The Inhibitory Effect of Telmisartan on the Metabolism of Arachidonic Acid by CYP2C9 and CYP2C8: An in Vitro Study

Yuka Kato, Yuji Mukai, Anders Rane, Nobuo Inotsume, and Takaki Toda

Division of Clinical Pharmacology, Hokkaido Pharmaceutical University School of Pharmacy; and Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska University Hospital, Karolinska Institute; Stockholm SE-14186, Sweden.

Received February 27, 2017; accepted May 18, 2017

Epoxyeicosatrienoic acids (EETs) are generated from arachidonic acid (AA) by CYPs. EETs comprise four regioisomers (14,15-, 11,12-, 8,9-, and 5,6-EET). EETs show potent physiological effects, including vasodilation, anti-inflammation, myocardial preconditioning, and anti-platelet aggregation effects. We recently demonstrated that telmisartan, one of angiotensin II receptor blockers, inhibits AA metabolism by CYP enzymes, including CYP2C8, CYP2C9, and CYP2J2. We conducted studies of AA metabolism using recombinant CYP enzymes to estimate the inhibition constant and the type of inhibition by telmisartan of CYP2C9 and CYP2C8. The contribution ratio (CR) of each CYP enzyme was investigated using human liver microsomes. Dixon and Lineweaver–Burk plots indicated that telmisartan is a mixed inhibitor of both CYP2C9 and CYP2C8; telmisartan did not show a time-dependent inhibition toward these CYP enzymes. Based on the CRs, both CYP2C9 and CYP2C8 are the key enzymes in the metabolism of AA in the human liver. Uptake of telmisartan in the liver by organic anion transporting polypeptide (OATP) IB3 and the non-linear metabolism in gastrointestinal tract augment the potential of the drug to inhibit the CYP enzymes in the liver.

Key words epoxyeicosatrienoic acid; arachidonic acid; telmisartan; drug–endogenous interaction

Arachidonic acid (AA) is a 20-carbon unsaturated fatty acid with double bonds at positions 14, 11, 8, and 5. AA is metabolized by cyclooxygenase and lipoygenase as well as several CYPs including CYP2C8, CYP2C9, and CYP2J2. The four epoxyeicosatrienoic acids (EETs), i.e., 14,15-, 11,12-, 8,9-, and 5,6-EET, are metabolites generated from AA by CYP enzymes. It has been reported that AA metabolism in human liver microsomes (HLMs) is catalyzed principally by CYP2C. Daikh et al. showed that CYP2C9 produces 14,15- and 11,12-EET at a ratio of 2.3:1.0 along with a small amount of 8,9-EET, whereas CYP2C8 generates 14,15- and 11,12-EET at a ratio of 1.3:1.0, typically without detectable 8,9- and 5,6-EET. EETs are generated in various tissues, including vascular endothelial cells, liver, heart, and kidney. It is known that EETs play a role in vasodilation by opening calcium-activated potassium channels, hyperpolarizing the membrane, and relaxing vascular smooth muscle. EETs also provide protective effects towards the cardiovascular system, such as anti-inflammation, cardiac preconditioning, and anti-platelet aggregation. EETs are rapidly metabolized to the corresponding dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). 14,15-EET is the preferred substrate for sEH and the production rates of 11,12- and 8,9-DHET are lower than that of 14,15-DHET. DHETs generally have less biological activity than EETs.

Telmisartan, one of the angiotensin II receptor blockers (ARBs), is known to prevent cardiovascular events by reducing blood pressure and to improve left ventricular remodeling induced by myocardial infarction (MI). However, several patients have experienced side effects while taking telmisartan, including MI at a rate of 39/2036 (1.9%) and anginapectoris at a rate of 18/2036 (0.9%) (http://www.ehealthme.com/drug/telmisartan/; accessed January 14, 2017).

Drug–drug interactions can cause serious adverse events and are generally well studied, whereas the interaction between drugs and endogenous substances such as AA remain to be determined. We previously developed a method for the simultaneous quantification of EETs and DHETs by LC-MS/MS. Using this method, we demonstrated that several ARBs inhibit AA metabolism in recombinant CYP (rCYP) 2C8, rCYP2C9, rCYP2J2, and HLMs, among the ARBs, telmisartan exhibited more potent inhibitory effect towards the CYP enzymes. Several studies have revealed that telmisartan inhibits CYP2C9 and CYP2J2 in HLMs. Ren et al. reported that telmisartan is a mixed inhibitor of CYP2J2. However, the mechanism by which telmisartan inhibits CYP2C8 has not been evaluated to date.

The aim of this study was to elucidate the features of telmisartan inhibition of AA metabolism. In addition, we calculated the contribution ratio (CR) of CYP2C9 and CYP2C8 in the metabolism of AA, revealing the significance of each CYP enzyme on drug–AA interaction.

MATERIALS AND METHODS

Chemicals Telmisartan, sulfaphenazole, rofecoxib, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Paclitaxel, diclofenac, and quercetin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Docetaxel trihydrate and 6α-hydroxypaclitaxel were purchased from Toronto Research Chemicals (Toronto, Canada) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), respectively. Recombinant CYP2C8 (rCYP2C8: 2000 pmol cytochrome P450/mL), recombinant CYP2C9*1 (rCYP2C9*1: 1000 pmol cytochrome P450/mL), insect cell control SUPERSOMES™,
150-donor pooled human liver microsomes, monoclonal antibody for human CYP2C8 (mAb-2C8) derived from mouse, and NADPH regeneration system solutions A and B were all obtained from Corning (Woburn, MA, U.S.A.). Polyclonal antibody for human CYP2C9 (pAb-2C9) was purchased from Nihon Nosan (Yokohama, Japan). The eight eicosanoids 14,15-, 11,12-, 8,9-, and 5,6-EET, and 14,15-, 11,12-, 8,9-, and 5,6-DHET, the corresponding deuterated eicosanoids 14,15-, 11,12-, 8,9-, and 5,6-EET-\text{d}_1, and 14,15-, 11,12-, and 8,9-DHET-\text{d}_1, used as internal standards, AA, and 4'-hydroxydiclofenac were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Since 5,6-DHET-\text{d}_1, is not commercially available, 8,9-DHET-\text{d}_1, was used as an internal standard for the determination of 5,6-DHET levels. OASIS® HLB cartridges were purchased from Waters (Milford, MA, U.S.A.). All other chemicals and solvents were HPLC grade or special grade.

**LC-MS/MS Analysis** The concentrations of EETs and DHETs were determined using the LC-MS/MS method described previously.\textsuperscript{20,21} An Agilent 1200 series HPLC system (Santa Clara, CA, U.S.A.) was used with a binary pump, a degasser, and a thermostatic autosampler. HPLC separation was achieved using an Ascentis Express C18 column (10 cm×2.1 mm, 2.7 μm, Sigma-Aldrich). Mobile phase A consisted of 0.1% formic acid in acetonitrile, mobile phase B consisted of 0.1% formic acid in water, and the flow rate was 0.3 mL/min. The linear gradient program was as follows: 50% B for 27 min, 50–90% B from 27 to 28 min, hold at 90% B from 28 to 35 min, 90–50% B from 35 to 36 min, and then re-equilibration at 50% B from 36 to 43 min. Mass spectrometric analysis was performed using a QTRAP® 3200 mass spectrometer (AB Sciex, Framingham, MA, U.S.A.). The ionization source was electrospray ionization, and EETs and DHETs were quantified using multiple reaction monitoring operated in the negative ion mode. The limit of quantitation of this method for EETs and DHETs were 5 and 2 nm, respectively.

**Kinetics Study of Arachidonic Acid Metabolism** The Michaelis–Menten kinetic parameters ($K_m$ and $V_{max}$) for AA metabolism were determined over the concentration range of 0–100 μM AA in an incubation study using rCYP2C8. After 3 min pre-incubation at 37°C, the reaction was initiated by addition of NADPH regeneration system solutions. Each incubation mixture consisted of AA, 50 mM phosphate buffer (pH 7.4), 2 pmol P450/rCYP2C8 (11 mg protein/mL), and NADPH regeneration system\textsuperscript{20} solutions A and B in a final volume of 200 μL. Samples were incubated for 30 min and the reaction was stopped with 50 μL of ice-cold acetonitrile. After adding a solution of internal standards, EETs and DHETs were extracted with ethyl acetate using an OASIS® HLB cartridge. Extracted samples were evaporated to dryness and reconstituted with 50 μL of 50% acetonitrile, and then 40 μL was injected into the LC-MS/MS system.

**Inhibition Study Using Telmisartan** Various final concentrations of telmisartan (0–100 μM) were incubated with rCYP2C9 or rCYP2C8 (3.9 or 6.4 mg protein/mL, respectively), 50 mM phosphate buffer (pH 7.4), and NADPH regeneration system\textsuperscript{21} solutions A and B to prepare a final volume of 200 μL. Since CYP2C9 is less active in phosphate buffer according to the package insert of rCYP2C9 (Corning), 100 mM Tris–HCl buffer (pH 7.4) was used for the experiments using rCYP2C9 instead of 50 mM phosphate buffer. After 3 min pre-incubation at 37°C, the reaction was initiated by the addition of AA. The AA concentrations were set at 10, 20, and 40 μM for the CYP2C8 experiments based on the $K_m$ value from the above-described kinetics studies, and at 7.5, 15, and 30 μM for the CYP2C9 experiments based on the $K_m$ values previously obtained (unpublished data). Sample purification and the quantification of EETs and DHETs were conducted as described above.

**Inhibition Study Using CYP Inhibitors and Anti-CYP Antibodies** Inhibition studies using CYP inhibitors or anti-CYP antibodies in HLMs were conducted to calculate the CR of CYP2C9 and CYP2C8 on AA metabolism. Sulfaphenazole (a typical inhibitor of CYP2C9) or quercetin (a typical inhibitor of CYP2C8) at concentrations of 10 μM was incubated with HLMs (0.25 mg protein) in 50 mM Tris–HCl buffer (pH 7.4). We used AA contained in HLMs as a substrate (approx. 20 μM). After pre-incubation at 37°C for 3 min, the reaction was started by adding 1 mM NADPH. The reaction mixture was incubated for 10 min, then 1 mL ice-cold ethanol was added to terminate the reaction. In accordance with the manufacturer’s instructions, pAb-2C9 (2.5 mg pAb protein/mg HLM protein) or mAb-2C8 (0.5 mg mAb protein/mg HLM protein) was pre-incubated with HLMs (0.25 mg protein for pAb-2C9 or 0.0625 mg protein for mAb-2C8) in 50 mM Tris–HCl buffer (pH 7.4) at room temperature for 10 min or 20 min on ice, respectively. The reactions were conducted as described for the inhibition study using CYP inhibitors. After centrifugation of the samples at 6490×g for 5 min, the supernatants were mounted onto OASIS® HLB cartridges. Sample purification was carried out as described above.

**Single Time Point Time-Dependent Inhibition Assay** Single time point time-dependent inhibition assays were conducted using the method previously reported.\textsuperscript{25,26} Enzymatic activity remaining after 30 min pre-incubation in the presence or absence of NADPH was determined and the percentage of CYP enzymatic activity loss (% activity loss) was calculated using Eq. 1:

$$% \text{ activity loss} = 100 \times \left\{ 1 - \left( \frac{A_{\text{telmisartan, NADPH+}}} {A_{\text{vehicle, NADPH+}}} \right) \left( \frac{A_{\text{vehicle, NADPH-}}} {A_{\text{telmisartan, NADPH-}}} \right) \right\}$$

where $A_{\text{telmisartan, NADPH+}}$ represents the production rate of metabolites after pre-incubation with telmisartan and NADPH, $A_{\text{vehicle, NADPH+}}$ represents the production rate of metabolites after pre-incubation without telmisartan but with NADPH, $A_{\text{telmisartan, NADPH-}}$ represents the production rate of metabolites after pre-incubation with telmisartan but without NADPH, and $A_{\text{vehicle, NADPH-}}$ represents the production rate of metabolites after pre-incubation without telmisartan or NADPH. The cut-off value of percentage activity loss was set at 20% as reported previously.\textsuperscript{25} HLMs (0.25 mg protein) were pre-incubated at 37°C for 30 min after adding 50 μM telmisartan with 1 mM NADPH or H$_2$O in 50 mM Tris–HCl buffer. After 30 min, 25 μL aliquots of the pre-incubation mixture were diluted 10-fold with a secondary incubation mixture containing fresh Tris–HCl buffer, 1 mM NADPH, and probe substrate (25 μM diclofenac for CYP2C9 or 30 μM paclitaxel for CYP2C8). Substrate concentrations were set at 4-fold the $K_m$ value to minimize the effect of reversible inhibition by telmisartan.\textsuperscript{25} The secondary incubation was carried out for
RESULTS

Kinetic Parameters of Arachidonic Acid Metabolism by CYP2C8  Incubation studies of AA with rCYP2C8 were used to determine the production rate of total eicosanoids, calculated as the sum of EETs and DHETs. The assay revealed that the production rate of total eicosanoids rose with increasing AA concentration, plateauing at AA concentrations of 20 mM ammonium acetate buffer (pH 5.0), 42.58, uM delivered at a flow rate of 0.9 mL/min. 4'-Hydroxydiclofenac, a metabolite of diclofenac, was also quantified by a UV detector at 280 nm. Mobile phase was acetonitrile–75 mM sodium acetate buffer (pH 5.0; 32 : 68, v/v) delivered at a flow rate of 0.8 mL/min.

Data Analysis  The $K_m$ and $V_{max}$ values were calculated by nonlinear regression analysis. The production rate of total eicosanoids in the presence of CYP inhibitors or anti-CYP antibodies were compared with that in samples lacking these inhibitors or antibodies. Dunnett’s test or t-test was performed using GraphPad Prism software version 6.0 (GraphPad Software Inc.; San Diego, CA, U.S.A.). To determine the $K_i$ value and the type of inhibition, the production rate of the 14,15- and 11,12-forms, calculated at the sum of the EET and the corresponding DHET of each regioisomer, were analyzed by Dixon plots27 and Lineweaver–Burk plots28 using SigmaPlot 12.5 (Systat Software, San Jose, CA, U.S.A.). The data were fitted to competitive, noncompetitive, and mixed inhibition model, and the best-fit model was selected based on Akaike’s information criteria (AIC) and $r^2$ squared ($r^2$).

DISCUSSION

In this study, we elucidated the mechanism of inhibition of AA metabolism by telmisartan. Incubation studies with rCYP2C8 produced by a baculovirus expression system revealed enzyme kinetic parameters for the AA metabolism of CYP2C8, was quantified by a UV detector at 240 nm. The mobile phase was acetonitrile and 20 mM ammonium acetate buffer (pH 5.0; 42 : 58, v/v) delivered at a flow rate of 0.9 mL/min. 4'-Hydroxydiclofenac, a metabolite of diclofenac, was also quantified by a UV detector at 280 nm.

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The production rate of total eicosanoids rose with increasing AA concentration, plateauing at AA concentrations of 20 mM ammonium acetate buffer (pH 5.0), 42.58, uM delivered at a flow rate of 0.9 mL/min. 4'-Hydroxydiclofenac, a metabolite of diclofenac, was also quantified by a UV detector at 280 nm. Mobile phase was acetonitrile–75 mM sodium acetate buffer (pH 5.0; 32 : 68, v/v) delivered at a flow rate of 0.8 mL/min. Both metabolites were separated using a Kinetix® C18 column (250×4.6 mm, 5 µm, Phenomenex, Torrance, CA, U.S.A.).

6α-hydroxylation via CYP2C8 were calculated as 5.8 and 8.1%, respectively. Since these values were less than 20%25, these results suggested that telmisartan is not a time-dependent inhibitor, and irreversible inhibition is not related to AA metabolism.

Contribution Ratios of CYP2C9 and CYP2C8 in AA Metabolism  The CRs of CYP2C9 and CYP2C8 in AA metabolism were determined from the inhibition fraction of each CYP enzyme by inhibition experiments using CYP inhibitors and anti-CYP antibodies. The inhibition fractions of CYP2C9 by sulfaphenazole and pAb-2C9 were calculated to be 52 and 58% (p<0.001, compared with control), respectively, whereas quercetin and mAb-2C8 inhibited CYP2C8 activity by 30 and 39% (p<0.001, compared with control), respectively (Fig. 4).

In bovine coronary arteries, all the regioisomers act as vasodilators.13) 14,15-EET has been shown to have cardiac preconditioning effects.15) 11,12-EET has been shown to exert

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anti-inflammatory effects by decreasing the expression of the cell adhesion molecule vascular cell adhesion molecule-1.14) The sum of the production ratio of 14,15- and 11,12-forms are 89% for CYP2C9 and 99% for CYP2C8 in this study, and therefore the data for the 8,9- and 5,6-forms were excluded from the analysis. DHETs were detected in the incubation samples, despite the absence of sEH in rCYPs. EETs and DHETs were not determined in rCYPs and control microsomes after incubation without AA (data not shown), and thus we considered that EETs and DHETs in the incubation samples were generated from added AA. The mechanism of DHETs production from EETs in rCYPs remains unclear. Therefore, we analyzed the sum of each EET and the corresponding DHET to determine the type of inhibition and $K_i$ values.

The calculated AIC values indicated that telmisartan is a noncompetitive inhibitor of CYP2C9 and a competitive inhibitor of CYP2C8. However, the absolute difference in AIC values between mixed and competitive or noncompetitive models was 2.0±0.6 (mean±S.D., range; 0.9–2.7). The AIC is considered to provide little evidence for model selection when the AIC difference between two models is less than 2.30) At the same time, the $r^2$ values suggested that the mixed inhibition model is the best-fit for both CYP enzymes.

We have already reported that the IC$_{50}$ values of telmisartan on AA metabolism in rCYP2C8, rCYP2C9, and HLMs were 49.5, 34.6, and 24.1 $\mu$M, respectively.21,22) These results suggested that telmisartan showed similar inhibitory effects in rCYP2C8, rCYP2C9, and HLMs. EETs are produced in several tissues3–12) and act as autocrine and paracrine factors that influence vascular tone.31) Since the liver contains abundant CYP enzymes and EETs constituted 14 to 28% of total metabolites of AA in HLMs,4) EETs produced in the liver seem to affect the circulation levels of EETs.

Ren et al. recently reported that telmisartan exhibits mixed inhibition of CYP2J2 by binding to the heme pocket.24) Telmisartan contains an imidazole ring (Fig. 5); thus, according to this mechanism, the imidazole nitrogen could bind to the heme in CYP2C9 and CYP2C8, thereby providing nonselective inhibition of enzyme activity. Therefore, we infer that telmisartan is a mixed inhibitor of AA metabolism by CYP2C9 and CYP2C8, causing allosteric inhibition of AA metabolism by these enzymes.

Polyclonal antibody against CYP2C9 was used to investigate the CR of CYP2C9 on AA metabolism, given that monoclonal antibody against human CYP2C9 is not commercially available. We confirmed that pAb-2C9 does not exhibit cross-reactivity to CYP2C8 at 2.5 mg pAb protein/mg HLM protein (data not shown). The estimated CRs of CYP2C in the present study were larger than the values reported by Lundblad et al.8) possibly due to the differences in the P450 content and the catalytic activities of HLMs32) between two studies. In the present study, the CR of CYP2C9 with anti-CYP antibodies

![Graphs](image-url)
was similar to that of CYP inhibitors (Fig. 4). The concentration of quercetin was limited to 10 µM to avoid non-specific inhibition; as a result, CYP2C8 inhibition was insufficient. Higher expression of CYP2C9 protein compared to CYP2C8 protein has been reported in HLMs, whereas CYP2J2 in the liver constitutes 1–2% of the total CYP content. Our results suggest that CYP2C9 plays a major role in AA metabolism in HLMs, consistent with a previous report. Furthermore, our results indicate that CYP2C8 is also the key enzyme in AA metabolism in the liver, since the CR value of CYP2C8 was calculated to be over 30%. Therefore, telmisartan–drug and telmisartan–endogenous substance interactions are presumed to be mediated via both CYP2C8 and CYP2C9.

Table 2. \( K_i \) Values of Telmisartan on the Metabolism of Arachidonic Acid

|                | CYP2C9 (\( n=3\)-5) | CYP2C8 (\( n=4\)-7) |
|----------------|---------------------|---------------------|
| 14,15-Form     | 30.5±13.4           | 27.2±8.4            |
| 11,12-Form     | 36.8±17.2           | 20.0±4.8            |

Table 2: \( K_i \) Values of Telmisartan on the Metabolism of Arachidonic Acid

Using a reported equation, the unbound maximum hepatic input concentration of telmisartan was calculated to be 0.12 µM after multiple doses of 160 mg telmisartan. This value is lower than calculated \( K_i \) values in rCYPs (Table 2). However, it has been reported that radioactivity of telmisartan in the liver was over 40-fold higher than that of plasma in rats and the saturation of OATP1B3 has not observed in clinical dosage of telmisartan. Therefore, the concentration of telmisartan in the liver is likely to be over \( K_i \) values, it is possible that high-dose telmisartan might inhibit the CYP enzymes in the liver. The implications for cardiovascular risk events at high doses of telmisartan remain to be studied.

In conclusion, our results suggest that telmisartan decreases
the production of EETs and DHETs from AA by acting as a mixed inhibitor of CYP2C9 and CYP2C8. The inhibitory effect of telmisartan would occur within its clinical dosage range. Our findings may have clinical implications since EETs have cardiovascular protective effects, including vasodilation, anti-inflammation, and myocardial preconditioning effects. Further studies are required to determine the plasma concentration of EETs and DHETs in patients taking telmisartan, and to elucidate the relationship between EET and DHET concentrations and the risk of cardiovascular events.

Acknowledgments The authors would like to thank Ms. Yuki Saeki, Ms. Nao Nakayama, Mr. Taishiro Oguro, and Mr. Toshinori Nitanda for their technical assistance.

Conflict of Interest The authors declare no conflict of interest.

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