POLYMORPHIC EXPRESSION OF CYP1A2 LEADING TO INTERINDIVIDUAL VARIABILITY IN METABOLISM OF A NOVEL BENZODIAZEPINE RECEPTOR PARTIAL INVERSE AGONIST IN DOGS

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ABSTRACT:

5-(3-Methoxyphenyl)-3-(5-methyl-1,2,4-oxadiazol-3-yl)-2-oxo-1,2-dihydro-1,6-naphthyridine (AC-3933) is a novel cognitive enhancer with central benzodiazepine receptor partial inverse agonistic activity. AC-3933 is predominantly metabolized to hydroxylated metabolite [SX-5745; 3-(5-hydroxymethyl-1,2,4-oxadiazol-3-yl)-5-(3-methoxyphenyl)-2-oxo-1,2-dihydro-1,6-naphthyridine] in dog. Initially, we found that there is considerable interindividual variability in AC-3933 hydroxylation in dogs and that dogs could be phenotyped as extensive metabolizer (EM) and poor metabolizer (PM). Then, to clarify the cause of AC-3933 polymorphic hydroxylation in dogs, in vitro studies were carried out using liver microsomes from EM and PM dogs. Our results show that AC-3933 hydroxylation clearance in PM dogs was much lower than that in EM dogs (0.2 versus 10.8–20.5 μl/min/mg, respectively). In addition, AC-3933 hydroxylation was significantly inhibited by α-naphthoflavone, a CYP1A inhibitor, and by anti-CYP1A2 antibodies, indicating that CYP1A2 was responsible for the polymorphic hydroxylation of AC-3933 in dogs. Furthermore, immunoblotting results have shown that although CYP1A2 protein was not detected in PM dogs (<0.86 pmol/mg), CYP1A2 content in EM dogs was prominent (6.1–13.0 pmol/mg). These results indicate that AC-3933 polymorphic hydroxylation arises from the polymorphic expression of CYP1A2 in dogs, which might involve genetic polymorphism of the CYP1A2 gene.

In the pharmaceutical industry, dogs are commonly used as a nonrodent species for toxicological and pharmacological studies of drug candidates. In addition, dog pharmacokinetic data along with in vitro metabolic data can be very useful for the prediction of human in vivo pharmacokinetics and interpretation of toxicity and efficacy results in both species. However, remarkable interindividual difference of drug concentration in plasma is frequently observed in dogs after drug administration (Paulson et al., 1999; Azuma et al., 2002). This variability of pharmacokinetics often affects the results of toxicological and pharmacological studies. Therefore, it is important for efficient and reliable preclinical studies to clarify the mechanism of pharmacokinetic variability and to remove the factors affecting it.

Cytochrome P450 (P450) plays a decisive role in the oxidative metabolism of xenobiotics and endogenous substances (Rendic and Di Carlo, 1997). In humans, many genetic polymorphisms of P450 have been reported, and some of them are considered important factors for interindividual variability of drug metabolism and pharmacokinetics (http://www.imm.ki.se/CYPPalleles/). On the other hand, in dogs several P450s have been cloned and sequenced, including CYP1A1/2 (Uchida et al., 1990), CYP2B11 (Graves et al., 1990), CYP2C21/41 (Uchida et al., 1990; Blaisdell et al., 1998), CYP2D15 (Sakamoto et al., 1995), CYP2E1 (Lankford et al., 2000), and CYP3A12/26 (Ciaccio et al., 1991; Fraser et al., 1997). However, the contribution of these P450s to the interindividual variability of pharmacokinetics in dogs is unknown.

5-(3-Methoxyphenyl)-3-(5-methyl-1,2,4-oxadiazol-3-yl)-2-oxo-1,2-dihydro-1,6-naphthyridine (AC-3933) is a novel cognitive enhancer with central benzodiazepine receptor partial inverse agonistic activity. The mechanism of AC-3933’s memory-improving action is based on enhancement of the cholinergic function through the allosteric reduction of γ-aminobutyric acid activity. In dogs, AC-3933 is metabolized to a major hydroxylated metabolite (SX-5745) and a minor demethylated metabolite (SX-5773), and subsequently, SX-5745 is reductively metabolized to SX-6088 (Fig. 1). This report describes a polymorphism in AC-3933 pharmacokinetics in dogs, and
we investigated the cause of the polymorphism using in vitro experiments.

Materials and Methods

Chemicals. [14C]AC-3933 (5-(3-methoxyphenyl)-3-(5-methyl-1,2,4-oxadiazol-3-yl)-2-oxo-1,2-dihydro-[7-14C]-1,6-naphthyridine) and AC-3933 were synthesized at Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). The specific activity of [14C]AC-3933 was 3.99 MBq/mg, and radiochemical purity was >99%. Ethoxyresorcin, 7-ethoxycoumarin, and aminopyrine were purchased from Sigma-Aldrich (St. Louis, MO). Phenacetin and quinidine were purchased from Wako Pure Chemicals (Osaka, Japan). Phenacetin was used for 10 min of the reaction mixture, and the eluate was evaporated to dryness. The residue was dissolved in 50 μl of the mobile phase, and an aliquot of the solution was analyzed by HPLC (model 1090 system; Agilent Technologies, Palo Alto, CA) using a reverse-phase column (Inertsil ODS-3V, 4.6 × 250 mm) at 40°C with a flow rate of 1.0 ml/min. The mobile phase consisted of solvent A (0.05% trifluoroacetic acid) and solvent B (acetonitrile), and the gradient used was as follows: 17% solvent B (0–6 min); 17 to 40% (6–16 min); 40 to 90% (16–19 min); 90 to 17% (19–21 min); and 17% (21–25 min). The wavelength of the detector was 330 nm. With this assay method, accurate determination was possible over the concentration range of 25 to 10,000 ng/ml of AC-3933 and SX-5745.

Preparation of Dog Liver Microsomes. Livers were obtained from two EM dogs (EM1 and EM2) and two PM dogs (PM1 and PM2). Each liver was homogenized in 3 volumes of 1.15% potassium chloride/10 mM potassium phosphate buffer (pH 7.4)/0.1 mM EDTA. The homogenate was centrifuged at 9000g for 20 min, and the supernatant was further centrifuged at 105,000g for 60 min. The pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4), and centrifuged at 105,000g for 60 min. The resulting pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA and stored at −80°C until use. Microsomal protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

In vitro Metabolism of AC-3933. [14C]AC-3933 (2.5, 5, 10, 20, 30, 40, and 50 μM) was incubated for 10 min at 37°C in 500 μl of a reaction mixture consisting of 50 mM phosphate buffer (pH 7.4), liver microsomes, and 0.8 mM NADPH. The reaction was started by adding NADPH and stopped by adding 1.5 ml of acetonitrile. After centrifugation at 900g for 10 min, the supernatant was evaporated to dryness, and the residue was dissolved in the mobile phase for HPLC analysis. HPLC was operated under the same conditions as above. AC-3933 and its metabolites (SX-5745 and SX-5773) were quantified by radioactivity using a scintillation cocktail (Ultima Flo-M, 2 ml/min; PerkinElmer Life and Analytical Sciences, Boston, MA) and a flow-scintillation detector (FLO-ONE/Beta A-515; PerkinElmer Life and Analytical Sciences). Assays were performed in triplicate.

Kinetic parameters were obtained by fitting metabolic velocity-concentration data into eq. 1 for hydroxylation (SX-5745 formation) or eq. 2 for demethylation (SX-5773 formation) using the nonlinear least-squares method and analysis software, Origin (OriginLab Corp., Northampton, MA):

\[ V = V_{\text{max}} \times S / (K_m + S) \]  
\[ V = C_{\text{Lmax}} \times S \]

where V is metabolic velocity; S, substrate concentration; \( V_{\text{max}} \), maximum reaction velocity; and \( K_m \), Michaelis-Menten constant. The intrinsic clearance (\( C_{\text{Lmax}} \)) for hydroxylation was obtained from \( V_{\text{max}} / K_m \).

Identification of P450 Isozyme Responsible for AC-3933 Hydroxylation. To identify the P450 isozyme responsible for AC-3933 hydroxylation,
chemical inhibition and immunoinhibition experiments were performed. The assay procedure was essentially the same as that described under In Vitro Metabolism of AC-3933, except that the AC-3933 concentration was 10 μM. For the chemical inhibition experiment, the following P450 inhibitors were used: 10 μM α-naphthoflavone as a CYP1A inhibitor, 10 μM sulfaphenazole as a CYP2C inhibitor, 1 μM quinidine as a CYP2D inhibitor, and 0.5 μM ketoconazole as a CYP3A inhibitor (Roussel et al., 1998; Tasaki et al., 1998; Bogaards et al., 2000). Each inhibitor was incubated in a reaction mixture containing pooled EM dog liver microsomes. For the immunoinhibition experiment, polyclonal anti-P450 antibodies against rat CYP1A1, CYP1A2, CYP2B1, CYP2C11, and CYP3A2 were used. Before the reaction was started, antibodies (0.43–2.15 mg of IgG/mg of microsomal protein) or corresponding preimmune sera were preincubated with pooled EM dog liver microsomes for 30 min at room temperature.

Measurement of Activity of P450s. Activities of EROD and POD, both of which reflect CYP1A2 activity (Chauvet et al., 1997; Graham et al., 2002), and activities of ECOD and APN, both of which reflect CYP2B and/or CYP3A activity (Nishibe and Hirata, 1995; Ma et al., 1998; Nakata et al., 2000), were determined. A reaction mixture consisting of 50 mM phosphate buffer (pH 7.4), 0.8 mM NADPH, EM or PM dog liver microsomes, and a substrate [ethoxyresorufin (0.01 mM), phenacetin (0.1 mM), ethoxycoumarin (0.1 mM), and aminopyrine (5 mM)] was incubated at 37°C for 15 min. EROD and ECOD activity was measured according to the method of Matsubara et al. (1983a,b). APN activity was measured according to the method of Nash (1953). POD activity was measured as follows: the reaction was started by adding NADPH and stopped by adding 1 ml of acetonitrile and 100 μl of antipyrine (10 μM) as internal standard. After centrifugation at 900g for 10 min, the supernatant was separated and evaporated to dryness, and the residue was dissolved in the mobile phase for HPLC analysis. HPLC analysis was performed under the same conditions as above. The mobile phase consisted of 10 to 50% solvent B (acetonitrile) and solvent A (0.05% trifluoroacetic acid) and the gradient used was as follows: 10 to 20% (21 min); 20 to 70% (21–50 min); 50 to 100% (50–21 min); 30 to 100% (21–22 min); and 100% (22–25 min). The wavelength of the detector was 245 nm. For the inhibition experiment of POD, 10 μM α-naphthoflavone was incubated in a reaction mixture containing PM and EM dog liver microsomes.

Immunoblotting. EM and PM dog liver microsomal proteins (5 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred electrically to a polyvinylidene difluoride membrane (Laemmli, 1970; Towbin et al., 1979). Polyclonal goat anti-CYP1A, -CYP2B, -CYP2C, and -CYP3A antibodies were used as primary antibodies. Bands that reacted with the primary antibodies were visualized using alkaline phosphatase-conjugated anti-goat IgG antibodies. CYP1A, CYP2B, CYP2C, and CYP3A content in EM and PM dog liver microsomes was measured using standard dog microsomes obtained from Daiichi Pure Chemicals Co., Ltd. Correlation coefficients of standard curves for CYP1A, CYP2B, CYP2C, and CYP3A were 0.980 (0.86–13.7 pmol/mg), 0.989 (3.28–52.5 pmol/mg), 0.942 (2.83–45.2 pmol/mg), and 0.957 (3.48–55.6 pmol/mg), respectively.

Results

In Vivo Pharmacokinetics. The concentrations of AC-3933, SX-5745, SX-5773, and SX-6088 in dog plasma after oral administration of 100 mg/kg AC-3933 are shown in Fig. 2, and the pharmacokinetic parameters are summarized in Table 1. There was considerable inter-individual variability in AC-3933 pharmacokinetics, and the dogs could be divided into two groups. In one group designated EM, Cmax and AUC0–24 of SX-5745 were the same or higher than those of AC-3933 (Fig. 2A). In the other group designated PM, in contrast, Cmax and AUC0–24 of SX-5745 were much lower than those of AC-3933 (Fig. 2B). However, Cmax ratio and AUC0–24 ratio of SX-6088 (a subsequent metabolite from SX-5745) to SX-5745 in EM dogs (0.341 ± 0.303 and 0.336 ± 0.297, respectively) were not significantly different from those in PM dogs (0.201 ± 0.045 and 0.224 ± 0.019, respectively). On the other hand, although Cmax and AUC0–24 of SX-5773, the other primary metabolite, in PM dogs were significantly higher than those in EM dogs, there was no significant difference in the Cmax ratio and AUC0–24 ratio of SX-5773 to AC-3933 between EM and PM dogs. These results indicated that the difference in pharmacokinetics of AC-3933 and SX-5745 between EM and PM dogs is due to the difference in AC-3933 hydroxylation, but not SX-5745 metabolism and not AC-3933 demethylation. In addition, Cmax ratio and AUC0–24 ratio of SX-5745 to AC-3933 were significantly different between EM and PM dogs, indicating that those parameters reflect the phenotype. C3h ratio of SX-5745 to AC-3933 was also significantly different between EM and PM dogs, and we decided that C3h ratio could be used for phenotyping instead of Cmax ratio and AUC0–24 ratio.

Phenotyping. To obtain liver samples from EM and PM dogs, phenotyping test were carried out in 20 dogs using C3h ratio of SX-5745 to AC-3933. As the histogram of logarithmically transformed C3h ratio showed bimodal distribution (Fig. 3), the dogs could be phenotyped as EM (n = 18) and PM (n = 2).

In Vitro Kinetics of AC-3933 Metabolism. Kinetic parameters for AC-3933 hydroxylation (SX-5745 formation) and demethylation (SX-5773 formation) in EM and PM dog liver microsomes are shown in Table 2. AC-3933 was mainly metabolized to SX-5745 in EM dog liver microsomes as well as in vivo (Fig. 4). AC-3933 hydroxylation clearance in PM dogs was ~50- to 100-fold lower than that in EM dogs. On the other hand, there was no difference in AC-3933 demethylation clearance between EM and PM dogs.

Identification of P450 Isozyme Responsible for AC-3933 Hydroxylation. To identify the P450 isozyme responsible for AC-3933 hydroxylation in dogs, the effects of P450 inhibitors and anti-P450 antibodies on AC-3933 hydroxylation in EM dog liver microsomes were investigated (Fig. 5). In the chemical inhibition experiment, α-naphthoflavone (CYP1A inhibitor) inhibited AC-3933 hydroxylation by 91%. On the other hand, sulfaphenazole (CYP2C inhibitor), quinidine (CYP2D inhibitor), and ketoconazole (CYP3A inhibitor) had no inhibitory effects on AC-3933 hydroxylation. In the immunoinhibition experiment, anti-CYP1A2 antibodies completely inhibited AC-3933 hydroxylation, whereas anti-P450 antibodies against CYP1A1, CYP2B1, CYP2C11, and CYP3A2 could not inhibit AC-3933 hydroxylation. These results strongly indicate that CYP1A2 is responsible for AC-3933 hydroxylation in dogs. Under these conditions, AC-3933 demethylation was not clearly inhibited by any of the inhibitors or antibodies.

![Fig. 2. Concentrations of AC-3933 and its metabolites in EM (A) and PM (B) dog plasma after oral administration of 100 mg/kg AC-3933.](image-url)
Pharmacokinetic parameters of AC-3933 and its metabolites after oral administration of 100 mg/kg AC-3933 in dogs

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Metabolites</th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>$AUC_{0-24}$ (ng·h/ml)</th>
<th>Ratios to AC-3933</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM ($n = 9$)</td>
<td>AC-3933</td>
<td>1395 ± 1101</td>
<td>15,923 ± 14,099</td>
<td>$C_{\text{max}}$</td>
</tr>
<tr>
<td>SX-5745</td>
<td>1656 ± 1244</td>
<td>19,504 ± 16,513</td>
<td>1.567 ± 0.878</td>
<td></td>
</tr>
<tr>
<td>SX-5773</td>
<td>42 ± 40</td>
<td>282 ± 509</td>
<td>1.479 ± 0.732</td>
<td></td>
</tr>
<tr>
<td>SX-6088</td>
<td>535 ± 530</td>
<td>6320 ± 6486</td>
<td>0.028 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>PM ($n = 3$)</td>
<td>AC-3933</td>
<td>6453 ± 1417**</td>
<td>83,108 ± 20,126*</td>
<td>$AUC_{0-24}$</td>
</tr>
<tr>
<td>SX-5745</td>
<td>172 ± 67**</td>
<td>2322 ± 912*</td>
<td>0.027 ± 0.006**</td>
<td></td>
</tr>
<tr>
<td>SX-5773</td>
<td>151 ± 55*</td>
<td>1978 ± 723**</td>
<td>0.026 ± 0.006**</td>
<td></td>
</tr>
<tr>
<td>SX-6088</td>
<td>35 ± 16*</td>
<td>531 ± 242</td>
<td>0.023 ± 0.003</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. Statistically significant difference from EM indicated as *$p < 0.05$ and **$p < 0.005$ by $t$ test.

In vitro kinetic parameters for AC-3933 metabolism in EM and PM dog liver microsomes

[$^{14}$C]AC-3933 (2.5, 5, 10, 20, 30, and 50 μM) was incubated for 10 min at 37°C with EM and PM dog liver microsomes in the presence of NADPH, and its metabolites were quantified by HPLC. For kinetic analysis, see Materials and Methods.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>$K_m$</th>
<th>$V_{\text{max}}$</th>
<th>$CL_{\text{int}}$</th>
<th>$CL_{\text{int}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM1</td>
<td>15.2</td>
<td>0.164</td>
<td>10.8</td>
<td>1.4</td>
</tr>
<tr>
<td>EM2</td>
<td>11.4</td>
<td>0.233</td>
<td>20.5</td>
<td>1.2</td>
</tr>
<tr>
<td>PM1</td>
<td>27.9</td>
<td>0.006</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>PM2</td>
<td>37.7</td>
<td>0.007</td>
<td>0.2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*K_m and $V_{\text{max}}$ for demethylation were not determined due to nonsaturable kinetics.

Discussion

In this study, we discovered that AC-3933 polymorphic hydroxylation arises from polymorphic expression of CYP1A2 in dogs. Indeed, the results of in vivo experiments indicated that the AC-3933 hydroxylation is polymorphic in dogs, and that dogs could be phenotyped as EM and PM. In vitro studies using liver microsomes from EM and PM dogs have shown a remarkable difference in the AC-3933 hydroxylation clearance between EM and PM dogs (Table 2). This difference correlated well with the difference in $C_{\text{max}}$ of SX-5745 to AC-3933 in plasma. In addition, AC-3933 hydroxylation in EM dogs was strongly inhibited by α-naphthoflavone, a CYP1A inhibitor.
and by anti-CYP1A2 antibodies (Fig. 5). Furthermore, markers’ activity for CYP1A2 in PM dogs was at least 2-fold lower than that in EM (Fig. 6). These results indicate that CYP1A2 is responsible for AC-3933 polymorphic hydroxylation in dogs and that CYP1A2 activity decreases in PM dogs. The decrease in CYP1A2 activity may be attributed to either a decrease in the expression of CYP1A2 or a decrease in its intrinsic activity. To test these assumptions, the expression levels of P450 proteins were examined using immunoblotting (Fig. 7). Remarkable differences were observed in the expression level of CYP1A-related protein between EM and PM dogs. In addition, the CYP1A-related protein detected by immunoblotting was possibly CYP1A2, because CYP1A2, but not CYP1A1, has been reported to be constitutively expressed in untreated dogs (Uchida et al., 1990; Graham et al., 2002). These results indicate that the decrease in CYP1A2 activity in PM dogs is caused by a decrease in the expression level of CYP1A2 protein and not by a decrease in its intrinsic activity.

Although CYP1A2 protein in PM dogs was undetectable in immunoblotting, CYP1A2 marker activity in PM dogs was only 2-fold lower than that in EM dogs. To resolve this discrepancy, we investigated the effects of α-naphthoflavone on POD activity in EM and PM dogs (Fig. 6A). In contrast with the inhibition in EM dogs, POD activity in PM dogs was activated by α-naphthoflavone, and the difference between EM and PM disappeared. It has been reported that α-naphthoflavone is not only a CYP1A inhibitor but also an activator of CYP2C and CYP3A (Sai et al., 2000). Therefore, our results indicated that POD activity in PM dogs is catalyzed by other P450s except CYP1A2, and that AC-3933 is a more specific substrate for dog CYP1A2 than phenacetin.

In support of in vivo pharmacokinetics, in vitro intrinsic clearance of AC-3933 demethylation was similar in EM and PM dogs and not correlated with the expression level and marker activities of CYP1A2. These results indicated that AC-3933 demethylation is catalyzed by not CYP1A2 but other P450s, and that the P450s are not essential for polymorphic hydroxylation of AC-3933 in dogs.

Recently, Azuma et al. (2002) reported interindividual differences in CYP1A content in dogs. However, this variation in CYP1A content (about 2-fold) was much less than that found in this study. This suggests that the mechanisms of variability observed in both studies are different and that the interindividual difference in CYP1A2 content found in this study would bring about more serious variability of pharmacokinetics, which can affect toxicological and pharmacological data in dogs.
Based on our result that CYP1A2 is polymorphically expressed in dogs, it is possible that there is a genetic polymorphism in dog CYP1A2 gene or related gene(s), i.e., molecules involved in transcription, translation, and post-translational modification of CYP1A2. Polymorphism of dog CYP1A2 gene and related genes has so far not been reported. However, in humans, interindividual differences in CYP1A2 content have been reported, and it is postulated that some genetic factors along with environmental factors are associated with these interindividual differences (Pelkonen et al., 2001). Although several mutations of human CYP1A2 gene that affect the inducibility of CYP1A2 have been reported (Nakajima et al., 1999; Sachse et al., 1999), the genetic factors relevant to CYP1A2 activity are not completely understood in humans. To identify the putative genetic factors inducing polymorphic expression of dog CYP1A2 might provide clues to not only the genotyping method for selection of PM and EM dogs, but also to the mechanism of interindividual difference in human CYP1A2. Additional molecular-based studies are needed to clarify the mechanism of CYP1A2 polymorphic expression in dogs.

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References
Blaisdell J, Goldstein JA, and Bia SA (1998) Isolation of a new canine cytochrome P450 cDNA from the cytochrome P450 2C subfamily (CYP2C41) and evidence for polymorphic differences in its expression. Drug Metab Dispos 26:278–283.