

Short Communication

Contributions of Arylacetamide Deacetylase and Carboxylesterase 2 to Flutamide Hydrolysis in Human Liver^S

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ABSTRACT:

Flutamide, an antiandrogen drug, is widely used for the treatment of prostate cancer. The major metabolic pathways of flutamide are hydroxylation and hydrolysis. The hydrolyzed metabolite, 5-amino-2-nitrobenzotrifluoride (FLU-1), is further metabolized to *N*-hydroxy FLU-1, an assumed hepatotoxicant. Our previous study demonstrated that arylacetamide deacetylase (AADAC), one of the major serine esterases expressed in the human liver and gastrointestinal tract, catalyzes the flutamide hydrolysis. However, the enzyme kinetics in human tissue microsomes were not consistent with the kinetics by recombinant human AADAC. Thus, it seemed that AADAC is not the sole enzyme responsible for flutamide hydrolysis in human. In the present study, we found that recombinant carboxylesterase (CES) 2 could hydrolyze flutamide at low concentrations of flutamide. In the inhibition assay, the flutamide hydro-

lysis activities at a flutamide concentration of 5 μ M in human liver and jejunum microsomes were strongly inhibited by a selective CES2 inhibitor, 10 μ M loperamide, with the residual activities of 22.9 \pm 3.5 and 18.6 \pm 0.7%, respectively. These results suggest that CES2 is also involved in the flutamide hydrolysis in human tissues. Using six individual human livers, the contributions of AADAC and CES2 to flutamide hydrolysis were estimated by using the relative activity factor. The relative contribution of CES2 was approximately 75 to 99% at the concentration of 5 μ M flutamide. In contrast, the relative contribution of AADAC increased in parallel with the concentration of flutamide. Thus, CES2, rather than AADAC, largely contributed to the flutamide hydrolysis in clinical therapeutics.

Introduction

Flutamide is a nonsteroidal antiandrogen drug that is used for the treatment of prostate cancer. However, flutamide occasionally causes severe hepatotoxicity (Thole et al., 2004). Flutamide itself is not toxic when used at the appropriate clinical dose, but bioactivation of flutamide has been considered to be the cause of flutamide-induced hepatotoxicity (Fau et al., 1994). Flutamide is mainly metabolized to 2-hydroxyflutamide and 5-amino-2-nitrobenzotrifluoride (FLU-1) by human CYP1A2 and esterase(s), respectively (Katchen and Buxbaum, 1975; Schulz et al., 1988). It has been suggested that 2-hydroxyflutamide is associated with the therapeutic effect of flutamide (Katchen and Buxbaum, 1975), whereas FLU-1 is considered to have no therapeutic effect (Aizawa et al., 2003). FLU-1 is further metabolized to *N*-hydroxy FLU-1 by human CYP3A4, and *N*-hydroxy FLU-1 is considered to be associated with hepatotoxicity (Ohbuchi et al., 2009).

Our previous study demonstrated that arylacetamide deacetylase (AADAC) catalyzed the flutamide hydrolysis (Watanabe et al., 2009). However, we noticed that when the flutamide hydrolase activity was measured using human liver microsomes (HLM) from an individual

sample, the Eadie-Hofstee plot was biphasic (Supplemental Fig. 1). This result suggests the involvement of enzyme(s) other than AADAC in flutamide hydrolysis in human.

Carboxylesterases (CES), especially CES1 and CES2 enzymes, are the major serine esterase responsible for the hydrolysis of various drugs and xenobiotics (Imai et al., 2006). CES1 is highly expressed in liver, whereas CES2 is expressed in jejunum and kidney as well as liver (Watanabe et al., 2009). Our previous study denied the involvement of major esterases other than AADAC in the flutamide hydrolysis, but the activity was measured at 500 μ M (Watanabe et al., 2009). Therefore, the involvement of other esterases at a low flutamide concentration remains unknown. In this study, we investigated the involvement of CES in the flutamide hydrolysis at a low flutamide concentration and estimated the contributions of AADAC and CES in liver.

Materials and Methods

Chemicals and Reagents. Flutamide, FLU-1, and rifampicin were purchased from Wako Pure Chemical Industries (Osaka, Japan). 25-Desacetylri-fampicin (25-deacetylri-fampicin), irinotecan hydrochloride (CPT-11), and 7-ethyl-10-hydroxycamptotesin (SN-38) were purchased from Toronto Research Chemicals (Toronto, Canada). Ethopropazine was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in this study were of analytical or the highest quality commercially available.

Human Tissues. HLM (pooled, $n = 50$), human liver cytosol (HLC) (pooled, $n = 22$), and human jejunum microsomes (HJM) (pooled, $n = 10$) were purchased from BD Gentest (Woburn, MA) and Tissue Transformation Technologies (Edison, NJ), respectively. Six individual human liver samples

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ABBREVIATIONS: FLU-1, 5-amino-2-nitrobenzotrifluoride; AADAC, arylacetamide deacetylase; HLM, human liver microsomes; CES, carboxylesterase; CPT-11, irinotecan hydrochloride; HLC, human liver cytosol; HJM, human jejunum microsomes; HLH, human liver homogenates; RAF, relative activity factor; $l_{inlet,u,max}$, the maximum value of the unbound concentration at the inlet to the liver.

were supplied by the National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Chiba, Japan). The use of the human livers was approved by the Ethics Committee of Kanazawa University (Kanazawa, Japan). The human liver homogenates (HLH) were prepared according to our previous report (Iwamura et al., 2012).

Flutamide, Rifampicin, and CPT-11 Hydrolase Activities. The flutamide hydrolase activity was determined according to Watanabe et al. (2009). The concentrations of enzyme sources were as follows: HLM, HLH, HJM, and Sf21 cell homogenates expressing human AADAC, CES1, and CES2 (Fukami et al., 2010; Watanabe et al., 2010), 0.4 mg/ml; and HLC, 1.0 mg/ml. An inhibitory analysis of flutamide hydrolysis was performed using loperamide, a potent CES2 inhibitor (Quinney et al., 2005). The concentrations of loperamide and flutamide were 10 and 5 μM , respectively. For kinetic analyses of the flutamide hydrolase activity, the parameters were estimated from the fitted curves using a computer program (KaleidaGraph; Synergy Software, Reading, PA) designed for nonlinear regression analysis.

The rifampicin hydrolase activity, which is a marker of AADAC enzyme activity, was determined according to our previous study (Nakajima et al., 2011). The concentration of enzyme sources (HLH and Sf21 cell homogenates expressing human AADAC) was 0.5 mg/ml. The substrate concentration of rifampicin was 50 μM .

The CPT-11 hydrolase activity, which was used as a marker of CES2 enzyme activity, was determined according to Takahashi et al. (2009) except for the mobile phase. The concentration of enzyme sources (HLH and Sf21 cell homogenates expressing human CES2) was 0.2 mg/ml. The mobile phase was 25% acetonitrile and 75% of 10 mM KH_2PO_4 containing 3 mM 1-heptanesulfonic acid sodium salt.

Contributions of AADAC and CES2 to Flutamide Hydrolase Activity in HLH. AADAC is expressed in liver microsomes, whereas CES2 is expressed in liver microsomes and cytosol (Xu et al., 2002). Therefore, their contributions to flutamide hydrolysis in human liver were analyzed using individual HLH samples. The percentage contributions of AADAC and CES2 to the flutamide hydrolase activity in individual HLH were estimated by applying the relative activity factor (RAF) as the ratio of activity values, as described previously (Watanabe et al., 2010). In this study, the RAF values for AADAC ($\text{RAF}_{\text{AADAC}}$) were determined as the ratios of the rifampicin hydrolase activities in HLH to the value by recombinant AADAC. The RAF values for CES2 (RAF_{CES2}) were determined as the ratios of the CPT-11 hydrolase activities in HLH to the value by recombinant CES2. Using the RAF, the predicted flutamide hydrolase activities by AADAC (V_{AADAC}) and CES2 (V_{CES2}) in HLH were expressed as follows: $V_{\text{AADAC}} = V_{\text{rec-AADAC}} \times \text{RAF}_{\text{AADAC}}$ and $V_{\text{CES2}} = V_{\text{rec-CES2}} \times \text{RAF}_{\text{CES2}}$, where $V_{\text{rec-AADAC}}$ and $V_{\text{rec-CES2}}$ are the flutamide hydrolase activities by recombinant AADAC and CES2, respectively. The contributions of AADAC and CES2 to the flutamide hydrolase activities in HLH were calculated using the following equations: Contribution of AADAC (%) = $(V_{\text{AADAC}}/V_{\text{HLH}}) \times 100$ and Contribution of CES2 (%) = $(V_{\text{CES2}}/V_{\text{HLH}}) \times 100$, where the V_{HLH} values are the flutamide hydrolase activities in HLH.

Statistical Analysis. Statistical analyses between multiple groups were performed using one-way analysis of variance, followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

Results and Discussion

The Eadie-Hofstee plot for the flutamide hydrolase activity in one individual HLM sample was biphasic (Supplemental Fig. 1), whereas the activity by recombinant AADAC was fitted to the Michaelis-Menten equation (Kobayashi et al., 2012). Our previous study denied the involvement of esterases other than AADAC in the flutamide hydrolysis by evaluating only at 500 μM (Watanabe et al., 2009). According to data for the Eadie-Hofstee plot (Supplemental Fig. 1), an enzyme with high affinity to flutamide hydrolysis seemed to be involved at flutamide concentrations lower than 75 μM . The flutamide hydrolase activity at 20 μM was detected by recombinant human CES2, although recombinant human CES1 showed activity similar to mock cells (Fig. 1A). By measuring the hydrolase activity of *p*-nitrophenyl acetate, a general esterase substrate, we confirmed

that recombinant CES1 and CES2 function normally (CES1, K_m : $150.8 \pm 17.8 \mu\text{M}$, V_{max} : $1145.6 \pm 36.4 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$; CES2, K_m : $123.7 \pm 13.3 \mu\text{M}$, V_{max} : $518.1 \pm 14.0 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) (data not shown). The activity at 20 μM in HLM was not inhibited by 10 μM ethopropazine, an inhibitor of butyrylcholinesterase (Yamaori et al., 2006), and was not activated by 1 mM CaCl_2 , which is required for exerting paraoxonase activity (Kuo and La Du, 1998) (data not shown). Thus, we first found the involvement of human CES2 in the flutamide hydrolysis. Considering the chemical structure of flutamide, this result was consistent with the notion that the preferential substrates for CES2 are compounds bearing a small acyl moiety and a bulky alcohol or amino groups (Imai et al., 2006).

Data for the flutamide hydrolase activity by recombinant human CES2 were fitted to the substrate inhibition equation with the K_m , V_{max} , and K_i values of $0.3 \pm 0.1 \text{ mM}$, $1113.1 \pm 206.4 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, and $2.3 \pm 0.3 \mu\text{M}$, respectively (Fig. 1B). The CL_{int} value calculated from V_{max}/K_m was $4.2 \pm 0.2 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. It is not surprising that data for flutamide hydrolase activity by recombinant human CES2 were fitted to the substrate inhibition equation. The prasugrel, a thienopyridine prodrug, hydrolase activity by human CES2 was also inhibited at high concentrations, although data for its hydrolysis were not fitted to a single substrate inhibition equation (Williams et al., 2008). In our previous studies (Watanabe et al., 2009; Kobayashi et al., 2012), the flutamide hydrolase activity in HLM was fitted to the Michaelis-Menten equation, and the Eadie-Hofstee plot was not obviously biphasic. Therefore, we mistakenly believed that AADAC was the major enzyme responsible for flutamide hydrolysis. However, when using other HLM samples (from a single donor), the Eadie-Hofstee plot was clearly biphasic (Supplemental Fig. 1). The expression ratio of CES2 and AADAC might cause a different Eadie-Hofstee plot.

To confirm the involvement of CES2 in flutamide hydrolysis, the inhibitory effects of loperamide, a potent CES2 inhibitor (Quinney et al., 2005), on the flutamide hydrolase activities by recombinant CES2, AADAC, HLM, HLC, and HJM were analyzed (Fig. 1C). The activity by recombinant CES2 was potently inhibited by 10 μM loperamide (percentage of control: $7.7 \pm 1.5\%$), whereas that by recombinant AADAC was weakly inhibited (percentage of control: $74.7 \pm 3.2\%$). In HLM, HLC, and HJM, the activities at the flutamide concentration of 5 μM were decreased to 22.9 ± 3.5 , 15.7 ± 4.0 , and $18.6 \pm 0.7\%$ of the controls by 10 μM loperamide, respectively. These results suggest that CES2 was largely involved in the flutamide hydrolysis at 5 μM in HLM, HLC, and HJM. In HLC, CES1 and CES2, but not AADAC, are expressed. From the finding that the activity in HLC was strongly inhibited by loperamide, soluble CES2 in cytosol would catalyze the flutamide hydrolysis similarly to microsomal membrane-bound CES2. In addition, this result also supports no involvement of CES1 in flutamide hydrolysis, although CES1 is highly expressed in liver (Watanabe et al., 2009).

The *in vivo* intrinsic clearance (CL_{int} , *in vivo*) values of flutamide hydrolysis in HLM and HJM were estimated using the following equation (Soars et al., 2002): CL_{int} , *in vivo* = $[\text{CL}_{\text{int}}] \times [\text{microsomal protein/tissue (mg/g)}] \times [\text{tissue/body weight (g/kg)}]$.

The CL_{int} values of flutamide hydrolysis in HLM and HJM were approximately 1.8 and 12.9 $\mu\text{l} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, respectively (Kobayashi et al., 2012). The CL_{int} , *in vivo* values were calculated using microsomal protein/liver of 40 mg/g and the liver weight of 1500 g (Hakooz et al., 2006), and microsomal protein/jejunum mucosa of 20.5 mg/g and the jejunum mucosa weight of 65.8 g [calculated from wet weight for jejunum (411 g) and jejunum mucosal mass (16% of total wet weight)] (Paine et al., 1997; Cubitt et al., 2009). According to these values, the CL_{int} , *in vivo* values of HLM and HJM

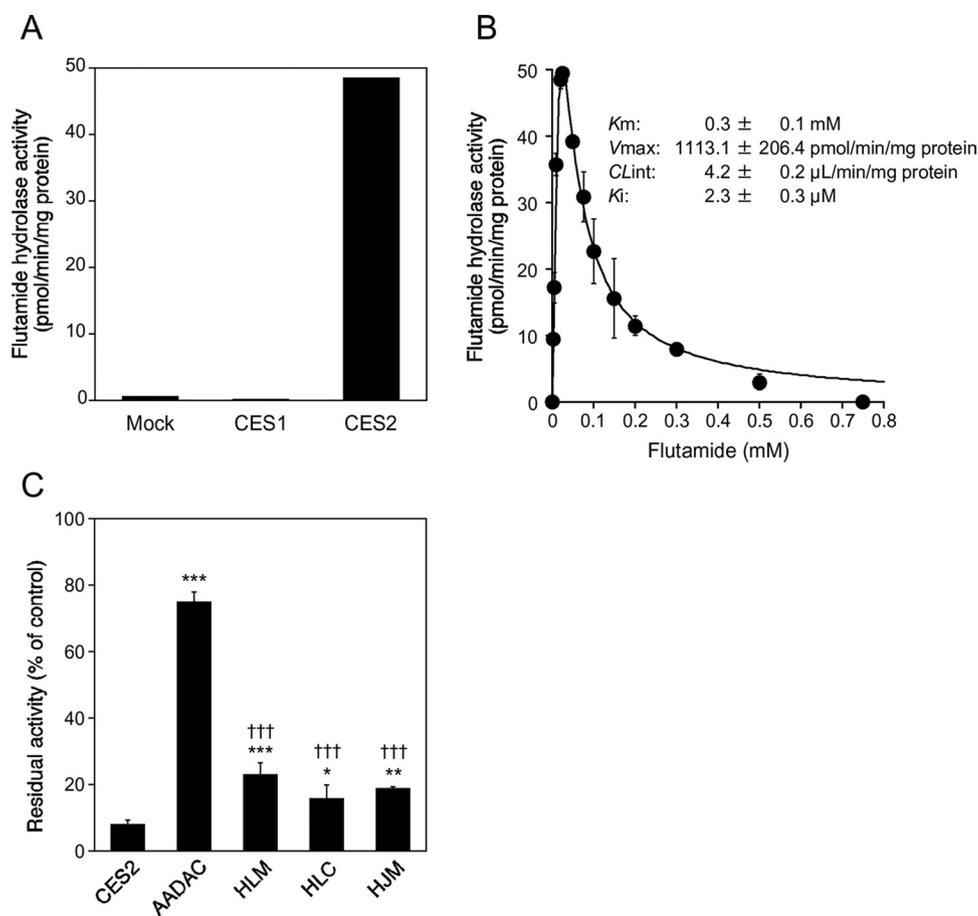


FIG. 1. A, flutamide hydrolyse activities by recombinant human CES1 and CES2. The Sf21 cells expressing these enzymes were incubated with 20 μ M flutamide. B, kinetic analysis of flutamide hydrolyse activity by recombinant human CES2 expressed in Sf21 cells. Total cell homogenates of Sf21 cells were incubated with 0.002 to 0.75 mM flutamide. Each data point represents the mean \pm S.D. of triplicate determinations. C, inhibitory effect of loperamide on flutamide hydrolyse activity. Flutamide hydrolyse activities by recombinant human CES2, AADAC, HLM, HLC, and HJM were determined at the substrate concentration of 5 μ M. The concentration of loperamide was 10 μ M. The control activities by CES2, AADAC, HLM, HLC, and HJM were 17.2 ± 2.3 pmol \cdot min $^{-1}$ \cdot mg protein $^{-1}$, 9.1 ± 0.2 pmol \cdot min $^{-1}$ \cdot mg protein $^{-1}$, 8.6 ± 0.3 pmol \cdot min $^{-1}$ \cdot mg protein $^{-1}$, 2.1 ± 0.0 pmol \cdot min $^{-1}$ \cdot mg protein $^{-1}$, and 64.4 ± 1.5 pmol \cdot min $^{-1}$ \cdot mg protein $^{-1}$, respectively. Each column represents the mean \pm S.D. of triplicate determinations. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ compared with recombinant CES2. †††, $P < 0.001$ compared with recombinant AADAC.

were calculated to be approximately 108.0 and 17.4 ml/min, respectively. Thus, the contribution of liver to flutamide hydrolysis would be higher than that of jejunum.

In addition, enzymes involved in drug hydrolysis are present beyond the liver and intestine. However, the results of our previous study (Kobayashi et al., 2012) found that flutamide hydrolyse activities in human kidney and pulmonary microsomes were extremely low compared with those in human liver and jejunum microsomes. Furthermore, the involvements of the representative esterases in plasma, butyrylcholinesterase and paraoxonase, in flutamide hydrolysis could be denied. The contributions of other tissues to flutamide hydrolysis would be low in the body.

To predict contributions of AADAC and CES2 quantitatively, the RAF values for AADAC and CES2 were calculated. The results of our recent study found that rifampicin hydrolysis is specifically catalyzed by AADAC (Nakajima et al., 2011). Data for the activity were fitted to the substrate inhibition equation with the maximum activity at 200 μ M (Nakajima et al., 2011). Thus, this study used 50 μ M rifampicin as the marker substrate for AADAC. As a marker of CES2 enzyme activity, the CPT-11 hydrolyse activity at 2 μ M was measured in a similar way to that in our previous study (Fukami et al., 2010).

Using the rifampicin and CPT-11 hydrolyse activities by recombinant AADAC and CES2 (41.77 and 2.99 pmol \cdot min $^{-1}$ \cdot mg protein $^{-1}$, respectively), the RAF_{AADAC} and RAF_{CES2} values in individual HLH were calculated as shown in Table 1. By applying these RAF values, the contributions of AADAC and CES2 at three flutamide concentrations (5, 50, and 200 μ M) in HLH were estimated according to the equations under *Contributions of AADAC and CES2 to Flutamide Hydrolyse Activity in HLH* (Table 1). The total contributions of AADAC and CES2 to flutamide hydrolysis were 79.6 to 170.4% in

individual HLH. A lot of data for total contributions exceed 100%. The possible causes were as follows: 1) although CPT-11 hydrolysis is considered to be mostly catalyzed by CES2 at a low concentration of CPT-11, CES1 might also participate with the reaction with lower catalytic efficiency compared with CES2, resulting in the overestimation of RAF_{CES2} values. However, in our previous study (Watanabe et al., 2010), CPT-11 hydrolyse activity by CES1 at a concentration of 2 μ M was undetectable. Furthermore, above our report (Watanabe et al., 2010) also showed that the CPT-11 hydrolyse activity at 2 μ M in HLM was potently inhibited by loperamide with IC_{50} value of 0.46 μ M; 2) some factors expressing in liver homogenates might interfere with the enzyme activities of AADAC and CES2. Therefore, the flutamide hydrolyse activity in liver homogenates might be decreased, resulting in the overestimation of total contributions, although the relative contributions of AADAC and CES2 were not significantly affected. The flutamide hydrolyse activities in HLH (V_{HLH}) are shown in Fig. 2A. The activities by AADAC (Fig. 2A, gray) and CES2 (Fig. 2A, black) in individual HLH were estimated using the contribution ratios shown in Table 1. Figure 2B shows AADAC and CES2 contributions calculated by normalizing the total contributions to 100%. Figure 2A clearly shows that the flutamide hydrolyse activity at 5 μ M depends on the CES2, and the relative contribution of CES2 was calculated to be more than 74% (Fig. 2B). At 50 μ M flutamide, the relative contributions of AADAC were calculated to be 38.9 to 79.9%, except in sample no. 1 (11.6%). At 200 μ M flutamide, AADAC was the principal enzyme catalyzing the flutamide hydrolysis, with relative contributions of more than 89%, except in sample no. 1 (62.7%). Thus, we found that CES2 and AADAC contribute to the flutamide hydrolysis in human liver at low and high concentrations of flutamide, respectively. The average relative contributions of AADAC/CES2 in

TABLE 1

RAF values calculated from the marker activity and the contributions of AADAC and CES2 to flutamide hydrolysis in six HLH

	Hydrolase Activity		RAF		Flutamide			Contribution		
	Rifampicin	CPT-11	AADAC	CES2	Concentration	Hydrolase Activity		AADAC	CES2	Total
						V_{rec}				
						AADAC	CES2			
	$pmol \cdot min^{-1} \cdot mg$ $protein^{-1}$				μM	$pmol \cdot min^{-1} \cdot mg$ $protein^{-1}$				%
rec-AADAC	41.77				5	3.1				
					50	87.6				
					200	389.0				
rec-CES2		2.99			5	16.0				
					50	38.9				
					200	13.5				
Liver homogenate										
1	1.00	1.23	0.024	0.412	5		4.5	1.6	146.7	148.3
					50		10.6	19.7	150.7	170.4
					200		10.5	89.0	53.1	142.1
2	3.27	0.94	0.089	0.314	5		3.5	7.7	142.7	150.5
					50		16.2	48.1	75.4	123.6
					200		40.5	85.5	10.5	96.0
3	10.55	0.43	0.253	0.143	5		3.2	24.3	72.1	96.4
					50		21.2	104.2	26.3	130.5
					200		76.0	129.3	2.5	131.8
4	6.39	0.87	0.153	0.292	5		5.1	9.3	92.2	101.5
					50		28.1	47.7	40.5	88.2
					200		56.4	105.5	7.0	112.4
5	5.94	0.42	0.142	0.140	5		3.4	13.0	66.6	79.6
					50		17.9	69.5	30.3	99.8
					200		53.4	103.6	3.5	107.1
6	5.69	0.52	0.136	0.172	5		2.1	20.4	134.7	155.1
					50		14.2	83.8	47.1	130.9
					200		54.6	97.1	4.3	101.4

HLH at 5, 50, and 200 μM flutamide were 11.7/88.3, 53.0/47.0, and 89.4/10.6%, respectively. Sample 1 showed the flutamide hydrolase activity at 5 μM with a similar value to those of the other samples, but it showed lower activity than the others at higher concentrations of flutamide (Fig. 4A). The results of a later study found that this sample

has a polymorphic allele of AADAC gene, which causes the decreased enzyme activity (Shimizu et al., 2012).

The maximum value of the unbound concentration at the inlet to the liver ($I_{inlet,u,max}$) (Fukami et al., 2010) was estimated using the following equation: $I_{inlet,u,max} = f_u \times [C_{max} + (Dose \times k_a \times$

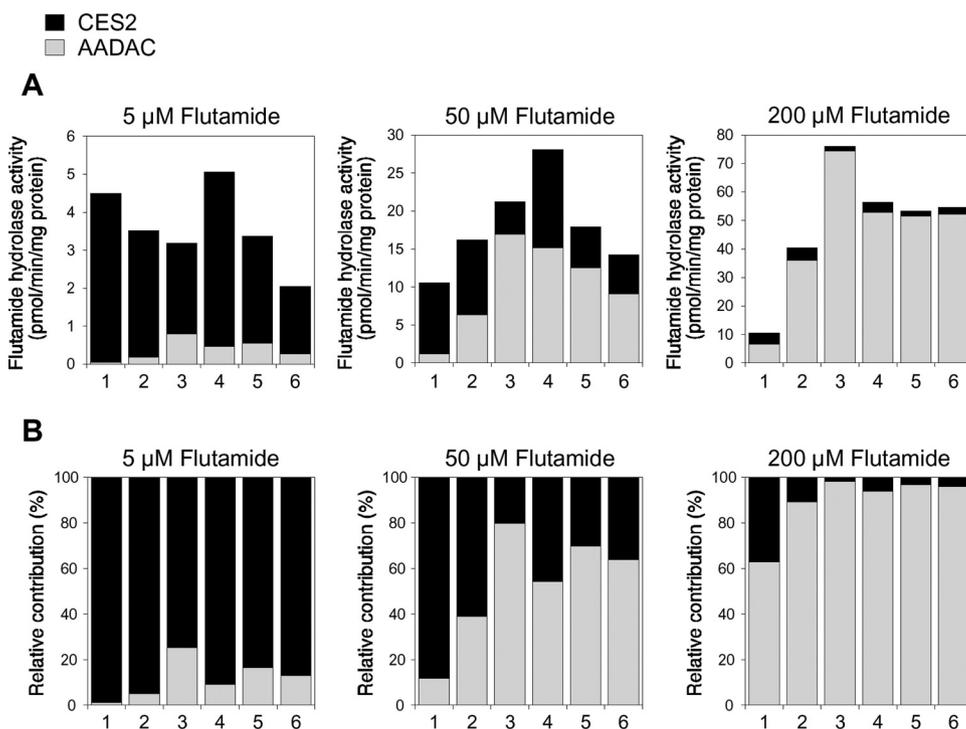


FIG. 2. A, flutamide hydrolase activities in individual HLH. HLH (0.4 mg/ml) were incubated with 5, 50, and 200 μM flutamide. Gray and black columns represent the activities by AADAC and CES2, respectively, based on the data of percentage contributions. The extrapolation of percentage contributions of AADAC and CES2 were determined by the RAF using HLH and recombinant human AADAC and CES2 from the homogenates of Sf21 cells. To determine the RAF values, 50 μM rifampicin and 2 μM CPT-11 were used as the marker substrates of AADAC and CES2, respectively. B, relative contributions of AADAC (gray) and CES2 (black) to flutamide hydrolysis in individual HLH. Based on the contributions shown in Table 1, total contributions were normalized to 100%. Each column represents the mean of duplicate determinations.

$F_a/Q_h]$, where f_u is the unbound fraction in the plasma, C_{max} is the maximum concentration in the blood, k_a is the first-order rate constant for gastrointestinal absorption, F_a is the fraction absorbed from the gastrointestinal tract into the portal vein, and Q_h is the hepatic blood flow rate (1450 ml/min). Because there are no F_a data for flutamide, a value of 1.0 was used for F_a to avoid underestimating the $l_{inlet,u,max}$ of flutamide. The $l_{inlet,u,max}$ value of flutamide was estimated to be 56 nM after oral dosing at 125 mg, the value of which is much lower than the concentration of flutamide used in this study (5–200 μ M). Therefore, CES2 is considered a major enzyme in flutamide hydrolysis in liver in clinical therapeutics, although AADAC also shows flutamide hydrolase activity. The activity by recombinant CES2 was fitted to the substrate inhibition equation with the highest activity at 20 μ M and almost no activity at 750 μ M. However, because the $l_{inlet,u,max}$ of flutamide was estimated to be 56 nM after oral dosing at 125 mg, this substrate inhibition pattern of CES2 would not have an effect on the hydrolysis potency of flutamide *in vivo*.

The small intestine is also involved in the first-pass metabolism after oral dosing. Although the contribution of the liver to flutamide hydrolysis was estimated to be higher than that of the intestine, it is conceivable that the small intestine is also important tissue for its hydrolysis. Data of the flutamide hydrolase activity in HLM seemed to be fitted to the Michaelis-Menten equation, whereas those in HJM were clearly fitted to the combined Michaelis-Menten equation/substrate inhibition equation (Kobayashi et al., 2012). Thus, CES2 would contribute to flutamide hydrolysis in human jejunum at a higher rate than in human liver.

Previous reports showed a 15- and 3-fold variation in the CES2 expression in the cytosolic and microsomal fractions of 13 liver samples (Xu et al., 2002). Zhu et al. (2000) reported that dexamethasone and rifampicin could induce human CES2 mRNA expression, probably by the activation of pregnane X receptor. In addition, several functionally deficient CES2 alleles were found in the Japanese population (Kubo et al., 2005). Thus, there may be inter- or intraindividual variations in CES2 expression and function that affect the flutamide pharmacokinetics. The flutamide-hydrolyzed metabolite, FLU-1, is further metabolized to *N*-hydroxy FLU-1 by human CYP3A4, and *N*-hydroxy FLU-1 is thought to be associated with hepatotoxicity (Ohbuchi et al., 2009). Further study is needed to clarify the association of CES2 and AADAC with flutamide-induced hepatotoxicity.

In conclusion, we found that human CES2, as well as AADAC, is involved in the flutamide hydrolysis, and CES2 is the principal enzyme in flutamide hydrolysis at a therapeutic dose. Because flutamide hydrolysis is associated with induced hepatotoxicity, this study provides useful information about the safe use of flutamide in a clinical setting.

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Authorship Contributions

Participated in research design: Kobayashi, Fukami, Nakajima, and Yokoi.

Conducted experiments: Kobayashi and Shimizu.

Contributed new reagents or analytic tools: Kobayashi.

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References

- Aizawa Y, Ikemoto I, Kishimoto K, Wada T, Yamazaki H, Ohishi Y, Kiyota H, Furuta N, Suzuki H, and Ueda M (2003) Flutamide-induced hepatic dysfunction in relation to steady-state plasma concentrations of flutamide and its metabolites. *Mol Cell Biochem* **252**:149–156.
- Cubitt HE, Houston JB, and Galetin A (2009) Relative importance of intestinal and hepatic glucuronidation-impact on the prediction of drug clearance. *Pharm Res* **26**:1073–1083.
- Fau D, Eugene D, Berson A, Letteron P, Fromenty B, Fisch C, and Pessayre D (1994) Toxicity of the antiandrogen flutamide in isolated rat hepatocytes. *J Pharmacol Exp Ther* **269**:954–962.
- Fukami T, Takahashi S, Nakagawa N, Maruichi T, Nakajima M, and Yokoi T (2010) *In vitro* evaluation of inhibitory effects of antidiabetic and antihyperlipidemic drugs on human carboxylesterase activities. *Drug Metab Dispos* **38**:2173–2178.
- Hakooz N, Ito K, Rawden H, Gill H, Lemmers L, Boobis AR, Edwards RJ, Carlile DJ, Lake BG, and Houston JB (2006) Determination of a human hepatic microsomal scaling factor for predicting *in vivo* drug clearance. *Pharm Res* **23**:533–539.
- Imai T, Taketani M, Shii M, Hosokawa M, and Chiba K (2006) Substrate specificity of carboxylesterase isozymes and their contribution to hydrolase activity in human liver and small intestine. *Drug Metab Dispos* **34**:1734–1741.
- Iwamura A, Fukami T, Higuchi R, Nakajima M, and Yokoi T (2012) Human α/β hydrolase domain containing 10 (ABHD10) is responsible enzyme for deglucuronidation of mycophenolic acid acyl-glucuronide in liver. *J Biol Chem* **287**:9240–9249.
- Katchen B and Buxbaum S (1975) Disposition of a new, nonsteroid, antiandrogen, α,α,α -trifluoro-2-methyl-4'-nitro-m-propionoluidide (Flutamide), in men following a single oral 200 mg dose. *J Clin Endocrinol Metab* **41**:373–379.
- Kobayashi Y, Fukami T, Nakajima A, Watanabe A, Nakajima M, and Yokoi T (2012) Species differences in tissue distribution and enzyme activities of arylacetamide deacetylase in human, rat, and mouse. *Drug Metab Dispos* **40**:671–679.
- Kubo T, Kim SR, Sai K, Saito Y, Nakajima T, Matsumoto K, Saito H, Shirao K, Yamamoto N, Minami H, et al. (2005) Functional characterization of three naturally occurring single nucleotide polymorphisms in the CES2 gene encoding carboxylesterase 2 (HCE-2). *Drug Metab Dispos* **33**:1482–1487.
- Kuo CL and La Du BN (1998) Calcium binding by human and rabbit serum paraoxonases. Structural stability and enzymatic activity. *Drug Metab Dispos* **26**:653–660.
- Nakajima A, Fukami T, Kobayashi Y, Watanabe A, Nakajima M, and Yokoi T (2011) Human arylacetamide deacetylase is responsible for deacetylation of rifamycins: rifampicin, rifabutin, and rifapentine. *Biochem Pharmacol* **82**:1747–1756.
- Ohbuchi M, Miyata M, Nagai D, Shimada M, Yoshinari K, and Yamazoe Y (2009) Role of enzymatic *N*-hydroxylation and reduction in flutamide metabolite-induced liver toxicity. *Drug Metab Dispos* **37**:97–105.
- Paine MF, Khalighi M, Fisher JM, Shen DD, Kunze KL, Marsh CL, Perkins JD, and Thummel KE (1997) Characterization of interintestinal and intraintraintestinal variations in human CYP3A-dependent metabolism. *J Pharmacol Exp Ther* **283**:1552–1562.
- Quinney SK, Sanghani SP, Davis WI, Hurley TD, Sun Z, Murry DJ, and Bosron WF (2005) Hydrolysis of capecitabine to 5'-deoxy-5-fluorocytidine by human carboxylesterases and inhibition by loperamide. *J Pharmacol Exp Ther* **313**:1011–1016.
- Schulz M, Schmoldt A, Donn F, and Becker H (1988) The pharmacokinetics of flutamide and its major metabolites after a single oral dose and during chronic treatment. *Eur J Clin Pharmacol* **34**:633–636.
- Shimizu M, Fukami T, Kobayashi Y, Takamiya M, Aoki Y, Nakajima M, and Yokoi T (2012) A novel polymorphic allele of human arylacetamide deacetylase leads to diminished enzyme activity. *Drug Metab Dispos* doi: 10.1124/dmd.112.044883.
- Soars MG, Burchell B, and Riley RJ (2002) *In vitro* analysis of human drug glucuronidation and prediction of *in vivo* metabolic clearance. *J Pharmacol Exp Ther* **301**:382–390.
- Takahashi S, Katoh M, Saitoh T, Nakajima M, and Yokoi T (2009) Different inhibitory effects in rat and human carboxylesterases. *Drug Metab Dispos* **37**:956–961.
- Thole Z, Manso G, Salgueiro E, Revuelta P, and Hidalgo A (2004) Hepatotoxicity induced by antiandrogens: a review of the literature. *Urol Int* **73**:289–295.
- Watanabe A, Fukami T, Nakajima M, Takamiya M, Aoki Y, and Yokoi T (2009) Human arylacetamide deacetylase is a principal enzyme in flutamide hydrolysis. *Drug Metab Dispos* **37**:1513–1520.
- Watanabe A, Fukami T, Takahashi S, Kobayashi Y, Nakagawa N, Nakajima M, and Yokoi T (2010) Arylacetamide deacetylase is a determinant enzyme for the difference in hydrolase activities of phenacetin and acetaminophen. *Drug Metab Dispos* **38**:1532–1537.
- Williams ET, Jones KO, Ponsler GD, Lowery SM, Perkins EJ, Wrigton SA, Ruterbories KJ, Kazui M, and Farid NA (2008) The biotransformation of prasugrel, a new thienopyridine prodrug, by the human carboxylesterases 1 and 2. *Drug Metab Dispos* **36**:1227–1232.
- Xu G, Zhang W, Ma MK, and McLeod HL (2002) Human carboxylesterase 2 is commonly expressed in tumor tissue and is correlated with activation of irinotecan. *Clin Cancer Res* **8**:2605–2611.
- Yamaori S, Fujiyama N, Kushihara M, Funahashi T, Kimura T, Yamamoto I, Sone T, Isobe M, Ohshima T, Matsumura K, et al. (2006) Involvement of human blood arylesterases and liver microsomal carboxylesterases in nafenostat hydrolysis. *Drug Metab Pharmacokin* **21**:147–155.
- Zhu W, Song L, Zhang H, Matoney L, LeCluyse E, and Yan B (2000) Dexamethasone differentially regulates expression of carboxylesterase genes in humans and rats. *Drug Metab Dispos* **28**:186–191.

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