Activation and deactivation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by cytochrome P450 enzymes and flavin-containing monooxygenases in common marmosets (Callithrix jacchus)

Shotaro Uehara, Yasuhiro Uno, Takashi Inoue, Norie Murayama, Makiko Shimizu, Erika Sasaki, and Hiroshi Yamazaki

Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan (S.U., N.M., M.S., H.Y.), Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd., Kainan, Wakayama, Japan (Y.U.), Department of Applied Developmental Biology, Central Institute for Experimental Animals, Kawasaki, Kanagawa (T.I., E.S.), Japan, and Keio Advanced Research Center, Keio University, Minato-ku, Tokyo, Japan (E.S.)
Running Title Page

Running title: MPTP activation by marmoset P450 2D6

*Correspondence author: Prof. Hiroshi Yamazaki

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

Number of Text Pages: 24
Number of Tables: 3
Number of Figures: 5
Number of References: 32
Number of Words in Abstract: 246
Number of Words in Introduction: 511
Number of Words in Discussion: 773

Abbreviations: FMO, flavin-containing monooxygenases (EC 1.14.13.8); MPDP+, 1-methyl-4-phenyl-2,3-dihydropyridinium ion; MPP+, 1-methyl-4-phenylpyridinium ion, PTP 4-phenyl-1,2,3,6-tetrahydropyridine; P450, cytochrome P450 (EC 1.14.14.1).
Abstract

The potential pro-neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces Parkinson-like syndromes in common marmosets, other primates, and humans. MPTP is metabolically activated to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺) and 1-methyl-4-phenylpyridinium (MPP⁺) ions by desaturation reactions; MPTP is deactivated to 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) by N-demethylation and to MPTP N-oxide. The roles of cytochromes P450 (P450s) and flavin-containing monooxygenases (FMOs) in the oxidative metabolism of MPTP-treated marmosets are not yet fully clarified. The aim of this study was to elucidate P450- and FMO-dependent MPTP metabolism in marmoset liver and brain. Rates of MPTP N-oxygenation in liver microsomes were similar to those in brain microsomes from 11 individual marmosets (substrate concentration, 50 μM) and were correlated with rates of benzydamine N-oxygenation (r = 0.75, p < 0.05); the reactions were inhibited by methimazole (10 μM). MPTP N-oxygenation was efficiently mediated by recombinantly expressed marmoset FMO3. Rates of PTP formation by MPTP N-demethylation in marmoset liver microsomes were correlated with bufuralol 1'-hydroxylation rates (r = 0.77, p < 0.01) and were suppressed by quinidine (1 μM), thereby indicating the importance of marmoset P450 2D6 in PTP formation. MPTP transformations to MPDP⁺ and MPP⁺ were efficiently catalyzed by recombinant marmoset P450 2D6 and human P450 1A2. These results indicated the contributions of multiple drug-metabolizing enzymes to MPTP oxidation, especially marmoset FMO3 in deactivation (N-oxygenation) and marmoset P450 2D6 for both MPTP deactivation and MPTP activation to MPDP⁺ and MPP⁺. The present findings provide a foundation for understanding MPTP metabolism and for the successful production of preclinical marmoset models.
Visual Abstract

Deactivation

PTP
marmoset P450 2D6
MPTP
MPTP N-oxide
marmoset FMO3

Activation

marmoset P450 2D6
MPDP⁺
MPP⁺
Neurotoxicity
Introduction

Cytochrome P450 (P450, EC 1.14.14.1) enzymes have been characterized with respect to drug metabolism and disposition (Johansson and Ingelman-Sundberg, 2011). Research has focused on the P450s as well as on another monooxygenase family, the flavin-containing monooxygenases (FMOs, EC 1.14.13.8) (Yamazaki and Shimizu, 2013), involved in the oxidation of a variety of compounds associated with pharmacological and/or toxicological effects in humans. The potential pro-neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) degenerates dopaminergic neurons and causes Parkinsonism in primates, including common marmosets, cynomolgus monkeys, and humans (Davis et al., 1997; Langston and Ballard, Jr., 1983; Ballard et al., 1985). Several metabolic pathways of MPTP have been reported and are summarized in Fig. 1. The 1-methyl-4-phenyl-2,3-dihydropyridinium ion (MPDP⁺, Fig. 1) is reportedly an unstable compound that undergoes further oxidation to form the active toxic 1-methyl-4-phenylpyridinium ion (MPP⁺) (Chiba et al., 1985). The metabolic transformation of MPTP to its toxic metabolite MPP⁺ via intermediate MPDP⁺ is mediated by monoamine oxidase or P450 2D6 in human brains (Davis et al., 1997; Langston and Ballard, Jr., 1983; Ballard et al., 1985). On the other hand, MPTP is also metabolized to non-neurotoxic 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) and MPTP N-oxide (Fig. 1) by P450 2D6 and FMO, respectively, in human livers (Herraiz et al., 2013; Bajpai et al., 2013).

The common marmoset (Callithrix jacchus) is a member of the New World non-human primate family Callitrichidae (Abbott et al., 2003; Mansfield, 2003; Carrion, Jr. and Patterson, 2012; Okano et al., 2012; Tokuno et al., 2012). The species has attracted considerable attention as a potentially useful animal model in fields such as neuroscience and drug
toxicology (Mansfield, 2003) because of its size, availability, unique biological characteristics (Abbott et al., 2003), and evidence of cross-reactivity with human cytokines and hormones (Okano et al., 2012). In particular, MPTP-treated marmosets have been used for preclinical studies on Parkinson disease (Eslamboli, 2005; Jenner, 2009). The parkinsonian marmosets have shown characteristic neural degeneration of nigrostriatal dopamine neurons and behavioral signs such as moving tremors, immobility, muscle rigidity, positional dysfunction, and L-dopa-induced dyskinesia (Ando et al., 2014; Ando et al., 2012). However, we do not yet have a comprehensive understanding of common marmoset drug metabolizing enzymes related to MPTP metabolism, partly because of the absence of an available reference genome sequence or enzyme characteristics. Although we recently carried out identification and quantitative analysis of tissue-specific mRNA transcripts of P450s and FMOs in the marmoset as an animal model in drug development (Shimizu et al., 2014), limited numbers of P450 isoforms (Uno et al., 2011) and no FMOs in the marmoset have been investigated in terms of their drug metabolizing activities so far.

The present study combined analyses to identify novel common marmoset FMO1/3 and P450 enzymes that have until now been unreported and to investigate MPTP oxidative metabolism. We report herein that marmoset FMO3 mainly contributed to deactivation of MPTP and that marmoset P450 2D6 was responsible for both deactivation and metabolic activation of MPTP to MPP⁺. The present findings should prove an important resource for future biomedical research and will facilitate use of the common marmoset as an animal preclinical Parkinsonism model.
Materials and Methods

Animals

Adult common marmosets (>2 years old) were purchased from CLEA Japan (Tokyo, Japan). The animals were maintained in cages (40×610×1578 mm) at 24–27°C and 40–60% relative air humidity with a 12/12-h light/dark cycle and had free access to a balanced diet (CMS-1M; CLEA Japan) with added vitamins and water. This study was approved by the animal ethics committees and gene recombination experiment safety management committees of the Central Institute for Experimental Animals and was performed in accordance with the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan (2006). Animal care was conducted in accordance with the recommendation of the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Resources, 2011). Brain and liver samples were collected from 11 common marmosets (five males and six females, 2–6 years of age) after euthanasia by exsanguination under ketamine (60 mg/kg) and isoflurane deep anesthesia as described previously (Shimizu et al., 2014).

Chemicals and enzymes

MPTP, MPP⁺, 7-ethoxyresorufin, chlorzoxazone, S-mephenytoin, warfarin, ketoconazole, sulfaphenazole, quinidine, and ticlopidine were purchased from Sigma-Aldrich (St Louis, MO), and coumarin, midazolam, and α-naphthoflavone were from Wako Pure Chemical Industries (Osaka, Japan). Paclitaxel, bufuralol hydrochloride, PTP, and MPTP N-oxide were obtained from Toronto Research Chemicals (Toronto, Canada), Corning (New York, NY), Tokyo Chemical Industry (Tokyo, Japan), and KNC Laboratories (Kobe, Japan), respectively. Microsomal fractions from marmoset tissue samples were prepared as described previously (Yamazaki et al., 2014; Yamazaki et al., 2002). Microsomal samples
from a baculovirus-insect cell line (Supersomes) expressing human P450 1A1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 were purchased from Corning. The other chemicals and reagents used were obtained in the highest grade commercially available.

**Molecular cloning of cDNAs for marmoset P450s and FMOs**

To obtain cDNAs of marmoset P450s and FMOs, reverse transcription (RT)-polymerase chain reaction (PCR) was performed using brain and liver total RNAs of marmosets. Total RNA was isolated from each tissue using RNeasy Mini Kits (QIAGEN, Valencia, CA) according to the protocols of the manufacturer’s instructions, and then a first-strand cDNA was prepared by RT reaction at 50°C for 1 h in a mixture containing 1 μg of total RNA, oligo(dT), and SuperScript III RT reverse transcriptase (Invitrogen, Carlsbad, CA). PCR was performed using KOD-plus-neo DNA polymerase (Toyobo, Osaka, Japan) with RT product as described by the protocols of the manufacturer’s instructions. Full-length cDNAs encoding marmoset P450s and FMOs were amplified by PCR with the following oligonucleotide primers: 5'-CAGATGGCATTGTCCCAGTTTGTTC-3' and 5'-CGGTGTCTTCTCACTGGAAGGAG-3' for P4501A2, 5'-ATGGAGCTCACCGTCTTCCTCTTC-3' and 5'-CTGGATGACCGGAATCTCTTGAC-3' for P450 2B6, 5'-GCTCATAGTTGTCTTAGTAAGAAGAGTAGGCTTCAAT-3' and 5'-TCAGACAGGAATGAGATCTGGTGTTACGGG-3' for P450 2C8, 5'-CAGGGGTGTCCAGAGGAGTTCAGT-3' and 5'-TCTAGCGGGGCACAGCACAAAG-3' for P450 2D6, 5'-ATGTCTGCCCTCGGCTAGACTGTG-3' and 5'-CATATGCAAAGAAAGGAATAGGTTTGAGGAA-3' for P450 2E1, and 5'-CGGAGGAGAGAGATAGTAGTGTAGT-3'.
5'-CTTAGGAAAATTCAGGCTCCACTTACA-3' for P450 3A4,
5'-CAACACGCACGTACAACACTGAAAGGAGA-3' and
5'-CTGGGGGCAAGCTTCTTGAAACA-3' for P450 3A5,
5'-ACAGCAGCACAAACTGAAAGGAAA-3' and
5'-CTGGGGCAACAGCTTTCTTGATGC-3' for P450 3A90,
5'-GAGAACATGGGCAAGCGAGTTG-3' and
5'-CCTTTAGGAAATCTTTTACTCATAGGAAAATCAG-3' for FMO1, and
5'-ATGGGGAAGAAAGTGGCCATCA-3' and
5'-ATGATGATTAGGTCAACACAAGGAAAACAG-3' for FMO3. PCR was performed using an Applied Biosystems Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA) consisting of an initial denaturation at 94°C for 2 min and 35 cycles of 98°C for 10 s, 60°C for 30 s, and 68°C for 2 min followed by a final extension at 68°C for 7 min. The PCR products were cloned into pCR4 vectors using a TOPO Cloning Kit (Invitrogen) according to the manufacturer’s instructions. Sequencing of the inserts was carried out using an ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) with an ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

Recombinant marmoset P450s and FMOs

Recombinant marmoset P450s (P450 1A2, 2B6, 2C8, 2D6, 2E1, 3A4, 3A5, and 3A90) and FMOs (FMO1 and FMO3) were heterologously expressed in *Escherichia coli* using expression plasmids. For P450s, the N-terminus modification was conducted by PCR using the forward and reverse primers, 5'-GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTTTTCAGCCACAGAGCTTCTCCT-3' and 5'-GCTCTAGACGGGTGTCTTCTCCTCATGGGAAGG-3' for P450 1A2, 5'-GGAATTCCATATGGCTCTGTATTAGCAGTTTTTTTCGACTCCTCACAGGCTTTT
TGC-3′ and 5′-GCTCTAGACTTCAGCGGCGGAGGA-3′ for P450 2B6, 5′-GGAATTCATATGGCTCTGTTATTAGCAGTTTTGTCTGTCTCTTTTTTTGCTTC TCTTTTCAC-3′ and 5′-GCTCTAGACAGAGGATGAACAGATCTGGTA-3′ for P450 2C8, 5′-GGAATTCATATGGCTCTGTTATTAGCAGTTTTTGACTGATATGCGGCC ATTCTCCTGCT-3′ and 5′-GCTCTAGACGAGTGTCCTCCTCACAATGTG-3′ for P450 2D6, 5′-GGAATTCATATGGCTCTGTTATTAGCAGTTTTTGCTGGCTGTGACAGTG-3′ and 5′-GCTCTAGACGCGGCGACAGCACA-3′ for P450 2E1, 5′-GGAATTCATATGGCTCTGTTATTAGCAGTTTTTGGTGAACCCCTGGCTTTCTCCTG-3′ and 5′-GCTCTAGAGCAAATTCAGGCTCCACTTACAGTC-3′ for P450 3A4, and 5′-GGAATTCATATGGCTCTGTTATTAGCAGTGGTTTTTGGTGAACCCCTGGCTTTCTCCTG-3′ and 5′-GCTCTAGACATTCATTTCTGAGTTTGATCCATCTCACA-3′ for P450 3A90, respectively. The NdeI and XbaI sites (underlined) in the forward and reverse primers, respectively, were used for subcloning of the product into pCW vectors that contained human NADPH-P450 reductase cDNA (Uno et al., 2010). Membrane preparation and measurement of P450 protein and reductase contents in each sample were performed as described previously (Uehara et al., 2010). Expected drug oxidation activities of marmoset P450 enzymes were confirmed with typical human P450 probe substrates (Uehara et al., 2011; Yamazaki et al., 2002).

For FMOs, PCR was carried out with the primers, 5′-ATGGGCAAGCGAGTTGTTGCCATTGT-3′ and 5′-CCGCTCGAGCTTATTTAGAAAATTTTACTCATAGAAATCAG-3′ for FMO1 and 5′-ATGGGGAAGAAAGTTGCCCATCA-3′ and
5′-CCGCTCGAGTTAGTGTAACCCAAGGAAAACAGCAA-3′ for FMO3. After restriction enzyme digestion using XhoI (the restriction site is underlined), the PCR products were subcloned to pET30 vectors (Novagen, Madison, WI) to provide a 6×His-tag at the N-terminus (Uno et al., 2013). Protein expression and E. coli membrane preparations were performed as described previously (Yamazaki et al., 2014) in a similar manner to that used for human FMO1 and FMO3 preparations. The final amount of recombinant FMO proteins in bacterial membranes were normalized to the flavin adenine dinucleotide contents (Yamazaki et al., 2014). Expected drug oxygenation activities of marmoset FMO1/3 were confirmed with the typical FMO probe substrate benzydamine (Yamazaki et al., 2014).

**MPTP oxidation catalyzed by marmoset P450s and FMOs**

The four metabolites of MPTP, namely PTP, MPTP N-oxide, MPDP⁺, and MPP⁺ (Fig. 1), were determined as described previously (Herraiz et al., 2013) with minor modifications. Briefly, reaction mixtures of 0.25 mL contained enzyme sources (microsomal preparations or E. coli membranes), 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 mM NADP⁺, 5 mM glucose 6-phosphate, 5 mM MgCl₂, and 1 U/ml glucose-6-phosphate dehydrogenase), and 1–2000 μM MPTP, unless otherwise specified. After incubation for 10 min at 37°C, the reaction was terminated by the addition of 25 μL of 60% (v/v) perchloric acid. The linearity for incubation time and protein concentrations were confirmed under the present conditions. The supernatant obtained by centrifugation (10,000 g, 5 min) was analyzed by high-performance liquid chromatography (HPLC) equipped with ultraviolet (at 290 nm) and fluorescence detection (excitation at 332 nm and emission at 344 nm) with a Mightysil RP-18 GP Aqua column (4.6 × 150 mm, 5 μm, Kanto Chemical, Tokyo, Japan) at a flow rate of 1.0 ml/min using a mobile phase consisting of 0.05 M ammonium acetate (pH 5.5) and acetonitrile. The gradient conditions for elution were 1–25.6%
acetonitrile (0–8 min), 25.6–72% acetonitrile (8–15 min), 72% acetonitrile (15–20 min), and 1% acetonitrile (20–30 min). PTP and MPTP N-oxide were quantified on the basis of their standard curve peak areas with F<sub>323/344</sub>. MPDP<sup>+</sup> was quantified on the basis of the standard curve peak areas at A<sub>290</sub> of MPP<sup>+</sup>.

The activities of 7-ethoxyresorufin O-deethylation, coumarin 7-hydroxylation, paclitaxel 6α-hydroxylation, warfarin 7-hydroxylation, S-mephenytoin 4′-hydroxylation, bufuralol 1′-hydroxylation, chlorzoxazone 6-hydroxylation, midazolam 1′-hydroxylation, and benzydamine N-oxygenation in brain or liver microsomes were determined as described previously (Uehara et al., 2011; Yamazaki et al., 2002). Drug and MPTP oxidation analyses were carried out in triplicate determinations. The kinetic analysis of MPTP oxidations was performed using a nonlinear regression analysis in Michaelis-Menten model (KaleidaGraph, Synergy Software, Reading, PA).
Results

**MPTP oxidation by marmoset liver and brain microsomes**

Representative HPLC chromatograms of MPTP metabolites in marmoset liver microsomes are shown in Figs. 2A and 2B after 10 min incubation of MPTP (50 μM) in the presence of an NADPH-generating system. The four metabolites of MPTP shown in Fig. 1 could be detected with ultraviolet (at 290 nm) and fluorescence detection (excitation at 332 nm and emission at 344 nm) in an analytical reverse phase HPLC system. To elucidate the metabolic pathways of MPTP in the marmoset, the formation rates of PTP, MPTP N-oxide, MPDP⁺, and MPP⁺ mediated by liver and brain microsomes from 11 individual marmosets were determined (Fig. 3). Although rates of MPTP N-oxygenation in brain microsomes were similar to those in liver microsomes from marmosets at a substrate concentration of 50 μM, formation rates of PTP, MPP⁺, and MPDP⁺ in brain microsomes were lower than those in liver microsomes. MPDP⁺ and MPP⁺ formation rates in liver microsomes (Figs. 3B and 3D) were approximately 2- and 4-fold faster, respectively, than PTP formation rates (Fig. 3A). The formation rates of four MPTP metabolites in individual marmosets varied less than 5-fold within the liver and brain microsomes analyzed in the present study (Fig. 3); larger inter-individual variations in the marmoset were observed for MPP⁺ formation in the brain (4.7-fold). Interestingly, PTP formation rates (Fig. 3A) correlated with MPDP⁺ formation rates (Fig. 3B) in marmoset liver microsomes ($r = 0.77$, $n = 11$, $p < 0.05$).

Correlation analyses revealed that MPTP N-demethylation (PTP formation) activities were correlated with activities of midazolam 1′-hydroxylation ($r = 0.92$, $n = 10$, $p < 0.001$), bufuralol 1′-hydroxylation ($r = 0.77$, $n = 10$, $p < 0.01$), and 7-ethoxyresorufin O-deethylation ($r = 0.73$, $n = 10$, $p < 0.05$) (Table 1). Significant correlation coefficients ($p < 0.05$) were also observed between MPDP⁺ and MPP⁺ formations and warfarin 7-hydroxylation activities,
between MPP⁺ formation and midazolam 1’-hydroxylation activities, and between MPTP 
N-oxygenation and benzodiazepine N-oxygenation (Table 1). No correlations were seen 
between MPTP metabolite formation and coumarin 7-hydroxylation, paclitaxel 
6α-hydroxylation, S-mephenytoin 4’-hydroxylation or chlorzoxazone hydroxylation activities 
under the present conditions.

The effects of P450 and FMO inhibitors (α-naphthoflavone, sulfaphenazole, ticlopidine, 
quindiuzide, ketoconazole and methimazole) on MPTP oxidation in liver microsomes were 
investigated at substrate concentrations of 50 μM of MPTP (Fig. 4). Quinidine (1–10 μM), 
a P450 2D inhibitor, strongly inhibited MPTP N-demethylation (PTP formation) (Fig. 4A) 
and moderately suppressed MPP⁺ formation (Fig. 4D) in marmoset liver microsomes. 
Ticlopidine (2–20 μM), a P450 2C inhibitor, moderately suppressed MPTP N-demethylation 
(Fig. 4A) and MPP⁺ formation (Fig. 4D) in liver microsomes. α-Naphthoflavone (0.2–5 
μM), a P450 1A inhibitor, partly suppressed MPDP⁺ formation (Fig. 4B) in liver microsomes. 
Methimazole (10–100 μM), a FMO substrate and inhibitor, strongly suppressed MPTP 
N-oxygenation in liver microsomes (Fig. 4C). In contrast, inhibitory effects of ketoconazole 
(0.02–0.2 μM) or sulfaphenazole (1–20 μM), inhibitors of P450 3A4 and 2C9, respectively, 
were not seen on MPTP oxidation under the present conditions.

**MPTP metabolism by recombinantly expressed P450s and FMOs**

MPTP (50 μM) was incubated with recombinant marmoset FMO1 and FMO3 and 
marmoset P450 1A2, 2B6, 2C8, 2D6, 3A4, 3A5, and 3A90, along with human FMO1 and 
FMO3 and human P450 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5. 
Marmoset FMO3 (Fig. 2C) catalyzed MPTP N-oxygenation (14.5 nmol/min/nmol FMO, 
Table 2) more efficiently than FMO1 did; a similar pattern was evident for human FMO3 
(78.0 nmol/min/nmol FMO) versus FMO1. Marmoset P450 2D6 (Fig. 2D) efficiently
catalyzed PTP formation (6.6 nmol/min/nmol P450), in a similar manner to human P450 2D6 (3.5 nmol/min/nmol P450) (Fig. 5). Marmoset P450 2D6 also mediated MPDP+ and MPP+ formation (2.0, and 5.7 nmol/min/nmol P450, respectively), but the rates of MPDP+ and MPP+ formation mediated by human P450 2D6 were much lower (Fig. 5). Marmoset P450 1A2 and 2C8 also mediated the formation of PTP, MPDP+, and MPP+, but to a lesser extent than P450 2D6. In addition, human P450 1A2 showed high activities for the formation of MPDP+ and MPP+, whereas human P450 2D6 showed much lower activities under the present conditions.

Kinetic parameters for MPTP oxidation by marmoset and human liver microsomes and recombinant P450 2D6 and FMO3 were determined. Apparent $K_m$ values for PTP formation by liver microsomes from marmosets and humans were 451 and 577 $\mu$M, respectively, and for recombinant marmoset and human P450 2D6, $K_m$ values were 71 and 125 $\mu$M, respectively (Table 3). The calculated $V_{max}/K_m$ values for PTP formation were 0.95 and 0.23 $\mu$L/min/mg protein and 220 and 92 $\mu$L/min/nmol P450, respectively. Marmoset P450 2D6 showed roughly 2-fold $V_{max}/K_m$ value for PTP formation to that of human P450 2D6. Apparently consistent $K_m$ values (162 $\mu$M and 137 $\mu$M) for MPTP N-oxygenations by marmoset liver microsomes and marmoset FMO3, respectively, were obtained in a similar manner to those in humans. Apparently consistent $K_m$ values (66 $\mu$M and 72 $\mu$M) for MPP+ formation by marmoset liver microsomes and marmoset P450 2D6 were observed with a high $V_{max}/K_m$ value (150 $\mu$L/min/nmol P450 2D6), but the $K_m$ value for MPP+ formation in human liver microsomes was much lower than that of human P450 2D6, resulting in a low $V_{max}/K_m$ value (8.5 $\mu$L/min/nmol) for human P450 2D6. In terms of kinetic parameters for MPDP+ formation, a similar pattern of differences between marmosets and humans was seen to that for MPP+ formation.
Discussion

Oxidation of potential pro-neurotoxin MPTP has been reported in various animal species, including mouse, rat, and human (Chiba et al., 1990; Coleman et al., 1996; Yoshihara et al., 2000). In vitro studies using rat liver microsomes have shown approximately 20 times faster MPTP N-oxygenation rates than MPTP N-demethylation rates (Cashman and Ziegler, 1986). In the present study, fast MPTP N-oxygenation reactions in vitro were confirmed in marmoset liver and brain microsomes (Fig. 3). These facts suggest that MPTP N-oxygenation might be the major detoxification pathway in most animal species, including common marmosets. MPTP N-oxygenation in marmoset liver microsomes was likely mediated by FMO3 because of suppression of the reaction by methimazole (an inhibitor for FMO, Fig. 4) and the high metabolic capacity of marmoset FMO3 compared to FMO1 (Table 3). In terms of FMO-mediated reactions, apparent $K_m$ values for NADPH-dependent drug oxygenations were generally in the range ~50–100 μM (Yamazaki et al., 2014), presumably because FMO exhibits a stable 4a-flavin hydroperoxide intermediate capable of oxygenating both nucleophiles and electrophiles in its catalytic cycle, even in the absence of an oxygenatable substrate (Jones and Ballou, 1986).

In the present study, the main roles of liver microsomal P450 2D6 and FMO3 in MPTP oxidation in common marmosets were demonstrated (Table 3 and Fig. 5). Marmoset liver microsomes were the main focus of this study to investigate P450-dependent metabolic activation of MPTP because efficient catalytic activities of liver microsomes with respect to MPTP oxidation were seen, in comparison with the generally lower activities for marmoset brain microsomes. It could be noted that the present correlation analysis using 10 marmoset liver microsomes with relatively less interindividual variations might not be predictive as the chemical inhibitor and recombinant enzyme experiments. Several lines of evidence in this
study using marmoset liver microsomes with correlation or chemical inhibition studies and experiments with recombinantly expressed P450 enzymes and FMO3 suggested that the MPTP N-demethylation, MPTP N-oxygenation, and MPDP⁺ and MPP⁺ formation reactions shown in Fig. 1 were mediated by multiple drug-metabolizing enzymes, such as P450 1A, 2C, 2D, and FMO in marmoset liver microsomes, but these reactions were mainly catalyzed by marmoset P450 2D6 or FMO3. Kinetic analysis of MPP⁺ formation revealed that the apparent $K_m$ values for marmoset liver microsomes and recombinant marmoset P450 2D6 were consistent (Table 3), although minor roles of other P450 isoforms could not be ruled out. It should be noted that occasional species differences were seen with regard to the roles of P450 enzymes between marmosets and humans (Fig. 5); human P450 1A2 seemed to have high capacity for MPDP⁺ and MPP⁺ formation under the present conditions. Although monoamine oxidase exists in another cellular fraction was not tested in the present study, these results collectively suggested that, in the marmoset, P450 2D6 is mainly responsible for both deactivation of MPTP to PTP and metabolic activation of MPTP to MPP⁺ to produce the preclinical marmoset model for Parkinson-like syndromes. In our preliminary study, we were unable to isolate P450 2D30 cDNA from marmoset livers by RT-PCR, and thus excluded P450 2D30 from further analysis. This was consisted with a reported finding that P450 2D30 was not isolated from another source of marmosets using the same P450 2D30 primer sets (Hichiya et al., 2004).

The present findings should provide a foundation for understanding MPTP metabolism and the successful production of preclinical marmoset Parkinsonism models. Although some differences were seen in this study in terms of major roles of drug-metabolizing enzymes involved in the MPTP activation and deactivation pathways in humans and marmosets, marmosets would be again recognized to be good preclinical model for Parkinson’s disease as reported (Ando et al., 2014; Ando et al., 2012). A variety of genetic
polymorphisms of \textit{P450 2D6} causing gene duplication, reduced function or loss of function in humans are well-known (Kiyotani et al., 2010). Recently, genetic polymorphisms of \textit{P450 2D} enzymes in cynomolgus and rhesus macaques (Uno et al., 2014) were also demonstrated. In the present study, the inter-individual variations for MPTP oxidations in marmoset liver microsomes (Fig. 3) were not as high as expected, presumably because no genetic variations for marmoset \textit{P450 2D6} were confirmed in our preliminary study using the present 11 marmosets. Further study will be of interest to find more efficient individual marmosets, i.e., rapid metabolizers of \textit{P450 2D6} substrates, to effectively produce the Parkinson-preclinical animal model.

In conclusion, the present results indicated the contributions of multiple drug-metabolizing enzymes in MPTP oxidation, especially marmoset FMO3 in MPTP deactivation (\textit{N}-oxygenation). In the marmoset, \textit{P450 2D6} was responsible for biotransformations including MPTP deactivation (\textit{N}-demethylation) and metabolic activation of MPTP (by desaturation reactions) leading to active neurotoxic compound MPP$^+$. 
Authorship contribution

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

Conducted experiments: Uehara, Uno, Murayama, and Shimizu.

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.


Coleman T, Ellis SW, Martin IJ, Lennard MS and Tucker GT (1996) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is N-demethylated by cytochromes P450 2D6, 1A2 and 3A4 -Implications for susceptibility to parkinson's disease. *J Pharmacol Exp Ther* 277:685-690.


Footnotes

This work was supported in part by a Grant-in-Aid for Scientific Research and also resulted from "Construction of System for Spread of Primate Model Animals" under the Strategic Research Program for Brain Sciences of the Ministry of Education, Culture, Sports, Science and Technology of Japan.
Figures legends

Fig. 1. Metabolic activation (desaturation reactions) and deactivation ($N$-demethylation and $N$-oxygenation) pathways of MPTP. MPDP$^+$, 1-methyl-4-phenyl-2,3-dihydropyridinium ion; MPP$^+$, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; and PTP, 4-phenyl-1,2,3,6-tetrahydropyridine.

Fig. 2. Representative HPLC chromatograms of MPTP oxidized by marmoset liver microsomes (A,B) and recombinant FMO3 (C) and P450 2D6 (D). MPTP and its metabolites were analyzed with a reverse-phase LC system using fluorescence and ultraviolet detection as described in Section 2.5.

Fig. 3. Variation in formation rates of PTP (A), MPDP$^+$ (B), MPTP $N$-oxide (C), and MPP$^+$ (D) from MPTP by liver and brain microsomes of 11 individual marmosets. MPTP (50 µM) was incubated with liver (■) and brain (□) microsomes (0.50 mg/mL) from 11 individual marmosets for 10 min in the presence of an NADPH-generating system. The individual marmosets were numbered in the increasing order of MPP formation rates in liver microsomes.

Fig. 4. Effects of chemical inhibitors on the formation rates of PTP (A), MPDP$^+$ (B), MPTP $N$-oxide (C), and MPP$^+$ (D) from MPTP in marmoset liver microsomes.
Percentages of the control (without inhibitors) are shown. α-Naphthoflavone, sulfaphenazole, ticlopidine, quinidine, ketoconazole, and methimazole, respectively, inhibit P450s 1A, 2C9, 2C, 2D, 3A4, and FMO.

Fig. 5. Formation of PTP (A,D), MPDP⁺ (B,E), and MPP⁺ (C,F) from MPTP mediated by marmoset (A–C) and human (D–F) recombinant P450 enzymes. Marmoset and human recombinant P450 enzymes (20 pmol/mL) were incubated with 50 µM MPTP at 37°C for 10 min in the presence of an NADPH-generating system.
<table>
<thead>
<tr>
<th>Probe reaction activity</th>
<th>P450 isoform</th>
<th>Correlation coefficient, r, for rates of formation of MPTP metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PTP</td>
</tr>
<tr>
<td>7-Ethoxyresorufin O-deethylation</td>
<td>1A</td>
<td>0.73⁺</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>2A</td>
<td>–0.16</td>
</tr>
<tr>
<td>Paclitaxel 6α-hydroxylation</td>
<td>2C</td>
<td>0.29</td>
</tr>
<tr>
<td>Warfarin 7-hydroxylation</td>
<td>2C</td>
<td>0.60</td>
</tr>
<tr>
<td>S-Mephenytoin 4’-hydroxylation</td>
<td>2C</td>
<td>0.30</td>
</tr>
<tr>
<td>Bufuralol 1’-hydroxylation</td>
<td>2D</td>
<td>0.77**</td>
</tr>
<tr>
<td>Chlorzoxazone hydroxylation</td>
<td>2E</td>
<td>–0.27</td>
</tr>
<tr>
<td>Midazolam 1’-hydroxylation</td>
<td>3A</td>
<td>0.92***</td>
</tr>
<tr>
<td>Benzydamine N-oxygenation</td>
<td>FMO</td>
<td>0.63</td>
</tr>
</tbody>
</table>

*The P450 isoform or FMO mainly responsible for catalyzing the probe reactions.

*, p < 0.05; **, p < 0.01; ***, p < 0.001.
Table 2. MPTP N-oxygenation catalyzed by marmoset and human FMO1 and FMO3.

<table>
<thead>
<tr>
<th></th>
<th>MPTP N-oxygenation, nmol/min/nmol FMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marmoset</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FMO1</td>
</tr>
<tr>
<td></td>
<td>FMO3</td>
</tr>
<tr>
<td>Human</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FMO1</td>
</tr>
<tr>
<td></td>
<td>FMO3</td>
</tr>
</tbody>
</table>

Marmoset and human recombinant FMOs (20 pmol/mL) were incubated with 200 µM MPTP at 37°C for 10 min in the presence of an NADPH-generating system. Formations of PTP, MPDP+, and MPP+ by FMO were below the detection limits (for MPDP+ and MPP+, <0.1 nmol/min/nmol FMO; for PTP, <0.01 nmol/min/nmol FMO).
Table 3. Kinetic parameters for MPTP oxidation by marmoset and human liver microsomes and recombinant P450 2D6 and FMO3.

<table>
<thead>
<tr>
<th></th>
<th>Marmoset</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver microsomes</td>
<td>P450 2D6</td>
</tr>
<tr>
<td>PTP formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ μM</td>
<td>451±99</td>
<td>71±7</td>
</tr>
<tr>
<td>$V_{max}$ nmol/min/mg</td>
<td>0.43±0.03</td>
<td>-</td>
</tr>
<tr>
<td>$V_{max}/K_m$ μl/min/mg</td>
<td>-</td>
<td>15.6±0.4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>220</td>
</tr>
<tr>
<td>MPTP N-oxide formation</td>
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<tr>
<td>$K_m$ μM</td>
<td>162±21</td>
<td>N.A.</td>
</tr>
<tr>
<td>$V_{max}$ nmol/min/mg</td>
<td>0.52±0.02</td>
<td>-</td>
</tr>
<tr>
<td>$V_{max}/K_m$ μl/min/mg</td>
<td>-</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1.3</td>
</tr>
<tr>
<td>MPDP$^+$ formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ μM</td>
<td>125±19</td>
<td>74±23</td>
</tr>
<tr>
<td>$V_{max}$ nmol/min/mg</td>
<td>0.23±0.01</td>
<td>-</td>
</tr>
<tr>
<td>$V_{max}/K_m$ μl/min/mg</td>
<td>-</td>
<td>5.5±0.4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>3.6</td>
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<tr>
<td>MPP$^+$ formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ μM</td>
<td>66±18</td>
<td>72±18</td>
</tr>
<tr>
<td>$V_{max}$ nmol/min/mg</td>
<td>0.23±0.02</td>
<td>-</td>
</tr>
<tr>
<td>$V_{max}/K_m$ μl/min/mg</td>
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<td>11±1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Kinetic parameters were calculated by non-linear regression analysis (mean ± SE, 10 points of substrate concentrations within the range 1–2,000 μM). NA, not available.
Activation

Neurotoxicity

Deactivation

PTP

MPTP

MPTP N-oxide

MPDP⁺

MPP⁺
Fig. 2

A

$F_{332/344}$

2.0 x 10^4

1.0 x 10^4

0

5

10

$t_R$, min

PTP

MPTP

N-Oxide

B

$A_{290}$

1.0 x 10^4

0.5 x 10^4

0

5

10

$t_R$, min

MPP+

MPDP+

MPTP

C

$F_{332/344}$

5.0 x 10^6

2.5 x 10^6

0

5

10

$t_R$, min

MPTP

N-Oxide

D

$A_{290}$

5.0 x 10^4

2.5 x 10^4

0

5

10

$t_R$, min

MPP+

MPDP+

MPTP
Fig. 3

Marmoset liver and brain microsomes
Fig. 4

(A) PTP formation (% of control activity)
(B) MPDP⁺ formation (% of control activity)
(C) MPTP N-oxide formation (% of control activity)
(D) MPP⁺ formation (% of control activity)

[Chemicals], μM

α-Naphthoflavone
Sulfaphenazole
Ticlopidine
Quinidine
Ketoconazole
Methimazole
Fig. 5

Marmoset P450 enzymes

Human P450 enzymes

PTP formation (nmol/min/nmol P450)

MPDP⁺ formation (nmol/min/nmol P450)

MPP⁺ formation (nmol/min/nmol P450)