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GENETIC POLYMORPHISM OF ALDEHYDE OXIDASE IN DONRYU RATS

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Running title: Genetic polymorphism of aldehyde oxidase in Donryu rats

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ABBREVIATIONS: MAO, monoamine oxidase; SD, Sprague-Dawley; PM, poor metabolizer; EM, extensive metabolizer; UM, ultrarapid metabolizer; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis;

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pI, isoelectric point; SNP, single nucleotide polymorphism

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ABSTRACT:

One of major metabolic pathways of RS-8359, a selective and reversible MAO-A inhibitor, is the aldehyde oxidase catalyzed 2-hydroxylation at the pyrimidine ring. Donryu rats showed a dimorphic pattern for the 2-oxidation activity with about 20~40-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 were referred to as ultrarapid metabolizers (UM). There was no significant difference in the expression levels of mRNA of aldehyde oxidase between the EM and PM rats. Analysis of nucleotide sequences demonstrated four substitutions, of which the substitutions at 377G>A and 2604C>T caused 110Gly-Ser and 852Ala-Val amino changes, respectively. Amino acid residue 110 is located very near the second Fe-S center of aldehyde oxidase. Its change from non-chiral Gly to chiral Ser might result in a conformational change of aldehyde oxidase protein with the shift of pI value from 5.0 in the EM rats to 6.2 in the PM rats. The 110Gly-Ser amino acid substitution (377G>A) might 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 and small individual variation.

RS-8359, [(±)-4-(4-cyanoanilino)-5,6-dihydro-7-hydroxy-7*H*-cyclopenta[*d*]-pyrimidine], is a reversible and selective MAO-A inhibitor (Yokoyama et al., 1989; Miura et al., 1993), which has been developed as an antidepressant (Puchler et al., 1997; Plenker et al., 1997). One of the major metabolic pathways of RS-8359 is aldehyde oxidase-catalyzed 2-oxidation 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 AUC(*R*)/AUC(*S*) ratio of 238 in monkeys and is 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 thirty-six volunteers

conducted in clinical trials of RS-8359 (Puchler et al., 1997) showed reasonable coefficients of variation for Cmax, 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., 1999; 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, 1987a, 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 et al., 2002; Kitamura et al., 2006). Further, aldehyde oxidase can catalyze the reduction of a variety of functional groups including sulfoxides, *N*-oxides, azo dyes, and *N*-hydroxycarbamoyl 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 (Kitamura et al., 1999a; Jordan et al., 1999) and famciclovir (Rashidi et al., 1997). A large rat strain variation was demonstrated in the oxidation activity of benzaldehyde (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 demonstrated in Wistar rats (Gluecksohn-Waelsch et al., 1967) and SD rats (Beedham et al., 1998). In the current study, we examined the mechanism of individual variations of aldehyde oxidase in Donryu rats using the technology of molecular biology.

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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 HPLC analysis, was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of reagent grade.

Preparation of Liver Cytosolic Fractions. Eight-week-old male rats of Crj: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 in rooms with a unidirectional airflow at a controlled temperature (22±2°C), relative humidity (50±10%), and 12-h light/dark cycles (07.00-19.00 hours). 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

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three volumes of 10 mM phosphate buffer (pH 7.4) containing 1.15% KCl and 100 μM phenylmethanesulfonyl fluoride by a Potter-Elvehjem Teflon homogenizer. The cytosolic fractions were prepared by successive centrifugation at 9000 *g* for 20 min and then at 105000 *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~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 $K_3Fe(CN)_6$, 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 5000 *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. x 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 Seisakusho 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 Seisakusho).

Purification of Aldehyde Oxidase. Before purification, aldehyde oxidase activity was measured in the liver cytosolic fractions from Crj: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 9000 *g*. 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 (3 x 22 cm) followed by Mono Q HR5/5 column (0.5 x 5.0 cm) chromatography. The purified enzymes showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed on PhastGel Gradient 4-15 (Amersham Bioscience, Uppsala, Sweden).

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 (Amersham Biosciences). A pI Calibration Kit 3-10 (Amersham Biosciences) was used for isoelectric point 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, St. Louis, MO). 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.

cDNA Synthesis and Real-time PCR. The PCR reaction and subsequent cloning was 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 using a TOPO TA Cloning Kit (Invitrogen). The resulting plasmids were purified using a Wizard Plus Minipreps DNA Purification System (Promega Co., Madison, WI). The DNA sequences of the products were determined by a CEQ 8000 Analysis System (Beckman-Coulter Inc., Fullerton, CA) with a DTCS 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 DNAs 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, USA) with SYBR Green PCR Master Mix (Applied Biosystems, 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 22~25, the RT-PCR products were confirmed by visualization on 1% TAE agarose gel with ethidium bromide staining. All 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 (Amersham Bioscience). The proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Daiichi Pure Chemicals Co. Ltd., Ibaraki, Japan) in transfer buffer (15% methanol containing Tris 25 mM and glycine 192 mM, pH 8.3). Detection of aldehyde oxidase was performed using an ECF Western Blotting Kit

(Amersham Bioscience). The membrane was blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS), 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 1:2500. The blocking and incubation at each immunoreaction step were performed at room temperature for 1 hr, and the membrane was washed two or three times with PBS containing 0.1% Tween-20 (PBST). The target proteins on the membrane were detected by the enhanced chemifluorescence (ECF) detection system (Amersham Bioscience). Relative densities were measured by a Fluoro-image Analyzer FLA-3000G (Fuji Photo Film Co. Ltd., Kanagawa, Japan). An HMW Calibration Kit (Amersham Bioscience) was used for the molecular weight standards.

Nucleotide Sequences of Aldehyde Oxidase cDNA. PCR fragments corresponding to 4032 bp 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 dNTP solution, 2.5 U Ex-Taq DNA

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polymerase (TaKaRa Bio Inc., Shiga, Japan), 2 mM $MgCl_2$, 1 μM of 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 Minipreps DNA Purification System (Promega). The insertion of aldehyde oxidase cDNA was confirmed by agarose gel electrophoresis after digestion with *Sac* I. The DNA sequences were determined by a CEQ 8000 Analysis System with a DTCS Quick Start Kit. 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±SE 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.

TABLE 1

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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 is 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 *V*max/*K*m values of the EM group were approximately 20~40-fold greater than were those of the PM group. Significantly larger *V*max values ($p < 0.0001$) were observed in the EM group than in the PM 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 due to the significant differences in the *V*max values. One rat of each sex among the EM rats showed an

extremely high *V*max value of 220~330 pmol/min/mg protein whereas their *K*m values (about 90 μM) were on an order similar to those of the other rats. The two individuals are referred to as ultrarapid metabolizers (UM).

FIG. 1

TABLE 2

Real-time PCR Analysis. Fig. 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.

FIG. 2

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Western Blot Analysis. The immunoreactive protein levels of aldehyde oxidase were measured by Western blot. After detection of immunoreactive bands by the enhanced chemifluorescence (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.

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 isoelecric point (pI) of the purified aldehyde oxidase was determined by isoelectric electrophoresis (Fig. 4). Different pIs were observed for the EM rats (5.0) and the PM rats (6.2).

FIG. 4

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. PMs, EMs, and UMs had SNP patterns as listed in Table 3. The SNPs at positions 377 and 2604 accompanied amino acid changes of 110Gly-Ser and 852Ala-Val, respectively. All rats used in this study were confirmed to show the respective SNPs according to their aldehyde oxidase activity. The UM and PM rats were homozygous at all four nucleotide positions whereas the EM rats were heterozygous. As demonstrated in this paper, the SNPs 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 SNPs, 1759T(SD)>C(Donryu), 2783C(SD)>A(Donryu), and 2935A(SD)>G(Donryu) were detected between the female SD rats and the Donryu PM rats.

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TABLE 3

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 poor metabolizers (PM), and extensive metabolizers (EM) of RS-8359. The EM rats had *K*m/*V*max values about 20~40-fold greater than did those of the PM rats that were primarily due to the difference in *V*max values. One rat among the EM rats of each sex showed an extremely high 2-oxidation activity that was referred to as an ultrarapid metabolizer (UM). The oxidation activity of the UM rat was not included in the summary of the kinetic parameters of the EM rats. As to the apparent polymorphism of aldehyde oxidase, there are at least two

reports. Using N^1-methylnicotinamide as a substrate in Wistar rats, Gluecksohn-Waelsch et al. (1967) reported that the activity was appreciable in only 36 out of 76 rats. Beedham et al. (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% that were roughly comparable to those already reported in Wistar and SD rats. In addition to a marked inter-strain difference in aldehyde oxidase in rats, a large intra-strain 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 human 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 SNPs at 377 and 2604 accompanied the respective amino acid changes of 110Gly-Ser and 852Ala-Val, as shown in Table 3. All rats used in this study showed the respective SNPs 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 changed from His to Asn compared to those of female SD rats reported by Wright et al (1998). However, the cDNA sequences in male and female Donryu rats were identical and differed only in ten positions when compared to male and female SD rats (Wright et al., 1998).

Aldehyde oxidase is a homodimer with a monomeric molecular weight of about

150 kDa; each monomer contains a molybdenum cofactor, a FAD, and two different 2Fe-2S redox centers (Beedham, 1998, 2002). The first and second Fe-S centers have been reported as located between amino acid residues 43-74 and 112-155, respectively. Five sites within the large molybdenum-pterin cofactor (MoCo) binding domain have been identified as follows: MoCo 1 between amino acid residues 799-806, MoCo 2 between 914-923, MoCo 3 between 1043-1045, MoCo 4 between 1079-1084, and MoCo 5 between 1263-1268 (Wright et al., 1998). Because the two amino acid substitutions confirmed in the present study were 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 non-chiral Gly in the UM rats to chiral Ser in the PM rats. The isoelectric point of aldehyde oxidase purified from the liver of the EM rats was at pH 5.0 compared to 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 aldehyde oxidase. The conformational change might affect the surface electric charge of that protein and cause the shift of isoelectric points. 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 in order 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 might be caused by the SNPs, 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 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 1332/1333 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 rat strain differences and species differences in aldehyde oxidase, and large individual variation in human. Aldehyde oxidase and its variants are being further characterized by *in vitro* expression experiments.

The conclusion is that the 110Gly-Ser amino acid substitution (377G>A) might 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 and small individual variation.

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Figure captions

FIG. 1. Individual and mean data of the 2-oxidation activity of the (*S*)-enantiomer of RS-8359 by aldehyde oxidase in 12 male Donryu rats.

FIG. 2. Real-time PCR analysis of aldehyde oxidase in 12 male and 11 female Donryu rats. The mRNA expression level of each rat was normalized by that of GAPDH. Statistically significant difference between the EM and PM rats: $*p < 0.05$, $**p < 0.01$.

FIG. 3. Western blot analysis of aldehyde oxidase in 12 male and 11 female Donryu rats.

FIG. 4. Isoelectric electrophoresis of aldehyde oxidase purified from (a) PM rats and (b) EM rats in the Donryu strain.

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TABLE 1

Primers used for real-time PCR and cloning of rat aldehyde oxidase

Primer	Position	Sequence	Tm	Product size
Cloning for Real-time				
Rat AO Forward	3452-3471	5'-ggatatttcaggggctacga-3'	62.6	694bp
Rat AO Reverse	4145-4123	5'-actctggctttcatatttagcat-3'	59.7	
GAPDH Forward	897-917	5'-taccagggctgccttctcttg-3'	67.5	693bp
GAPDH Reverse	1589-1570	5'-aggcggcatgtcagatccac-3'	69.7	
Real-time PCR				
Rat AO Forward	3795-3814	5'-ccccatccgaacactcaaac-3'	66.5	81bp
Rat AO Reverse	3875-3856	5'-gaaataccgaacagcccagg-3'	65.4	
GAPDH Forward	945-964	5'-cgacccttcattgacctca-3'	67.3	81bp
GAPDH Reverse	1025-1005	5'-ttgactgtgccgttgaacttg-3'	66.1	
Cloning				
Rat AO Forward-*Acc65*		5'-gc*ggtacc*gacctcgtcgtcatggatcc-3'	68.4	4032bp
Rat AO Reverse-*Sal*		5'-gc*gtcgac*actcacacaggtatgttcc-3'	73.8	
Sequencing				
Rat AO Forward 1	351-372	5'-ttcaggagaggatcgccaagtg-3'	69.6	
Rat AO Forward 2	751-775	5'-gttctacagtaatagaatgacatgg-3'	57.3	
Rat AO Forward 3	1130-1150	5'-agacatctggactcggatctg-3'	63.2	
Rat AO Forward 4	1546-1568	5'-agtcacccttgcaggttcagctc-3'	69.8	
Rat AO Reverse 1	2562-2542	5'-aacatgtcttcccctcgttcc-3'	66.2	
Rat AO Reverse 2	2935-2912	5'-ttccttgcttgtaatgggtattatc-3'	59.8	
Rat AO Reverse 3	3384-3365	5'-ttcttgctgatgatgggctc-3'	65.2	
Rat AO Reverse 4	3703-3679	5'-agagtagctcagctcctctatcgtg-3'	65.1	

Reference sequences are GenBank accession number AF110478 for aldehyde oxidase 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.

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TABLE 2

Kinetic parameters for the 2-oxidation activity of the (S)-enantiomer of RS-8359 by low and high activity group of Donryu strain rats

Gender	Group	*K*m (μM)	*V*max (pmol/min/mg protein)	*V*max/*K*m (ml/min/mg protein)
Male	UM (1)	90.0	333	3.70
	PM (6)	87.0±5.63	5.1±0.23	0.067±0.004
	EM (5)	53.9±6.81*	125.0±6.67**	2.40±0.184**
Female	UM (1)	86.7	222	2.56
	PM (6)	100.3±15.5	6.7±0.75	0.07±0.005
	EM (4)	79.0±6.07	105.9±15.1**	1.34±0.094**

Each value represents the mean±S.E. of four to six rats. The figures in parentheses show the number of rats studied.

*p<0.01, **p<0.0001 compared with the low activity group.

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TABLE 3

The nucleotide differences in the AO cDNA between the UM, EM and PM groups of Donryu rats and between Donryu and Sprauge-Dawley rats

Nucleotide No. in male SD[a)]	SD[a)] male	SD[b)] female	Donryu male		
			UM (1)	EM (5)	PM (6)
133	A	G	A	A/G	G
377	**A**	**A**	**G (Gly)**	**G/A (Gly/Ser)**	**A (Ser)**
405	C	G	G	G	G
408	G	T	T	T	T
1679	T	C	C	C	C
1759	T	T	C	C	C
1994	A	G	G	G	G
2563	G	A	A	A	A
2604	**T**	**T**	**C (Ala)**	**C/T (Ala/Val)**	**T (Val)**
2783	C	C	A	A	A
2872	T	C	C	C	C
2935	A	A	A	A/G	G
3739	G	A	A	A	A
3875	C	T	T	T	T
3993	G	C	C	C	C

The nucleotide sequences of Donryu rats were numbered with the reference to those of male SD rats (Wright et al., 1998).The number of rats for the analysis of nucleotide sequences is shown in the parentheses. Nucleotide substitutions accompanying amino acid changes were indicated by bold alphabets. The cDNA sequences in male and female Donryu rats were identical and differed only in ten positions when compared to male and female SD rats.

[a)] GenBank accession number is AF110478.

[b)] GenBank accession number is AF110477.

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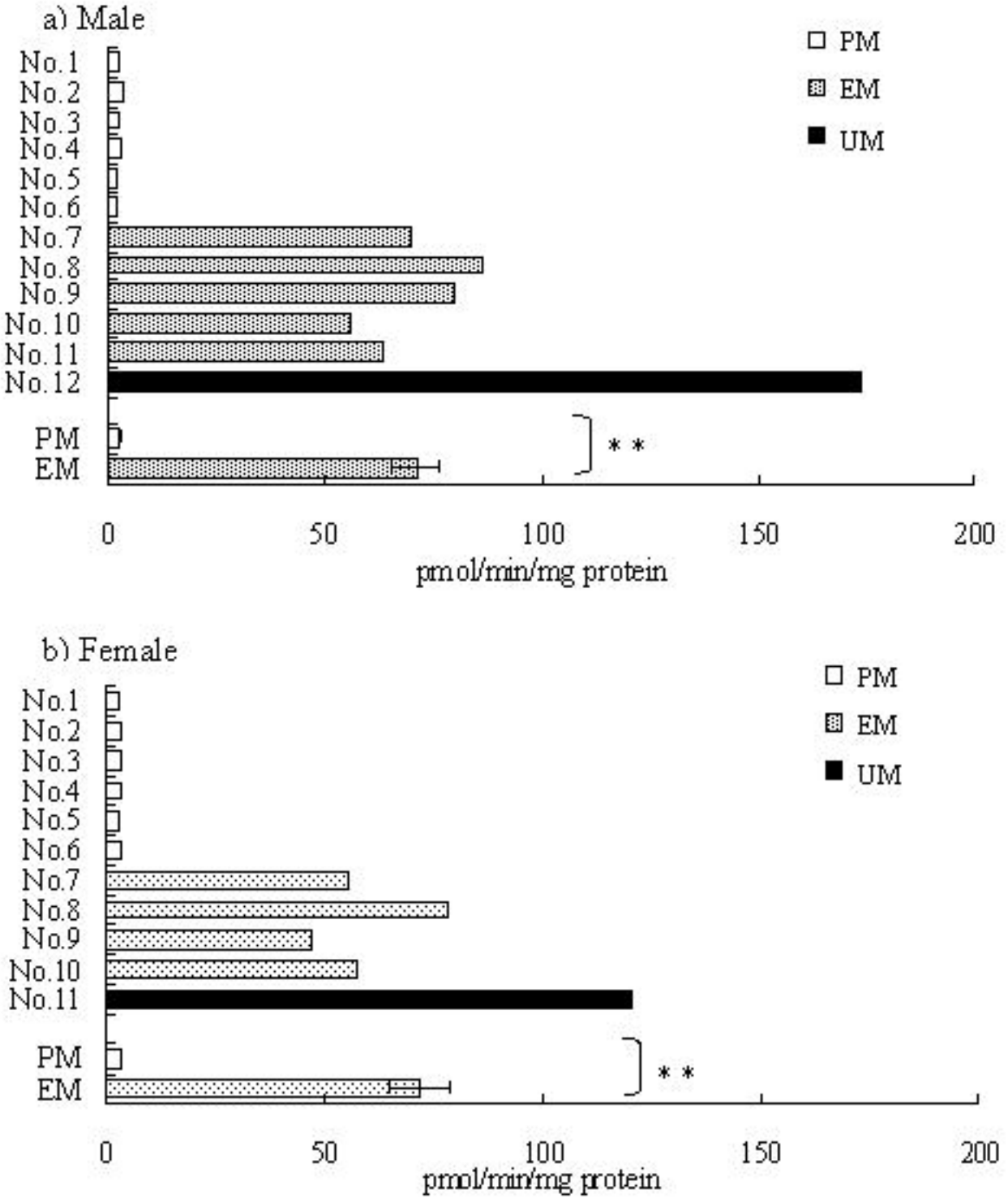

Fig. 1

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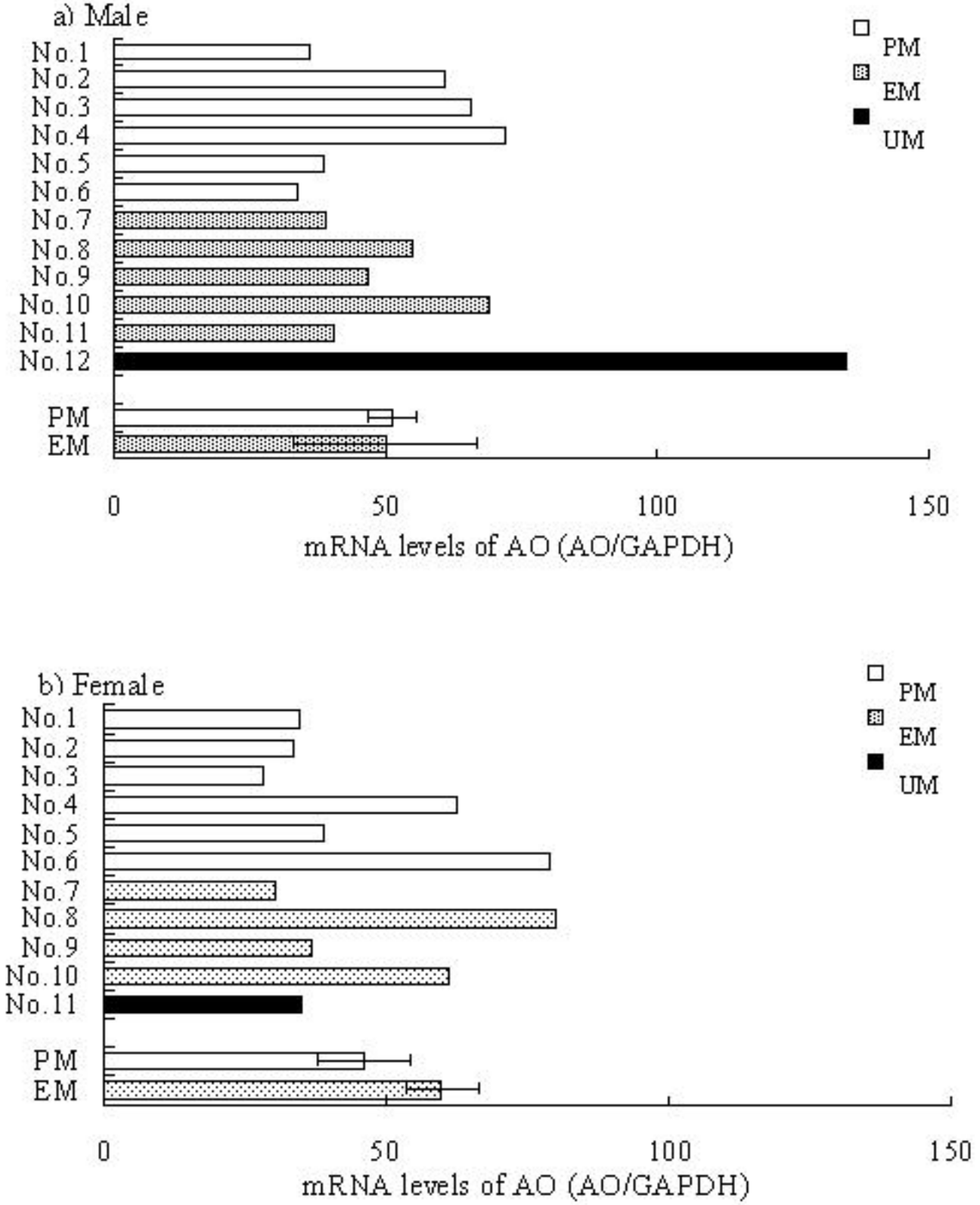

Fig. 2

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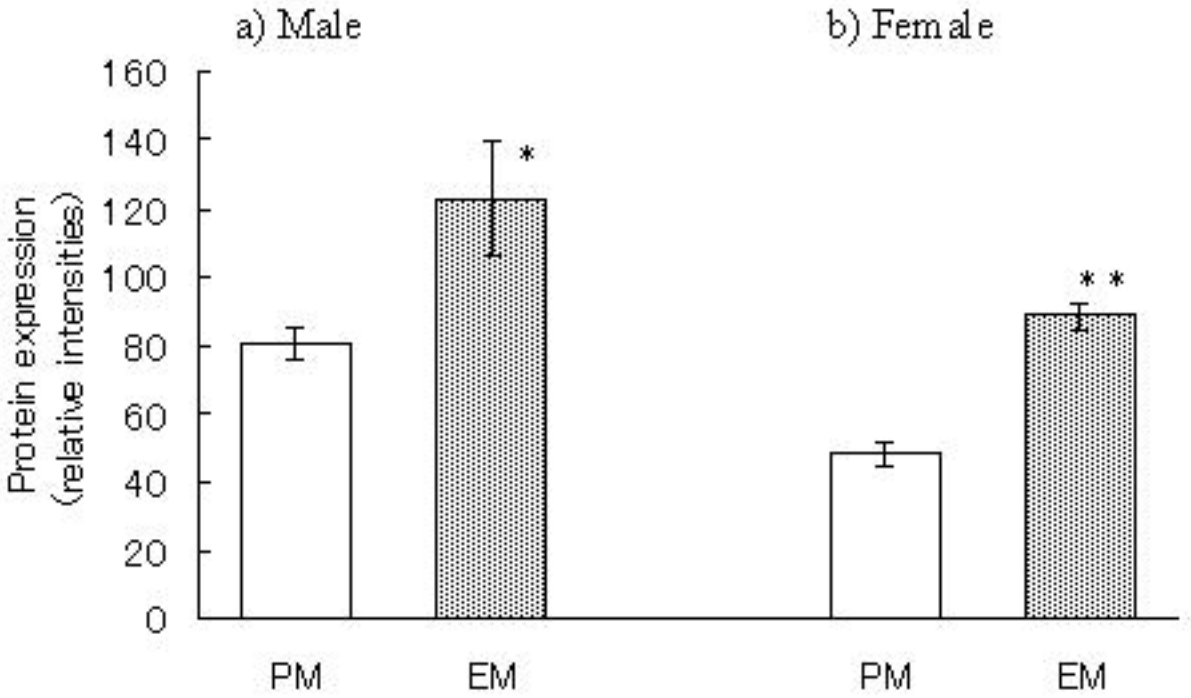

Fig. 3

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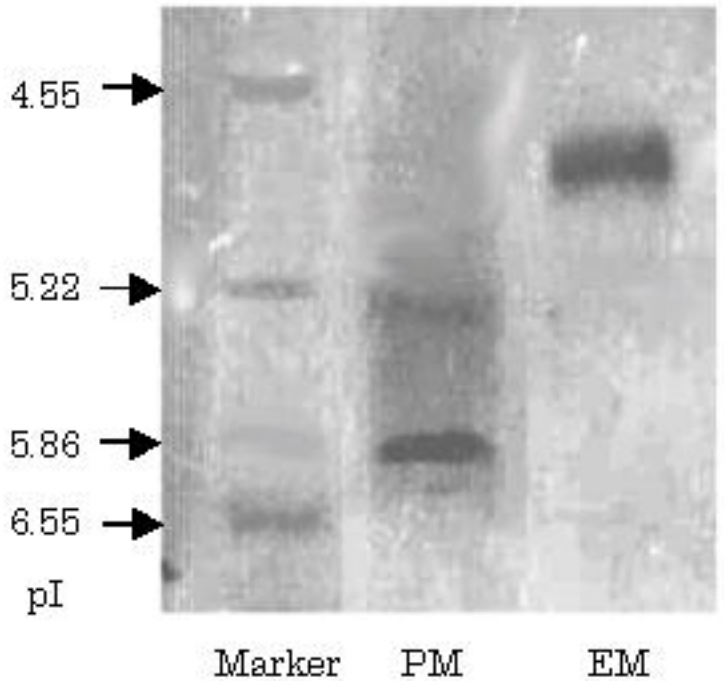

Fig. 4