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**Short communication**

# Marmoset flavin-containing monooxygenase 3 in liver is a major benzydamine and sulindac sulfide oxygenase

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**Running Title Page**

**Running title**: Cloning of marmoset FMO cDNAs

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**Abbreviations:** FMO, flavin-containing monooxygenase (EC 1.14.13.8); HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; RT, reverse transcription.

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**Abstract**

Common marmosets (*Callithrix jacchus*) are potentially primate models for preclinical drug metabolism studies because the molecular characteristics of cytochrome P450 (P450) enzymes have similarities between this species and humans. However, characterization of non-P450 enzymes have not been clarified in marmosets. Here, we report characterization of flavin-containing monooxygenase (FMO) 1-5 identified in marmoset tissues. Marmoset FMO forms shared high amino acid sequence identities (93-95%) and phylogenetic closeness with human homologue FMO forms. FMO1 and FMO3 mRNA were abundantly expressed in livers and kidneys among five marmoset tissues examined, where FMO3 protein were detected by immunoblotting. FMO inhibition assays using preheated tissue microsomes indicated that benzydamine *N*-oxygenation and sulindac sulfide *S*-oxygenation in marmoset livers was mainly catalyzed by FMO3, the major hepatic FMO. Marmoset FMO3 protein heterologously expressed in *Escherichia coli* effectively catalyzed benzydamine *N*-oxygenation and sulindac sulfide *S*-oxygenation comparable to marmoset liver microsomes. These results indicated that FMO3 enzyme expressed in marmoset livers mainly metabolized benzydamine and sulindac sulfide, typical human FMO substrates, suggesting its importance for FMO-dependent drug metabolism in marmosets.

## Introduction

Flavin-containing monooxygenase (FMO, EC 1.14.13.8) is a family of xenobiotic-metabolizing enzymes involved in the oxygenation of a broad range of chemicals containing nitrogen, sulfur, or phosphorous (Krueger and Williams, 2005). In humans, functional genes *FMO 1-5* (Lawton et al., 1994) and a nonfunctional pseudogene *FMO6* (Hines et al., 2002) have been identified, and their mRNAs expressed in various tissues. FMO3 is considered a major functional FMO enzyme in the human liver and contributed to the metabolism of the anti-inflammatory drugs benzydamine and sulindac sulfide and diet-derived trimethylamine (Shimizu et al., 2015).

The common marmoset (*Callithrix jacchus*) is a useful non-human primate species for pharmacokinetics studies due to its significant similarity of cytochrome P450 (EC 1.14.14.1) characteristic features to humans (Uno et al., 2016). *De novo* transcriptome analysis indicated that *FMO1*-, *FMO3*-, *FMO4*-, and *FMO5*-like genes were expressed in marmoset livers, kidneys, and intestines (Shimizu et al., 2014). FMO3 effectively catalyzed the *N*-oxygenation of potential proneurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in marmoset livers (Uehara et al., 2015). Only two FMO forms have been identified, however, little information is available about the molecular characteristics in marmosets (Uehara et al., 2015).

In this study, we isolated three marmoset FMO cDNA based on *FMO* gene cluster organization, and analyzed them for their sequence identity, tissue expression, and catalytic activities using recombinant proteins heterologously expressed in *Escherichia coli*. This work is of importance for understanding the fundamental characteristics and functions of marmoset FMO for the use of marmoset as non-human primate models in preclinical drug development.

## Materials and Methods

Detailed methods are shown separately in the Supplemental Data. FMO cDNAs were isolated by reverse transcription (RT)-polymerase chain reaction (PCR) with cDNA libraries transcribed from total RNA from marmoset tissues as described (Uehara et al., 2015). The structure of primate *FMO* gene clusters was determined by BLAT (UCSC Genome Bioinformatics, University of California, Santa Cruz, CA). Multiple alignment of amino acid sequences and phylogenetic analysis were performed by Genetyx system (Software Development, Tokyo, Japan) and DNASIS Pro (Hitachi Software, Tokyo, Japan), respectively. FMO amino acid sequences used were from GenBank (National Center for Biotechnology Information, Bethesda, MD). Pooled liver microsomes from marmosets (5 males) were purchased from Corning Life Sciences (Woburn, MA). Pooled microsomes of brains, lungs, livers, kidneys, and small intestines were prepared from tissue samples of 20 marmosets (10 males and 10 females, >2 years old) at the Central Institution for Experimental Animals (Kawasaki, Japan) as described (Uehara et al., 2016a) under the approval of the Institutional Animal Care and Use Committee. FMO mRNA distribution in brains, lungs, livers, kidneys, and small intestines, each pooled from 6 male and 6 female adult marmosets (>2 years old), was analyzed by real-time RT-PCR as described (Uehara et al., 2016b). Recombinant marmoset FMO1 and FMO3 were heterologously expressed in *Escherichia coli* using pET30 vectors (Novagen, Madison, WI) as described (Yamazaki et al., 2014). Tissue microsomes (10 μg), microsomes from five individual livers, and recombinant FMO3 protein (0.1 pmol) were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel by electrophoresis, and transferred to a polyvinylidene difluoride membrane (Uehara et al., 2016a). Benzydamine *N*-oxygenation and sulindac sulfide *S*-oxygenation activities by recombinant FMO proteins and tissue microsomes were measured by HPLC as described (Yamazaki et al., 2014). For FMO inactivation, liver or kidney microsomes were preheated at 45°C for 5 min without NADPH-

generating system (Taniguchi-Takizawa et al., 2015). All other reagents used were the highest quality commercially available.

**Results and Discussion**

The analysis of the genome sequence of the common marmoset showed *FMO 1-4* and *FMO6* genes were localized in the gene cluster and *FMO5* gene was localized outside this cluster, in marmoset chromosome 18 (Supplemental Fig. 1). Marmoset *FMO* genes had one-to-one orthologous relationships to human *FMO* genes, even though *FMO* gene cluster organization is different between marmosets and humans. We successfully isolated FMO1-5 cDNA in marmoset livers by RT-PCR. Marmoset FMO 1-5 contained open reading frames of 532-556 amino acid residues, respectively, (Supplemental Fig. 2) and had functionally important regions (FAD- and NADPH-pyrophosphate-binding sites), and the two characteristic FMO pentapeptides (EGLEP and FATGY). Marmoset FMO 1-5 shared high amino acid sequence identities (93-95%) with human FMO counterparts (Supplemental Table 1) and phylogenetically more closely clustered with the corresponding primate orthologs than other species (Supplemental Fig. 3). Interestingly, in cynomolgus monkeys, FMO6 is a functional enzyme that is widely expressed in kidneys, hearts, testis, uterus, and livers (Uno et al., 2013) and although we have tried, we failed to clone FMO6 cDNA from marmoset tissues.

To investigate the tissue distribution of FMO1-5 mRNAs and proteins in marmosets, real-time RT-PCR was performed to measure expression levels of FMO1-5 in pooled brains, lungs, livers, kidneys, and small intestines. FMO3 mRNA was the most abundant in livers and kidneys, followed by lungs (Fig. 1A), whereas FMO1 mRNA was also expressed abundantly in livers and kidneys, but not as much as FMO3 mRNA, the same as results previously reported by *de novo* transcriptome analysis (Shimizu et al., 2014). Indeed, FMO3 protein (~50 kDa) were immunologically detected with anti-human FMO3 antibodies in pooled marmoset livers and kidneys (Fig. 1B), with a small non-specific unknown band. No immunoreactive bands in these tissue microsomes were seen with commercial anti-human FMO1 antibodies (results not

shown). Similar to marmosets, FMO3 is postnatally expressed in kidneys of rabbits and rats (Ripp et al., 1999). FMO1 has been reportedly expressed postnatally in livers of dogs (Lattard et al., 2002), rabbits (Shehin-Johnson et al., 1995), rats (Novick et al., 2009), and mice (Itoh et al., 1997). Human FMO1, FMO2, and FMO3 have been predominantly expressed in kidneys, lungs, and livers, respectively, whereas FMO4 and FMO5 have been widely expressed in various tissues (Zhang and Cashman, 2006; Uno et al., 2013). In humans, FMO1 is expressed in fetal liver, but its expression is rapidly extinguished after birth (Koukouritaki et al., 2002). In mice, apparent sex differences of FMO3 protein expression level in livers (Ripp et al., 1999) were found, but not in marmosets (Fig. 1A). Marmoset FMO2 and FMO5 mRNA were dominantly expressed in lungs and livers, whereas FMO4 mRNA was expressed in livers, kidneys, and small intestines at very low levels, similar to human and cynomolgus monkey FMO forms (Zhang and Cashman, 2006; Uno et al., 2013). These results suggested that tissue distribution of FMO3 and FMO1 were partially different between marmosets and humans.

To assess the importance of FMO forms in drug oxidation in marmoset livers, drug oxygenation activities by liver microsomes preheated for FMO inhibition were measured. Preheat-sensitive benzydamine *N*-oxygenation and sulindac sulfide *S*-oxygenation activities in marmoset liver and kidney microsomes was found (Supplemental Table 1). Recombinant FMO3-mediated benzydamine *N*-oxygenation activity and its $V_{max}/K_m$ value were higher than those by recombinant FMO1, suggesting that FMO3 abundantly expressed in livers plays a role for the *N*-oxygenation of benzydamine in marmoset livers (Table 1), similar to human livers (Taniguchi-Takizawa et al., 2015). Kinetic analyses also indicated that marmoset liver microsomes effectively catalyzed sulindac sulfide *S*-oxygenation ($V_{max}/K_m$, 0.12 mL/min/mg protein) (Table 1), compared with those of human ($V_{max}/K_m$, 0.02 mL/min/mg protein) as previously reported (Yamazaki et al., 2014). Marmoset FMO3 was catalytically efficient ($V_{max}/K_m$, 0.80 mL/min/nmol) for sulindac sulfide *S*-oxygenation, and showed a low $K_m$ value

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(38 μM), comparable to marmoset liver microsomes (23 μM), similar to human FMO3 (Yamazaki et al., 2014). Marmoset FMO1 showed low $K_m$ (43 μM) and high $V_{max}/K_m$ (0.53 mL/min/nmol), compared with human FMO1 ($K_m$, 280 μM; $V_{max}/K_m$, 0.01 mL/min/nmol). Considering this together with tissue distribution, FMO1 might account for the potential species differences of sulindac sulfide *S*-oxygenation rates between marmoset and human livers. These results indicated that benzydamine *N*-oxygenation and sulindac sulfide *S*-oxygenation in marmoset livers were mainly catalyzed by FMO3.

In summary, marmoset FMO1-5 had amino acid sequences highly identical (>93%) to human FMO1-5, and a phylogenetically close relationship with the human FMO1-5. Different to those of humans, FMO3 and FMO1 mRNA was abundant in marmoset livers and kidneys among five FMO forms. Recombinant marmoset FMO3 enzymes heterologously expressed in *Escherichia coli* effectively metabolized typical human FMO substrates, benzydamine and sulindac sulfide. These results indicated that marmoset FMO1-5 have sequence similarities with those of humans, but partially different from human in terms of tissue expression. Importantly, marmoset and human FMO3, a major hepatic FMO in both species, had similar enzymatic properties, suggesting the similarity of FMO-dependent drug metabolism for marmosets and humans.

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## Authorship contribution

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

Conducted experiments: Uehara and Shimizu.

Contributed new reagents or analytic tools: Inoue and Sasaki.

Performed data analysis: Uehara, Shimizu, Uno, and Yamazaki.

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

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**Footnotes**

Shotaro Uehara, Makiko Shimizu, and Yasuhiro Uno equally contributed.

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## Legends for figures

**Fig. 1. Determination of FMO mRNA (A) and FMO protein (B, C) levels in marmoset tissues.**

Expression levels of marmoset FMO 1-5 mRNAs in five marmoset tissues (each pool of six male and six female marmosets) were measured by real-time RT-PCR (A). Raw values of target gene expression were normalized with 18S rRNA level. Each datum point represents the average and standard deviation of triplicate determinations from three representative experiments. Pooled tissue microsomes from marmosets (20 μg/lane) (B) and recombinant marmoset FMO3 protein (0.1 pmol of FMO3/lane), individual liver microsomes from marmosets (male, lanes 1-2; female, lanes 3–5) (20 μg/lane) (C) were analyzed by immunoblotting using anti-human FMO3 antibodies. Protein disulfide isomerase (PDI) expression was assessed as a loading control.

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**Table 1**

**Kinetic parameters of benzydamine *N*-oxygenation and sulindac sulfide *S*-oxygenation by recombinant FMO proteins and liver microsomes from marmosets**

| Enzyme source | Benzydamine *N*-oxygenation | | | Sulindac sulfide *S*-oxygenation | | |
|---|---|---|---|---|---|---|
| | $K_m$ | $V_{max}$ | $V_{max}/K_m$ | $K_m$ | $V_{max}$ | $V_{max}/K_m$ |
| | *μM* | *nmol/min/ mg protein* | *ml/min/mg protein* | *μM* | *nmol/min/ mg protein* | *ml/min/mg protein* |
| Liver microsomes | 47 ± 9 | 3.8 ± 0.2 | 0.081 | 23 ± 14 | 2.7 ± 0.4 | 0.12 |
| | | *nmol/min/ nmol FMO* | *ml/min/nmol FMO* | | *nmol/min/ nmol FMO* | *ml/min/nmol FMO* |
| Marmoset FMO1 | 40 ± 10 | 0.76 ± 0.04 | 0.019 | 43 ± 15 | 23 ± 2 | 0.53 |
| Marmoset FMO3 | 21 ± 4 | 27 ± 1 | 1.3 | 38 ± 13 | 31 ± 2 | 0.80 |

Each substrate (10-1000 μM) was incubated with liver microsomes (0.05 mg protein) and recombinant proteins (10 pmol equivalent) at 37°C for 10-15 min in the presence of an NADPH-generating system. Kinetic parameters were calculated from fitted curves by non-linear regression (mean ± SE).

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**Figure 1**