Role of Human Liver Cytochrome P450 2C9 in the Metabolism of a Novel α4β1/α4β7 Dual Antagonist, TR-14035

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Summary: The metabolism of a novel dual antagonist for α4β1/α4β7 integrin, TR-14035, and the role of polymorphic enzyme responsible for this metabolism were investigated. Human liver microsomes catalyzed the NADPH-dependent metabolism of TR-14035 to a primary metabolite, O-desmethyl TR-14035. This formation was completely blocked by both sulfinpyrazone, a selective CYP2C9 inhibitor, and CYP2C9 antibody, whereas potent inhibitors selective for other CYPs exhibited little effects. Of 12 recombinant CYPs examined, O-desmethyl metabolite was principally formed by CYP2C9. CYP1A1, an extrahepatic enzyme, also had this activity (about one-fourth of CYP2C9). Utilizing recombinant CYP2C9*1, K_m and V_max/K_m values of 23.3 μM and 0.284 μL/min/pmol CYP2C9, respectively, were obtained for the O-desmethyl formation, which were quite similar to those in CYP2C9*2 enzyme. In contrast, V_max/K_m value in recombinant CYP2C9*3 was approximately one-sixth of CYP2C9*1 and *2. In agreement, kinetics studies using human liver microsomes with CYP2C9*1/*1, *2/*2 and *3/*3 genotypes revealed that the V_max/K_m value in *2/*2 microsomes was comparable to that in wild type microsomes, in contrast, that in *3/*3 microsomes was reduced. These results demonstrate CYP2C9 is a primary enzyme mediating the O-desmethylation of TR-14035 in human liver. In homozygotes of CYP2C9*3, the metabolic clearance of TR-14035 should be decreased compared with homozygotes of CYP2C9*1 or 2.

Key words: α4β1/α4β7 antagonist; CYP2C9; genetic polymorphism; metabolism; human liver microsome

Introduction

The α4 integrins, α4β1 and α4β7, expressed on leukocytes, can bind to vascular cell adhesion molecule-1 and mucosal addressin cell adhesion molecule, respectively, localized on endothelial venules. This interaction is critical for the firm adhesion of leukocytes to the endothelium, necessary for the subsequent transendothelial migration through the vessel wall. Therefore, α4β1 and α4β7 integrins are key regulators of physiologic and pathologic responses in inflammation and autoimmune diseases. Antagonists of α4β1 and α4β7 may have therapeutic applications in a number of diseases with an inflammatory component including inflammatory bowel disease, asthma, multiple sclerosis and rheumatoid arthritis.

TR-14035, N-(2,6-dichlorobenzoyl)-4-(2,6-dimethoxyphenyl)-L-phenylalanine (Fig. 1), is the first orally bioavailable chemical entity shown to be a potent and dual antagonist for α4β1/α4β7 integrin-mediated cell adhesion.1) Our previous studies2-3) demonstrate that a primary metabolic route of TR-14035 includes O-demethylation on a dimethoxy-biphenyl ring in the rat and dog. The resultant O-desmethyl metabolite is further conjugated with sulfate in the rat, whereas in the dog, no sulfate conjugate is detected due to lack of this in vitro conjugation activity in liver cytosols. In addition, in vitro metabolism studies using human liver subcellular fractions indicate a principal metabolite of TR-14035 is O-desmethyl derivative, and not followed by sulfate conjugation. To date, cytochrome P450 (CYP) isoform(s) responsible for this desmethylation of TR-14035 have not been identified.

CYP2C9, one of major CYPs found in the human
liver, is a polymorphic enzyme contributing to the metabolism of a wide range of clinically important drugs, including tolbutamide, phenytoin, S-warfarin, losartan, and numerous nonsteroidal anti-inflammatory drugs.22) Many studies have been investigated effects of CYP2C9 variants on catalytic activity of substrates. CYP2C9*2 enzyme (Cys144 allelic variant) expressed in recombinant system displays a decrease in intrinsic clearance of several drugs, including S-warfarin, tolbutamide, losartan and celecoxib when compared with the wild-type, CYP2C9*1,2,3) whereas gives the similar intrinsic clearance on the metabolism of diclofenac, torsemide and lornoxicam.3,4) On the other hand, CYP2C9*3 (Leu359 allelic variant) has demonstrated reduced clearances with all CYP2C9 substrates evaluated.2,3,5)

The objectives of the present study were to identify the CYP isoform(s) responsible for the O-desmethylation of TR-14035, and to evaluate the role of CYP2C9 polymorphism in vitro.

Materials and Methods

Chemicals: TR-14035 and O-desmethyl TR-14035 (Fig. 1) were synthesized at Tanabe Seiyaku Co. and Tanabe Research Laboratories, USA, Inc., respectively. TR-14085 synthesized at Tanabe Research Laboratories was used as an internal standard for HPLC. Furafylline, coumarin and omeprazole were purchased from Ultrafine Chemical Ltd (Manchester, UK), Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Sulfaphenazole, quinidine, treloandamycin, glucose 6-phosphate and β-nicotinamide adenine dinucleotide (β-NADP+) were obtained from Sigma-Aldrich (St. Louis, MO). Glucose 6-phosphate dehydrogenase was obtained from Roche Diagnostics (Mannheim, Germany). A polyclonal antibody raised against human CYP2C9, which recognize only human CYP2C9, was purchased from Nihon Nosan Kogyo (Yokohama, Japan). Human liver microsomes (individual organ donor subjects H023, H042, H066, H103 and HK27) were purchased from BD Gentest (Woburn, MA). The information of CYP2C9 genotypes and CYP2C9 protein contents in the microsomes was provided by BD Gentest and shown in Table 2. Microsomes prepared from baculovirus-infected insect cells expressing CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9*1, 2C9*2, 2C9*3, 2C19, 2D6, 2E1, 3A4 and 4A11, respectively, were obtained from BD Gentest. All recombinant CYPs were coexpressed with NADPH-P450 reductase. All other reagents and solvents were of analytical grade and commercially available.

Incubation conditions: Briefly, the incubation mixture consisted of the following: 50 mM Tris-HCl buffer (pH 7.4), 5 mM magnesium chloride, 1 mM EDTA, 1 mM β-NADP+, 10 mM glucose 6-phosphate, 2 units/mL glucose 6-phosphate dehydrogenase and 0.05 mg microsomal protein or 5 pmol cDNA-expressed CYP protein in a final volume of 0.25 mL. Following a 5-min preincubation at 37°C, the reaction was initiated by the addition of TR-14035 (1–100 μM (ºKm), final volume in incubation). Acetonitrile showed no effect on O-desmethylation activity of TR-14035 up to 2% (v/v) (data not shown). After an additional 20-min incubation, the reaction was terminated by the addition of 0.1 mL of ice-cold acetonitrile and cooling on ice. Aliquots (10 μL) of TR-14085 (20 μg/mL in acetonitrile) were added as an internal standard. In each case, the resulting mixture was centrifuged at 10,000g for 10 min, and the supernatant was filtered before HPLC analysis.

Inhibition experiments with CYP isoform-selective chemical inhibitors were carried out at a final TR-14035 concentration of 10 μM (ºKm). Where appropriate, the final concentration of the inhibitors was chosen on the basis of established Ki values to ensure maximal inhibition (> 80%) of each CYP form.6–9) Inhibitors dissolved in acetonitrile, in which case, the final volume of acetonitrile in assy was kept to 1.25% (v/v). In the case of the mechanism-based inhibitors, furafylline and troleandomycin, the incubation mixtures were preincubated at 37°C for 15 min with an NADPH-generating system before the addition of TR-14035.

For immunoinhibition studies, human liver microsomes were preincubated with anti-CYP2C9 antibody or control serum for 30 min at room temperature, and
then the incubations were performed with 10 μM TR-14035 in a manner to that described above.

**HPLC analysis:** TR-14035 and its metabolites were separated on a reverse-phase C18 (Symmetry C18, 4.6 × 150 mm, 3.5 μm; Waters, Milford, MA) column using Waters HPLC system (pump, 600E; autosampler, 717; UV detector, 484) with a column oven temperature set at 40°C. The mobile phase consisted of 10% (v/v) aqueous acetonitrile containing 0.05% (v/v) trifluoroacetic acid (solvent A) and 80% (v/v) aqueous acetonitrile containing 0.05% (v/v) trifluoroacetic acid (solvent B), and delivered at a flow rate of 1.0 mL/min. The initial mobile phase consisted of 30% of solvent B, which increased linearly to 85% over 25 min, and the elutions of unchanged TR-14035, O-desmethyl TR-14035 and the internal standard (TR-14085) were monitored at 215 nm. TR-14035, O-desmethyl TR-14035 and the internal standard were eluted at 18.8, 13.7 and 21.3 min, respectively, under these conditions (Fig. 2(B)).

**Kinetic analysis:** Estimated kinetic parameters for the O-desmethylation of TR-14035 were obtained from the following Michaelis-Menten equation: 
\[
\frac{v}{K_m + S} = \frac{V_{max} \times S}{(K_m + S)},
\]
where \(v\) is the O-desmethylation rate of substrate (pmol/min/mg protein), \(S\) is the substrate concentration in incubation medium (μM), \(K_m\) is the Michaelis-Menten constant (μM), and \(V_{max}\) is the maximum O-desmethylation rate (pmol/min/mg protein). To calculate the kinetic parameters, the above equation was fitted to uptake data sets by an iterative nonlinear least squares method using a MULTI program.\(^{10}\) The input data were weighted as the reciprocal of the observed values, and the Damping Gauss-Newton Method algorithm was used for fitting.

**Results**

**Metabolism by human liver microsomes:** A typical HPLC chromatogram of the supernatant following a 20-min incubation of 10 μM TR-14035 with human liver microsomes in the presence of an NADPH-generating system is shown in Fig. 2(C). After incubation, one major metabolite was detected and identified as an O-desmethyl derivative of TR-14035 by liquid chromatography-mass spectrometry analysis.

**Metabolism by recombinant human cytochromes P450:** The O-desmethylation activities of TR-14035 by recombinant human CYP enzymes are depicted in Fig. 3. TR-14035 was found to be principally metabolized by CYP2C9*1, followed by CYP1A1 (approximately one-fourth of CYP2C9*1). A low rate of the O-desmethylation was observed for CYP2C8 only at a higher substrate concentration, and no activities were observed for CYPs 1A2, 1B1, 2A6, 2B6, 2C19, 2D6, 2E1, 3A4 and 4A11.

**Kinetics of TR-14035 O-desmethylation:** Kinetics of O-desmethylation of TR-14035 was investigated with each recombinant CYP2C9*1, *2 and *3 using Michaelis-Menten and Eadie-Hofstee plots (Fig. 4(A)). The \(K_m\), \(V_{max}\) and \(V_{max}/K_m\) values, estimated by fitting to the Michaelis-Menten equation, are summarized in Table 1. Apparent \(K_m\), \(V_{max}\) and \(V_{max}/K_m\) values in

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Fig. 2. Representative HPLC chromatograms of incubation blank (A), incubation blank spiked with TR-14035, O-desmethyl TR-14035 and internal standard (B) and after incubation of 10 μM TR-14035 with NADPH-fortified human liver microsomes (C).

Fig. 3. O-Desmethylation of TR-14035 by each recombinant human CYP.

Recombinant CYP isozymes (20 pmol P450/mL) were incubated with TR-14035 (1 and 10 μM) for 20 min. Each bar is the mean of two determinations.
CYP2C9*1 enzyme were estimated to be 23.3 μM, 6.63 pmol/min/pmol CYP2C9 and 0.284 μL/min/pmol CYP2C9, respectively. Those in CYP2C9*2 were 21.9 μM, 5.70 pmol/min/pmol CYP2C9 and 0.260 μL/min/pmol CYP2C9, respectively, which were quite similar to the values in CYP2C9*1. By contrast, obviously higher $K_m$ and lower $V_{max}$ were noticed in CYP2C9*3 enzyme, resulting in an approximately one-sixth $V_{max}/K_m$ value in CYP2C9*3 compared with those in CYP2C9*1 and *2.

Moreover, kinetics of TR-14035 metabolism was also investigated in human liver microsomes with different CYP2C9 genotypes (Fig. 4B). The $K_m$ and $V_{max}$ values are summarized in Table 2 with the content of CYP2C9 in each liver microsome. In all cases, analyses of Eadie-Hofstee plot revealed that single enzyme would be involved in the O-desmethylation of TR-14035. Apparent $K_m$ and $V_{max}$ values in the CYP2C9*1/*1 genotype ranged from 19.4 to 22.2 μM and 460 to 797 pmol/min/mg protein, respectively. Those in CYP2C9*2/*2 microsomes were calculated to be 49.1 μM and 1179 pmol/min/mg protein, respectively, and the resulting $V_{max}/K_m$ value was similar to that in CYP2C9*1/*1 microsomes. By contrast, CYP2C9*3/*3 microsomes exhibited an obvious decrease of $V_{max}/K_m$ value than CYP2C9*1/*1 and *2/*2 microsomes.

Chemical inhibition and immuno inhibition studies: Sulfaphenazole (0.5 and 5 μM), a selective inhibitor of CYP2C9, was shown to be remarkable decreases on O-desmethylation of TR-14035 (Fig. 5). This phenomenon appeared in human liver microsomes with wild, *2/*2 and *3/*3 genotypes in principal. But, the inhibition potential in microsomes with *3/*3 genotype tends to be lower than wild and *2/*2 genotypes. In contrast, other selective chemical inhibitors, including furafylline (CYP1A2-selective), coumarin (CYP2A6-selective), omeprazole (CYP2C19-selective), quinidine (CYP2D6-selective) and troleandomycin (CYP3A4-selective), exhibited a little or no effect on O-desmethylation activities.

Antibody against human CYP2C9 inhibited the formation of O-desmethyl TR-14035 in human liver microsomes with wild, *2/*2 and *3/*3 genotypes, yielding 95% or more inhibition in a concentration-dependent manner (Fig. 6). In contrast, control serum had no effect on metabolism of TR-14035.

### Discussion

In the present study, TR-14035 was predominantly biotransformed to an O-desmethylated metabolite by human hepatic microsomal fractions incubated with an
NADPH-generating system, and its formation seemed to be primarily mediated by CYP2C9 enzyme in human liver. The involvement of CYP2C9 was well supported by the following evidences. First, metabolic activity of TR-14035 in CYP2C9*1-expressing cells was 4- and 26-fold more extensive than those in CYP1A1 and CYP2C8, respectively (Fig. 3). Whereas CYP2C9 enzyme was found to be one of major CYPs in human liver, no CYP1A1 protein was detected in human liver.11) In addition, other CYPs lacked this metabolic activity. Second, the formation of O-desmethyl metabolite in human liver microsomes showed saturation kinetics with respect to substrate concentration, and the linearity in the Eadie-Hofstee plot suggested the involvement of single CYP isozyme on O-desmethyla-
tion of TR-14035 (Fig. 4). Additionally, the apparent $K_m$ estimated in human liver microsomes revealed a quite similarity with the $K_m$ in recombinant CYP2C9.
enzymes (Tables 1 and 2). Third, both sulfaphenazole, a selective inhibitor for CYP2C9, and antibody against CYP2C9 completely inhibited the O-desmethyl formation (Figs. 5 and 6).

In general, it is important to assess the role of polymorphic CYP2C9 on metabolism of a new chemical entity for predicting the impact of CYP2C9 genotype on the overall pharmacokinetics. Therefore, in this study, we have also investigated the role of this polymorphic enzyme on metabolism of TR-14035 using human liver microsomes with different genotypes and recombinant wild and variant CYP2C9s.

Human liver microsomes with CYP2C9*2/*2 genotype and recombinant CYP2C9*2 enzymes have been reported to reduce intrinsic clearances of some substrates, including phenytoin, S-warfarin and tolbutamide.2,3 In addition to in vitro studies, many clinical evaluation studies were undertaken to investigate the role of CYP2C9 polymorphism in metabolic clearance of substrates. For example, the CYP2C9-mediated clearance of phenytoin, one of narrow therapeutic index agents, was significantly lower in a CYP2C9*2 homozygous subject than in CYP2C9*1 homozygous subjects,12) and the phenytoin dose required to achieve a therapeutic serum concentration for CYP2C9*2 homozygous subjects was 36% lower than the dose required for wild type subjects.13) By contrast, metabolic clearances of other substrates, cyclophosphamide, ifosfamide, diclofenac, torsemide and lornoxicam in recombinant CYP2C9*2 enzymes were not significantly reduced compared with wild type enzymes.3,4,14) These observations suggest that effect of the substitution Arg144 to Cys144 in CYP2C9 on metabolic clearance of substrates is distinctly substrate-dependent. Based on above information, the substitutive effect on CYP2C9 alignment was examined in the O-desmethylation of TR-14035. Tables 1 and 2 clearly indicate similar intrinsic clearances between wild type CYP2C9*1 and variant type CYP2C9*2 on O-desmethyl formation of TR-14035, suggesting that no significant difference should be observed in TR-14035 elimination between wild type subjects and CYP2C9*2 homozygotes.

Many reports agree that the substitution Ile159 to Leu159 in CYP2C9 is highly important in terms of a decrease of drug metabolic activity.2,3,3) Also, on clinical pharmacokinetic studies, metabolic clearances of phenytoin, glipizide, S-warfarin and losartan in CYP2C9*3 homozygotes were significantly decreased compared with wild type subjects.15-18) Crystal structure of CYP2C9, recently determined,19,24) shows that amino acid 359 on helix K of CYP2C9 exists near the substrate recognition site 5 (Leu166 and Pro167) and 6 (C-terminal β-sheet 4). This evidence suggests the amino acid substitution from Ile159 to Leu159 may induce a conformational change in the substrate recognition site, resulting in a remarkable decrease of the binding affinity and/or metabolic capacity of substrates. In this study, obvious higher Km and lower Vmax on O-desmethyl formation of TR-14035 were found in CYP2C9*3/*3 liver microsomes and recombinant CYP2C9*3 enzymes, and its Vmax/Km in recombinant CYP2C9*3 was approximately 6-times lower than recombinant CYP2C9*1 and *2 (Table 1). In other CYP2C9 substrates, the Km and Vmax in recombinant CYP2C9*3 was also reported to be increased and reduced, respectively, resulting in approximately 6- to 20-times lower Vmax/Km.3) Therefore, these results indicated that homozygotes of CYP2C9*3 allele would cause a reduction in CYP2C9-mediated clearance of TR-14035 as well as other CYP2C9 substrates.

Some CYP2C9 substrates, including phenytoin, S-warfarin and glipizide, have a narrow therapeutic index and no or little elimination pathway with the exception of hepatic CYP2C9-mediated metabolic disposition, and thus homozygotes of CYP2C9*3 allele may experience severe drug-induced toxicity.20) Figure 3 shows CYP1A1 was also responsible for the metabolism of TR-14035, but its activity was lower than CYP2C9. Human CYP1A1, expressed predominantly in extra-hepatic organs, most notably lung, kidney, skin and placenta, is responsible for the metabolic activation of polycyclic aromatic hydrocarbon carcinogens.21) It is well-known that CYP1A1 can also mediate the biotransformation of some drugs, including R-warfarin, amiodarone, tamoxifen and propranolol,22) but the involvement in total body clearances of them seems to be essentially limited. Therefore, the involvement of CYP1A1 in TR-14035 clearance will be slight compared with that of CYP2C9, though a clinical evaluation should be necessary for identification of their involvement. This indicates that clinical pharmacokinetic profile of TR-14035 might be affected by the substitution Ile159 to Leu159 of CYP2C9.

We found sulfaphenazole and anti-human CYP2C9 antibody exhibited inhibitory effects on TR-14035 metabolism in human liver microsomes with not only wild/wild genotype, but also *2/*2 and *3/*3 genotypes (Figs. 5 and 6), suggesting CYP2C9 is a primary enzyme responsible for the metabolism of TR-14035 in not only wild subjects but also CYP2C9*2 and *3 homozygous subjects. However, inhibition potentials by sulfaphenazole were somewhat different among CYP2C9 genotypes, namely, inhibitory effect by sulfaphenazole was poorer in microsomes with *3/*3 genotype. Crystal structure of CYP2C9 indicates amino acid 144 on helix D is far from the substrate recognition site and haem, by contrast, amino acid 359 stays near the site.19,24) These reports are speculated that the substitution Arg144 to Cys144 dose not affect the structure around a substrate recognition site and haem, whereas,
the substitution Ile$^{359}$ to Leu$^{359}$ causes a conformational alteration of a substrate recognition site and affects the substrate/inhibitor interaction with the recognition site. This supports poor inhibitory effect in microsomes with *3/*3 genotype and similar inhibitory effect between microsomes with wild/wild and *2/*2 genotype in this study. Although sulfaphenazole seems to be useful for inhibition study with CYP2C9*2 and *3 enzymes as well as CYP2C9*1, it is necessary for careful use due to a different inhibition potential in *3/*3 genotype. In addition, the difference of inhibitory effect might reflect a clinical drug-drug interaction between TR-14035 and CYP2C9 inhibitor. However, a clinical evaluation should be conducted to verify its potential.

In summary, O-desmethylation of TR-14035 was mainly catalyzed by CYP2C9 in human liver. CYP1A1, expressed in extrahepatic tissues, also had this metabolic activity. Intrinsic activities of this formation in CYP2C9*2/*2 liver microsomes and recombinant CYP2C9*2 enzymes were consistent with those in wild type, respectively. By contrast, obviously higher $K_m$ and lower $V_{max}$ were observed in CYP2C9*3/*3 microsomes and recombinant CYP2C9*3. These results suggest that the metabolic clearance of TR-14035 should alter in homozygotes of CYP2C9*3 allele, but never in homozygotes of CYP2C9*2 allele.

References


