Drug Metabolism and Pharmacokinetics 30 (2015) 247–252

Contents lists available at ScienceDirect

Drug Metabolism and Pharmacokinetics

journal homepage: http://www.journals.elsevier.com/drug-metabolism-and-pharmacokinetics

Regular article

Functional characterization of 20 allelic variants of CYP1A2

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ARTICLE INFO

Article history:
Received 20 November 2014
Received in revised form
19 March 2015
Accepted 19 March 2015
Available online 27 March 2015

Keywords:
Cytochrome P450
CYP1A2
Genetic polymorphism
Phenacetin
7-Ethoxyresorufin

ABSTRACT

Genetic variations in cytochrome P450 1A2 (CYP1A2) are associated with interindividual variability in the metabolism and efficacy of many medications. Twenty CYP1A2 variants harboring amino acid substitutions were analyzed for functional changes in enzymatic activity. Recombinant CYP1A2 variant proteins were heterologously expressed in COS-7 cells. Enzyme kinetic analyses were performed with two representative CYP1A2 substrates, phenacetin and 7-ethoxyresorufin. Among the 20 CYP1A2 allelic variants, CYP1A2*4, CYP1A2*6, CYP1A2*8, CYP1A2*15, CYP1A2*16, and CYP1A2*21 were inactive toward both substrates. CYP1A2*11 showed markedly reduced activity, but the changes in K_m were different between the substrates. CYP1A2*14 and CYP1A2*20 exhibited increased activity compared to the wild-type enzyme, CYP1A2*1. This comprehensive *in vitro* assessment provided insight into the specific metabolic activities of CYP1A2 proteins encoded by variant alleles, which may to be valuable when interpreting the results of *in vivo* studies.

1. Introduction

CYP1A2 is a major form of cytochrome P450, which metabolizes approximately 9% of all medications including theophylline, olanzapine, and clozapine [1]. Interindividual variability in P450 activity is an important contributor to interindividual differences in drug efficacy and adverse effects. CYP1A2 activity has up to 60-fold interindividual variability [2,3], which is due to both genetic and non-genetic factors. Genetic factors account for 35–77% of interindividual variability in CYP1A2 activity [4]. Genetic polymorphisms in the *CYP1A2* gene have been studied and 40 CYP1A2 variant alleles have been recognized by the human CYP Allele Nomenclature Committee (http://www.cypalleles.ki.se/cyp1a2.htm).

Since smoking induces CYP1A2 activity through the polycyclic aromatic hydrocarbons found in tobacco smoke [5], genetic polymorphisms in the transcriptional regulatory regions have been extensively studied in smokers. The most common polymorphism -163C>A (CYP1A2*1F) was associated with increased inducibility of CYP1A2 [6]. This polymorphism was reported to reduce weight-normalized olanzapine serum concentration, which could alter efficacy [7]. Higher serum olanzapine concentrations led to better symptom improvement in schizophrenic patients. The -3860G>A (CYP1A2*1C) polymorphism is a causal factor for decreased CYP1A2 inducibility [8], and -3860G>A carriers showed increased severity of tardive dyskinesia, a movement disorder induced by long-term treatment with antipsychotic drugs [9]. Asthma patients carrying the -2964G>A polymorphism homozygously showed significantly lower theophylline clearance [10]. The -2467delT (CYP1A2*1D) polymorphism was associated with a decreased risk of disease severity in patients with COPD [11]. The blood theophylline levels of patients carrying the "del/del" mutation at -2467 were higher than that of the patients carrying "T/del" or "T/T." These reports indicated that CYP1A2 genetic polymorphisms could affect clinical responses to a number of different medications.

Of the 40 recognized allelic variants of CYP1A2, 20 are non-synonymous polymorphisms. These 20 polymorphisms that produce amino acid substitutions could result in changes in protein conformation and enzymatic activity. It is particularly challenging to characterize the function of these alleles *in vivo* since the number of patients carrying these non-synonymous alleles is small. Therefore, *in vitro* characterization of CYP1A2 activity by expression of recombinant enzymes is essential to facilitate our understanding of the interindividual variability in responses to drugs.

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http://dx.doi.org/10.1016/j.dmpk.2015.03.001

The activity of several non-synonymous CYP1A2 variant proteins has been determined *in vitro*. Zhou et al. expressed CYP1A2*3, CYP1A2*4, CYP1A2*5, and CYP1A2*6 in *E. coli* and measured protein expression and enzymatic activity [12]. Saito et al. and Murayama et al. determined the catalytic activity of CYP1A2*8–CYP1A2*16 expressed in Chinese hamster V79 cells [13,14]. Palma et al. characterized the activities of CYP1A2*4, CYP1A2*5, CYP1A2*8, CYP1A2*9, CYP1A2*12, and CYP1A2*13 with a diverse group of substrates with and without co-expression of cytochrome b_5 in *E. coli* [15,16]. Although these reports demonstrated the existence of the CYP1A2 polymorphisms that alter enzymatic activity, the kinetic parameters (Michaelis constant (K_m), maximal initial velocity (V_{max}), and intrinsic clearance (CL_{int}, V_{max}/K_m)) of the CYP1A2 variant proteins were not consistent with previous reports. This discrepancy may be due to differences in expression systems and reaction conditions (e.g., reaction time and substrate concentration).

In the present study, *CYP1A2*1–CYP1A2*21* polymorphisms (except splicing defect variant *CYP1A2*7)* were evaluated after expression in COS-7 cells. Five of these polymorphisms (*CYP1A2*17–CYP1A2*21)* were previously uncharacterized. Phenacetin and 7-ethoxyresorufin were used as representative CYP1A2 substrates to determine whether any functional changes observed are substrate-dependent.

2. Materials and methods

2.1. Chemicals

Phenacetin was purchased from Nacalai Tesque (Kyoto, Japan). 4-Acetamidophenol, 7-ethoxyresorufin, and resorufin were purchased from Sigma–Aldrich (St. Louis, MO, USA). 3-Acetamidophenol was obtained from Tokyo Chemical Industry (Tokyo, Japan). CYP1A2 baculosomes were obtained from Invitrogen, Life Technologies (Carlsbad, CA, USA). Oxidized β-nicotinamide-adenine dinucleotide phosphate (NADP+), glucose-6-phosphate (G6P), and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast (Tokyo, Japan). Polyclonal anti-human CYP1A2 antibodies were purchased from Nosan Corporation (Kanagawa, Japan). Polyclonal anti-calnexin antibodies were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Polyclonal goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase were obtained from DakoCytomation (Glostrup, Denmark) and Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. CYP1A2 cDNA cloning and construction of expression vectors

CYP1A2 cDNA fragments from a human liver cDNA library (Takara, Shiga, Japan) were amplified by the polymerase chain reaction with the forward primer 5′-CACCATGGCATTGTCCCAGTCTGTTC-3′ and the reverse primer 5′-TCAGTTGATGGAGAAGCGCAGCC-3′ using PfuUltra High-Fidelity DNA polymerase (Agilent Technologies, Santa Clara, CA, USA). The underlined sequence in the forward primer was introduced for directional TOPO cloning. The amplified fragments were subcloned into the pENTR/D-TOPO vector (Invitrogen). Plasmids carrying *CYP1A2*1* cDNA were used as a template to generate various *CYP1A2* constructs (*CYP1A2*2–CYP1A2*6* and *CYP1A2*8–CYP1A2*21*) using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. All prepared constructs were confirmed by direct sequencing. Wild-type and variant *CYP1A2* cDNA sequences were subsequently subcloned into the mammalian expression vector pcDNA3.4 (Invitrogen) by TA cloning.

2.3. Determination of protein expression levels by immunoblotting

Expression of CYP1A2 variant proteins in COS-7 cells and preparation of microsomal fractions were performed as previously described [17]. COS-7 microsomal fractions (5 μg of microsomal protein) were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels, and western blotting was performed according to standard procedures. The recombinant CYP1A2 Baculosomes Reagent (Invitrogen) was used as the standard (range, 0.009–0.138 pmol) in each gel to quantify the content of CYP1A2 protein. The CYP1A2 protein was detected using polyclonal anti-human CYP1A2 antibodies (diluted at 1:1000) and horseradish peroxidase-conjugated goat anti-rabbit IgG (DakoCytomation) (diluted at 1:10,000). Western blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA). Calnexin was detected with polyclonal anti-calnexin antibodies (diluted at 1:5000), and horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) (diluted at 1:10,000). Immunoblots were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific). Chemiluminescence was quantified using a ChemiDoc XRS$^+$ with the help of Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Phenacetin O-deethylation assay

Phenacetin *O*-deethylation by CYP1A2 was measured as reported previously, with several modifications [13]. The incubation mixture consisted of the microsomal fraction (50 μg microsomal protein, 1.1–26.2 pmol CYP1A2/mg microsomal protein), phenacetin, an NADPH-generating system (1.3 mM NADP$^+$, 3.3 mM G6P, 3.3 mM magnesium chloride, and 0.4 units/mL G6PDH), and 50 mM potassium phosphate buffer (pH 7.4). The total incubation volume was 150 μL. Phenacetin (5, 10, 25, 50, 100, 200, 400, or 600 μM) was incubated with the enzyme to determine the kinetics of phenacetin *O*-deethylation. Following incubation at 37 °C for 1 min, reactions were initiated by addition of the microsomal fraction, and the mixture was incubated at 37 °C for 40 min. Reactions were terminated by addition of 150 μL of methanol containing 0.2 μM 3-acetamidophenol, the internal standard. Determination of phenacetin *O*-deethylation in the microsomal fractions (50 μg microsomal protein containing CYP1A2*1, CYP1A2*4, CYP1A2*9, CYP1A2*10, or CYP1A2*14) revealed that 4-acetamidophenol formation was linear up to 40 min. When the reaction was performed for 40 min with 20–50 microsomal μg protein, 4-acetamidophenol (acetaminophen) formation was linear up to 50 μg of microsomal protein containing CYP1A2*1 (data not shown).

After the addition of 300 μL of potassium phosphate buffer (20 mM, pH 5.4) and the removal of proteins by centrifugation (10,000× *g* for 3 min), 50 μL of the supernatant was subjected to high-performance liquid chromatography (HPLC). The HPLC system consisted of a Waters 2695 Separations Module, a Waters 2487 dual λ absorbance detector (Waters, Milford, MA, USA) and a COSMOSIL 5C_{18}-AR-II column (4.6 × 150 mm; temperature, 40 °C; Nacalai Tesque). The mobile phase consisted of 20 mM potassium phosphate buffer (pH 5.4)-acetonitrile (97:3 v/v) for the first 15 min, which was subsequently replaced by H_2O-acetonitrile (50:50 v/v) from 15 to 19 min and restored to 20 mM potassium phosphate buffer (pH 5.4)-acetonitrile (97:3 v/v) from 19 to 30 min. Elution was performed at a flow rate of 1.0 mL/min. The quantity of 4-acetamidophenol was determined by measuring the absorbance at 245 nm. The retention times of 4-acetamidophenol and 3-acetamidophenol were 8.7 min and 14.6 min, respectively. The lower limit of quantification for 4-acetamidophenol was 50 nM.