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Short communication

Molecular cloning and characterization of marmoset aldehyde oxidase

Shotaro Uehara, Yasuhiro Uno, Eriko Okamoto, Takashi Inoue, Erika Sasaki, and Hiroshi Yamazaki

Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan (SU, EO, and HY); Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd., Kainan, Wakayama, Japan (YU); Department of Applied Developmental Biology (TI) and Center of Applied Developmental Biology (ES), Central Institute for Experimental Animals, Kawasaki, Japan; and Keio Advanced Research Center, Keio University, Minato-ku, Tokyo, Japan (ES)

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Running Title Page

Running title: Cloning of marmoset AOX1 cDNA

Correspondence author: Hiroshi Yamazaki, PhD, Professor

Showa Pharmaceutical University, 3-3165 Higashi-tamagawa Gakuen, Machida, Tokyo 194-8543, Japan. Phone: +81-42-721-1406; Fax: +81-42-721-1406. E-mail: hyamazak@ac.shoyaku.ac.jp

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Abbreviations: AOX, aldehyde oxidase (EC 1.2.3.1); HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; RT, reverse transcription.

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Abstract

Common marmosets (*Callithrix jacchus*), New world monkeys, are a promising primate model for preclinical drug metabolism studies due to the similarities of cytochrome P450 (P450) enzyme function to those of humans. Despite an increasing number of drug candidates catalyzed by non-P450 enzymes, drug metabolizing enzymes other than P450s have been hardly identified or characterized in marmosets. In this study, we identified *aldehyde oxidase* (*AOX*) *1* gene by marmoset genome analysis. AOX1 cDNA was cloned from marmoset livers by reverse transcription-polymerase chain reaction. Deduced amino acid sequences of AOX1 cDNA showed high sequence identities (92–93%) with primate AOX1s. Phylogenetic analysis showed that marmoset AOX1 was closely clustered with primate AOX1s, unlike non-primate animal model AOX1s of pig, rabbit, rat, and mouse, used in drug metabolism. The tissue expression analyses by real-time RT-PCR and immunoblotting showed that marmoset AOX1 mRNA and protein were abundantly expressed in livers, similar to cynomolgus monkeys and humans. Marmoset AOX1 heterologously expressed in *Escherichia coli* catalyzed the oxidation of carbazeran and phthalazine, typical AOX1 substrates, similar to cynomolgus monkey and human AOX1s. Human and marmoset AOX1 effectively catalyzed phthalazine oxidation when assessed with Michaelis-Menten kinetics, but cynomolgus monkey AOX1 catalyzed this reaction with cooperative kinetics with high capacity. These results indicated that tissue distribution and enzymatic function of AOX1 enzyme is similar between marmosets and humans, suggesting that marmosets are a suitable primate model for AOX-dependent drug metabolism in preclinical studies.

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Introduction

The Old World primates, cynomolgus monkeys (*Macaca fascicularis*) and rhesus monkeys (*Macaca mulatta*), and the New World primate, common marmosets (*Callithrix jacchus*), have been widely used (Uno et al., 2016) and have shown potential (Sasaki, 2015) as non-human primate models in preclinical testing of drug candidates. Aldehyde oxidase (AOX, EC 1.2.3.1) is a molybdenum cofactor-containing drug-metabolizing enzyme involved in the oxidation of a variety of aldehydes and heterocyclic-containing drug molecules (Garattini et al., 2009). Marked interspecies differences of AOX activities in livers have been reported (Garattini and Terao, 2012) resulting in a lack of clinical translation of candidate drugs attributed to decreased rat AOX activity or missing dog *AOX* gene (Crouch et al., 2017). Oxidation activities of *N*-phenylquinolinium chloride in rat and dog livers have been shown to be low and negligible, respectively, whereas baboon livers have showed high aldehyde oxidase activity; these oxidation activities by marmoset and guinea pig livers are reportedly similar to human livers (Beedham et al., 1987). A selective inhibitor of the sodium/hydrogen exchanger, zoniporide, have been converted to 2-oxozoniporide in liver cytosolic fractions from humans and rats, but not dogs (Dalvie et al., 2010). In terms of cytochrome P450 (EC 1.14.14.1) catalytic function, marmosets could be one of the animal models for human drug metabolism (Uno et al., 2016). On the other hand, the establishment of appropriate surrogate animal species for AOX-dependent drug metabolism has been desired, but little information is available about the molecular characteristics of marmoset AOX.

In this study, we isolated marmoset AOX1 cDNA based on the marmoset genome and analyzed the tissue expression specificity and catalytic activity using recombinant AOX protein. This work

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provides information about the molecular basis and characteristics of marmoset AOX1 for the utilization of marmosets in pharmacokinetic and toxicokinetic preclinical testing.

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Materials and Methods

Chemicals and enzymes

Phthalazine and phthalazone were purchased from Tokyo Kasei (Tokyo, Japan). Carbazeran and 4-hydroxycarbazeran were purchased from Toronto Research Chemicals (Toronto, Canada). All DNA oligonucleotides were supplied by Sigma Genosys (Ishikari, Japan). Caucasian liver cytosolic fractions pooled from 10 humans (mixed gender) previously prepared (Shimada et al., 1994) were used under the approval of the ethics committee of Showa Pharmaceutical University. Marmoset liver cytosolic fractions were prepared from 10 marmosets (9 males and 1 female, >2 years of age) raised at the Central Institution for Experimental Animals (Kawasaki, Japan). Liver cytosolic fractions were also prepared from cynomolgus monkeys (10 males, 3-8 years old, Shin Nippon Biomedical Laboratories, Ltd., Kainan, Japan) and dogs (2 males, sexually mature; Xenotech Lenexa, KS). Brain, lung, liver, kidney, and jejunum tissues were obtained from marmosets, and brain, lung, heart, liver, kidney, jejunum, adrenal gland, testis, ovary, uterus, and nasal tissues were also collected from cynomolgus monkeys. This study was reviewed and approved by the Institutional Animal Care and Use Committees of the Central Institution for Experimental Animals and Shin Nippon Biomedical Laboratories, Ltd. Anti-human AOX1 antibodies (ab197828) and goat anti-rabbit IgG-horseradish peroxidase (A0545) were purchased from Abcam (Cambridge, MA) and Sigma-Aldrich, respectively. All other reagents were of the highest quality commercially available.

AOX1 cDNA cloning

Human, cynomolgus monkey, and marmoset AOX1 cDNAs were cloned by (RT)-polymerase chain

reaction (PCR). Human, cynomolgus monkey, and marmoset liver cDNAs were prepared with total RNA extracted from livers using SuperScript III RT reverse transcriptase (Invitrogen, Carlsbad, CA), oligo (dT) primer (Invitrogen), and RNase OUT (Invitrogen) following the manufacturer's instructions. PCR was performed using KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) with an ABI GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). PCR conditions consisted of an initial denaturation step at 98°C for 2 minutes, 35 cycles of denaturation at 98°C for 15 seconds, annealing at 65°C for 30 seconds, and extension at 68°C for 2.5 minutes, and a final extension at 68°C for 7 minutes. PCR primers were designed based on genome sequences of humans, cynomolgus monkeys, and marmosets; hAOX1 (5rt1) 5′-ATGGACCGGGCGTCCGAGCTGCTCTTC-3′ and hAOX1 (3rt1) 5′-GATTGCCATCTGGGAAGAGGCACTCTGTTTTCTC-3′ for human AOX1, mfAOX1 (5rt1) 5′-CAATGGACAGGGCGTCCGAACTGCT-3′ and mfAOX1 (3rt1) 5′-CATTGCCATCTGGGAAGAGGCACTCCA-3′ for cynomolgus monkey AOX1, and cjAOX1 (5rt1) 5′-ATGGACCGGACGCCGGAGCTGCTCTTCTAC-3′ and cjAOX1 (3rt1) 5′-CTGCCGTCTGGGAAGGCATGCCATTTTCTC-3′ for marmoset AOX1. PCR products were purified and cloned into pCR4 vectors using a Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen). Insert DNA fragments were sequenced with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

Enzyme assay

Phthalazine oxidation activities by recombinant AOX proteins and liver cytosolic fractions were measured by HPLC (Prominence-I LC-2030C HPLC system, Shimadzu, Kyoto, Japan) as described

previously (Panoutsopoulos and Beedham, 2004) with some minor changes. Briefly, the incubation mixture consisted of AOX1 protein (arbitrary volume) or 0.50 mg/mL liver cytosolic fractions, 0.44-80 μM phthalazine, and 100 μM potassium phosphate buffer (pH 7.4) in a total volume of 0.25 mL. The reaction was incubated at 37°C for 3 min and terminated with 25 μL of 60% trichloroacetic acid. The resultant solutions were centrifuged at 10,000g for 5 min. Supernatants (100 μL) were analyzed by reversed-phase HPLC on a C_{18} column (5 μm, 250 × 4.6 mm, Mightysil aqua) using a mobile phase gradient consisting of acetonitrile and 0.1% acetic acid at a flow rate of 1.2 mL/min with ultraviolet monitoring at 285 nm. The gradient elution conditions were 0% acetonitrile (0–7 minutes), 10–15% acetonitrile (15–19 minutes), 15% acetonitrile (19–23 minutes), and 0% acetonitrile (23.1–30 minutes). The standard calibration curve indicated linearity over the concentration range of 0.20–150 μg/ml of phthalazone. The enzymatic activities were calculated based on the standard curve prepared with dilution series of parent drug phthalazone. Kinetic parameters for phthalazine oxidation were estimated from the fitted curves employing Michaelis-Menten equations or Hill equations using the KaleidaGraph program (Synergy Software, Reading, PA). Carbazeran oxidation activities by recombinant AOX proteins and liver cytosolic fractions were measured by an HPLC system coupled with an API4000 (AB Sciex, Foster City, CA) as described previously (Kaye et al., 1985; Manevski et al., 2014) with some minor changes. Briefly, the incubation mixtures consisted of approximately 40 pmol/mL AOX1 protein (arbitrary volume) or 0.50 mg/mL liver cytosolic fractions, 5.0 μM carbazeran, and 100 μM potassium phosphate buffer (pH 7.4) in a total volume of 0.25 mL. The reaction was incubated at 37°C for 2.5 min and terminated with 500 μL of acetonitrile containing 5 μM dextrorphan as an internal standard. The resultant solutions were centrifuged at 10,000 *g* for 5 min. Supernatants (5-10 μL) were analyzed by reversed-phase HPLC on a C_{18} column

(3 μm, 2.0 × 3.5 mm, Cadenza CD-C18) using an isocratic mobile phase [0.1% formic acid in water and 0.1% formic acid in acetonitrile (80:20, v/v)] at a flow rate of 0.2 mL/min. The metabolite and internal standard were detected in the positive ion electrospray ionization mode and identified using the transitions *m/z* 377.2→288.1 for 4-hydroxycarbazeran and *m/z* 258.1→157.1 for dextrorphan. The peak area ratio of the analyte versus the internal standard was determined for each injection sample and used to quantify the amount of metabolite formed. The standard calibration curve indicated linearity over the concentration range of 4.0–1000 ng/ml. The enzymatic activities were calculated based on standard curves prepared with dilution series of 4-hydroxycarbazeran.

Results and Discussion

The homology search of the genome sequences of the common marmoset showed *AOX1*, *AOX2*, and *AOX4* genes were localized in the *AOX* gene cluster in marmoset chromosome 6 (Supplemental Fig. 1). Multiple *AOX* genes contained complete open reading frames in rats, dogs, marmosets, and cynomolgus monkeys, unlike humans. Among experimental animals, *AOX1* gene was a pseudogene in dogs, different from cynomolgus monkeys, marmosets, and rats. The presence of *AOX3* gene in marmosets was not determined clearly because of the five large gaps (>1900 bp) between *AOX1* and *AOX4* genes (Supplemental Fig. 1). Marmoset AOX1 cDNA from livers were successfully cloned by RT-PCR. Marmoset AOX1 cDNA contained the open reading frame of 1337 amino acid residues, including highly conserved amino acids involved in the interactions with the 2Fe-2S prosthetic groups, the FAD cofactor (the molybdenum cofactor), and characteristic and functional key residues of AOX proteins (Supplemental Fig. 2). The exon-intron structure of marmoset *AOX1* gene was determined using BLAT by aligning the marmoset AOX1 cDNA sequence with the marmoset genome. Marmoset *AOX1* gene spanned >89.7 kb and consisted of thirty-five exons (Supplemental Table 1), similar to cynomolgus monkey and human *AOX1* genes. The sizes of exons and introns ranged from ≥45 to 228 bp and 301 to ≥10775 bp, respectively. The results indicated that marmoset *AOX1* possesses a similar gene structure to *AOX1* of cynomolgus monkeys and humans. Deduced amino acids of marmoset AOX1 cDNA showed high sequence identities with primate AOX1 (92–93%) (Table 2), but to a lesser extent with pig (85%), rabbit (82%), rat (81%), and mouse (82%) AOX1. Phylogenetic analysis indicated that marmoset AOX1 was more closely clustered with human AOX1 than pig, rabbit, and rodent AOX1 (Supplemental Fig. 3). These results

suggest an evolutionary closeness of marmoset AOX1 to human AOX1 as compared with AOX1 of non-primate species.

Tissue distribution of AOX1 mRNAs in marmosets and cynomolgus monkeys was investigated by real-time RT-PCR. Marmoset AOX1 mRNA was the most abundant in livers among brains, lungs, livers, kidneys, and small intestines (Supplemental Fig. 4A). Cynomolgus monkey AOX1 mRNA was also most abundant in livers, followed by adrenal glands (Supplemental Fig. 4B), among eleven tissues. Similarly, AOX1 mRNA was abundant in livers and adrenal glands among 23 human tissues (Nishimura and Naito, 2006). Additionally, the protein expression of liver cytosolic AOX1 was investigated by immunoblotting using anti-human AOX1 antibodies cross-reacting with AOX1 of marmosets and cynomolgus monkeys (Fig. 1). AOX1 proteins (~150 kDa) were immunologically detected in liver cytosolic fractions from humans, rhesus monkeys, cynomolgus monkeys, and marmosets, but not dogs (Fig. 1A). AOX1 proteins were expressed in individual liver cytosolic fractions from marmosets, cynomolgus monkeys, and rhesus monkeys (Fig. 1B, 1C). In cynomolgus monkeys, AOX1 protein expression levels in liver cytosolic fractions were not substantially different between the animals bred in Cambodia, China, and Indonesia (Fig. 1B). These results suggested that the tissue distribution of AOX1 is similar between marmosets, cynomolgus monkeys, and humans.

To assess catalytic activities of marmoset AOX1, phthalazine and carbazeran oxidation activities by marmoset liver cytosolic fractions and recombinant AOX1 protein were measured. Phthalazine and carbazeran oxidation activities by liver cytosolic fractions from marmosets and cynomolgus monkeys were higher than those of humans (Supplemental Table 3). Kinetic analyses for phthalazine oxidations were performed using liver cytosolic fractions from humans, cynomolgus monkeys, and

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marmosets and recombinant AOX1 enzymes (Table 1, Supplemental Fig. 5). Liver cytosolic fractions from humans and marmosets showed Michaelis-Menten kinetics with comparable low K_m values (4.7 μM for humans, 5.7 μM for marmosets) and different V_{max} values (0.11 nmol/min/mg protein for humans, 5.0 nmol/min/mg protein for marmosets) (Table 1, Supplemental Fig. 5A and 5E). Marmoset AOX1 enzyme had high affinity (K_m values, 1.3 μM) for phthalazine oxidation, comparable to liver cytosolic fractions from marmosets, suggesting AOX1 is a major aldehyde oxidase in marmoset livers (Table 1, Supplemental Fig. 5B and 5F). Because precise molecular expression levels in histidine-tagged AOX protein were unknown under any standard proteins, the arbitrary units were applied in this study (Table 1). On the other hand, liver cytosolic fractions from cynomolgus monkeys showed cooperative kinetics for phthalazine oxidation with high V_{max} values (83 nmol/min/mg protein) (Table 1, Supplemental Fig. 5C). Cooperativity was also observed in recombinant cynomolgus monkey AOX1 (Table 1, Supplemental Fig. 5D).

A recent report has indicated that cynomolgus monkeys are a suitable surrogate model for AOX metabolism of the EGFR inhibitor BIBX1382 in terms of its *in vivo* clearance and metabolite profile (Hutzler et al., 2014). AOX-dependent oxidation of a multityrosine kinase inhibitor lenvatinib has been catalyzed by liver 9000 *g* supernatant fractions from only cynomolgus monkeys and humans, but not by those of rats or dogs (Inoue et al., 2014). The predicting of human *in vivo* clearance values by scaling using *in vitro* intrinsic clearance values of five AOX substrates indicated that guinea pigs and monkeys reportedly represent better models of AOX-mediated drug clearances in humans (Crouch et al., 2017). Therefore, these differences of kinetics for AOX1-dependent phthalazine oxidation in liver cytosolic fractions between humans and cynomolgus monkeys might be minor

species differences. *In vitro* hepatic oxidation activities of *N*-phenylquinolinium chloride catalyzed by AOX were similar to human in marmosets and guinea pigs, unlike rats, guinea pig, dogs, and baboons (Beedham et al., 1987). In this study, marmoset liver cytosol and recombinant marmoset AOX1 metabolized human AOX1 substrates, phthalazine and carbazeran (Table 1, Supplemental Table 3). These results indicated the similarity of enzymatic functions between human and marmoset AOX1 enzymes, suggesting that marmosets would also be a suitable primate model for AOX-dependent drug oxidation in preclinical testing.

In conclusion, we identified a novel AOX1 in marmosets, highly homologous to AOX1 of other primate species. Although *in vivo* pharmacokinetics of representative human AOX substrates in the marmoset have not been currently investigated, similar tissue expression patterns and oxidation activities towards carbazeran and phthalazine (a typical human AOX1 substrate) of marmoset AOX1 to human AOX1 suggest functional similarities of AOX-dependent drug oxidations between marmosets and humans *in vitro* situation.

Authorship contribution

Participated in research design: Uehara, Uno, and Yamazaki.

Conducted experiments: Uehara, Uno, and Okamoto.

Contributed new reagents or analytic tools: Inoue and Sasaki.

Performed data analysis: Uehara, Uno, and Yamazaki.

Wrote or contributed to the writing of the manuscript: Uehara, Uno, and Yamazaki.

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Footnotes

Shotaro Uehara and Yasuhiro Uno equally contributed.

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Legends for figures

Fig. 1. Immunochemical detection of AOX1 proteins in liver cytosolic fractions from marmosets and cynomolgus monkeys.

Anti-human AOX1 antibodies cross-reacted with AOX1 proteins in liver cytosolic fractions (20 μg) from marmosets, cynomolgus monkeys, and humans, but not in liver cytosolic fractions from dogs (A). AOX1 proteins were detected in individual liver cytosolic fractions from 9 cynomolgus monkeys (lanes 1-3, bred in China; lanes 4-6, bred in Indonesia; lanes 7-9, bred in Indonesia), 3 rhesus monkeys (lanes 9-11) (B) and 10 marmosets (C). Anti-human AOX1 antibodies cross-reacted with recombinant histidine-tagged AOX1 proteins (20 μg) of marmosets, cynomolgus monkeys, and humans (D).

Table 1. Kinetic parameters for phthalazine oxidations by liver cytosolic fractions from marmosets, cynomolgus monkeys, and recombinant AOX1 enzymes

Enzyme source	K_m or S_{50}	Hill coefficient	V_{max}	V_{max}/K_m or V_{max}/S_{50}
Liver cytosolic fraction	*μM*		*nmol/min/mg protein*	*mL/min/mg protein*
Humans	4.7 ± 1.6	-	0.11 ± 0.01	0.023
Cynomolgus monkeys	32 ± 6	1.4 ± 0.2	83 ± 9	2.6
Marmosets	5.7 ± 0.3	-	5.0 ± 0.1	0.88
Recombinant enzymes	*μM*		*nmol/min/arbitrary units* [a]	*mL/min/arbitrary units* [a]
Human AOX1	23 ± 2	-	6.5 ± 0.2 (2.3[b])	0.28
Cynomolgus monkey AOX1	5.8 ± 0.3	1.3 ± 0.1	69 ± 1	12
Marmoset AOX1	1.3 ± 0.3	-	1.4 ± 0.1	1.1

Kinetic parameters were determined by non-linear regression analysis (mean ± standard error, n = 14 points of substrate concentrations of 0.44–80 μM, in duplicate determinations) employing the equation, $v = V_{max} \times [S]/(K_m + [S])$ for Michaelis-Menten equation and $v = V_{max} \times [S]^n/([S_{50}]^n + [S]^n)$ for Hill equation. [a] Relative phthalazine oxidation activities were shown on the bases of recombinant human AOX1 activity (at substrate concentration of 5.0 μM) as 1, because of unknown precise molecular expression level without standard proteins. [b] A number in parenthesis (2.3 nmol/min/nmol of AOX1) was a reported V_{max} value (Hartmann et al., 2012) for phthalazine oxidation by recombinant human AOX1.

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Figure 1