Inhibitory Effects of Angiotensin Receptor Blockers on CYP2C9 Activity in Human Liver Microsomes

Emi KAMIYAMA*, Yasushi YOSHIGAE, Atsushi KASUYA, Makoto TAKEI, Atsushi KURIHARA and Toshihiko IKEDA

Drug Metabolism and Pharmacokinetics Research Laboratories, Sankyo Co., Ltd., Tokyo, Japan

Summary: We investigated the inhibitory effects of the angiotensin receptor blockers (ARBs), candesartan, irbesartan, losartan, losartan active metabolite (EXP-3174), olmesartan, telmisartan and valsartan (0.3–300 μM), on the CYP2C9 activity in human liver microsomes using (S)-(−)-warfarin as a typical CYP2C9 substrate.

Except for olmesartan and valsartan, these ARBs inhibited the activity of 7-hydroxylation of (S)-(−)-warfarin with IC50 values of 39.5–116 μM. Of six synthetic derivatives of olmesartan, five compounds which possess either alkyl groups or a chloro group at the same position as that of the hydroxyisopropyl group in olmesartan inhibited CYP2C9 activity with IC50 values of 21.7–161 μM. Olmesartan and the olmesartan analogue, RNH-6272, both having a hydroxyisopropyl group, showed no inhibition, indicating that the hydrophilicity of this group greatly contributes to the lack of CYP2C9 inhibition by these two compounds. A three-dimensional model for docking between EXP-3174 and CYP2C9 indicated that the chloro group of EXP-3174 is oriented to a hydrophobic pocket in the CYP2C9 active site, indicating that the lipophilicity of the group present in ARBs at the position corresponding to that of the hydroxyisopropyl group in olmesartan is important in inhibiting CYP2C9 activity.

Key words: angiotensin receptor blocker; CYP2C9; human liver microsome; warfarin; inhibition; IC50; lipophilicity

Introduction

Angiotensin II type 1 receptor blockers (ARBs) have been used in the treatment of hypertension not only in monotherapy but also in combination therapy with other antihypertensive drugs such as a calcium channel blocker or an angiotensin I converting enzyme inhibitor.1,2) Currently, candesartan cilexetil (pharmacologically active metabolite: candesartan), irbesartan, losartan (pharmacologically active metabolite: EXP-3174), olmesartan medoxomil (pharmacologically active metabolite: olmesartan), telmisartan and valsartan are marketed and used clinically (Fig. 1). Hypertensive patients are generally senile and are often afflicted with other diseases, most typically with hyperlipidemia and diabetes mellitus. They are generally receiving multidrug therapy, and therefore, it is important to investigate the possibility of drug-drug interaction between antihypertensive drugs including ARBs and concomitant drugs.

Most ARBs are known to be metabolized by CYP2C9,1–5) one of the major isoforms of cytochrome P450 (P450) in human liver microsome, thereby catalyzing the oxidation of many clinically important drugs.6) In particular, losartan is converted to the pharmacologically active metabolite EXP-3174 by mainly CYP2C9,7,8) and one of metabolites of irbesartan catalyzed by CYP2C9 has very low pharmacological activity.3,9) On the other hand, candesartan and valsartan are metabolized to a pharmacologically inactive metabolite, albeit slowly, by the action of CYP2C9.5,10–12) Among ARBs, only two drugs, telmisartan and olmesartan are inert as substrates of P450 isoforms, including CYP2C9.2,13,14) Telmisartan is partially metabolized by glucuronidation,3,5,13) and olmesartan is excreted as unchanged olmesartan without undergoing metabolism.14) Thus, CYP2C9 is involved in the metabolism of most ARBs, but the contribution of this enzyme is different in each ARB. All ARBs commonly possess a 4-(2'-tetrazolyl)biphenyl group or its bioisosteric 4-(2'-...
carboxy)biphenyl group in their molecules (Fig. 1), though their metabolic fates through oxidation by CYP2C9 are different, probably due to a part of the molecule other than the common group having a different affinity to CYP2C9.

Many models have been proposed for the ligand recognition of CYP2C9, and recently, the crystal structures of CYP2C9-warfarin complex and CYP2C9-(S)-flurbiprofen complex have been determined.\textsuperscript{15,16} In the structure of CYP2C9-flurbiprofen complex, the flurbiprofen molecule was located at a position in which the hydroxylation site is 4.9 Å from the heme iron and the carboxyl group interacts with Arg108 and Asn204 of CYP2C9, showing good agreement with the pharmacophore models and the results of site-directed mutagenesis studies.\textsuperscript{16} These resent progress of three dimensional structure models of CYP2C9 are very helpful to understand the relationship between structures of ligands (substrates or inhibitors) and their affinity to the enzyme.

In the present study, we focus on relationship between structures of ARBs and their inhibitory effect on CYP2C9 activities. The $IC_{50}$ values of seven ARBs in the inhibition of CYP2C9 activity in human liver microsomes were determined using (S)-(−)-warfarin as the substrate. No marked inhibition of the CYP2C9 activity was observed in the case of olmesartan and valsartan. Since various synthetic derivatives of olmesartan are available, we also determined the $IC_{50}$ values of these olmesartan analogues and employ docking model of ligand-enzyme complex in order to investigate the relationship between their structures and affinity to CYP2C9 enzyme.

**Materials and Methods**

**Chemicals:** RNH-6270 (olmesartan, content 96.0% by HPLC), RNH-6272 (content 98.9% by HPLC), RNH-6384 (content 96.1% by HPLC), RNH-6390 (con-
CYP2C9 inhibition assay: The activity of CYP2C9 was measured as the activity of warfarin 7-hydroxylase. The incubation mixture (225 μL) consisted of 0.5 mg protein/mL of human liver microsomes, 10 mM of potassium phosphate buffer (pH 7.4), varying concentrations of ARBs in dimethyl sulfoxide (final concentration: 0, 0.3, 1, 3, 10, 30, 100 and 300 μM) and 5 μM of (S)-(−)-warfarin in methanol in each well of a 96-well plate. After pre-incubation of the mixture at 37°C for 3 min, a reaction was started by the addition of 2 mM NADPH (25 μL). After incubation for 40 min, a 100 μL-aliquot of the incubation mixture was collected, and added to a mixture of 200 μL of methanol containing 1 μM of coumachlorine as the internal standard substance to stop the reaction. This mixture was centrifuged at 3,000 rpm for 10 min at 4°C to precipitate the protein. The assays were carried out in quadruplicate for ARB or duplicate for olmesartan analogue. The supernatant fractions were applied to an HPLC system consisting of an Alliance 2795 separation module (Waters Corporation, Milford, MA, USA) interfaced to a Quattro LC (Waters Corporation). The samples were separated on an Inertsil ODS-3 column (GL Sciences Inc., Tokyo, Japan) maintained at 40°C using a mobile phase of methanol/10 mM ammonium acetate (70/30, v/v) at pH 4.0 when candesartan was used as an inhibitor or at pH 5.15 when olmesartan analogues were used as inhibitors, or using a mobile phase of methanol/10 mM ammonium acetate (65/35, v/v) at pH 4.0 when other ARBs were used as inhibitors. The ionization modes for determination of 7-hydroxywarfarin and coumachlorine were ESI-negative. The capillary voltage, source block temperature and desolvation temperature were 3.5 kV, 120°C and 350°C, respectively. Each optimized multiple reaction monitoring condition was used. Inhibitory activity was expressed as a percentage against the activity of the control incubations (no inhibitor). The IC_{50} values were calculated with the computer software WinNonlin Professional (version 3.1, Pharsight Corporation, Mountain View, CA, USA) and Microsoft Office Excel 2000 and 2003 (Microsoft Corporation). For determination of K_{i} value of losartan, varying concentrations of losartan (final concentration: 3, 10, 30 and 100 μM) and (S)-(−)-warfarin (final concentration: 2.5, 5 and 10 μM) were used, and formation of 7-hydroxywarfarin was determined as described above. The apparent K_{i} value of losartan was estimated from Dixon plots.

Measurement of LogD: The pH-dependent partition coefficient, LogD, was determined at 25°C under varying pH conditions by a shake-flask method using 1-octanol as the organic phase and Britton-Robinson buffer as the aqueous phase (pH: 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 7.4).

Each ARB (5 mg) was dissolved in either 20 mL of 1-
octanol saturated with the Britton-Robinson buffer or in 20 mL of the Britton-Robinson buffer saturated with 1-octanol, as a stock solution. A 4 mL-aliquot of the stock solution was added to a flask containing 4 mL of the counter solution, 1-octanol or the Britton-Robinson buffer. The flask containing the two phases was shaken using a seesehen device (FNX-11-20) for 5 min, and centrifuged (3000 rpm) for 10 min. After separation of both solutions, each layer was collected, and diluted with an equal volume of acetone. The concentrations of ARBs were determined by the HPLC method described below. The measurements were performed in triplicate under each pH condition.

The samples were analyzed on an Alliance 2695 separation module (Waters Corporation) equipped with a photodiode array detector, 2996 (Waters Corporation). Chromatographic separation was carried out using an XTerra MS C18 column (100 mm in length, with an internal diameter of 4.6 mm and a particle size of 3.5 μm, Waters Corporation) maintained at 40°C with the mobile phase consisting of solvent A (one volume of phosphoric acid in 1000 volumes of water) and solvent B (one volume of phosphoric acid in 1000 volumes of acetonitrile). A gradient elution mode using a mixture of solvent A and solvent B with a different time program was adopted for the analysis of each ARB. The flow rate was 1.2 mL/min.

The LogD values for the ARBs were calculated as a partition between the Britton-Robinson buffer and 1-octanol according to the equation below, where Co is the concentration in the organic phase and Cw is the concentration in the aqueous phase.

\[
\text{LogD} = \log \left( \frac{C_o}{C_w} \right)
\]

**Calculation of CLogP values:** The CLogP values were calculated with the computer software CLOGP (Daylight Version 4.82, Daylight Chemical Information Systems, Inc., Aliso Viejo, CA, USA).

**Docking model analysis:** Docking studies employing AUTODOCK (Version 3.0, Scripps Research Institute, La Jolla, CA, USA) were carried out to obtain model structures of CYP2C9-EXP-3174 complex. Automated dockings were performed using the CYP2C9 structure from flurbiprofen-complex. Among the ten structures obtained from randomly seeded searches using a Lamarckian genetic algorithm, three had a binding mode in which the site of hydroxylation, the n-butyl group of EXP-3174, was positioned about 3.5 Å from the heme iron and in which the tetrazole group formed a salt bridge with Arg-109.

**Results and Discussion**

**Inhibitory effects of ARBs on 7-hydroxylation of S-(−)-warfarin:** Experiments to examine the inhibitory effects of seven ARBs (candesartan, irbesartan, losartan, EXP-3174, olmesartan, telmisartan and valsartan) on the CYP2C9-mediated 7-hydroxylation of S-(−)-warfarin were carried out using human liver microsomes, as shown in Fig. 3. The IC50 values of the ARBs were 39.5 μM for losartan, 41.9 μM for telmisartan, 48.9 μM for EXP-3174, 57.7 μM for candesartan and 116 μM for irbesartan. Interestingly, the IC50 value (41.9 μM) of telmisartan, which does not serve as a substrate of CYP2C9, was the same level as that (39.5 μM) of losartan, indicating that telmisartan has significant affinity to CYP2C9. On the other hand, olmesartan and valsartan, which are a non-substrate and a poor substrate of P450, respectively, showed no inhibition in the range of concentration investigated (0.3—300 μM).

The losartan showed the lowest IC50 values and we estimated Ki value of losartan to assess clinical risk of drug-drug interaction, as shown in Fig. 4. From the Dixon plot (Fig. 4), apparent Ki value of losartan was estimated to be 5.75 μM and apparent Ki value based on free concentration was calculated to be 4.6 μM from the binding ratio of losartan in microsomal protein (20%). This free Ki value was more than 500 times higher than free plasma concentration of losartan (8.3 nM) after oral administration of a 50 mg losartan tablet (Cmax of total concentration: 642 nM, plasma free fraction; 0.013). Even taking account the maximum concentration input into the liver, it is unlikely that the effective inhibitory concentrations would be attained in clinical treatment of losartan. Similar to that, we think other ARBs (candesartan, irbesartan and telmisartan) also are unlikely to show significant clinical risk of CYP2C9 inhibition taken together the consideration of relatively low in vitro inhibitory potential and low plasma free concentration due to strong protein binding of these drugs.

As described above, the inhibitory effects on CYP2C9 activity were strikingly different among these ARBs, although the ARBs investigated have similar structures commonly containing the 4-(2'-tetrazolyl)biphenyl group or its bioisosteric 4-(2'-carboxy)biphenyl group. It should also be noted that the small difference in the chemical structure between EXP-3174 and olmesartan, as shown in Fig. 1, made a large difference in the affinity to CYP2C9.

**Physicochemical properties:** The relationship in the ARBs between their lipophilicity and affinity for CYP2C9 was examined under varying pH conditions (Fig. 5). The LogD value (~−0.85) of olmesartan, which showed almost no affinity to CYP2C9 in the inhibition experiment, was lower at pH 3–5 than the values of the other ARBs (candesartan: 1.55–3.3, losartan: 2.7–3.3, EXP-3174:1.4–3.3, irbesartan: 2.89–3.04, telmisartan: 2.6–3.3, valsartan: 1.65–2.98). At pH 7.4, on the other hand, the LogD values of candesartan, EXP-3174, olmesartan and valsartan were almost at the
same levels and were all negative values (−1.3 ~ −0.79). These four ARBs have both a 4-(2’-tetrazolyl) biphenyl group and a carboxylic acid group in their structures, and these two acidic groups were regarded to dissociate at pH 7.4, giving a highly hydrophilic nature to the molecule.

The CLogP is regarded as representing the lipophilicity of the molecule under an undissociated condition, and this value of olmesartan was 2.8 and was lower by about one than those of the other ARBs (losartan: 4.1, EXP-3174: 4.6, valsartan: 4.9, candesartan: 5.4, irbesartan: 6.0, telmisartan: 7.5). These results for LogD and CLogP indicate that olmesartan is much more hydrophilic compared to other ARBs, quite conceivably due to the presence of one more polar group, hydroxyisopropyl group, in addition to a 4-(2’-tetrazolyl) biphenyl group, a carboxylic acid group and an imidazolyl group. The CLogP value of telmisartan was 7.5 and was highest among all the ARBs, indicating the highest lipophilicity of telmisartan among the ARBs.

It is well known that affinity to P450 as a substrate is, in general, correlates with lipophilicity, which is typical.
ly represented by LogD or LogP. Thus, we examined the correlation of the LogD/CLogP values with the IC50 values in the inhibition of the 7-hydroxylation of (S)-(-)-warfarin. Although the CLogP values apparently correlated with the IC50 values, there was no statistically significant correlation, with the IC50 values of losartan and EXP-3174 being outliers in the correlation (r²: 0.1918, Fig. 6). As mentioned below we found that interactions between the chloro group and hydrophobic pocket of the CYP2C9 may result their low IC50 values, and when losartan and EXP-3174 were excluded in the correlation analysis, a statistically significant correlation between the IC50 and CLogP values was observed (r²: 0.6699), with increasing lipophilicity being associated with decreasing inhibition of the (S)-(-)-warfarin hydroxylation. This correlation indicates that the almost complete lack of affinity of olmesartan or the highest affinity of telmisartan to CYP2C9 is either due to high hydrophilicity or to the highest lipophilicity among the ARBs.

**Inhibitory effects of olmesartan analogues on 7-hydroxylation of S(-)-warfarin:** As described above, olmesartan showed the lowest CLogP value among the ARBs and almost no inhibition to CYP2C9 activity, which is most likely due to the presence of a hydroxyisopropyl group in the molecule. To study the contribution of the hydroxyisopropyl group to the decreased affinity of olmesartan to CYP2C9, five olmesartan analogues with the hydroxyisopropyl group being substituted with either alkyl groups or a chloro group (RNH-6384, RNH-6390, RNH-6391, RNH-6458 and RNH-8239, Fig. 2) and one olmesartan analogue with a propyl group at the imidazole ring, which was substituted with a butyl group while the other parts of the molecule were unchanged (RNH-6272, Fig. 2), were examined in terms of their inhibitory effects on the (S)-(-)-warfarin hydroxylation, as shown in Fig. 7. No marked inhibition on the hydroxylation of (S)-(-)-warfarin was observed by the addition of olmesartan and RNH-6272, both having a hydroxyisopropyl group (Fig. 7). For RNH-6384, RNH-6390, RNH-6391 or RNH-6458, which has an isopropyl group, an ethyl group, a methyl...
Inhibitory Effect of Angiotensin Receptor Blockers to CYP2C9

Fig. 6. Correlation between CLogP and IC50 in CYP2C9 inhibition.
The lower line represents the CLogP-IC50 correlation including all the ARBs (candesartan: ○, irbesartan: ◇, losartan: ▲, EXP-3174: △, olmesartan: ●, telmisartan: ■ and valsartan: ○) and the upper line represents the correlation including ARBs other than losartan and EXP-3174.

Docking models of EXP-3174 to CYP2C9:

The difference in the chemical structure between EXP-3174, which is a CYP2C9 inhibitor, and olmesartan, which is a non-inhibitor of CYP2C9, is small as EXP-3174 has a chloro group and a butyl group in the imidazole ring, and olmesartan correspondingly has a hydroxyisopropyl group and propyl group (Fig. 1). Therefore, the effect of the chloro group was examined based on docking models of EXP-3174 to CYP2C9 generated by using AUTODOCK so that the binding mode of EXP-3174 is consistent with its known site of metabolism. In these models, the chloro group of EXP-3174 formed a hydrophobic interaction with the Ala477 and Ile205 of the enzyme. It was assumed that olmesartan, having a hydroxyisopropyl group instead of a chloro group, would show only weak interaction with the hydrophobic pocket when it was bound to the enzyme in a manner similar to EXP-3174. These results indicated that the affinity of ARBs to CYP2C9 is determined not only by the lipophilicity of the whole molecule but also by the lipophilicity of the group present at the same position as that of the hydroxyisopropyl group in olmesartan or the chloro group in losartan and EXP-3174. In fact, RNH-6384, RNH-6390, RNH-6391 and RNH-6458, which have different alkyl groups at the same position, showed an inhibitory effect on (S)-(−)-warfarin hydroxylation.

Valsartan also had no inhibitory effect on the CYP2C9 activity in the present study. Since the synthetic derivatives of valsartan were not available, we were unable to investigate the structure-CYP2C9 inhibition relationship, though we assume that the lack of an imidazole ring in its structure allowed the free rotation of the groups other than the 4-(2'-tetrazolyl)biphenyl group. This would explain the low affinity of this drug to CYP2C9, resulting in the lack of CYP2C9 inhibition.

In the present study, among the seven clinically available ARBs investigated, candesartan, irbesartan, losartan, EXP-3174 and telmisartan moderately inhibited CYP2C9 in vitro. However, it is unlikely that effective concentrations for CYP2C9 inhibition would be attained in clinical treatment with losartan and other ARBs taking into the consideration their relatively high inhibitory concentrations compared to their low free plasma concentrations in vivo. ARBs are known to show the most potent inhibitory effect on CYP2C9 among P450s, and therefore in this study we focus on their inhibitory effect on this enzyme. However, the risk
Fig. 7. Inhibitory effect of RNH-6270 analogues on 7-hydroxylation of S-(−)-warfarin in human liver microsomes. The final concentration of S-(−)-warfarin was 5 μM. The reaction mixture was incubated at 37°C for 40 min. Each symbol represents the mean of duplicate determinations.

of clinical drug-drug interaction of these drugs based on other P450s needs to be further examined because among ARBs losartan and irbesartan also inhibited other P450s including CYP3A4, which is expressed in the intestine as well as the liver.

Among the ARBs we tested, olmesartan and valsartan showed very low affinity to CYP2C9 in the in vitro assay. It was previously shown that these drugs also hardly affect other P450s activities in vitro.22,23) From the inhibitory effect of olmesartan analogs and the docking model analysis, the low affinity of olmesartan to CYP2C9 was considered to be due to the presence of an additional polar group in its molecule, namely a hydroxyisopropyl group, which is inept at favorably interacting with the hydrophobic pocket of CYP2C9. These observations indicated that part of the chemical structure of ARBs greatly affects their affinity to CYP2C9 enzyme.

References


