Genetic Polymorphism of Aldehyde Oxidase in Donryu Rats

Kunio Itoh, Akiko Masubuchi, Takamitsu Sasaki, Mayuko Adachi, Nobuaki Watanabe, Kiyoshi Nagata, Yasushi Yamazoe, Masahiro Hiratsuka, Michinao Mizugaki, and Yorihisa Tanaka

Department of Drug Metabolism and Pharmacokinetics (K.I., A.M., T.S., M.A., Y.T.) and Department of Clinical Pharmaceutics (M.H., M.M.), Tohoku Pharmaceutical University, Sendai, Japan; Department of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (K.N., Y.Y.); and Drug Metabolism and Pharmacokinetics Research Laboratories, Sankyo Co. Ltd., Tokyo, Japan (N.W.)

Received June 14, 2006; accepted February 7, 2007

ABSTRACT:

One of major metabolic pathways of [(±)-4-(4-cyanoanilino)-5,6-dihydro-7-hydroxy-7H-cyclopenta[d]-pyrimidine] (RS-8359), a selective and reversible monoamine oxidase type A inhibitor, is the aldehyde oxidase-catalyzed 2-hydroxylation on the pyrimidine ring. Donryu rats showed a dimorphic pattern for the 2-oxidation activity with about 20- to 40-fold variations in the V_{max}/K_{m} values between a low and a high activity group. The rats were classified as extensive metabolizers (EM) and poor metabolizers (PM) of RS-8359, of which ratios were approximately 1:1. One rat among the EM rats of each sex showed extremely high activity, and they were referred to as ultrarapid metabolizers. There was no significant difference in the expression levels of mRNA of aldehyde oxidase between the EM and PM rats. Analysis of nucleotide sequences showed four substitutions, of which the substitutions at 377G>A and 2604C>T caused 110Gly-Ser and 852 Ala-Val amino acid changes, respectively. Amino acid residue 110 is located very near the second Fe-S center of aldehyde oxidase. Its change from nonchiral Gly to chiral Ser may result in a conformational change of aldehyde oxidase protein with the shift of isoelectric point value from 5.0 in the EM rats to 6.2 in the PM rats. The 110Gly-Ser amino acid substitution (377G>A) may be primarily responsible for the variations of aldehyde oxidase activity observed in Donryu rats, in addition to the difference of expression levels of aldehyde oxidase protein. If a new drug candidate is primarily metabolized by aldehyde oxidase, attention should be given to using a rat strain with high aldehyde oxidase activity and small individual variation.

RS-8359 is a reversible and selective monoamine oxidase type A (MAO-A) inhibitor (Yokoyama et al., 1989; Miura et al., 1993), which has been developed as an antidepressant (Plenker et al., 1997; Puchler et al., 1997). One of the major metabolic pathways of RS-8359 is aldehyde oxidase-catalyzed 2-hydroxylation on the pyrimidine ring to give the 2-keto metabolite, which is preferential in rats, monkeys, and humans (Itoh et al., 2005). Other pathways were hydroxylation on the cyclopentanol ring to cis-diol and trans-diol catalyzed by cytochrome P450, which is preferential in mice (Itoh et al., 2006), and glucuronidation catalyzed by UDP-glucuronosyl transferase, which is preferential in dogs (Iwabuchi et al., 1998). All of these major metabolic events proceed with high enantioselectivity for the (S)-enantiomer that leads to more rapid disappearance of the (S)-enantiomer from plasma in every animal species. In particular, monkeys and humans have an extremely high aldehyde oxidase activity that results in an area under the curve AUC(S)/AUC(R) ratio of 238 in monkeys and a nearly negligible (S)-enantiomer in human plasma (Takasaki et al., 2005). There were no large variations in the in vitro 2-oxidation activity of RS-8359 using five human liver cytosol samples. The pharmacokinetic profiles of RS-8359 in 36 volunteers conducted in clinical trials of RS-8359 (Puchler et al., 1997) showed reasonable coefficients of variation for C_{max}, AUC, and half-life. These results suggest that there is no appreciable polymorphism in the aldehyde oxidase-catalyzed 2-oxidation of RS-8359, although the sample number was small. Indeed, genetic polymorphism of aldehyde oxidase has not been reported in humans (Beedham et al., 2003), whereas a large variation of in vitro activity has been well known (Kitamura et al., 1999a; Al-Salmy, 2001).

Aldehyde oxidase (EC 1.2.3.1) is a major member of a relatively small family of molybdenum hydroxylases that include xanthine oxidase (Beedham, 1985, 1987, 1997, 2002; Kitamura et al., 2006). Aldehyde oxidase catalyzes the oxidation of a wide range of endogenous and exogenous aldehydes and N-heterocyclic aromatic compounds. N-Heterocyclic drugs such as methotrexate, 6-mercaptopurine, cinchona alkaloids, and famciclovir are oxidized by this enzyme (Beedham, 2002; Kitamura et al., 2006). Furthermore, aldehyde oxidase can catalyze the reduction of a variety of functional groups, including sulfoxides, N-oxides, azo dyes, and N-hydroxycarbamoyl derivatives of drug metabolites. It is, therefore, important to study the genetic polymorphism of aldehyde oxidase for new drug candidates.
substituents in the presence of an appropriate donor (Kitamura et al., 2006).

Marked species differences have been well documented for the aldehyde oxidase-catalyzed metabolism of drugs, including methotrexate (Jordan et al., 1999; Kitamura et al., 1999a) and famiclovir (Rashidi et al., 1997). A large rat strain variation was shown in the oxidation activity of benzaaldehyde (Sugihara et al., 1995) and methotrexate (Kitamura et al., 1999b). The genetic variation in aldehyde oxidase was also reported by the electrophoresis method (Kunieda et al., 1999). Similar to those reports, we observed remarkable species differences and rat strain differences in the metabolism of the (S)-enantiomer of RS-8359 (Itoh et al., 2006; Sasaki et al., 2006). During the study of rat strain differences in the 2-oxidation activity of RS-8359, we were aware of the individual variations in Donryu rats, as shown in Wistar rats (Gluecksohn-Waelsch et al., 1967) and Sprague-Dawley (SD) rats (Beedham, 1998). In the current study, we examined the mechanism of individual variations of aldehyde oxidase in Donryu rats using the technology of molecular biology.

Materials and Methods

Chemicals and Reagents. RS-8359, its (S)-enantiomer, and the 2-keto metabolite were supplied by Ube Kosan Co. Ltd. (Yamaguchi, Japan). Hydrocortisone, an internal standard of high-performance liquid chromatography (HPLC) analysis, was purchased from Sigma Chemical Co. (St. Louis, MO). All the other reagents were of reagent grade.

Preparation of Liver Cytosolic Fractions. Eight-week-old male rats of Crl:Donryu and Iar:Wistar (Wistar-Imamichi) strains were purchased from Charles River Japan (Yokohama, Japan) and Imamichi Institute for Animal Reproductions (Saitama, Japan), respectively. The animals were housed according to the Guidelines for Animal Experimentation (Tohoku Pharmaceutical University) in cages with a unidirectional airflow at a controlled temperature (22 ± 2°C), relative humidity (50 ± 10%), and 12-h light/dark cycles (7:00 AM to 7:00 PM). Tap water was available ad libitum, and CE2 food (Clea Japan, Tokyo, Japan) was available ad libitum except for overnight fasting before use. The animals were sacrificed by bleeding from the carotid artery under anesthesia, and their livers were immediately extracted. The livers were homogenized in three volumes of 10 mM phosphate buffer (pH 7.4) containing 1.15% KCl and 100 mM phenylmethylsulfonyl fluoride by a Potter-Elvehjem Teflon (DuPont, Wilmington, DE) homogenizer. The cytosolic fractions were prepared by successive centrifugation at 9000 g for 10 min and then at 105,000 g for 60 min. The protein concentration was determined using BCA Protein Assay Reagent (Pierce Biotech, Rockford, IL) with bovine serum albumin as the standard.

Enzyme Activity Assay. The (S)-enantiomer of RS-8359 (3.1 μM to 0.2 mM) was incubated at 37°C for 10 min in a reaction mixture (0.25 ml) consisting of 80 mM phosphate buffer (pH 7.4), 1.0 mM KFe(CN)6, 0.13 mM EDTA, and prepared cytosol (20 mg/ml, 0.10 ml). The reaction was stopped by the addition of acetonitrile (0.50 ml) containing 0.2 mg/ml of hydrocortisone as an internal standard, after which the mixture was centrifuged at 9000 g for 3 min. Aliquots (25 μl) of the supernatant were analyzed for quantification of the oxidation product by reverse-phase HPLC on a YMC ODS A-312 column (6.0 mm i.d. × 150 mm, YMC Co., Ltd., Kyoto, Japan). A mobile phase was composed of acetonitrile/0.5% ammonium acetate (14:86); the flow rate was 1.0 ml/min. The HPLC instrument was a Shimadzu model 6A High Performance Liquid Chromatograph System (Shimadzu Seikakuso Co. Ltd., Kyoto, Japan). The peaks were monitored for absorbance at 315 nm, and the peak area was calculated on a Chromatopak C-R4A (Shimadzu Seikakuso).

Purification of Aldehyde Oxidase. Before purification, aldehyde oxidase activity was measured in the liver cytosolic fractions from Crl:Donryu rats. The rats were divided into low and high aldehyde oxidase activity groups, and then the enzyme was purified from each group according to the method described previously (Itoh et al., 2005). Briefly, the cytosolic fraction was kept at 60°C for 10 min, and then the precipitated proteins were separated by centrifugation for 10 min at 9000g. Ammonium sulfate was added to the supernatant to 50% saturation. The protein precipitates were collected by centrifugation, dissolved in 10 mM phosphate buffer (pH 7.4), and filtered through a 0.45-μm disk filter. The filtrate was applied to a Benzamidine Sepharose 6B column (5 × 22 cm), followed by Mono Q H5/5 column (0.5 × 5.0 cm) chromatography. The purified enzymes showed a single band on SDS-polyacryamide gel electrophoresis (PAGE) performed on PhastGel Gradient 4-15 (GE Healthcare Bio-Science, Piscataway, NJ).

Isoelectric Electrophoresis. Electrophoresis of the purified aldehyde oxidase (1 μg) was performed on PhastGel IEF 3-9 (Amersham Biosciences). The gels were stained for protein with Coomassie Tablet PhastGel Blue R-350 (GE Healthcare Bio-Science). A isolectric point (pH) Calibration Kit 3-10 (GE Healthcare Bio-Science) was used for pl markers.

Preparation of Anti-Rat Liver Aldehyde Oxidase Antisera. Aldehyde oxidase was isolated and purified from liver cytosolic fractions of Wistar-Imamichi rats according to the method described previously (Itoh et al., 2005). The antibody preparation was conducted at Trans Genic Inc. (Kumamoto, Japan). The purified enzyme (1.0 mg protein/ml) was emulsified with an equal volume of Freund’s complete adjuvant (Sigma). Each of two rabbits was immunized with 1 ml of immunogen by intradermal injections every 4 weeks. Four months after the first immunization, blood was taken by cardiac puncture, and antisera were prepared by a conventional method. Pooled antisera were stored at −80°C until use for Western blot analysis.

dDNA Synthesis and Real-Time Polymerase Chain Reaction. The polymerase chain reaction (PCR) reaction and subsequent cloning were performed to generate the standard for quantitative PCR of aldehyde oxidase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as follows. Total RNA was isolated from rat liver with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. An aliquot of 1 μg of total RNA was used to synthesize the first-strand cDNA with SuperScript II (Invitrogen). PCR amplification was conducted with cDNA (1 μg), Ex-Taq polymerase, and respective oligonucleotide primers for aldehyde oxidase and GAPDH, as shown in Table 1. The primers for aldehyde oxidase were designed with reference to the nucleotide sequences of male SD rat aldehyde oxidase (Wright et al., 1999).

The PCR conditions were as follows: denaturation at 95°C for 1 min, 25 amplification cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min, and a final extension of 72°C for 5 min. The amplified DNA fragment (1 μg) was subcloned in the pCRII-TOPO vector with a TOPO TA Cloning Kit (Invitrogen). The resulting plasmids were purified using a Wizard Plus MiniPreps DNA Purification System (Promega, Madison, WI). The DNA sequences of the products were determined by a CEQ 8000 Analysis System (Beckman-Coulter Inc., Fullerton, CA) with a Dye Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter) according to the recommended protocol. The respective standard curves for aldehyde oxidase and GAPDH were constructed using serial dilutions of plasmid DNA to determine the amount of template in each reaction. Plasmid DNA were linearized and quantified by spectrophotometry for amplification.

Quantitative real-time PCR analyses were performed using the PE ABI 7700 PRISM Sequence Detection System (Perkin-Elmer Life Science, Boston, MA) with SYBR Green FastStart DNA MultiMix (Roche Molecular Biochemicals, Warrington, UK), reverse-transcribed cDNA (1 μg), and gene-specific primers, shown in Table 1, which were designed using Primer Express Software (PE Applied Biosystems). The PCR reactions were performed at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. At Ct 22 to 25, the reverse transcription-PCR products were confirmed by visualization on 1% Tris-acetate-EDTA agarose gel with ethidium bromide staining. All the reactions were performed in triplicate to confirm reproducibility and included a negative control without template to verify that no primer-dimers were being generated.

Western Blot Analysis. Cytosolic proteins were separated by SDS-PAGE, which was performed on PhastGel gradient 8-25 in PhastGel SDS buffer strips (GE Healthcare Bio-Science). The proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Duichii Pure Chemicals Co., Ltd., Ibaraki, Japan) in transfer buffer (15% methanol containing 25 mM Tris and 192 mM glycine, pH 8.3). Detection of aldehyde oxidase was performed using an enhanced chemiluminescence (ECL) Western Blotting Kit (GE Healthcare Bio-Science). The membrane was blocked with 5% nonfat dry milk in phosphate-buffered saline and then incubated successively with a primary rabbit anti-rat aldehyde oxidase antibody at 0.5 μg/ml, a secondary antibody (anti-rabbit fluorescein-linked whole antibody) at a dilution of 1:600, and a tertiary antibody (anti-fluorescein alkaline phosphatase conjugate) at a dilution of 1:2000.
of 1:2500. The blocking and incubation at each immunoreaction step were performed at room temperature for 1 h, and the membrane was washed two or three times with phosphate-buffered saline containing 0.1% Tween-20. The target proteins on the membrane were detected by the ECF detection system (GE Healthcare Bio-Science). Relative densities were measured by a Fluoroimage Analyzer FLA-3000G (Fuji Photo Film Co. Ltd., Kanagawa, Japan). A high molecular weight calibration kit (GE Healthcare Bio-Science) was used for the molecular weight standards.

**Nucleotide Sequences of Aldehyde Oxidase cDNA.** PCR fragments corresponding to 4032 bp base pair of aldehyde oxidase were prepared using the cDNA synthesized above as the template. The primers used are listed in Table 1. The reaction was performed in a 50-μl solution containing 1× Ex-Taq buffer, 200 μM deoxynucleoside-5’-triphosphate solution, 2.5 U Ex-Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), 2 mM MgCl₂, 1 μM each primer, and 1 μl of the cDNA template. PCR amplification was performed using a PCR thermal cycler MP (TaKaRa) under the following conditions: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 4 min, and a final extension of 72°C for 7 min. The amplified DNA fragments were cloned with the TOPO TA Cloning Kit (Invitrogen). The resulting plasmids were purified using a Wizard Plus Miniprep DNA Purification System (Promega). The insertion of aldehyde oxidase cDNA was confirmed by agarose gel electrophoresis after digestion with SacI. The DNA sequences were determined by a CEQ 8000 Analysis System with a Dye Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter). In addition to the M13 sequence primer, the primers listed in Table 1 were used for sequencing.

**Statistical Analysis.** The results are expressed as the mean ± S.E. for the number of experiments. Statistical significance was compared between low and high activity groups by a Student’s t-test. Values with p < 0.05 were considered statistically significant.

**Results**

**Individual Variations of Aldehyde Oxidase Activity in Donryu Rats.** Aldehyde oxidase activity was determined in the liver cytosolic fractions from 12 male and 11 female Donryu rats using the (S)-enantiomer of RS-8359 as a substrate. The enzyme activity (pmol/min/mg protein) of individual rats together with their average data are shown in Fig. 1. The results clearly indicate the presence of two groups with low and high activity at a frequency ratio of about 1:1.

The two groups were classified as poor metabolizers (PM) and extensive metabolizers (EM) of RS-8359. The kinetic parameters calculated from Michaelis-Menten plots are summarized in Table 2. The Vₘₐₓ/Kₘ values of the EM group were approximately 20- to 40-fold greater than those of the PM group. Significantly larger Vₘₐₓ values (p < 0.0001) were observed in the EM group than in the PM group.

<table>
<thead>
<tr>
<th>Primer used for real-time PCR and cloning of rat aldehyde oxidase</th>
<th>Position</th>
<th>Sequence</th>
<th>Tₘₐₓ</th>
<th>Product Size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat AO forward</td>
<td>3452–3471</td>
<td>5’-ggatatcatcgggctacgc-3’</td>
<td>62.6</td>
<td>694</td>
</tr>
<tr>
<td>Rat AO reverse</td>
<td>4148–4123</td>
<td>5’-agccggcttgcatccaggt-3’</td>
<td>57.9</td>
<td>693</td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>897–917</td>
<td>5’-tcacaggtgccctctgctg-3’</td>
<td>67.5</td>
<td>693</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>1589–1570</td>
<td>5’-agccggcttgcatccaggt-3’</td>
<td>69.7</td>
<td>693</td>
</tr>
</tbody>
</table>

Reference sequences are GenBank accession number AF110478 for aldehyde oxidase (AO) and GenBank accession Number AF106860 for GAPDH. As to the primers for cloning of rat aldehyde oxidase cDNA, a recognized restriction site is indicated by italic, and the initiation and stop codons are indicated by underline, respectively.
group. The $K_m$ values were significantly smaller ($p < 0.05$) in the EM rats than in the PM rats in males, whereas there were no significant differences in females. Thus, the differences in the intrinsic clearance were essentially a result of the significant differences in the $V_{\text{max}}$ values. One rat of each sex among the EM rats showed an extremely high $V_{\text{max}}$ value of 220 to 330 pmol/min/mg protein, whereas their $K_m$ values (about 90 $\mu$M) were on an order similar to those of the other rats. The two individuals are referred to as ultrarapid metabolizers (UM).

**Real-Time PCR Analysis.** Figure 2 shows the individual data of the mRNA expression levels of aldehyde oxidase normalized by that of GAPDH and the average data. No significant correlation was observed in the mRNA expression levels between the PM and EM rats.

**Western Blot Analysis.** The immunoreactive protein levels of aldehyde oxidase were measured by Western blot analysis. After detection of immunoreactive bands by the ECF system, their relative densities were measured by FLA-3000G (Fuji Photo Film, Tokyo, Japan). The relative density of the EM rats was stronger than that of the PM rats ($p < 0.05$ in males and $p < 0.01$ in females), as shown in Fig. 3.

**Isoelectric Electrophoresis.** Aldehyde oxidase purified from the respective liver cytosolic fractions of the PM and EM Donryu rats exhibited one band on SDS-PAGE. The $p$I of the purified aldehyde oxidase was determined by isoelectric electrophoresis (Fig. 4). Different $p$I s were observed for the EM rats (5.0) and the PM rats (6.2).

**Nucleotide Sequences of Aldehyde Oxidase from PM and EM Rats.** With reference to the reported nucleotide sequences of aldehyde oxidase of the SD rats (AOX1 cDNA; GenBank accession number AF110478), full-length cDNA was cloned and sequenced from the livers of 12 PM rats (6 males and 6 females), 9 EM rats (5 males and 4 females), and 2 UM rats (1 male and 1 female) from the Donryu strain. Four nucleotide substitutions were observed among the three phenotypes. PM, EM, and UM had single nucleotide polymorphism (SNP) patterns as listed in Table 3. The SNP at positions 377 and 2604 accompanied amino acid changes of Arg to Cys (377) and Asn to Ser (2604), respectively. All the rats used in this study were confirmed to show the respective SNP according to their aldehyde oxidase activity. The UM and PM rats were homzygous at all four nucleotide positions, whereas the EM rats were heterozygous. As shown in this article, the SNP at positions 377 and 2604 were in accordance with the aldehyde oxidase activity. Both the male and female rats showed the same nucleotide substitutions and amino acid changes. Concomitantly, three SNP, 1759T(SD)$\rightarrow$C(Donryu), 2783C(SD)$\rightarrow$A(Donryu), and 2935A(SD)$\rightarrow$G(Donryu), were detected between the female SD rats and the Donryu PM rats.

**Discussion.**

One of the major metabolic pathways of RS-8359 is the 2-oxidation catalyzed by aldehyde oxidase (Iwabuchi et al., 1998; Takasaki et al., 2005). During the study of rat strain differences in the activity, we were aware of the individual variations in Donryu rats and carefully investigated the phenomenon from the perspective of molecular biology.

The 2-oxidation activity was clearly divided into low and high activity groups with a frequency ratio of approximately 1:1. This indicated that Donryu rats showed a dimorphic pattern for the 2-oxidation activity, and so they were classified as PM and EM of RS-8359. The EM rats had $K_m$/$V_{\text{max}}$ values greater than did those of the PM rats that were primarily caused by the difference in $V_{\text{max}}$ values. One rat among the EM rats of each sex showed an extremely high 2-oxidation activity that was referred to as a UM. The oxidation activity of the UM rat was not included in the
sohn-Waelsch et al. (1967) reported that the activity was appreciable using polymorphism of aldehyde oxidase, there are at least two reports. Using N\textsuperscript{-}methylnicotinamide as a substrate in Wistar rats, Gluecksohn-Waelsch et al. (1967) reported that the activity was appreciable in only 36 of 76 rats. Beedham (1998) also showed that 60% of SD rats were deficient of the functional activity of aldehyde oxidase. The occurrence frequency of PM in Donryu rats was approximately 60%, roughly comparable with those already reported in Wistar and SD rats. In addition to a marked interstrain difference in aldehyde oxidase in rats, a large intrastain difference is obvious in SD, Wistar, and Donryu rats. Generally, a proper selection of animal models is needed for the pharmacological, toxicological, and pharmacokinetic studies during development of a new drug. If a new drug candidate is primarily metabolized by aldehyde oxidase, attention should be given to using a rat strain that has high aldehyde oxidase activity as in humans and small individual variation.

The levels of mRNA and protein were analyzed by real-time PCR and Western blot, respectively, to look for the reasons of polymorphism of aldehyde oxidase in Donryu rats. The mRNA levels showed no significant differences between the EM and PM rats ($p = 0.452$ in males and $p = 0.339$ in females). On the other hand, the expression levels of immunoreactive protein were significantly higher in the EM rats than were those in the PM rats ($p < 0.05$ in male and $p < 0.01$ in female). However, the PM rats still maintained approximately 60% of aldehyde oxidase protein level of the EM rats. This suggests that the low expression levels of aldehyde oxidase protein only partially explain the extremely low aldehyde oxidase activity in the PM rats. Analysis of the full-length nucleotide sequences of the open reading frame of aldehyde oxidase revealed the four nucleotide substitutions between the UM, EM, and PM Donryu rats regardless of sex. Among those, the SNP at 377 and 2604 accompanied the respective amino acid changes of 110Gly-Ser and 852 Ala-Val, as shown in Table 3. All the rats used in this study showed the respective SNP according to their aldehyde oxidase activity. Thus, the UM and PM rats were homozygous at all four nucleotide positions, whereas the EM rats were heterozygous. In the amino acid sequences of aldehyde oxidase of the Donryu PM rats, only one among 1333 amino acids was substituted from nonchiral Gly in the UM rats to chiral Ser in the PM rats.

**TABLE 3**

| Nucleotide differences in the aldehyde oxidase cDNA between the UM, EM, and PM groups of Donryu rats and between Donryu and SD rats |

<table>
<thead>
<tr>
<th>Nucleotide No. in Male SD</th>
<th>Malea</th>
<th>Femaleb</th>
<th>Donryu Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>133</td>
<td>A</td>
<td>G</td>
<td>A</td>
</tr>
<tr>
<td>377</td>
<td>A</td>
<td>A</td>
<td>G (Gly)</td>
</tr>
<tr>
<td>405</td>
<td>C</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>408</td>
<td>G</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>1679</td>
<td>T</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>1759</td>
<td>T</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>1994</td>
<td>A</td>
<td>G</td>
<td>G</td>
</tr>
<tr>
<td>2563</td>
<td>G</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>2604</td>
<td>T</td>
<td>T</td>
<td>C (Ala)</td>
</tr>
<tr>
<td>2783</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>2872</td>
<td>T</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>2935</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>3739</td>
<td>G</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>3875</td>
<td>C</td>
<td>T</td>
<td>T</td>
</tr>
<tr>
<td>3993</td>
<td>G</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

\* GenBank accession number is AF110478. 
\* GenBank accession number is AF110477.

The nucleotide sequences of Donryu rats were numbered with the reference to those of male SD rats (Wright et al., 1999). The number of rats for the analysis of nucleotide sequences in male and female Donryu rats were identical and differed only in 10 positions when compared with male and female SD rats.

The study was not included in these MoCo binding regions, it does not seem that the marked decrease of catalytic activity might be caused by direct steric change of the enzyme’s active centers. Amino acid residue 852 is located between MoCo 1 and MoCo 2. The change from Ala to Val at that point does not seem to largely affect the structure of aldehyde oxidase because the two amino acids have very similar physicochemical properties. On the other hand, amino acid residue 110 is located very near the second Fe-S center and is substituted from nonchiral Gly in the UM rats to chiral Ser in the PM rats. The pH of aldehyde oxidase purified from the liver of the EM rats was at pH 5.0 compared with that from the PM rats at pH 6.2. This finding suggests that the amino acid changes from Gly to Ser near the second Fe-S center may result in the conformational change of alde-
hyde oxidase. The conformational change may affect the surface electric charge of that protein and cause the shift of pI. As a result, the severe decrease in enzyme activity could be produced in the PM rats. Indeed, amino acid 110 is conserved in human, monkey, bovine, rabbit, rat, and mouse and seems to be important for function of aldehyde oxidase through possibly maintaining its conformational structures.

The liver cytosol from the PM rats was treated with dithiothreitol for reduction of disulfide bond and 4,4′-dithiodipyridine for oxidation of thiol groups to investigate whether the activity will be changed. The low activity did not increase at all by either treatment (data not shown). The results suggest that the variations in the 2-oxidation of (S)-RS-8359 may be caused by the SNP, but not by the redox status of aldehyde oxidase reported by Wright et al. (1999) to distinguish male and female forms of aldehyde oxidase in SD rat. Different mechanisms were suggested between the individual variations of male and female forms of aldehyde oxidase in Donryu rats and the sex differences of aldehyde oxidase in SD rats.

A recent study by Sasaki et al. (2006) revealed the low 2-oxidation activity of the (S)-enantiomer in the SD strain. The amino acid sequences of aldehyde oxidase of female SD rats have 1332 of 1333 identities to those of the Donryu PM rats, and both have 110Ser and 852Val sequences. This finding suggests the importance of amino acid residues 110 and 852, especially 110, for the functional expression of aldehyde oxidase activity. We are interested in whether the SNP at 377 is responsible, as well as in rat strain differences and species differences in aldehyde oxidase activity, and large individual variation in humans. Aldehyde oxidase and its variants are being further characterized by in vitro expression experiments.

We conclude that the 110Gly-Ser amino acid substitution (377G>A) may be importantly responsible for the variations of aldehyde oxidase activity observed in Donryu rats, in addition to the difference of expression levels of aldehyde oxidase protein. If a new drug candidate is primarily metabolized by aldehyde oxidase, attention should be given to using a rat strain with high aldehyde oxidase activity and small individual variation.

Acknowledgments. We thank Dr. T. Ikeda, Director of the Drug Metabolism and Pharmacokinetics Research Laboratories, Sankyo Co. Ltd., and Drs. K. Nishimura and Y. Kawahara, former directors of the Research Laboratories, for their encouragement.

References


Address correspondence to: Yorihisa Tanaka, Department of Drug Metabolism and Pharmacokinetics, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan. E-mail: ytanaka@tohoku-pharm.ac.jp