Short Communication

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

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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 hydrolysis activities at a flutamide concentration of 5 μM in human liver and jejunum microsomes were strongly inhibited by a selective CES2 inhibitor, 10 μM loperamide, with the residual activities of 22.9 ± 3.5 and 18.6 ± 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 μM 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 (Obuchi 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 hydrolysis 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 μM (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 human liver.

Materials and Methods

Chemicals and Reagents. Flutamide, FLU-1, and rifampicin were purchased from Wako Pure Chemical Industries (Osaka, Japan). 25-Desacetylritampicin (25-deacetylrifampicin), irinotecan hydrochloride (CPT-11), and 7-ethyl-10-hydroxycamptothecin (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...
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 (HLLH) 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, HLM, 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.

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 (HLLH 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 (HLLH and SF21 cell homogenates expressing human CES2) was 0.2 mg/ml. The mobile phase was 25% acetonitrile and 75% of 10 mM KH2PO4 containing 3 mM 1-heptanesulfonic acid sodium salt.

Contributions of AADAC and CES2 to Flutamide Hydrolase Activity in HLLH. AADAC is expressed in liver microsomes, whereas CES2 is expressed in liver microsomes and cytosol (Xu et al., 2002). Their contributions to flutamide hydrolysis in human liver were analyzed using individual HLLH samples. The percentage contributions of AADAC and CES2 to the flutamide hydrolase activity in individual HLLH 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 (RAF<sub>AADAC</sub>) were determined as the ratios of the rifampicin hydrolase activities in HLLH to the value by recombinant AADAC. The RAF values for CES2 (RAF<sub>CES2</sub>) were determined as the ratios of the CPT-11 hydrolase activities in HLLH to the value by recombinant CES2. Using the RAF, the predicted percentages 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<sub>int</sub>, in vivo) values of flutamide hydrolysis in HLM and HJM were estimated using the following equation (Soars et al., 2002): CL<sub>int</sub>, in vivo = [CL<sub>int</sub>]<sub>tissue</sub> × [microsomal protein/tissue (mg/g)] × [tissue/body weight (g/kg)].

The CL<sub>int</sub> values of flutamide hydrolysis in HLM and HJM were approximately 1.8 and 12.9 μl · min<sup>−1</sup> · mg protein<sup>−1</sup>, respectively (Kobayashi et al., 2012). The CL<sub>int</sub> values in vivo 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<sub>int</sub>, in vivo values of HLM and HJM
respectively), the RAFAADAC and RAFCES2 values in individual HLH (Table 1). The total contributions of Hydrolase Activity in HLH equations under Contributions of AADAC and CES2 to Flutamide hydrolysis were 79.6 to 170.4% in /H9262 to the substrate inhibition equation with the maximum activity at 200 by AADAC (Nakajima et al., 2011). Data for the activity were fitted RAF values for AADAC and CES2 were calculated. The results of our would be low in the body. The contributions of other tissues to flutamide hydrolysis be denied. The contributions of other tissues to flutamide hydrolysis could thermore, the involvements of the representative esterases in plasma, butyrylcholinesterase and paraoxonase, in flutamide hydrolysis could 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 μM (Nakajima et al., 2011). Thus, this study used 50 μM rifampicin as the marker substrate for AADAC. As a marker of CES2 enzyme activity, the CPT-11 hydrolyase activity at 2 μM was measured in a similar way to that in our previous study (Fukami et al., 2010).

Using the rifampicin and CPT-11 hydrolyase activities by recombinant human CES1 and CES2, the Sf21 cells expressing these enzymes were incubated with 20 μM flutamide. A, kinetic analysis of flutamide hydrolysis 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 ± S.D. of triplicate determinations. B, inhibitory effect of loperamide on flutamide hydrolyase activity. Flutamide hydrolyase activities by recombinant human CES2, AADAC, HLM, HLC, and HJM were determined at the substrate concentration of 5 μM. The concentration of loperamide was 10 μM. The control activities by CES2, AADAC, HLM, HLC, and HJM were 17.2 ± 2.3 pmol·min⁻¹·mg protein⁻¹, 9.1 ± 0.2 pmol·min⁻¹·mg protein⁻¹, 8.6 ± 0.3 pmol·min⁻¹·mg protein⁻¹, 2.1 ± 0.0 pmol·min⁻¹·mg protein⁻¹, and 64.4 ± 1.5 pmol·min⁻¹·mg protein⁻¹, respectively. Each column represents the mean ± 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 hydrolyase 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.

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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:

$$I_{inlet,u,max} = \frac{C_{max} \times (Dose \times k_a)}{f_{u}}$$

<table>
<thead>
<tr>
<th>TABLE 1</th>
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RAF values calculated from the marker activity and the contributions of AADAC and CES2 to flutamide hydrolysis in six HLH

<table>
<thead>
<tr>
<th>Hydrolase Activity</th>
<th>RAF</th>
<th>Flutamide Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>CPT-11</td>
<td>AADAC</td>
</tr>
<tr>
<td>Concentration (μM)</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Hydrolase Activity (pmol·min⁻¹·mg protein⁻¹)</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Vrec</td>
<td>AADAC</td>
<td>CES2</td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.23</td>
</tr>
<tr>
<td>2</td>
<td>3.27</td>
<td>0.94</td>
</tr>
<tr>
<td>3</td>
<td>10.55</td>
<td>0.43</td>
</tr>
<tr>
<td>4</td>
<td>6.39</td>
<td>0.87</td>
</tr>
<tr>
<td>5</td>
<td>5.94</td>
<td>0.42</td>
</tr>
<tr>
<td>6</td>
<td>5.69</td>
<td>0.52</td>
</tr>
<tr>
<td>Liver homogenate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.23</td>
</tr>
<tr>
<td>2</td>
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$\text{FIG. 2. A, flutamide hydrolase activities in individual HLH. HLH (0.4 mg/ml) were incubated with 5, 50, and 200 μM flutamide. Gray and black columns represent the activities by AADAC and CES2, respectively, based on the data of percentage contributions. The extrapolation of percentage contributions of AADAC and CES2 were determined by the RAF using HLH and recombinant human AADAC and CES2 from the homogenates of Sf21 cells. To determine the RAF values, 50 μM rifampicin and 2 μM CPT-11 were used as the marker substrates of AADAC and CES2, respectively. B, relative contributions of AADAC (gray) and CES2 (black) to flutamide hydrolysis in individual HLH. Based on the contributions shown in Table 1, total contributions were normalized to 100%. Each column represents the mean of duplicate determinations.}$
In the intestinal tract, the absorption of flutamide is slower than in the liver. Ces2 is considered to be a major enzyme in flutamide hydrolysis in vivo. Previous reports showed a 15- and 3-fold variation in the CES2 expression and function that affect the rate of flutamide hydrolysis. This study contributes to the understanding of the contribution of the liver to 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.

Acknowledgments

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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.

Performed data analysis: Kobayashi and Fukami.

Wrote or contributed to the writing of the manuscript: Kobayashi, Fukami, and Yokoi.

References


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Supplemental Fig. 1. Kinetic analysis of the flutamide hydrolase activity in one individual HLM sample. HLM (0.4 mg/ml) was incubated with 0.005 – 0.75 mM flutamide. Each data point represents the mean ± SD of triplicate determinations.