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Comparison of substrate specificity among human arylacetamide deacetylase and carboxylesterases

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ABSTRACT

Human arylacetamide deacetylase (AADAC) is an esterase responsible for the hydrolysis of some drugs, including flutamide, indiplon, phenacetin, and rifamycins. AADAC is highly expressed in the human liver, where carboxylesterase (CES) enzymes, namely, CES1 and CES2, are also expressed. It is generally recognized that CES1 prefers compounds with a large acyl moiety and a small alcohol or amine moiety as substrates, whereas CES2 prefers compounds with a small acyl moiety and a large alcohol or amine moiety. In a comparison of the chemical structures of known AADAC substrates, AADAC most likely prefers compounds with the same characteristics as does CES2. However, the substrate specificity of human AADAC has not been fully clarified. To expand the knowledge of substrates of human AADAC, we measured its hydrolase activities toward 13 compounds, including known human CES1 and CES2 substrates, using recombinant enzymes expressed in Sf21 cells. Recombinant AADAC catalyzed the hydrolysis of fluorescein diacetate, *N*-monoacetyldapsone, and propanil, which possess notably small acyl moieties, and these substrates were also hydrolyzed by CES2. However, AADAC could not hydrolyze another CES2 substrate, procaine, which possesses a moderately small acyl moiety. In addition, AADAC did not hydrolyze several known CES1 substrates, including clopidogrel and oseltamivir, which have large acyl moieties and small alcohol moieties. Collectively, these results suggest that AADAC prefers compounds with smaller acyl moieties than does CES2. The role of AADAC in the hydrolysis of drugs has been clarified. For this reason, AADAC should receive attention in ADMET studies during drug development.

1. Introduction

Drug-metabolizing enzymes are involved in the detoxification of drugs and the activation of prodrugs. Esterases contribute to the hydrolysis of clinically therapeutic drugs that contain ester, amide, and thioester bonds. Among the esterases, carboxylesterases (CES) are the most well-studied and recognized enzymes that catalyze the hydrolysis of various xenobiotic and endogenous compounds (Fukami and Yokoi, 2012). In humans, CES1 and CES2 isoforms are important contributors to drug hydrolysis. These isoforms exhibit different tissue distributions and substrate specificities. CES1 is primarily expressed in the human liver, whereas CES2 is expressed in the liver and in extrahepatic tissues, including the gastrointestinal tract and kidneys (Imai et al., 2006; Xu et al., 2002). It has been demonstrated that CES1 prefers substrates with a small alcohol or amine moiety and a large acyl moiety, whereas CES2 prefers those with a large alcohol or amine moiety and a small acyl moiety (Imai et al., 2006).

We have demonstrated that AADAC is responsible for the hydrolysis of several drugs, such as flutamide (Watanabe et al., 2009), phenacetin (Watanabe et al., 2010), rifamycins (rifampicin, rifabutin, and rifapentine) (Nakajima et al., 2011), and indiplon (Shimizu et al., 2014). AADAC is primarily expressed in the liver and gastrointestinal tract (Watanabe et al., 2009), where it is localized to the endoplasmic reticulum membrane (Frick et al., 2004). A common structural characteristic of the substrates of human AADAC is the possession of a small acyl moiety, such as an acetyl or isopropyl group. However, because the known AADAC substrates are highly limited, it has not been established whether AADAC prefers compounds with small acyl moieties. In addition, it has not been determined whether AADAC can hydrolyze

Abbreviations: AADAC, arylacetamide deacetylase; CES, carboxylesterase; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; HIM, human intestinal microsomes; HJM, human jejunum microsomes; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; NAT, *N*-acetyltransferase; SN-38, 7-ethyl-10-hydroxycamptothecin.

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compounds known to be CES substrates. To address these issues, we evaluated the hydrolase activities among AADAC, CES1, and CES2 and compared their substrate specificities using various compounds, including known CES1 and CES2 substrates.

2. Materials and methods

2.1. Chemicals and reagents

Fluorescein diacetate, fluorescein, imidapril hydrochloride, mycophenolic acid, 2-cyclohexyl-2-phenylglycolic acid, *p*-aminobenzoic acid, propanil, 3,4-dichloroaniline, and temocapril hydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). Clofibrate, clofibric acid, dapsone, fenofibrate, oxybutynin chloride, and procaine hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO). *N*-acetyldapsone (*N*-monoacetyldapsone), clopidogrel hydrogen sulfate, clopidogrel carboxylic acid, 7-ethyl-10-hydroxycamptothecin (SN-38), fenofibric acid, imidaprilat, irinotecan hydrochloride trihydrate, and temocaprilat were purchased from Toronto Research Chemicals (Toronto, Canada). Mycophenolate mofetil was purchased from Cayman Chemical Company (Ann Arbor, MI). Osertamivir carboxylate was purchased from Medchemexpress (Princeton, NJ). Osertamivir phosphate was purchased from LKT Laboratories (St. Paul, MN). Recombinant human AADAC, CES1, and CES2, expressed in baculovirus-infected insect cells, and mock transfections were previously prepared (Fukami et al., 2010; Watanabe et al., 2010). All other chemicals were of analytical grade or the highest quality commercially available.

2.2. Hydrolase activities of various compounds

The hydrolase activities of various compounds were measured using recombinant human AADAC, CES1, and CES2. The products of mock-infected cells were used as a control. The concentrations of the substrates were set at approximately the *Km* and 1/10 of the *Km* values obtained using human liver microsomes (HLM) (Table 1), as we previously found that the kinetics of flutamide hydrolase activity by recombinant CES2 were fitted to the substrate inhibition equation and the maximum activity was observed at a substrate concentration of 1/10 of the *Km* obtained in HLM (Kobayashi et al., 2012). For substrates with no literature-reported values for *Km* in HLM, we determined the *Km* values using HLM (Supplemental Fig. S1). The assay conditions for each activity were determined to maintain linearity with respect to protein concentration and incubation time. The HPLC conditions are shown in Table 2. For all assays, the flow rate was 1.0 ml/min and the column temperature was 35 °C. The quantification of the metabolites was performed by comparing the HPLC peak heights to those of authentic standards.

2.2.1. *N*-acetyldapsone hydrolase activity

The *N*-acetydapsone hydrolase activity was determined as follows: a typical incubation mixture (final volume, 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4) and the enzyme sources (0.4 mg/ml). *N*-acetyldapsone was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 20 μM or 200 μM *N*-acetyldapsone after a 2-min preincubation at 37 °C. After a 30-min incubation at 37 °C, the reaction was terminated by the addition of 200 μl of ice-cold methanol. The protein was removed by centrifugation at 10,000 rpm for 5 min, and a 60-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment consisted of an L-7100 pump (Hitachi, Tokyo, Japan), an L-7200 autosampler (Hitachi), an L-7405 UV detector (Hitachi), and a D-2500 HPLC Chromato-Integrator (Hitachi) equipped with a WakoPak eco-ODS column (4.6 × 150 mm ID, 5 μm, Wako). *N*-acetyldapsone undergoes a low level of nonenzymatic hydrolysis. The content of dapsone in the mixture incubated without the enzyme sources was therefore subtracted from those containing the enzyme sources.

2.2.2. Clofibrate hydrolase activity

The clofibrate hydrolase activity was determined by a method similar to that outlined in Section 2.2.1. The concentration of the enzyme sources was 0.03 mg/ml. Clofibrate was dissolved in DMSO. The final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 1 μM or 10 μM clofibrate after a 2-min preincubation at 37 °C. After a 45-s incubation at 37 °C, the reaction was terminated by the addition of 200 μl of ice-cold acetonitrile. The mixture was centrifuged, and an 80-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment was the same as that described in Section 2.2.1 except that an L-7400 UV detector (Hitachi) and a D-7500 HPLC Chromato-Integrator (Hitachi) were used.

Table 1
Km values for hydrolase activity in HLM.

Substrate	*Km* values	Reference
N-acetyldapsone	231 ± 26 μM[a]	
Clofibrate	12.4 ± 0.7 μM[a]	
Clopidogrel	58 μM	Tang et al. (2006)
Fenofibrate	4.1 ± 0.2 μM[a]	
Fluorescein diacetate	4.9 ± 0.3 μM[a]	
Imidapril	245 μM	Takahashi et al. (2008)
Irinotecan	23.3 ± 5.3 μM	Haaz et al. (1997)
Mycophenolate mofetil	992 ± 69 μM	Fujiyama et al. (2010)
Oseltamivir	2.3 ± 0.1 mM[a]	
Oxybutynin	22 μM	Sato et al. (2012)
Procaine	0.8 ± 0.2 mM[a]	
Propanil	303 ± 21 μM[a]	
Temocapril	576 ± 33.9 μM	Imai et al. (2005)

[a] Data are shown in Supplemental Fig. 1.

Table 2
HPLC conditions for measuring each metabolite.

Substrate	Metabolite	Mobile phase	Wavelength (nm)
N-acetyldapsone	Dapsone	21% Methanol/20 mM ammonium formate (pH 3.5)	254
Clofibrate	Clofibric acid	40% Acetonitrile/20 mM citric acid (pH 3.9)	230
Clopidogrel	Clopidogrel carboxylic acid	40% Acetonitrile/0.05% trifluoroacetic acid	230
Fenofibrate	Fenofibric acid	45% Acetonitrile/20 mM citric acid (pH 3.9)	287
Fluorescein diacetate	Fluorescein	40% Methanol/30 mM potassium phosphate buffer (pH 7.4)	490
Mycophenolate mofetil	Mycophenolic acid	50% Methanol/0.1% perchloric acid	215
Oseltamivir	Oseltamivir carboxylate	44% Methanol/20 mM potassium dihydrogenphosphate (pH 2.5)	220
Procaine	*p*-Aminobenzoic acid	5% Methanol/20 mM ammonium acetate (pH 4.0)	280
Propanil	3,4-Dichloroaniline	40% Methanol/10 mM potassium dihydrogenphosphate	245
Temocapril	Temocaprilat	28% Acetonitrile/0.2% phosphoric acid	258

2.2.3. Clopidogrel hydrolase activity

The clopidogrel hydrolase activity was determined in a manner similar to Section 2.2.1. The concentration of the enzyme sources was 0.4 mg/ml. Clopidogrel hydrogen sulfate was dissolved in water. The reaction was initiated by the addition of 5 μM or 50 μM clopidogrel after a 2-min preincubation at 37 °C. After a 3-min incubation at 37 °C, the reaction was terminated by the addition of 200 μl of ice-cold acetonitrile. The mixture was centrifuged, and a 50-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment was the same as that described in Section 2.2.1.

2.2.4. Fenofibrate hydrolase activity

The fenofibrate hydrolase activity was determined in a manner similar to Section 2.2.1. The concentration of the enzyme sources was 0.025 mg/ml. Fenofibrate was dissolved in DMSO. The final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 0.5 μM or 5 μM fenofibrate after a 2-min preincubation at 37 °C. After a 15-s incubation at 37 °C, the reaction was terminated by the addition of 200 μl of ice-cold acetonitrile. The mixture was centrifuged, and a 50-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment was the same as that described in Section 2.2.1.

2.2.5. Fluorescein diacetate hydrolase activity

The fluorescein diacetate hydrolase activity was determined in a manner similar to that described in Section 2.2.1. The concentration of the enzyme sources was 0.01 mg/ml. Fluorescein diacetate was dissolved in DMSO. The final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 0.5 μM or 5 μM fluorescein diacetate after a 2-min preincubation at 37 °C. After a 3-min incubation at 37 °C, the reaction was terminated by the addition of 200 μl of ice-cold methanol. The mixture was centrifuged, and a 30-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment consisted of a Chromaster 5110 pump (Hitachi), a Chromaster 5210 autosampler (Hitachi), and a Chromaster 5420 UV–VIS detector (Hitachi) equipped with an Inertsil ODS-3 column (4.6 × 250 mm ID, 5 μm, GL Science, Tokyo, Japan). Fluorescein diacetate is nonenzymatically hydrolyzed. The content of fluorescein in the mixture incubated without the enzyme sources was therefore subtracted from those with the enzyme sources.

2.2.6. Mycophenolate mofetil hydrolase activity

The mycophenolate mofetil hydrolase activity was determined in a manner similar to that outlined in Section 2.2.1. The concentration of the enzyme sources was 0.08 mg/ml. Mycophenolate mofetil was dissolved in DMSO. The final concentration of DMSO in the incubation mixture was 1.0%. The reaction was initiated by the addition of 100 μM or 1 mM mycophenolate mofetil after a 2-min preincubation at 37 °C. After a 2-min incubation at 37 °C, the reaction was terminated by the addition of 200 μl of ice-cold methanol. After centrifugation, a 40-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment was the same as that described in 5) except that a WakoPak eco-ODS column (4.6 × 150 mm ID, 5 μm, Wako) was used. Mycophenolate mofetil undergoes nonenzymatic hydrolysis. The content of mycophenolic acid in the mixture incubated without the enzyme sources was therefore subtracted from those with the enzyme sources.

2.2.7. Oseltamivir hydrolase activity

The oseltamivir hydrolase activity was determined in a manner similar to that outlined in Section 2.2.1. The concentration of the enzyme sources was 0.1 mg/ml. Oseltamivir phosphate was dissolved in water. The reaction was initiated by the addition of 200 μM or 2 mM oseltamivir after a 2-min preincubation at 37 °C. After a 30-min incubation at 37 °C, the reaction was terminated by the addition of 200 μl of ice-cold acetonitrile. The mixture was centrifuged, and a 50-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment was the same as that described in Section 2.2.1 except that an Inertsil ODS-3 column (4.6 × 250 mm ID, 5 μm, GL Science) was used.

2.2.8. Procaine hydrolase activity

The procaine hydrolase activity was determined in a manner similar to that described in Section 2.2.1. The concentration of the enzyme sources was 0.8 mg/ml. Procaine hydrochloride was dissolved in water. The reaction was initiated by the addition of 100 μM or 1 mM procaine after a 2-min preincubation at 37 °C. After a 30-min incubation at 37 °C, the reaction was terminated by the addition of 200 μl of ice-cold methanol. The mixture was centrifuged, and a 60-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment was the same as that described in Section 2.2.1. Procaine undergoes a low level of nonenzymatic hydrolysis. The content of *p*-aminobenzoic acid in the mixture incubated without the enzyme sources was therefore subtracted from those with the enzyme sources.

2.2.9. Propanil hydrolase activity

The propanil hydrolase activity was determined in a manner similar to that described in Section 2.2.1. The concentration of the enzyme sources was 0.2 mg/ml. Propanil was dissolved in DMSO. The reaction was initiated by the addition of 30 μM or 300 μM propanil after a 2-min preincubation at 37 °C. After a 20-min incubation at 37 °C, the reaction was terminated by the addition of 10 μl of 60% perchloric acid. The mixture was centrifuged, and a 50-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment was the same as that described in Section 2.2.2 except that a Mightysil RP-18 (4.6 × 150 mm ID, 5 μm, Kanto Chemicals, Tokyo, Japan) was used. Because propanil is nonenzymatically hydrolyzed at a low rate, the content of 3,4-dichloroaniline in the mixture incubated without the enzyme sources was subtracted from those with the enzyme sources.

2.2.10. Temocapril hydrolase activity

The temocapril hydrolase activity was determined in a manner similar to that outlined in Section 2.2.1. The concentration of the enzyme sources was 0.05 mg/ml. Temocapril hydrochloride was dissolved in water. The reaction was initiated by the addition of 50 μM or 500 μM temocapril after a 2-min preincubation at 37 °C. After a 10-min incubation at 37 °C, the reaction was terminated by the addition of 200 μl of ice-cold acetonitrile. The mixture was centrifuged, and a 40-μl aliquot of the supernatant was subjected to HPLC. The HPLC equipment was the same as that described in Section 2.2.1.

2.2.11. Other hydrolase activities

Imidapril, irinotecan, and oxybutynin hydrolase activities were measured according to our previous studies (Takahashi et al., 2008, 2009; Maruichi et al., 2010). The concentrations of the enzyme sources were 0.2 mg/ml, 0.2 mg/ml, and 0.5 mg/ml for imidapril, irinotecan, and oxybutynin hydrolase activities, respectively.

3. Results and discussion

3.1. Hydrolase activities of 13 compounds by recombinant human AADAC, CES1, and CES2

To determine the substrate specificities of human AADAC and compare them with those of CES1 and CES2, we measured the hydrolase activities of 13 compounds, including known CES1 and

CES2 substrates. On the basis of the results obtained, the substrates were divided into 5 groups as follows.

3.1.1. Substrates hydrolyzed by AADAC and CES2

N-acetyldapsone and fluorescein diacetate were efficiently hydrolyzed by both AADAC and CES2 (Fig. 1A and B).

N-acetyldapsone is an acetyl metabolite of dapsone, which is an agent for the treatment of dermatitis herpetiformis. Dapsone is acetylated by *N*-acetyltransferases (NATs), especially NAT2, to *N*-acetyldapsone (Palamanda et al., 1995), and it is known that the produced *N*-acetyldapsone is efficiently hydrolyzed in humans (Gelber et al., 1971). In this study, we first found that the hydrolysis of *N*-acetyldapsone is catalyzed by human AADAC and CES2. Human AADAC had been identified as an enzyme that catalyzes the deacetylation of the carcinogen 2-acetylaminofluorene, which is an acetyl metabolite of aminofluorene (Probst et al., 1991). Thus, the cases of 2-acetylaminofluorene and *N*-acetyldapsone suggested that AADAC competes against the NAT activity. However, it has been reported that the acetyl metabolites of other known NAT substrates, including sulfamethazine, *p*-aminobenzoic acid, procainamide, and *p*-aminosalicyclic acid, are not hydrolyzed by AADAC (Probst et al., 1991). It remains to be determined whether human CES2 can catalyze the hydrolysis of these acetyl metabolites.

Fluorescein diacetate is considered to be a probe substrate for human CES2 (Wang et al., 2011). The present study found that AADAC also hydrolyzes fluorescein diacetate. Therefore, this compound would be inappropriate as a probe for CES2.

3.1.2. A substrate hydrolyzed by all esterases

Propanil was hydrolyzed by all esterases (Fig. 1C). Propanil, an herbicide, is hydrolyzed to 3,4-dichloroaniline, which is followed by its metabolism by P450 enzymes to *N*-hydroxy-3,4-dichloroaniline and 6-hydroxy-3,4-dichloroaniline, which oxidize hemoglobin to methemoglobin (Singleton and Murphy, 1973; McMillan et al., 1990). Therefore, all three enzymes, AADAC, CES1, and CES2, are associated with the incidence of the propanil-induced methemoglobinemia.

3.1.3. A substrate efficiently hydrolyzed by CES2

Among the 13 compounds investigated in this study, procaine was efficiently hydrolyzed only by CES2 (Fig. 1D). Procaine, a local anesthetic agent, is hydrolyzed in the human plasma and liver. Butyrylcholinesterase is considered to be the enzyme responsible for its hydrolysis in human plasma (Foldes et al., 1953), whereas CES2 is involved in its hydrolysis in the human liver (Ross et al., 2012). Therefore, investigators use procaine as a probe substrate for CES2 (Jewell et al., 2007; Ross et al., 2012). In this study, we found that procaine was efficiently hydrolyzed by CES2, although CES1 and AADAC also showed low levels of activity for this substrate (Fig. 1D). Thus, the use of procaine is appropriate as a probe substrate for human CES2.

3.1.4. A substrate hydrolyzed by both CES1 and CES2

Only irinotecan was efficiently hydrolyzed by both CES1 and CES2 but not by AADAC (Fig. 1E). Irinotecan, a prodrug of antineoplastic agents, is hydrolyzed to the pharmacologically active metabolite SN-38. It is generally appreciated that CES2 efficiently hydrolyzes irinotecan in humans (Xu et al., 2002) because the catalytic efficiency of irinotecan hydrolysis by CES2 is 60 times higher than that by CES1 (Humerickhouse et al., 2000). Our study confirmed that the irinotecan hydrolase activity of recombinant CES2 was higher than that of recombinant CES1. The results also demonstrated that AADAC does not participate in the activation of irinotecan.

3.1.5. Substrates efficiently hydrolyzed by CES1

Among the 13 compounds investigated in this study, 8 compounds (clofibrate, clopidogrel, fenofibrate, imidapril, mycophenolate mofetil, oseltamivir, oxybutynin, and temocapril) were efficiently hydrolyzed only by CES1 (Fig. 1F–M).

Clofibrate and fenofibrate, prodrugs of lipid-lowering agents, are pharmacologically activated through hydrolysis. Previously, we found that fenofibrate is hydrolyzed by HLM but not by human jejunum microsomes (HJM) (Fukami et al., 2010). CES1 is abundantly expressed in the human liver but not in the intestine (Watanabe et al., 2009), whereas CES2 and AADAC are expressed in both the human liver and intestine. Thus, it is reasonable that these prodrugs are specifically hydrolyzed by CES1.

Clopidogrel, an antiplatelet agent, is pharmacologically activated to its 5-thiol metabolite by several cytochrome P450 (CYP) enzymes, including CYP2C19 and CYP3A4, but approximately 85% of absorbed clopidogrel is hydrolyzed to an inactive metabolite, clopidogrel carboxylic acid (Hagihara et al., 2009). Tang et al. (2006) reported that clopidogrel is hydrolyzed by HLM but not by HJM. In addition, CES1 showed hydrolase activity against this compound, whereas CES2 did not. Based on this and the observation that AADAC did not hydrolyze clopidogrel (Fig. 1G), the results indicate that CES1 is the enzyme responsible for its hydrolysis in humans.

Imidapril and temocapril, inhibitors of an angiotensin-converting enzyme, are also prodrugs and are hydrolyzed to the pharmacologically active forms imidaprilat and temocaprilat, respectively. Because their hydrolysis was detected in HLM but not in HJM (Imai et al., 2005; Takahashi et al., 2009), the hydrolysis of imidapril and temocapril in humans is presumed to be due to the activity of CES1.

Mycophenolate mofetil, a prodrug of an immunosuppressant agent, is rapidly hydrolyzed to the pharmacologically active form mycophenolic acid after oral dosing. Fujiyama et al. (2010) reported that mycophenolate mofetil is hydrolyzed in both HLM and human intestinal microsomes (HIM), but the activity in HIM (Vmax: 15.6 ± 3.0 nmol/min/mg protein) was much lower than that in HLM (3073 ± 224 nmol/min/mg protein). In addition, these investigators measured its hydrolase activity using the S9 fraction of COS-1 cells expressing CES1 or CES2 and showed that the activity for CES1 (Vmax: 313 ± 10 nmol/min/mg protein) was much higher than that for CES2 (12.3 ± 0.4 nmol/min/mg protein). The results of our study were consistent with those by Fujiyama et al. (2010). AADAC and CES2 showed marginal activity, but CES1 is a major enzyme that catalyzes the hydrolysis of mycophenolate mofetil.

Oseltamivir, a prodrug for the treatment and prophylaxis of both influenza virus A and B infections, is hydrolyzed to oseltamivir carboxylate, the pharmacologically active form after oral dosing. Because it has been reported that oseltamivir is hydrolyzed in HLM, but not in HIM (Shi et al., 2006), CES1 is presumed to be the enzyme responsible for oseltamivir hydrolysis. In the present study, the hydrolysis of this compound was only observed for CES1.

Oxybutynin, an antimuscarinic agent used for the control of overactive bladder, is primarily metabolized to *N*-desethyloxybutynin by CYP3A4, but is also hydrolyzed to 2-cyclohexyl-2-phenylglycolic acid (Mizushima et al., 2007). Using purified human CES enzymes, Takai et al. (1997) reported that CES2 specifically hydrolyzes oxybutynin. However, Sato et al. (2012) recently used recombinant CES enzymes to demonstrate that CES1 hydrolyzes oxybutynin with a higher efficiency (CLint: 18 μl/min/mg protein) than CES2 (0.51 μl/min/mg protein). Because it has been reported that HLM exhibited a much higher oxybutynin hydrolase activity (773.5 pmol/min/mg protein at 200 μM) than HJM (36.8 pmol/min/mg protein) (Takahashi et al., 2008), CES1, rather than CES2, was considered to be responsible

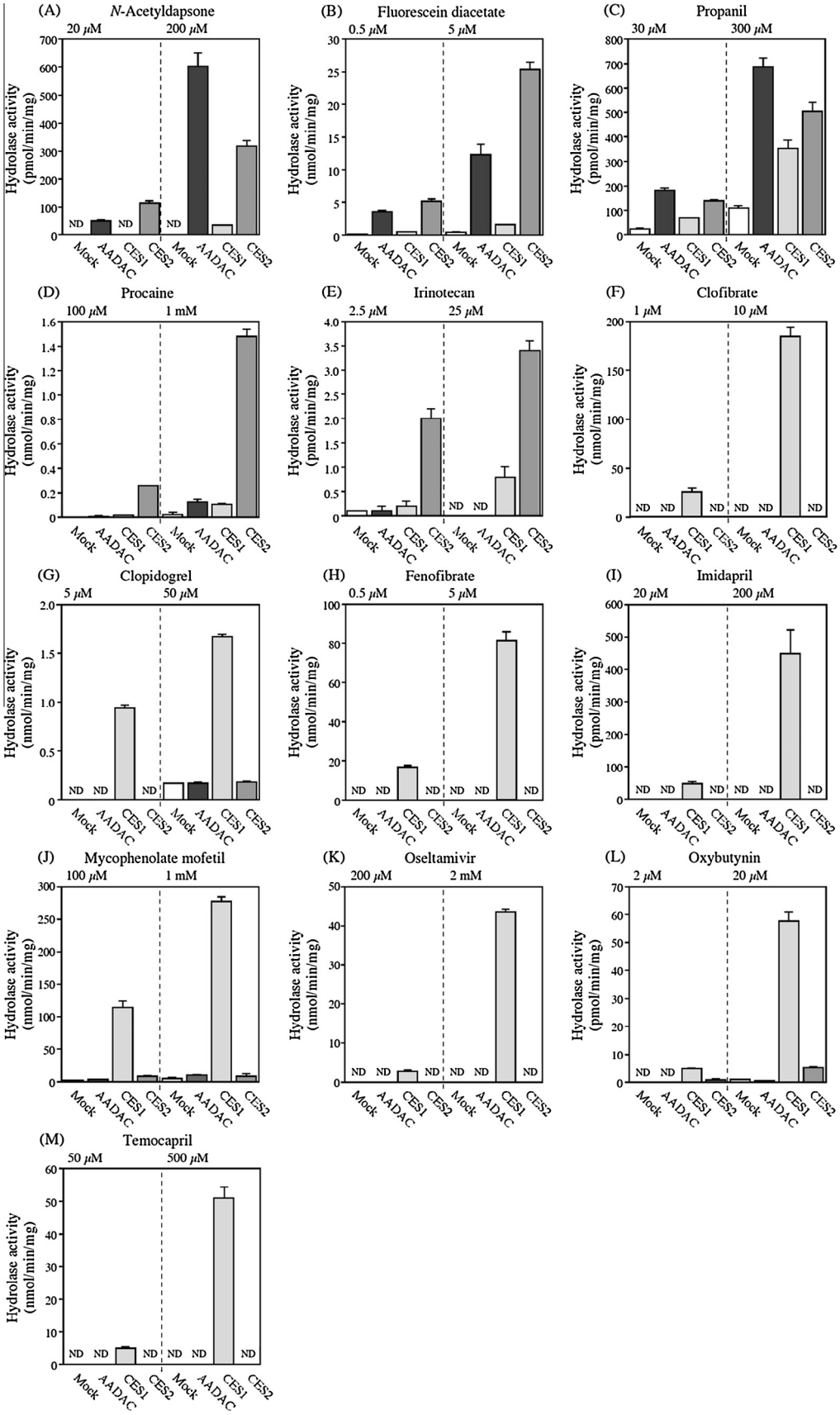

Fig. 1. Hydrolase activities of 13 compounds for recombinant human AADAC, CES1, and CES2. (A) *N*-acetyldapsone, (B) fluorescein diacetate, (C) propanil, (D) procaine, (E) irinotecan, (F) clofibrate, (G) clopidogrel, (H) fenofibrate, (I) imidapril, (J) mycophenolate mofetil, (K) oseltamivir, (L) oxybutynin, and (M) temocapril hydrolase activities were measured using the methods described in the Materials and Methods section. The substrate concentrations were set at approximately 1/10 of the *Km* and *Km* values obtained in HLM. Each column represents the mean ± SD of three independent determinations. ND, not detectable.

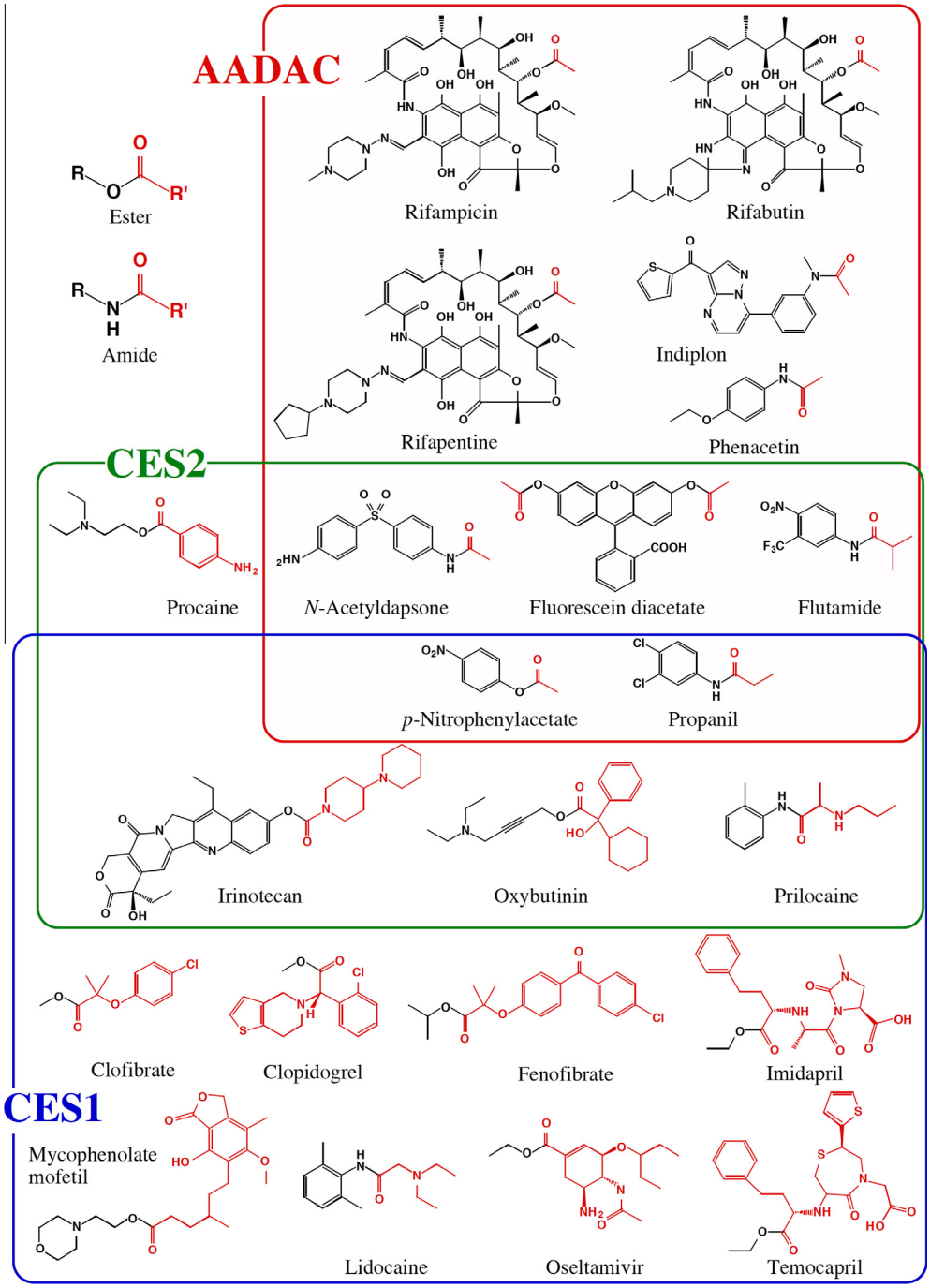

Fig. 2. Chemical structures of the compounds hydrolyzed by human AADAC, CES1, and CES2. The esterases involved in the hydrolysis of flutamide, indiplon, lidocaine, *p*-nitrophenyl acetate, phenacetin, prilocaine, rifampicin, rifabutin, and rifapentine were analyzed in our previous studies (Watanabe et al., 2009, 2010; Nakajima et al., 2011; Kobayashi et al., 2012; Higuchi et al., 2013; Shimizu et al., 2014). An acyl moiety in each compound is indicated in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for oxybutynin hydrolysis in humans. The present study revealed that CES1 showed substantially higher activities than CES2 for both 2 and 20 μM oxybutynin (Fig. 1L). In addition, because we found that human AADAC does not catalyze oxybutynin hydrolysis, CES1 is predicted to be the enzyme responsible for oxybutynin hydrolysis in humans.

3.2. Substrate specificity of human AADAC, CES1, and CES2

For the 13 compounds analyzed in this study and 9 compounds analyzed in our previous studies, the esterases involved in their hydrolysis are summarized in Fig. 2. We previously found that phenacetin, rifampicin, rifabutin, rifapentine, and indiplon are efficiently hydrolyzed by human AADAC but not by CES1 and CES2 (Watanabe et al., 2010; Nakajima et al., 2011; Shimizu et al., 2014). Flutamide is hydrolyzed by AADAC, but at low concentrations, flutamide is also efficiently hydrolyzed by CES2 (Watanabe et al., 2009; Kobayashi et al., 2012). Lidocaine is specifically hydrolyzed by CES1, whereas prilocaine is hydrolyzed by both CES1 and CES2 (Higuchi et al., 2013). *p*-Nitrophenyl acetate is hydrolyzed by all three esterases (Watanabe et al., 2009).

It has been recognized that human CES1 prefers substrates with a small alcohol or amine moiety and a large acyl moiety, whereas CES2 prefers those with a large alcohol or amine moiety and a

small acyl moiety (Imai et al., 2006). According to Fig. 2, AADAC appears to prefer substrates with smaller acyl moieties than does CES2. The crystal structure of human AADAC remains to be analyzed, and it is therefore not yet clear why AADAC prefers substrates with relatively small acyl moieties. Nevertheless, the findings in this study provide new knowledge regarding the substrate specificities of human AADAC.

4. Conclusions

In this study, we determined that human AADAC prefers substrates with smaller acyl moieties compared with those utilized by human CES1 and CES2. These esterases contribute strongly to drug hydrolysis, resulting in the detoxification of xenobiotics, the activation of prodrugs, or drug-induced toxicity. The information presented in this study will expand our knowledge of the substrate specificities of human AADAC, CES1, and CES2.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejps.2015.07.006.

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