In Vitro Inhibition of CYP2C9-Mediated Warfarin 7-Hydroxylation by Iguratimod: Possible Mechanism of Iguratimod–Warfarin Interaction

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Iguratimod is a novel disease-modifying antirheumatic drug. A blue letter (safety advisory) for drug interaction between iguratimod and warfarin was issued by the Ministry of Health, Labour and Welfare of Japan in May 2013. Iguratimod may affect warfarin metabolism catalyzed by CYP. However, it is not clear whether iguratimod inhibits warfarin oxidation. This study was performed to investigate the effects of iguratimod on warfarin 7-hydroxylation with human liver microsomes (HLMs) and recombinant CYP enzymes. Iguratimod concentration-dependently inhibited R,S-warfarin 7-hydroxylase activity of HLMs with an IC₅₀ value of 15.2 μM. The inhibitory effect was examined with S-warfarin and R-warfarin to determine which enantiomer was more potently inhibited by iguratimod. Iguratimod potently inhibited the S-warfarin 7-hydroxylase activity of HLMs with an IC₅₀ value of 14.1 μM, but showed only slight inhibition of R-warfarin 7-hydroxylase. Furthermore, iguratimod inhibited the S-warfarin 7-hydroxylase activity of recombinant CYP2C9.1 (rCYP2C9.1) and rCYP2C9.3 in a concentration-dependent manner with IC₅₀ values of 10.8 and 20.1 μM, respectively. Kinetic analysis of the inhibition of S-warfarin 7-hydroxylation by iguratimod indicated competitive-type inhibition for HLMs and rCYP2C9.1 but mixed-type inhibition for rCYP2C9.3. The Kᵢ values for HLMs, rCYP2C9.1, and rCYP2C9.3 were 6.74, 4.23, and 14.2 μM, respectively. Iguratimod did not exert metabolism-dependent inhibition of S-warfarin 7-hydroxylation. These results indicated that iguratimod is a potent direct inhibitor of CYP2C9-mediated warfarin 7-hydroxylation and that its inhibitory effect on CYP2C9.1 was more sensitive than that on CYP2C9.3.

Key words: iguratimod; warfarin; CYP2C9; inhibition; drug–drug interaction; polymorphism

Iguratimod is a novel disease-modifying antirheumatic drug, which has been used for the treatment of rheumatoid arthritis exclusively in Japan and China. It is notable that iguratimod exerts a beneficial effect in rheumatoid arthritis patients with inadequate response to methotrexate. Iguratimod is extensively metabolized by the liver, and several metabolites of iguratimod have been characterized in plasma from healthy male subjects following oral administration of the drug. (Fig. 1). The major metabolites are a deformylated form (M1) and its N-acetylated form (M2). The other metabolites are p-hydroxylated forms in the 6-phenoxyl groups of iguratimod, M1, and M2, which have been termed M4, M5, and M3, respectively. CYP is unlikely to be involved in the formation of M1 from iguratimod because M1 is formed regardless of recombinant CYP isoforms examined and the presence or absence of reduced nicotinamide adenine dinucleotide phosphate (NADPH). M1 was suggested to be metabolized by N-acetyltransferase to produce M2. On the other hand, iguratimod is metabolized by multiple isoforms, including CYP1A2, CYP2C9, and CYP3A4, to produce M4 and/or M5.

When manufacturing approval was granted in Japan, the instructions indicated that care should be taken on combined use of iguratimod with warfarin based on the observation that iguratimod prolonged prothrombin time in rats coadministered warfarin. Despite this instruction, a few patients with severe bleeding were reported in a blue letter (safety advisory) for drug interaction between iguratimod and warfarin issued by the Ministry of Health, Labour and Welfare of Japan in May 2013. Consequently, the use of iguratimod in combination with warfarin has been changed from a precaution to contraindication. Warfarin is a racemic mixture of S-warfarin and R-warfarin. S-Warfarin exerts a 3- to 5-fold more potent anticoagulant effect than R-warfarin. It has been reported that S-warfarin is mainly metabolized by CYP2C9, whereas R-warfarin is predominantly metabolized by CYP1A2 and CYP3A4. However, the mechanism underlying iguratimod–warfarin interaction remains unclear.

The in vitro inhibitory effect of iguratimod was examined in human liver microsomes (HLMs), as described in a report on the deliberation results for iguratimod published by the Ministry of Health, Labour and Welfare of Japan. Iguratimod at a concentration of 100 μM inhibited CYP2C9 activity by approximately 50%. Based on the inhibitory effect and maximum unbound hepatic input concentration, it was estimated that the possibility of interactions between iguratimod and drugs metabolized by CYP2C9 is low. We note here that tolbutamide was used as a probe substrate for CYP2C9 in this previous study, as described in an in-house document of Toyama Chemical Co., Ltd. The inhibitory potencies of CYP2C9 inhibitors have been shown to vary depending on the substrate used. Surprisingly, about one third of CYP2C9 inhibitors tested exhibited at least 5-fold higher Ki values for tolbutamide 4-hydroxylation than for S-warfarin 7-hydroxylation. Thus, the inhibitory effect of iguratimod on CYP2C9 may differ between tolbutamide and S-warfarin. However, it remains unclear whether iguratimod inhibits warfarin oxidation.

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In this study, we investigated the inhibitory effect of iguratimod on warfarin oxidation. Our results indicated that iguratimod potently inhibits CYP2C9-mediated warfarin 7-hydroxylation. In addition, this study suggests that the CYP2C9 genetic polymorphism may influence the inhibitory potency of iguratimod. The potential for in vivo interactions between iguratimod and warfarin is also discussed.

MATERIALS AND METHODS

Materials Iguratimod was purchased from ChemScene (Monmouth Junction, NJ, U.S.A.). Other chemicals were obtained from the following sources: R,S-warfarin, S-warfarin, and R-warfarin from Cayman Chemical (Ann Arbor, MI, U.S.A.); microsomes from baculovirus-infected insect cells expressing CYP2C9.1 or CYP2C9.3, each with NADPH-CYP reductase (Supersomes®), HLMs (50-donor pool, Catalog# 452156), 7-hydroxywarfarin, and sulfaphenazole from BD Gentest (Woburn, MA, U.S.A.); α-naphthoflavone from Sigma-Aldrich (St. Louis, MO, U.S.A.); ketoconazole from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate, and glucose-6-phosphate dehydrogenase from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals and solvents used were of the highest quality commercially available.

Enzyme Assay The 7-hydroxylase activities for R,S-warfarin, S-warfarin, and R-warfarin were determined as reported previously10) with minor modifications. HLMs (20 µg protein for R,S-warfarin and S-warfarin or 40 µg protein for R-warfarin), rCYP2C9.1 (4 pmol), and rCYP2C9.3 (8 pmol) were used as enzyme sources. The incubation mixture consisted of an enzyme source, warfarin (3 µM R,S-warfarin and S-warfarin or 30 µM R-warfarin), an NADPH-generating system (0.5 mM NADP, 10 mM glucose 6-phosphate, 10 mM magnesium chloride, and 1 unit/mL glucose-6-phosphate dehydrogenase), and 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 200 µL. Following prewarming at 37°C for 5 min, reactions were initiated by addition of the NADPH-generating system. Incubations were carried out at 37°C for 20 min (40 min for R-warfarin) and terminated by adding 10 µL of 70% (v/v) perchloric acid. After removal of protein by centrifugation, 50 µL of the supernatant was subjected to high-performance liquid chromatography (L-2100 pump, L-2200 autosampler, L-2300 column oven, and L-2480 fluorescence detector; Hitachi, Tokyo, Japan) with a Mightysil RP-18 GP II column (2.0×150 mm, 5 µm; Kanto Chemical Co., Inc., Tokyo, Japan) with a Mightysil RP-18 GP II column maintained in a column oven at 35°C. The mobile phase was a mixture of 36% (v/v) acetonitrile containing 0.04% (v/v) orthophosphoric acid. Elution was performed at a flow rate of 0.3 mL/min. The formation of 7-hydroxywarfarin was monitored at an excitation wavelength of 320 nm and emission wavelength of 415 nm.

Inhibition Studies Each enzyme source was incubated with a substrate in the presence of iguratimod (2.5–100 µM), sulfaphenazole (0.1–10 µM), α-naphthoflavone (0.1 µM), or ketoconazole (3 µM) in the same manner as described for the enzyme assays. All compounds were dissolved in dimethylsulfoxide and added to the incubation mixture at a final dimethylsulfoxide concentration of ≤0.5%. The IC₅₀ value was calculated by nonlinear regression analysis with GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA, U.S.A.), using the dose–response with variable slope function.

The effects of four different inhibitor concentrations on S-warfarin 7-hydroxylase activity were examined at four or five substrate concentrations to characterize the enzyme kinetics for the inhibition of CYP2C9 by iguratimod. The apparent Kᵢ values and the mode of inhibition were determined by nonlinear regression analysis for competitive or mixed inhibition with GraphPad Prism 5.02 (GraphPad Software Inc.). Akaike’s information criterion was used as a measure of goodness of...
fit. The mode of inhibition was verified by visual inspection of Lineweaver–Burk plots of the enzyme kinetic data.

To identify potential metabolism-dependent inhibition of CYP2C9 by iguratimod, inhibition experiments were performed as described below. The preincubation mixture contained an enzyme source, iguratimod (2.5–100 μM), the NADPH-generating system, and 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 180 μL. After prewarming at 37°C for 5 min, reactions were initiated by addition of the NADPH-generating system at the end of 5-min preincubation mixture (final substrate concentration 3 μM). Incubations were conducted in the same manner as described in the enzyme assay. Coincubation in which S-warfarin was added together with the NADPH-generating system at the end of 5-min pre-warming was also carried out.

**Prediction of in Vivo Drug Interactions for CYP2C9**

The in vivo inhibition potency was estimated by the methods of the interview form of KOLBET® Tablets 25 mg3 and Obach et al.11) The maximum unbound hepatic input concentration, $C_{\text{max},u,\text{inlet}}$, was determined using the following equation11:

$$C_{\text{max},u,\text{inlet}} = (C_{\text{max}} \cdot R_B + D \cdot F_s \cdot k_i / Q_b) \cdot f_u / R_B$$  \hspace{1cm} (1)

In Eq. 1, $C_{\text{max}}$ is defined as the maximum systemic plasma concentration after repeated oral administration to healthy elderly subjects (1.72 μg/mL), $R_B$ is the ratio of blood to plasma concentration (0.60), $D$ is the oral dose (25 mg), $F_s$ is the fraction of the oral dose absorbed (unity), $k_i$ is the first-order absorption rate constant (0.1 min⁻¹), $Q_b$ is the hepatic blood flow (1610 mL/min), and $f_u$ is the fraction unbound in the plasma (0.07). The ratio of the area under the curve (AUC) with inhibitor to control AUC was estimated using the following equation:

$$\frac{AUC_{\text{inhibited}}}{AUC_{\text{control}}} = 1 / [(f_{\text{uncYP2C9}} / (1 + [I]_{\text{in vivo}} / K_i^u)) + (1 - f_{\text{uncYP2C9}})]$$  \hspace{1cm} (2)

In Eq. 2, $AUC_{\text{inhibited}}$ is the AUC for a given substrate probe in the presence of an inhibitor, and $AUC_{\text{control}}$ is the AUC for the same probe substrate without inhibitor. The fraction of metabolism of the probe substrate by CYP2C9, the concentration of inhibitor in vivo, and the magnitude of the potency of the inhibitor unbound in HLMs are represented by $f_{\text{uncYP2C9}}$, $[I]_{\text{in vivo}}$, and $K_i^u$, respectively. The value used for $f_{\text{uncYP2C9}}$ of S-warfarin is 0.91.11) $[I]_{\text{in vivo}}$ corresponds to $C_{\text{max},u,\text{inlet}}$ described in Eq. 1. When it is assumed that the fraction of iguratimod unbound to liver microsomal proteins, $f_{u\text{HLM}}$, is equal to $f_u$, $K_i^u$ is represented by apparent $K_i$.3)

**RESULTS**

**Effects of Igruratimod on Warfarin 7-Hydroxylase Activities** The effects of iguratimod on R,S-warfarin 7-hydroxylation were examined with HLMs. Igruratimod inhibited the R,S-warfarin 7-hydroxylase activity in a concentration-dependent manner with an IC₅₀ value of 15.2 μM (Fig. 2A). Next, the effects of iguratimod on S-warfarin and R-warfarin 7-hydroxylation catalyzed by HLMs were investigated to clarify which enantiomer was more potently inhibited by iguratimod. Igruratimod effectively inhibited the S-warfarin 7-hydroxylation activity with an IC₅₀ value of 14.1 μM (Fig. 2A). In contrast, iguratimod slightly inhibited R-warfarin 7-hydroxylation activity.

Sulfaphenazole, a CYP2C9-selective inhibitor, potently inhibited R,S-warfarin and S-warfarin 7-hydroxylation activities of HLMs with IC₅₀ values of 0.246 and 0.232 μM, respectively (Fig. 2B). The inhibitory effect of sulfaphenazole on S-warfarin 7-hydroxylation catalyzed by HLMs in this study was comparable to those reported previously.12,13) Sulfaphenazole also inhibited HLMs-mediated R-warfarin 7-hydroxylation by approximately 40% (Fig. 2B). To identify the CYP isoforms responsible for R-warfarin 7-hydroxylation, an inhibition study was conducted with α-naphthoflavone and ketoconazole. α-Naphthoflavone (0.1 μM) and ketoconazole (3 μM) inhibited R-warfarin 7-hydroxylation activity of HLMs by about 35% (data not shown), suggesting that CYP1A2 and CYP3A4 as well as CYP2C9 may be responsible for R-warfarin 7-hydroxylation in HLMs.

It has been reported that S-warfarin 7-hydroxylation is predominantly catalyzed by CYP2C9.8,10) Thus, the effects of iguratimod on S-warfarin 7-hydroxylation were examined with rCYP2C9.1 and rCYP2C9.3 (Ile359Leu), the major allelic variant found in the Japanese population.14) Igruratimod inhibited S-warfarin 7-hydroxylation activities of rCYP2C9.1 and rCYP2C9.3 in a concentration-dependent manner with IC₅₀ values of 10.8 and 20.1 μM, respectively (Fig. 3).

**Kinetic Analysis of Igruratimod-Mediated Inhibition of S-Warfarin 7-Hydroxylase Activity** Kinetic analyses of the inhibition were carried out to characterize the modes of inhibition of CYP2C9 by iguratimod. Igruratimod showed...
competitive inhibition of 7-warfarin 7-hydroxylase activity of HLMs (Fig. 4). The mode of inhibition of rCYP2C9.1 by iguratimod was consistent with that of HLMs. On the other hand, iguratimod inhibited the activity of rCYP2C9.3 in a mixed manner. The apparent \( K_i \) values for HLMs, rCYP2C9.1, and rCYP2C9.3 were 6.74, 4.23, and 14.2 \( \mu \)M, respectively (Table 1).

The effects of iguratimod on in vitro intrinsic clearance of S-warfarin 7-hydroxylation were investigated. The \( V_{\text{max}}/K_m \) values, intrinsic clearances, for HLMs, rCYP2C9.1, and rCYP2C9.3 were decreased as the concentration of iguratimod increased (Table 2). Iguratimod at a concentration of 5 \( \mu \)M reduced the \( V_{\text{max}}/K_m \) values for HLMs, rCYP2C9.1, and rCYP2C9.3 by approximately 35%, 58%, and 20%, respectively.

Metabolism-Dependent Inhibition of 7-Hydroxylase Activity by Iguratimod

The effects of preincubation on inhibition by iguratimod were examined to determine whether iguratimod inhibits CYP2C9-mediated 7-warfarin 7-hydroxylation in a metabolism-dependent manner. A 20-min preincubation of iguratimod in the presence of NADPH did not alter the inhibitory effect on CYP2C9-mediated 7-warfarin 7-hydroxylation.

Table 1. Apparent \( K_i \) Values and Modes of Inhibition of S-Warfarin 7-Hydroxylation by Iguratimod

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>( K_i (\mu M) )</th>
<th>Modes of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLMs</td>
<td>6.74</td>
<td>Competitive</td>
</tr>
<tr>
<td>rCYP2C9.1</td>
<td>4.23</td>
<td>Competitive</td>
</tr>
<tr>
<td>rCYP2C9.3</td>
<td>14.2</td>
<td>Mixed</td>
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</table>

Table 2. Kinetic Parameters for Inhibition of S-Warfarin 7-Hydroxylation by Iguratimod

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>([I] (\mu M))</th>
<th>( K_m (\mu M) )</th>
<th>( V_{\text{max}} (\mu \text{mol/min/mg protein}) )</th>
<th>( V_{\text{max}}/K_m (\mu \text{L/min/mg protein}) )</th>
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</thead>
<tbody>
<tr>
<td>HLMs</td>
<td>0</td>
<td>6.68</td>
<td>5.97</td>
<td>0.894</td>
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<tr>
<td></td>
<td>5</td>
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<td>6.08</td>
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<tr>
<td></td>
<td>10</td>
<td>13.7</td>
<td>5.64</td>
<td>0.412</td>
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<tr>
<td></td>
<td>20</td>
<td>23.4</td>
<td>6.24</td>
<td>0.267</td>
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<td>18.6</td>
<td>5.90</td>
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<tr>
<td></td>
<td>2.5</td>
<td>5.38</td>
<td>19.4</td>
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<tr>
<td></td>
<td>5</td>
<td>7.68</td>
<td>18.9</td>
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<tr>
<td></td>
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<td>10.9</td>
<td>19.0</td>
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<tr>
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<td>11.0</td>
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<tr>
<td></td>
<td>5</td>
<td>25.1</td>
<td>10.1</td>
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<td>10</td>
<td>30.7</td>
<td>9.69</td>
<td>0.316</td>
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<td></td>
<td>20</td>
<td>46.3</td>
<td>9.43</td>
<td>0.204</td>
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not potentiate the inhibition of S-warfarin 7-hydroxylation activity of HLMs, rCYP2C9.1, and rCYP2C9.3 (Fig. 5).

Estimation of in Vivo Inhibitory Potency of Iguratimod on CYP2C9-Catalyzed S-Warfarin Metabolism Based on the apparent \(K_i\) value for HLMs obtained in this study and \(C_{\text{max}, \text{u, inlet}}\), we predicted in vivo metabolic interaction between iguratimod and S-warfarin. \(C_{\text{max}, \text{u, inlet}}\) was estimated to be 0.302 \(\mu\)g/mL (0.806 \(\mu\)M) on repeated oral administration to healthy elderly subjects.\(^7\) As calculated from the \(C_{\text{max}, \text{u, inlet}}\) value and \(K_i, \text{u}\) value for HLMs, the ratio of \(AUC_{\text{inhibited}}\) to \(AUC_{\text{control}}\) was estimated to be 2.3.

DISCUSSION

In the present study, we examined the inhibitory effects of iguratimod on warfarin oxidation to clarify the mechanism underlying drug interaction between iguratimod and warfarin. Iguratimod efficiently inhibited 7-hydroxylase activities of \(R, S\)-warfarin and S-warfarin in HLMs and/or recombinant enzymes. However, metabolism-dependent inhibition of S-warfarin 7-hydroxylation by iguratimod was not observed under the present conditions. These results suggest that iguratimod itself may inhibit the activity of CYP2C9.

It was reported previously that \(R\)-warfarin is mainly metabolized by CYP1A2 to form 6-, 7-, and 8-hydroxylated metabolites and by CYP3A4 to produce a 10-hydroxylated metabolite.\(^6\) However, these metabolites, except 7-hydroxylwarfarin, were not detected under the present conditions (data not shown), as determined by high-performance liquid chromatography with UV detection at 205 nm.\(^9\) Therefore, we investigated the effects of iguratimod on \(R\)-warfarin 7-hydroxylase activity of HLMs. The results indicated that iguratimod exhibited a slight inhibitory effect against \(R\)-warfarin 7-hydroxylation. Inhibition studies with sulfaphenazole, \(\alpha\)-naphthoflavone, and ketoconazole suggested that CYP2C9, CYP1A2, and CYP3A4 may be involved in \(R\)-warfarin 7-hydroxylation in HLMs. Iguratimod exerts no or less inhibitory effect on CYP1A2 and CYP3A4 activities.\(^7\) These findings support our observation of the slight inhibition of \(R\)-warfarin 7-hydroxylation by iguratimod.

In this study, the apparent \(K_i\) values of iguratimod were determined in HLMs and rCYP2C9s. It has been shown that estimation of \(K_i\) values of CYP2C9 inhibitors is affected by protein binding of these inhibitors in enzyme sources.\(^{16,17}\) Baba et al.\(^{16}\) reported that an apparent \(K_i\) value (0.188 \(\mu\)M) of miconazole for HLMs-mediated tolbutamide 4-hydroxylation was largely different from the \(K_i, \text{u}\) value (0.0024 \(\mu\)M) due to a very low unbound fraction (0.95–1.88%) of miconazole in an incubation mixture containing HLMs. If iguratimod binds to proteins of HLMs and rCYP2C9s, it is possible to overestimate the apparent \(K_i\) values of iguratimod. However, the unbound fraction of iguratimod is not available in the present study because protein binding of iguratimod in these enzyme sources has not been determined. Therefore, we expediently assumed that the fraction of iguratimod unbound to liver microsomal proteins, \(f_{\text{u, inlet}}\), is equal to \(f_{\text{u, uHLMs}}\), to predict in vivo metabolic interaction between iguratimod and S-warfarin, as described in the interview form of KOLBET\(^9\) Tablets 25 mg.\(^3\)

In this study, prediction of in vivo drug interactions suggested that coadministration of iguratimod may elevate the \(AUC\) of S-warfarin by 130%. A previous clinical study showed that the \(AUC\) of S-warfarin increased by 57% during capcitabine treatment, where the prothrombin time expressed as the international normalized ratio (PT-INR) was elevated by 91\%.\(^8\) It should be noted that the true anticoagulant effect of warfarin is likely to be underestimated because some patients with increased PT-INR were administered vitamin K for safety reasons. Taken together, it is suggested that iguratimod may inhibit warfarin metabolism and thus elevate PT-INR.

In addition to iguratimod, inhibition studies with its metabolites have been conducted previously by Toyama Chemical Co., Ltd. The metabolites of iguratimod, i.e., M1, M2, M3, M4, and M5, at a concentration of 100 \(\mu\)M inhibited tolbutamide 4-hydroxylation activity of HLMs by 34.3%, 44.9%, 19.3%, 29.3%, and 29.1%, respectively, as described in an in-house document of Toyama Chemical Co., Ltd. Among the metabolites, M4 is formed directly from iguratimod by several CYP isoforms, including CYP2C9 (Fig. 1). In this study, iguratimod did not exhibit the metabolism-dependent inhibition of S-warfarin 7-hydroxylation catalyzed by HLMs and rCYP2C9 enzymes. These findings suggest that iguratimod metabolites, such as M4 and reactive intermediates, are unlikely to contribute to iguratimod-mediated inhibition of S-warfarin 7-hydroxylation. On the other hand, the inhibitory effect of M2 on CYP2C9 activity is comparable to that of iguratimod.\(^3\) As the maximum systemic plasma concentration of M2 after repeated oral administration is approximately 2-fold higher than that of iguratimod,\(^3\) it is possible that M2 as well as iguratimod plays an important role in inhibition of CYP2C9-mediated warfarin oxidations. However, there is no
Ile359Leu substitution. Residue 359 in CYP2C9 is positioned in 3% of the Japanese population. The CYP2C9*3 allele encodes a missense mutation of the CYP2C9 gene found in 3% of the Japanese population. The CYP2C9*3 allele decreases the catalytic activity by up to approximately 90%. In addition, the Ile359Leu substitution influences the inhibitory effects of CYP2C9 inhibitors on the catalytic activity. Our study showed that the apparent K value of iguratimod for rCYP2C9.3 was about 3-fold higher than that for rCYP2C9.1. Furthermore, the decreased rate of intrinsic clearance of S-warfarin 7-hydroxylation was less dramatic for rCYP2C9.3 compared with rCYP2C9.1. These results suggest that the inhibitory potency of iguratimod against warfarin metabolism may be lower in subjects harboring the CYP2C9*3 allele than in those with CYP2C9*1/*1.

Many CYP2C9 inhibitors show substrate-dependent inhibitory effects. Kumar et al. examined the correlation of inhibitory potency of 28 compounds with 5 commonly used in vitro probe substrates for CYP2C9. They found that approximately one third of the CYP2C9 inhibitors investigated showed at least 5-fold higher K values for tolbutamide 4-hydroxylation than for S-warfarin 7-hydroxylation. In particular, the inhibitory effects of indomethacin and ketocnazole on S-warfarin 7-hydroxylation were 22-fold higher than those on tolbutamide 4-hydroxylation. Independent studies have shown that the inhibitory potency of nicardipine against S-warfarin 7-hydroxylation is 3-fold higher than that against tolbutamide 4-hydroxylation. These findings indicate that tolbutamide is not appropriate as a CYP2C9 probe to evaluate in vitro drug interactions. However, tolbutamide has been commonly used as a probe substrate for CYP2C9 by pharmaceutical companies, whereas S-warfarin is used infrequently. To avoid false-negative results, the in vitro inhibitory potency obtained with tolbutamide should not be extrapolated to the potential of in vivo drug interactions for CYP2C9. As such extrapolation has been done, close attention should be paid to this interpretation.

In conclusion, we demonstrated that iguratimod showed potent direct inhibition of CYP2C9-mediated warfarin 7-hydroxylation. This study indicated that the inhibitory effect of iguratimod on CYP2C9.1 is stronger than that on CYP2C9.3. Our study provided useful information regarding in vivo interactions between iguratimod and warfarin.

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**Conflict of Interest** The authors declare no conflict of interest.

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