IN VITRO METABOLISM OF THE CALMODULIN ANTAGONIST DY-9760e (3-[2-[4-(3-CHLORO-2-METHYLPHENYL)-1-PIPERAZINYL]ETHYL]-5,6-DIMETHOXY-1-(4-IMIDAZOYL METHYL)-1H-INDAZOLE DIHYDROCHLORIDE 3.5 HYDRATE) BY HUMAN LIVER MICROSOMES: INVOLVEMENT OF CYTOCHROMES P450 IN ATYPICAL KINETICS AND POTENTIAL DRUG INTERACTIONS

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ABSTRACT:

Human cytochrome P450 (P450) isozyme(s) responsible for metabolism of the calmodulin antagonist 3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole dihydrochloride 3.5 hydrate (DY-9760e) and kinetic profiles for formation of its six primary metabolites [M3, M5, M6, M7, M8, and M9] by human liver microsomes were identified using human liver microsomes and recombinant P450 enzymes. In vitro experiments, including an immunoinhibition study, correlation analysis, and reactions with recombinant P450 enzymes, revealed that CYP3A4 is the primary P450 isozyme responsible for the formation of the DY-9760e metabolites, except for M5, which is metabolized by CYP2C9. Additionally, at clinically relevant concentrations, CYP2C8 and 2C19 make some contribution to the formation of M3 and M5, respectively. The formation rates of DY-9760e metabolites except for M8 by human liver microsomes are not consistent with a Michaelis-Menten kinetics model, but are better described by a substrate inhibition model. In contrast, the enzyme kinetics for all metabolites formed by recombinant CYP3A4 can be described by an autoactivation model or a mixed model of autoactivation and biphasic kinetics. Inhibition of human P450 enzymes by DY-9760e in human liver microsomes was also investigated. DY-9760e is a very potent competitive inhibitor of CYP2C8, C9, and 2D6 (K_i 0.25–1.7 μM), a mixed competitive and noncompetitive inhibitor of CYP2C9 (K_i 11.4–20.1 μM), and a moderate inhibitor of CYP1A2 and 3A4 (K_i 11.4–20.1 μM), suggesting a high possibility for human drug-drug interaction.

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DY-9760e, 3-[2-[4-(3-chloro-2-methyl phenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-[4-(imidazolylmethyl)]-1H-indazole dihydrochloride 3.5 hydrate (Fig. 1), is a novel calmodulin antagonist developed to treat acute ischemic stroke. It inhibits calmodulin-dependent enzymes and in so doing exerts neuroprotective effects in animals with induced strokes (Sugimura et al., 1997; Sato et al., 1999, 2003, 2004; Fukunaga et al., 2000; Takagi et al., 2001; Hashiguchi et al., 2003). In experimental model rats with transient focal ischemia, DY-9760e significantly reduces the infarct volume when infused for 6 h from 1 h after the middle cerebral artery occlusion and also suppresses the increase in brain water content. Results of a previous study show that DY-9760e is metabolized by human liver microsomes in vitro, and the major metabolites of DY-9760e are identified as N-dealkylated (DY-9836), 4-hydroxylated (M3), O-demethylated (M5 and M7), and imidazole-oxidized derivatives (M6 and M8) (Fig. 1; Tachibana et al., 2005b). The results of pharmacokinetic studies in rats after intravenous administration of DY-9760e show that DY-9760e is extensively metabolized in the liver, and most of the administered DY-9760e is excreted into bile as glucuronide conjugates of hydroxylated and O-demethylated metabolites (Tachibana et al., 2005a,b). Therefore, DY-9760e is eliminated mainly through hepatic metabolic clearance.

Although the general outline of DY-9760e metabolism is known, more specific information is needed to understand its complex metabolism. Thus, this study had two purposes. One purpose of this study was to identify which human P450 isozyme(s) are involved in the formation of the primary metabolites of DY-9760e, and to determine the enzyme kinetics for the formation of metabolites using human liver microsomes and microsomes expressing recombinant human P450 enzymes. These studies provide essential information for determining the contribution of each P450 isozyme to DY-9760e metabolism. The second purpose of the present study was to estimate how
potently DY-9760e inhibits P450 enzymes in vitro. DY-9760e bears an imidazole ring in its structure. Results of several previous studies have shown that some compounds containing the imidazole ring, such as ketoconazole, sulfaphenazole, and miconazole, are potent inhibitors of CYP3A4 and 2C9 (Wilkinson et al., 1974; Rogerson et al., 1977; Ahmed et al., 1995; Halpert, 1995; Venkatakrishnan et al., 2000). To use DY-9760e safely and predict possible drug-drug interactions in humans, the inhibitory effects of DY-9760e against CYP1A2-, 2C9-, 2C19-, 2D6-, 2E1-, and 3A4-catalyzed metabolic reactions were investigated in vitro using human liver microsomes.

Materials and Methods

Chemicals. DY-9760e, DY-9836 (an N-dealkylated derivative), D91-6505a (M5), D91-4389a (M3), and DX-9194, used as an internal standard for LC-MS analysis, were synthesized by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and NADPH were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). 7-Ethoxyresorufin and resorufin sodium were purchased from Sigma-Aldrich (St. Louis, MO); tolbutamide was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); and 4-hydroxytobutamide, (S)-mephenytoin, 4'-hydroxymephenytoin, bufuralol hydrochloride, midazolam hydrochloride, and 1'-hydroxymidazolam were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). All other chemical reagents were analytical grade.

Microsomes and Antiserum. Human liver microsomes (pool of 16 donors; 0.432 nmol P450/mg protein) were purchased from XenoTech LLC (Kansas City, KS). In kinetic analysis experiments, human liver microsomes (pool of 11 donors; 0.550 nmol P450/mg protein) obtained from BD Gentest (Woburn, MA) were used. A bank of fully characterized human liver microsomes (0.5 mg/ml) was used in correlation analysis experiments. Microsomes prepared from insect cells transfected with a baculovirus coexpressing one of the following recombinant human P450 enzymes: CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9*1 ([Arg144] + cytochrome b5; hereafter, b5), 2C9*2 [Cys144], 2C18, 2C19, 2D6*1, 2E1+b5, 3A4, 3A4+b5, 3A5, and 4A11, and human NADPH-cytochrome P450 reductase (Supersomes) were obtained from BD Gentest.

Anti-human P450 polyclonal rabbit antiserum (anti-human CYP1A1, 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) were purchased from Nihon Nosan Kogyo K.K. (Yokohama, Japan). Nonimmune rabbit serum was obtained from Invitrogen (Carlsbad, CA).

Microsomal Incubations. Each reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.4), 0.25 mg/ml human liver microsomes, DY-9760e dissolved in methanol (1%, v/v), 12 mM glucose 6-phosphate, 2.2 units/ml glucose-6-phosphate dehydrogenase, 5 mM MgCl2, and 1 mM NADPH in a total volume of 400 μl. An initial experiment was conducted to find the optimal microsomal protein concentration and incubation time that resulted in a linear reaction velocity. After a 5-min preincubation period at 37°C, the reactions were started by the addition of NADPH and carried out at 37°C for 10 min. The reactions were terminated by adding 1 ml of acetonitrile, after which 100 μl of the internal standard solution (2 g/ml DX-9194) was added. After centrifugation at 1500 g for 10 min, each supernatant was evaporated to dryness and dissolved in 400 μl of a 60:40 (v/v) mixture of 10 mM ammonium acetate (adjusted to pH 7.4) and acetonitrile. A 20-μl aliquot of each reconstituted mixture was separately loaded onto the LC-MS system as described under “Analytical Procedure.” Since chemically synthetic compounds of M6, M7, and M8 were not available, the concentration of M7 was calculated using the calibration curve for its isomer, D91-6505a (M5), and the concentrations of M6 and M8 were calculated from the calibration curve for DY-9836.

Immunoinhibition Study. Pooled human liver microsomes (0.25 mg/ml), anti-human P450 polyclonal antiserum diluted with nonimmune rabbit serum, and 50 mM Tris-HCl buffer (pH 7.4) were combined and incubated for 10 min at 37°C. The reactions were terminated by adding 1 ml of acetonitrile, after which 100 μl of the internal standard solution (2 μg/ml DX-9194) was added. After centrifugation at 1500 g for 10 min, each supernatant was evaporated to dryness and dissolved in 400 μl of a 60:40 (v/v) mixture of 10 mM ammonium acetate (adjusted to pH 7.4) and acetonitrile. A 20-μl aliquot of each reconstituted mixture was separately loaded onto the LC-MS system as described under “Analytical Procedure.” Since chemically synthetic compounds of M6, M7, and M8 were not available, the concentration of M7 was calculated using the calibration curve for its isomer, D91-6505a (M5), and the concentrations of M6 and M8 were calculated from the calibration curve for DY-9836.

Correlation Analysis with a Panel of Human Liver Microsomes. DY-9760e (6.3 μM) was incubated with a panel of characterized human liver microsomes (0.5 mg/ml). Reaction velocities for the formation of the DY-
9760e metabolites were correlated against the marker activities specific for each P450 isozyme in the same microsomal reaction mixtures. The P450-specific marker activities of the individual microsomes were described in the data sheet provided by the manufacturer. Pearson’s correlation coefficient and corresponding p values for the correlation were used to assess the relationship between the P450 marker activities and the formation rates of DY-9760e metabolites. The two-tailed statistically significant difference was set at a p value of 0.05. Statistical analysis was performed using EXSAS ver. 5.00 (Arm Corp., Osaka, Japan) based on SAS release 6.12 (SAS Institute Japan, Tokyo, Japan).

Reactions with Recombinant Human P450 Enzymes (Initial Screen Test). DY-9760e at two concentrations, 6.3 and 50 μM, was incubated with a set of 15 recombinant human P450-expressing microsomes (Supremes) in 400-μl volume reaction mixtures. After a 2-min warming period at 37°C, the reaction was started by the addition of ice-cold microsomes (50 pmol of P450), and the reaction was conducted for 30 min at 37°C.

Analytical Procedure. LC-MS in selected ion monitoring mode was used to analyze the DY-9760e metabolites in the microsomal reaction mixtures. The instruments used for LC-atmospheric pressure chemical ionization-MS were a Finnigan LCQ ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA) equipped with an Alliance 2690 HPLC system (Waters, Milford, MA). Chromatographic separation of metabolites was carried out on a Symmetry C18 column (5 μm, 150 mm × 4.6 mm i.d.; Waters) at 40°C. A linear gradient of 30 to 60% acetonitrile in 10 mM ammonium acetate (adjusted to pH 7.4) over 30 min was used as the column eluent at a flow rate of 1 ml/min.


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The enzyme kinetics of DY-9760e metabolism by pooled human liver microsomes or recombinant P450 enzymes.

Enzyme Kinetics by Human Liver Microsomes or Recombinant P450 Enzymes. The enzyme kinetics of DY-9760e metabolism by pooled human liver microsomes and seven recombinant human P450 enzymes (CYP1A1, 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4) was investigated at the concentrations of DY-9760e ranging from 0.4 to 500 μM. Formation of all the metabolites by 50 pmol of recombinant P450 enzymes was linear over time to 10 min; therefore, incubations were conducted for this time period. Both [V/S]-[V] plots (Eadie-Hofstee plot) and [S]-[V] plots were used to analyze the kinetic data. Appropriate enzyme kinetics models to define the substrate concentration-velocity functions were determined from the Eadie-Hofstee plots. The data were fitted to various enzyme kinetics models using the GraFit 5.0 software (Erithacus Software Ltd., Horley, UK), designed for nonlinear regression analysis. The kinetic parameters were calculated after finding the model that best fit the data from among the following five equations: the single-site Michaelis-Menten model

\[ v = V_{\text{max}} \cdot \frac{[S]}{K_m + [S]} \]  

the two-site Michaelis-Menten model

\[ v = V_{\text{max}1} \cdot \frac{[S]}{K_{m1} + [S]} + V_{\text{max}2} \cdot \frac{[S]}{K_{m2} + [S]} \]  

the modified two-site Michaelis-Menten model (biphasic model; Clarke, 1998)

\[ v = (V_{\text{max1}} \cdot \frac{[S]}{K_{m1}} + CL_{\text{int}, \text{low affinity}} \cdot [S])/(K_{m1} + [S]) \]  

if \([S] \ll K_{m1}\) in eq. 3,

\[ v = V_{\text{max1}} \cdot \frac{[S]}{K_{m1} + [S]} + CL_{\text{int}, \text{low affinity}} \cdot [S] \]  

the substrate inhibition model

\[ v = V_{\text{max}1} \cdot \frac{[S]}{K_{m1} + [S]} + [S] / K_a \]  

where \(K_a\) is the substrate inhibition constant, and the autoactivation model (Hill equation)

\[ v = V_{\text{max}} \cdot \frac{[S]^n}{K_m + [S]^n} \]  

where n is the Hill coefficient.

Inhibition of P450 Enzymes by DY-9760e. To determine the effects of DY-9760e on each P450 isozyme, P450 isoform-specific substrates were incubated at 37°C with human liver microsomes in the presence or absence of varying concentrations of DY-9760e. Metabolic activities specific for each P450 isozyme were as follows: 7-ethoxycoumarin O-deethylase for CYP1A2 (Leclercq et al., 1996), tolbutamide 4-methylhydroxylase for CYP2C8/9 (Chen et al., 1993), (S)-mephenytoin 4-hydroxylation for CYP2C19 (Meier et al., 1985), bufuralol 1′-hydroxylation for CYP2D6 (Kronbach et al., 1987), chlorozoxazone 6-hydroxylation for CYP2E1 (Peter et al., 1990), and midazolam 1′-hydroxylation for CYP3A4 (Kronbach et al., 1989). These activities were determined using published methods after some modification. The amount of metabolite produced in each reaction mixture was analyzed by HPLC. Instruments used for HPLC were controlled by a Hitachi D-7000 system manager (Hitachi, Ltd., Tokyo, Japan) and consisted of an L-7100 pump, an L-7610 degasser, an L-7300 column oven, an L-7200 autosampler, an L-7455 UV detector, and an L-7480 fluorescence detector. Lineweaver-Burk plots (L-B plots) were used to analyze the mechanism of inhibition, and \(K_i\) values were determined by Dixon plots. When the inhibitory mechanism was found to be a mixed type of competitive and noncompetitive inhibitions, both \(K_i\) and \(K_{m1}\) values were determined. \(K_i\) represents the dissociation constant of the interaction between the enzyme-substrate complex and inhibitor. For \(K_{m1}\) determination, the \(y\)-intercept of the L-B plot was plotted against inhibitor concentration, and the intercept on the \(x\)-axis of a linear regression line was obtained (Palmer, 1991).

Results

Enzyme Kinetic Studies Using Human Liver Microsomes. The [S]-[V] plots for the formation of each DY-9760e metabolite by human liver microsomes are shown in Fig. 2, and the kinetic values are shown in Table 1. Kinetic analysis revealed that the formation rates of M3, M5, M6, M7, and DY-9836 by human liver microsomes were not consistent with a Michaelis-Menten kinetic model, but were better described by a substrate inhibition model (eq. 4). The formation of M3, M5, and M6 could not be completely fitted to the substrate inhibition model over a wide range of DY-9760e concentrations from 0.4 to 500 μM. Therefore, taking the human plasma level of DY-9760e into consideration, the data for DY-9760e concentrations greater than 200 or 500 μM were excluded from the nonlinear regression analysis to obtain the best fit at low substrate concentrations. In contrast, the formation rate of M8 was consistent with a single-enzyme Michaelis-Menten model over the entire substrate concentration range (eq. 1). The \(K_m\) values of all metabolites were similar, ranging between 1.2 and 3.6 μM. These values suggest a high affinity of the metabolic enzymes for DY-9760e. The substrate inhibition constant \(K_{m1}\) ranged from 197 to 1713 μM; as a consequence, DY-9760e is thought to have little inhibitory effect on its own metabolism in vivo at the clinically effective dose.

Immunoinhibition. The inhibitory effects of rabbit anti-human P450 antisera on DY-9760e metabolism are shown in Fig. 3. Anti-human CYP1A1/2, 2A6, 2D6, and 2E1 antisera had no inhibitory effects (data not shown). The formation of M3, M6, M7, and DY-9836 were strongly inhibited by anti-CYP3A4 antisera in a concentration-dependent manner. However, the formation of M5 was not inhibited by anti-CYP3A4 antisera. Instead, anti-CYP2C9 antisera inhibited the formation of M5 in a concentration-dependent manner. Although anti-CYP1A1, -2C8, and -2C19 antisera also inhibited the formation of M5, this inhibition was relatively weak (50%–70% of control activity remaining).

Correlation Studies. The correlation coefficients between the activities of each DY-9760e metabolite formation and the substrate specific for each P450 tested in a bank of characterized human liver microsomes are summarized in Table 2. The formation rates of M3, M6, M7, M8, and DY-9836 varied over a 7-fold range, and the rates for these metabolites correlated significantly \((r = 0.947–0.973; \ p < 0.001)\) with CYP3A4/5-selective testosterone 6β-hydroxylation activ-
ity at a DY-9760e concentration of 6.3 μM. A significant correlation with paclitaxel 6α-hydroxylation activity catalyzed by CYP2C8 was also observed for the formation of M3, M7, M8, and DY-9836, but was relatively weak ($r = 0.503–0.682$). The formation rate of M5 had a 3-fold sample-to-sample variation and correlated significantly with the diclofenac 4'-hydroxylation catalyzed by CYP2C9 ($r = 0.860; p < 0.001$).

### Enzyme Kinetic Studies Using Human Recombinant P450 Enzymes.

From the results of the initial screen with 15 different recombinant human P450 enzymes, CYP3A4 activity in the presence of $b_5$ for the formation of M3, M6, M7, M8, and DY-9836 was much higher than that of any other P450 enzymes examined, and the formation of M5 was catalyzed by recombinant CYP2C9, 2C19, 2D6, and 3A4 (data not shown). In addition to these P450 enzymes, CYP1A1, 1A2, and 2C8 possess relatively high DY-9760e metabolic activities; therefore, enzyme kinetics values for metabolite formation by these seven recombinant human P450 enzymes were obtained. The [S]-[V] plots for the formation of each metabolite by recombinant CYP3A4+$b_5$ are shown in Fig. 4, and all kinetics values are shown in Table 3.

Most data sets fit one of the Michaelis-Menten models (eqs. 1 and 2), but there were several data sets that could not be fit to the classical hyperbolic curve in [S]-[V] plots. These data sets represented convex or sigmoidal curves that resulted from substrate inhibition (eq. 4) and autoactivation (eq. 5), respectively. Substrate inhibition and autoactivation kinetics were also discernible from characteristic curved lines in Eadie-Hofstee plots. Kinetics data for M3 formation by CYP1A1 and 2C8, M5 formation by CYP2C9 and 2C19, and DY-9836 formation by CYP2C19 represented convex curves, which fit a substrate

### Table 1

<table>
<thead>
<tr>
<th>Metabolite Pathway</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
<th>$V_{max}/K_m$ (pmol/min/nmol P450)</th>
<th>$K_i$ (μM)</th>
<th>Kinetic Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3 Phenyl hydroxylation</td>
<td>1.6</td>
<td>67.6</td>
<td>122.9</td>
<td>76.8</td>
<td>284.6</td>
</tr>
<tr>
<td>M5 5-Demethylation</td>
<td>1.2</td>
<td>13.8</td>
<td>25.1</td>
<td>20.9</td>
<td>197.2</td>
</tr>
<tr>
<td>M6 Imidazole oxidation</td>
<td>2.5</td>
<td>59.8</td>
<td>108.7</td>
<td>43.5</td>
<td>256.4</td>
</tr>
<tr>
<td>M7 6-Demethylation</td>
<td>2.8</td>
<td>20.3</td>
<td>36.9</td>
<td>13.2</td>
<td>1713.3</td>
</tr>
<tr>
<td>M8 Imidazole oxidation</td>
<td>2.8</td>
<td>105.9</td>
<td>192.5</td>
<td>68.8</td>
<td>Michaelis-Menten</td>
</tr>
<tr>
<td>DY-9836 N-Dealkylation</td>
<td>3.6</td>
<td>69.8</td>
<td>126.9</td>
<td>35.3</td>
<td>1106.4</td>
</tr>
</tbody>
</table>

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**Fig. 2.** The formation rates of M3, M5, M6, M7, M8, and DY-9836 by pooled human liver microsomes. Lines represent the best fit to either a substrate inhibition model (M3, M5, M6, M7, and DY-9836) or a single-enzyme Michaelis-Menten model (M8). Data points represented by open circles in the plots of M3, M5, and M6 were excluded from the fitting lines. Insets, Eadie-Hofstee plots.
inhibition model. In contrast, the enzyme kinetics for all metabolites formed by CYP3A4 was described by a sigmoidal curve with aut activation and fit a Hill model (eq. 5; Fig. 4). Eadie-Hofstee plots also exhibited curved lines characteristic of autoactivation (inset graphs in Fig. 4). For the formation of M5 by CYP3A4, the rates best fit the following equation, derived simply by combining eqs. 3 and (5): 

\[
V = \frac{V_{\text{max}} \cdot [S]^n}{[K_m] + [S]^n} + \frac{CL_{\text{int, low affinity}} \cdot [S]}{[S]}
\]  

(6)

For the formation of M3 and M6 by CYP3A4, the $[S]^{-1}[V]$ plots showed sigmoidal curves at low substrate concentrations ($\leq 50 \mu\text{M}$), but rates decreased as substrate concentrations increased (inset graphs in Fig. 4). These results suggest that both autoactivation and substrate inhibition occur in one metabolic reaction, but no single model best describes these reactions. Therefore, curve fitting was carried out after excluding the data points for concentrations greater than 100 $\mu\text{M}$, because therapeutic plasma concentrations of DY-9760e are thought to rarely exceed 50 $\mu\text{M}$. Further analysis also revealed that the rate of CYP2C8-catalyzed M5 formation fits a biphasic model using eq. 3.

The enzyme kinetics analysis revealed the following information.

1) For the formation of M3, M6, M8, and DY-9836, CYP3A4 showed higher $V_{\text{max}}/[K_m]$ values than did any other P450 enzymes. 2) CYP2C9, 2C19, and 2D6 showed low $K_m$ (<1.0 $\mu\text{M}$) and high $V_{\text{max}}$ values for the formation of M5. 3) For the formation of M7, CYP2C19 and 2D6 showed higher $V_{\text{max}}/[K_m]$ values than did CYP3A4, resulting from their high substrate affinity, $K_m \leq 0.6 \mu\text{M}$. However, the $V_{\text{max}}$ values of these P450s are very low ($\leq 22 \text{pmol/min/mmol P450}$). In contrast, CYP3A4 also showed a high capacity, with a $V_{\text{max}}$ of 308.5 pmol/min/mmol P450 but with a moderate affinity of $K_m = 14.6 \mu\text{M}$. 4) For

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**TABLE 2**

Correlation of DY-9760e metabolism with specific P450 isozyme activity in human liver microsomes from 16 individual donors.

<table>
<thead>
<tr>
<th>P450 Isozyme Activity</th>
<th>P450</th>
<th>P40</th>
<th>M3</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
<th>DY-9836</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Ethoxyresorfin O-dealkylation</td>
<td>CYP1A2</td>
<td>0.003</td>
<td>-0.230</td>
<td>-0.067</td>
<td>0.078</td>
<td>0.009</td>
<td>0.020</td>
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<tr>
<td>Coumarin 7-hydroxylation</td>
<td>CYP2B6</td>
<td>0.539*</td>
<td>-0.472</td>
<td>0.412</td>
<td>0.443</td>
<td>0.438</td>
<td>0.420</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel 6α-hydroxylation</td>
<td>CYP3A4</td>
<td>0.682**</td>
<td>0.364</td>
<td>0.377</td>
<td>0.522*</td>
<td>0.550*</td>
<td>0.503*</td>
<td></td>
</tr>
<tr>
<td>Diclofenac 4α-hydroxylation</td>
<td>CYP1A2</td>
<td>0.358</td>
<td>0.860***</td>
<td>0.219</td>
<td>0.245</td>
<td>0.309</td>
<td>0.231</td>
<td></td>
</tr>
<tr>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>CYP2B6</td>
<td>0.184</td>
<td>-0.124</td>
<td>0.050</td>
<td>0.115</td>
<td>0.117</td>
<td>0.087</td>
<td></td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylation</td>
<td>CYP3A4</td>
<td>0.958***</td>
<td>-0.215</td>
<td>0.947***</td>
<td>0.973***</td>
<td>0.957***</td>
<td>0.966***</td>
<td></td>
</tr>
<tr>
<td>Lauric acid 12-hydroxylation</td>
<td>CYP4A9/11</td>
<td>0.160</td>
<td>0.188</td>
<td>0.031</td>
<td>0.210</td>
<td>0.138</td>
<td>0.132</td>
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</tr>
</tbody>
</table>

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. 

**FIG. 3.** Effects of anti-human P450 antiseraum (■, anti-CYP1A1; ▲, anti-CYP2B6; ○, anti-CYP3A4) on the formation of DY-9760e metabolites by human liver microsomes.
the formation of M3, CYP2C8 also showed a high catalytic activity of 118.2 μl/min/nmol P450, the same activity as CYP3A4 (129.9 μl/min/nmol P450). However, the Vmax value of CYP3A4 was 3-fold higher than that of CYP2C8.

Inhibition of P450 Enzymes by DY-9760e. Before each P450 inhibition study, preliminary experiments were conducted using a single substrate concentration around its Km value and 1, 10, and 100 μM DY-9760e. The result shows that DY-9760e is a potent inhibitor of CYP2C19, 2C9, and 2D6 and a moderate inhibitor of CYP3A4 and 1A2, with little effect on the CYP2E1 activity (data not shown). Using these preliminary data, appropriate substrate and inhibitor concentrations were selected for the determination of a precise inhibition constant (Ki value) for five different P450 isozyme-specific activities in human liver microsomes. Ki values were determined from Dixon plots as shown in Fig. 5. DY-9760e shows strong inhibitory effects on tolbutamide 4-methylhydroxylation catalyzed by CYP2C8/9 (Ki = 1.7 μM), (S)-mephentoin 4'-hydroxylation catalyzed by CYP2C19 (Ki = 2.5 μM), and bufuralol 1'‐hydroxylation catalyzed by CYP2D6 (Ki = 0.25 μM). The effects of DY-9760e on 7-ethoxyresorufin and O-deethylation catalyzed by CYP1A2 (Ki = 22.2 μM), midazolam 1'-hydroxylation (Ki = 11.7 μM) catalyzed by CYP3A4 are moderate. The mechanisms of inhibition of DY-9760e, judging from the L-B plots, are competitive for CYP1A2, 2C8/9, 2D6, and 3A4. As for CYP2C19, the L-B plots for each DY-9760e concentration showed a common intercept left of the y-axis and above the x-axis, indicating a mixed competitive and noncompetitive inhibition mechanism. The Ki value was 9.3 μM, as determined from the secondary plot shown in Fig. 5F.

Discussion

Results of pharmacokinetics studies in rats and monkeys revealed that DY-9760e is eliminated mainly through hepatic metabolic clearance (Tachibana et al., 2005a). Furthermore, a phase I pharmacokinetics study in healthy men revealed that the fraction of the dose excreted in the urine as unchanged DY-9760e is low, accounting for less than 1.5% of the administered dose (Daiichi Pharmaceutical, data on file). Although the full in vivo metabolic profile of DY-9760e in humans has not yet been identified, results from an in vitro metabolic study using human liver microsomes show that DY-9760e is extensively metabolized (Tachibana et al., 2005b). Therefore, in the present study, the P450 isozymes involved in the formation of the metabolites by human liver microsomes were identified.

The results from the immunoinhibition study (Fig. 3) and P450 activity correlation study (Table 2) suggest that CYP3A4 is responsible for the majority of DY-9760e metabolism, with the exception of the formation of M5 (5-demethylated derivative), which is mainly catalyzed by CYP2C9. However, reactions of DY-9760e with recombinant human P450 enzymes revealed that other P450 isozymes can also metabolize DY-9760e. Enzyme kinetic analysis using human liver microsomes (Table 1) and recombinant P450 enzymes (Table 3) yielded much information on the contribution of P450 enzymes to DY-9760e metabolism. A substrate inhibition or Michaelis-Menten kinetics model results in the best fitting for the formation of all DY-9760e metabolites in human liver microsomes (Fig. 2), whereas autoactivation, which is observed by recombinant CYP3A4 (Fig. 4), does not occur. This might have resulted from the contribution of P450 isozymes other than CYP3A4 at low substrate concentrations in
human liver microsomes. Moreover, the kinetics of the formation of M3, M5, and M6 in human liver microsomes is unique; substrate inhibition occurs at the concentrations of 25 to 200 μM, but the reaction velocity increases again at concentrations greater than 100 to 200 μM. These kinetic profiles derived from human liver microsomes might not reflect a reaction of a single enzyme but, rather, combined reactions by more than one enzyme. As shown in Table 3, for the M3 formation with CYP2C8-selective activity (Table 2) was statistically significant, although it was relatively weak (r = 0.682). Therefore, in addition to CYP3A4, CYP2C8 makes some contribution to the formation of M3 at low substrate concentrations. For the formation of M5, CYP2C9 mainly catalyzes this reaction, but CYP2C19 showed higher V_{max}/K_m values and lower K_m values than those of CYP2C9. These results suggest that the contribution of CYP2C19 is not negligible at low substrate concentrations. CYP2D6 can catalyze the formation of M5; however, its contribution would be only minor because of low relative abundance in the liver.

The contributions of specific P450 enzymes to the total metabolic clearance can be estimated from their relative abundance in the liver or relative activity factors (Crespi, 1995) during in vitro studies. Determining the contribution of each P450 isozyme at various substrate concentrations will be important to understand the complicated metabolic reaction. According to the relative activity factor approach, the contribution of each P450 to DY-9760e metabolism in human liver microsomes was estimated, and the results confirmed the contribution of CYP2C8 for the M3 formation and CYP2C19 for the M5 formation at clinically relevant concentrations (about 2 μM) (data not shown).

For the last several years, a large number of examples of atypical enzyme kinetics have been observed in drug metabolism reactions (Ekins et al., 1998; Houston and Kenworthy, 2000; Kenworthy et al., 2001; Shou et al., 2001; Hutzler and Tracy 2002; Tracy 2003; Atkins, 2004). To avoid mis-estimation of kinetic parameters, it is necessary to apply an appropriate kinetic model to the in vitro data, especially when extrapolating in vitro findings to the in vivo conditions. Three kinds of atypical kinetic models (autoactivation, substrate inhibition, and biphasic) are needed to explain the metabolism of DY-9760e. Interestingly, the kinetics for M5 formation by CYP3A4 was best described by a mixed model of autoactivation and biphasic kinetics (Fig. 4). A nonexhaustive literature search suggests that this is the first example of a combination model with autoactivation kinetics by the CYP3A4 enzyme. However, it must be noted that the catalytic activity of CYP3A4 in vitro system can be influenced by various incubation conditions. The atypical kinetics of DY-9760e metabolism by CYP3A4 are observed in the presence of b₄ and magnesium, both of which have been shown to influence the CYP3A4 activity (Guengerich et al., 1986; Yamazaki et al., 1995). Maenpaa et al. (1998) have reported that the buffer conditions, ionic strength, and source of reducing agents affected the midazolam hydroxylation activity. Furthermore, the kinetics of the oxidation of pyrene by CYP3A4 is sigmoidal in the absence of magnesium but biphasic in the presence of magnesium (Schrag and Wienkers, 2000). Although it is difficult to determine the optimal conditions reflecting in vivo situations, further investigation will be needed using various incubation conditions.

It has been hypothesized that most atypical enzyme kinetics are
caused by the simultaneous binding of more than one substrate molecule to an enzyme’s active site, although several other hypotheses, including an allosteric model, have been proposed (Ueng et al., 1997; Korzekwa et al., 1998). If the substrate binds to two or more sites, one of which triggers positive or negative cooperativity, the resulting kinetics might exhibit autoactivation or substrate inhibition, respectively. The formation rates of M3 and M6 by recombinant CYP3A4 can be fitted to an autoactivation model at $K_{1/2}^{A}$ but substrate inhibition subsequently occurred at $K_{1/2}^{B}$. If these results are correct, and not artifacts, DY-9760e is the substance to exert both positive and negative cooperativity on CYP3A4.

Figure 5 shows that DY-9760e potently inhibits CYP2D6, 2C8/9, and 2C19 specific activities with low $K_i$ values. The low $K_i$ values for these P450 isozymes are a reflection of the low $K_m$ values. In contrast, the inhibitory effects on CYP1A2 and 3A4 are relatively moderate, with $K_i$ values of greater than 10 $\mu$M, although CYP3A4 is the major contributor to the metabolism of DY-9760e with high capacity. Kenworthy et al. (1999) have shown that more than one probe substrate should be used when estimating inhibitory constants for inhibitors of CYP3A4, because $K_i$ values for inhibitors of CYP3A4 may be different depending on which probe substrate is being used. Therefore, the inhibitory effect on testosterone 6β-hydroxylation activity in addition to midazolam 1′-hydroxylation was tested using the method described previously (Tachibana and Tanaka, 2001). DY-9760e showed a similar inhibitory effect on testosterone 6β-hydroxylation with a $K_i$ value of 15 $\mu$M (data not shown).

Compounds with chemically similar structures often show characteristics in common with the inhibitors of P450 enzymes (Halpert, 1995; Ortiz de Montellano and Correia, 1995). DY-9760e contains both an imidazole ring, like ketoconazole and miconazole (CYP3A4 and 2C9 inhibitors), and methoxy groups such as quinidine (a CYP2D6 inhibitor). These chemical structures appear to contribute to the strong inhibitory effects of DY-9760e on P450 enzymes. Further investigation will be needed concerning the relationship between the chemical structure of DY-9760e and its strong inhibitory effects on each P450 isozyme.

In clinical situations, the human plasma concentrations of DY-9760e will be kept under 1 $\mu$g/ml (about 2 $\mu$M), but the drug concentrations around metabolic enzymes in the liver would be higher than the plasma concentrations. The in vitro data from the present study demonstrated that DY-9760e at therapeutically relevant concentrations might be a strong inhibitor of CYP2C9, 2C19, and 2D6. Clinical investigations of DY-9760e with drugs that might be concomitantly administered are required to predict drug-drug interactions in humans.

In conclusion, CYP3A4 and 2C9 are significantly involved in DY-9760e metabolism in the human liver. In addition, CYP2C8 and 2C19 make minor contributions to DY-9760e metabolism. The kinetic profile of DY-9760e can be described by a non-Michaelis-Menten model, including substrate inhibition, autoactivation, and biphasic metabolism. DY-9760e appears to be a broad-spectrum inhibitor of the P450 enzymes; thus, clinically significant drug-drug interactions could occur between DY-9760e and substrates of CYP2C9, 2D6, and 2C19.
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References


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