_001003085.1 and XM_014114117.1 for dog CES1 and CES2, respectively) were confirmed by DNA sequence analysis (FASMAC, Kanagawa, Japan). The pFastBac1 vector containing dog CES1 or CES2 cDNA was transformed into DH10Bac-competent cells, and other steps were performed according to a previously described method (Iwamura et al., 2012). The protein concentrations were determined according to the method of Bradford (1976) using γ-globulin as a standard.

2.3. Hydrolase activities of various compounds

2.3.1. PNPA hydrolase activity

The PNPA hydrolase activity was measured according to a previously reported method (Watanabe et al., 2009) with slight modifications (the concentration of enzyme sources: 0.1 mg/mL; substrate concentration: 100 μM; incubation time: 1 min).

2.3.2. Fenofibrate hydrolase activity

The fenofibrate hydrolase activity was measured using high-performance liquid chromatography (HPLC) according to a previously reported method (Fukami et al., 2015) with slight modifications [the concentration of enzyme sources: 0.1 mg/mL (HLM, HIM, DLM, and DIM) or 0.025 mg/mL (Sf21 cell homogenates expressing dog CES1 and AADAC: 0.1 mg/mL); incubation time: 15 s (HLM, HIM, DLM, and DIM) or 30 s (Sf21 cell homogenates expressing dog CES1 and AADAC)]. Incubation time for each enzyme source was set within the linearity for the formation of metabolite.

2.3.3. Imidapril hydrolase activity

The imidapril hydrolase activity was measured according to a previously reported method (Takahashi et al., 2009) with the following slight modifications. A typical incubation mixture (final volume of 0.2 mL) contained 100 mM potassium phosphate buffer, pH 7.4, and 0.2 mg/mL enzyme sources. The reaction was initiated by the addition of 200 μM imidapril and terminated by the addition of 200 μL of ice-cold acetonitrile. The mixture after incubation was centrifuged at 12,000*g* for 5 min and a 50-μL aliquot of the supernatant was subjected to HPLC. The HPLC analysis was performed using an L-7100 pump (Hitachi, Tokyo, Japan), L-7200 autosampler (Hitachi), L-7405 UV detector (Hitachi), and D-2500 Chromato-Integrator (Hitachi) equipped with a Symmetry C8 column (5-μm particle size, 4.6 mm i.d. × 250 mm; Waters, Milford, MA). The eluent was monitored at 215 nm. The mobile phase was 23% methanol containing 10 mM potassium dihydrophosphate (pH 2.7). The flow rate was 1.0 mL/min. The column temperature was 40 °C. Because imidapril is somewhat non-enzymatically hydrolyzed, the content of imidaprilat in the mixture