IN VITRO INTERACTIONS BETWEEN A POTENTIAL MUSCLE RELAXANT E2101 AND HUMAN CYTOCHROMES P450

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ABSTRACT:
E2101 or N-methyl-[1-[1-[2-fluorophenethyl]piperidine-4-yl]-1H-indol-6-yl] acetamide, an antagonist of 5-hydroxytryptamine receptor subtypes 1A and 2, is currently under development for the potential treatment of skeletal muscle associated spasticity. Here we characterized the in vitro metabolism of E2101 using human liver enzymes including human liver microsomal preparations, human liver S9 fractions, and individual forms of recombinant cytochromes P450 (P450s). Our results showed that E2101 was metabolized by P450s to form monohydroxylated (M1 and M2), dihydroxylated (M3), and N-dealkylated metabolites (M4). The structures of these major microsomal metabolites were proposed based on LC/MS/MS analyses. All four metabolites, M1–M4, were formed by CYP3A4. Metabolites, M1, M2, and M4, were also formed by CYP2C19 and M2 and M3 by CYP2D6. The potential P450 inhibition and induction of E2101 were also evaluated. E2101 was determined to be a competitive inhibitor of CYP2C19 and CYP2D6 with Ki of 15 and 48 μM, respectively, as determined by both Dixon plots and simultaneously nonlinear regression analyses. Induction of major P450 expression was not detected immunochemically after 72-h exposure to 10 or 50 μM E2101 in primary hepatocyte cultures obtained from three subjects. Taken together, E2101 is expected to metabolically interact with major human P450 enzymes including CYP2C19, CYP2D6, and CYP3A4, and a low risk of drug-drug interaction would be anticipated in clinical studies.
vitro with emphases on the potential enzyme inhibition and induction. A variety of enzyme preparations, including recombinant enzymes, liver subcellular fractions, and primary hepatocytes were used in the study. The results of this present study are expected to be useful for the prediction of potential drug–drug interactions for future clinical applications of E2101.

Experimental Procedures

Materials. Chemicals. ER2101, or N-methyl-[1-[(2-fluorophenethyl)piperidine-4-yl]-1H-indol-6-yl] acetamide, was obtained from Tsukuba Research Laboratories of Eisai Co., Ltd. (Ibaraki, Japan). (±)-Bufuralol, (±)-1-hydroxybufuralol, 6-hydroxychlorozoxazone, S-mephenytoin, 4′-hydroxy-\(S\)-mephenytoin, and monohydroxylated warfarin metabolites (6-, 7- and 10-hydroxywarfarin) were purchased from Gentest Corp. (Woburn, MA). Chlorozoxazone, coumarin, albendazole, \(R\)-propranolol, \(R\)-chlorowarfarin, rifampicin, NADPH, TRIZMA, magnesium chloride, potassium phosphates, ketoconazole, quinidine, and \(R\)-warfarin were obtained from Sigma-Aldrich (St. Louis, MO). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was purchased from National Cancer Institute Chemical Carcinogen Reference Standard Repositories (Midwest Research Institute, Kansas City, MO). Optically pure \(R\) and \(S\)-warfarin were prepared from the racemic mixture by the differential crystallization method (West et al., 1961). The purity of warfarin enantiomers was at least 98% as determined by chiral HPLC, mass spectrometer, and NMR analyses. All solvents used for the HPLC analyses were HPLC grade.

Enzymes and hepatocytes. Pooled human liver microsomal preparations and \(S\) fractions were purchased from Gentest Corp. The insect microsomal preparations containing cDNA-expressed recombinant human P450s, and the control insect microsomal preparations were also purchased from Gentest Corp. Primary cultures of human hepatocytes from three female Caucasian donors (ages 55, 56, and 79) and the culture media were purchased from In Vitro Technologies Inc. (Baltimore, MD). These donors did not have recorded liver diseases and damages. Two of the donors were smokers (ages 55 and 79).

Others. Polyclonal goat anti-human CYP1A1/2 and anti-human CYP3A4 antibodies, and monoclonal mouse anti-human CYP2D6 antibodies were obtained from Gentest Corp. Polyclonal rabbit anti-human CYP2C19 antibodies were from Research Diagnostics Inc. (Flanders, NJ). All secondary antibodies were from Sigma-Aldrich. The electrophoresis apparatus and accessories were obtained from Bio-Rad (Hercules, CA) or Pierce Chemical (Rockford, IL). The antibiotics (streptomycin/penicillin) and buffers for the electrophoresis were purchased from Invitrogen (Carlsbad, CA) or Bio-Rad.

Metabolite and Metabolic Enzyme Identification. The metabolism of E2101 was determined by the disappearance of E2101 or the appearance of E2101 metabolites in the reaction mixtures compared with the respective controls. E2101 was incubated in the reconstituted in vitro reaction systems containing pooled human liver \(S\) fractions, microsomal preparations, or recombinant hepatic P450 forms including CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. The reaction mixture (total volume 250 \(\mu\)l) contained 0.5 mg of liver microsomal protein, 50 or 25 pmol of recombinant P450, and E2101 (50–80 \(\mu\)M) in 50 mM Tris buffer containing 15 mM MgCl\(_2\) (pH 7.4). After being incubated at 37°C with gentle shaking for 1 min, the reaction was initiated by adding 25 \(\mu\)l of NADPH solution (20 mg/ml) and carried out for 4 h. The reaction mixture was transferred to a 96-well plate, and the supernatants were filtered through syringe filters (13 mm, 0.45 \(\mu\)m). The filtrates were analyzed by LC/MS (ion trap) or LC/MS/MS.

P450 Inhibition Assays. The incubations were carried out in test tubes (12.0 × 75 mm). The incubation mixtures (250 \(\mu\)l) contained 0.5 mg of HLM protein, 50 (CYP1A2 and CYP2E1) or 25 pmol (CYP2C9, CYP2C19, CYP2D6, and CYP3A4) of recombinant human P450s, 10 or 50 \(\mu\)M E2101, probe substrate (\(R\), \(S\)-warfarin, chlorozoxazone, \(S\)-mephenytoin, or bufuralol) at different concentrations, and 0.5 mg of NADPH in 50 mM Tris buffer containing 15 mM MgCl\(_2\) (pH 7.4). E2101 was added to the reaction mixture 5 min prior to the addition of the probe substrate. After being incubated in a 37°C waterbath with gentle shaking for 1 min, the reaction was initiated by adding 25 \(\mu\)l of NADPH solution (20 mg/ml) and carried out for 15 to 60 min. The reaction mixture containing only the probe substrate was used as the control. After the incubation, the reaction mixture was extracted with mixing with 250 \(\mu\)l of methanol containing the appropriate internal standard (IS). After vortex mixing and centrifuging in a desktop centrifuge at 14,000 rpm for 5 min, the supernatant was filtered through a syringe filter (13 mm, 0.45 \(\mu\)m) into a HPLC vial. The filtrates were analyzed using LC/UV/fluorimetric detection and LC/MS/MS.

If inhibition was detected, the inhibition potency was determined using recombinant P450s. E2101 appeared to inhibit the activities of CYP2D6 and CYP2C19. Therefore, three concentrations of \(S\)-mephenytoin (80, 30, and 20 \(\mu\)M) and bufuralol (5, 10, and 40 \(\mu\)M), and a range of concentrations (0–200 \(\mu\)M) of E2101 were used for the construction of Dixon plots and simultaneously nonlinear regression analyses (SNLR). The incubation conditions and sample preparations were the same as previously described.

The quantification was based on the calibration curves, and the quality control samples were applied to ensure the quality of the experiments. Samples for the standard curves and quality control were prepared in the same manner as those of the reaction samples.

P450 Induction Assays. Hepatocyte treatment and sample preparation. Upon the reception of the primary culture of human hepatocytes in 6-well plates, the culture media containing streptomyacin/penicillin were refreshed (2 ml/well). After being acclimatized in 5% CO\(_2\) and 37°C overnight, the cells were treated with the vehicle solution (negative control), the prototypic P450 inducers including TCDD (0.4 \(\mu\)M) for CYP1A1, rifampicin (50 \(\mu\)M) for CYP3A and possibly CYP2F2 (Feng et al., 1998; Xu et al., 2000; Gerbal-Chaloin et al., 2001; Liu et al., 2001), and E2101 (10 and 50 \(\mu\)M) for 72 h. E2101 stock solution was added to the culture media at a 1:40 ratio (v/v). The culture media and testing compounds were replenished every 24 h. At the end of the treatment, the cells were harvested into 1 ml of phosphate-buffered saline (PBS) after being washed twice. The cells were precipitated by centrifugation and resuspended in PBS (approximately 50 \(\mu\)l) for 60 min. The membrane was blocked by 5% nonfat dried milk blotting buffer (PBS containing 0.05% Tween 20) at 4°C overnight.

Western immuno blotting analysis. Proteins were resolved in a 12% SDS-polyacrylamide gel electrophoresis gel using a mini gel apparatus at a constant voltage (60 mV/gel) for 70 to 80 min and transferred onto a polyvinylidene difluoride membrane using a membrane-transferring unit at a constant voltage (60 mV/membrane) for 60 min. The membrane was blocked by 5% nonfat dried milk blotting buffer (PBS containing 0.05% Tween 20) at 4°C overnight. The membrane was rinsed with the blotting buffer and probed by 1:1000 diluted anti-human P450 antibodies in 2.5% nonfat dried milk blotting buffer for 1 h at room temperature. The membrane was rinsed six times (10 min each time) with the blotting buffer and exposed to 1:10,000 diluted secondary antibodies labeled with horseradish peroxidase for 1 h at room temperature. After being extensively rinsed with the blotting buffer, the membrane was exposed to the substrate of peroxidase (enhanced chemiluminescence reagent).

P450 proteins were detected by the fluorescence using an X-ray developer.

Instrumentation. Identification of metabolites and enzymes. LC/MS/MS systems were applied for the metabolite identification. The operation conditions were described as follows.

LC/MS was performed with LCQ ion trap mass spectrometer (Thermo Finnigan/GC & GC/MS Div., Austin TX). Hewlett Packard 1100 HPLC system (Hewlett Packard GmbH, Waldbronn, Germany) consisted of a binary pump, an autosampler, a column compartment unit, and an online variable wavelength UV detector. The detector was monitored at 270 nm, which is the UV\(_{max}\) of E2101 predetermined by scanning between 225 to 400 nm using a photodiode array detector (PDA). The metabolites were separated on a Hewlett Packard Eclipse C\(_18\) column (150 × 2.1 mm). The mobile phases were 10 mM ammonium acetate at pH 4.8 (A), and acetonitrile (B). The gradient (B) was 10% (0–6 min), 22% (12–22 min), 90% (25–30 min), and 10% (31 min and after). The flow rate was 0.25 ml/min. Software Navigator (version 1.2, Thermo Finnigan/GC & GC/MS Div.) was used to control the HPLC and MS and to acquire the data. The MS was operated at positive electrospray ionization (ESI) with 5.2 kV ionization potential and 220°C heated capillary temperature. The product ion spectra were generated under 24 V collision energy, which was optimized for the fragmentation of E2101.

LC/MS/MS was performed with Sciex API 2000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA):
The metabolites were separated on a Supelco Discovery C_{18} column (150 × 2.1 mm; Sigma-Aldrich), and the mobile phase described previously was run at 1.0 ml/min with a 1:10 split. The gradient (B) was 20% (0–6 min), 60% (14–18 min), 95% (20–24 min), and 20% (25 min and after). The operation of the HPLC and the MS/MS was controlled by MacChrom (version 1.6; Applied Biosystems, Foster City, CA). The MS/MS was operated at positive ESI with 5.0 kV ionization potential and 400°C ion source temperature. The product ion spectra were generated applying collision-induced dissociation (CID) with optimized ion optic parameter settings.

**P450 Substrate Assays.** CYP1A2/2C9/3A4-mediated warfarin 6-, 7-, 8-, and 10-hydroxylation were as described by Brian et al., 1990; Rettie et al., 1992; and Zhang et al., 1995. The system consisted of a Hewlett Packard 1100 HPLC system with a binary pump, an autosampler, a column compartment unit, a PDA, and a fluorescence detector. The system was controlled by ChemStation (version 6.03, Hewlett-Packard). Warfarin and the metabolites were resolved on a Hewlett Packard Zorbax ODS C_{18} column (250 × 4.6 mm). The flow rate was 1 ml/min. The mobile phases were 250 mM ammonium acetate at pH 4.9 (A) and 100% acetonitrile (B). The gradient (B) was 10% (0 min), 40% (5–10 min), 60% (16–19 min), and 90% (22–26 min), 10% (27 min and after), 6-, 8-, and 10-Hydroxywarfarin were monitored by UV absorbance at 313 nm and 7-hydroxywarfarin by the fluorescence at EX=250 nm/EM=340 nm.

CYP2E1-mediated chloroxazone 6-hydroxylation was as described by Court et al. (1997). The equipment was similar to the system described for warfarin hydroxylations, except the substitution of a PDA by a variable wavelength detector. The HPLC column and mobile phases were also the same as for the warfarin assay. The gradient (B) was 10% (0 min), 40% (6–14 min), and 90% (16–20 min), 10% (21 min and after). 6-Hydroxychloroxazone was monitored by UV absorbance at 280 nm.

CYP2D6-mediated bufuralol 1'-hydroxylation was as described by Boobis et al. (1985). The HPLC system described was interfaced with a Sciei API 2000 triple quadrupole mass spectrometer. A Supelco Discovery C_{18} column (150 × 2.1 mm), and isotropic mobile phase was used. The mobile phase, 10 mM ammonium acetate-acetonitrile/60:40, was run at 0.2 ml/min. The operation of the LC/MS/MS was controlled by MacChrom (version 1.6). The MS/MS was operated at positive ESI with 5.5 kV ionization potential and 500°C ion source temperature. Multiple reaction monitoring (MRM) was applied for the quantification. The MRM transition ions were m/z: 278→186 for 1'-hydroxybufuralol, and m/z 266→234 for the IS alendazole.

CYP2C19-mediated S-mephenytoin 4'-hydroxylation was as described by Goldstein et al. (1994). The LC/MS/MS system, including the separation column, described for the bufuralol hydroxylation was used. The isotropic mobile phase, 100 mM formic acid-acetonitrile/75:25, was run at 0.25 ml/min. The operation of the LC/MS/MS was controlled by MacChrom (version 1.6). The MS/MS was operated at positive ESI with a 5.5 kV ionization potential and 100°C ion source temperature. MRM was applied for the quantification. The MRM transition ions were m/z: 235→150 for 4'-hydroxymephenytoin, and m/z 260→183 for the IS R-propranolol.

**Data Analysis.** Data were acquired and analyzed by ChemStation (version 6.03, Hewlett Packard) for 6-, 7-, and 10-hydroxywarfarin and 6-hydroxychloroxazone and MacChrom (version 1.6) for 1'-hydroxybufuralol and 4'-hydroxymephenytoin. Quantification was based on peak area ratios of metabolites over the respective IS against the respective concentration of the standards. The metabolite curves were generated by linear regression with (6-, 7-, and 10-hydroxywarfarin, 1’-hydroxybufuralol and 4’-hydroxymephenytoin) or without (6-hydroxychloroxazone) a weighting factor (1/x²). The metabolic rates were determined using Excel (Microsoft Office 97; Microsoft Corporation, Redmond, WA) or SigmaPlot (version 6.00; SPSS Inc., Chicago, IL). Apparent inhibition constants (K_i) were estimated by Dixon plots generated by the linear regression analyses and by SNLR analyses applying the reversible inhibition models of Michaelis-Menten kinetics (Engel, 1996). The equations of velocity or turnover rate derived from these models are as follows:

\[
V = V_{\max}(1 + K_{i}S/(1 + I/K_{i}))
\]

\[
V = V_{\max}(1 + (1 + K_{i} + K_{i}S))
\]

\[
V = V_{\max}(1 + K_{i}/S)/(1 + I/K_{i})
\]

\[
V = V_{\max}[(1 + I/K_{i})' + (1 + K_{i}/S)(1 + I/K_{i})]
\]

Equation 1 is for the competitive inhibition model, eq 2 is for the uncompetitive model, eq 3 is for the noncompetitive model, and eq 4 is for the mixed inhibition model. S is the substrate concentration, and I is the inhibitor concentration. V_{\max} is the maximum turnover rate, and K_{i} is the substrate concentration at which the turnover rate is half of the maximum. K_{i} is the competitive inhibition constant, whereas K_{i}' is the uncompetitive inhibition constant. K_i is the dissociation constant of the enzyme-substrate complex.

Statistical analyses were performed using SigmaPlot and SigmaStat (version 2.03; SPSS Inc.). PowerPoint (Microsoft Corporation) was applied for the reconstruction of the images of Western immunoblots after being scanned.

**Results**

**Identification of Metabolites and Metabolic P450 Forms. Metabolite identification.** Several metabolites were detected in the reconstituted systems containing pooled HLM or HLS9 using mass spectrometers. Formation of these metabolites was NADPH-dependent indicating the involvement of P450s. The possible biotransformations were mono-oxidations at several positions, N-dealkylation at either the piperidinyl or methyl amido moiety, and multiple oxidations at different sites such as sequential mono-oxidations to form diol metabolites.

The identification of the major metabolites was undertaken using MS/MS spectral analyses. These metabolites (M1–M4), listed in Table 1, were detected online by both UV absorbance at 270 nm (Fig. 1) and MS total ion current scanned between 60 to 1800 atomic mass units. The MS/MS fragmentation patterns of the phase I metabolites and parent compound are often similar. The MS and the MS/MS product ion spectrum of E2101, serving as references for the spectral interpretation for the metabolites, were first determined (Fig. 2). The predominant MS ion at m/z 394 was the MH⁺ ion of E2101, and its intensive MS/MS product ions at m/z 178, 206, 123, and 229 were likely formed after the CID fragmentations at the positions proposed in Fig. 2. Two of the major metabolites (M1 and M2) formed in reconstituted systems containing HLM or HLS9 exhibited the MH⁺ ion at m/z 410. Apparently, these were the monohydroxylated metab-

**Table 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Major Metabolite</th>
<th>Relative Amount (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>E2101</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>M1</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>M2</td>
<td>30</td>
<td>7</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>M3</td>
<td>N.D.</td>
<td>2</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>M4</td>
<td>11</td>
<td>6</td>
</tr>
</tbody>
</table>

N.D., not detected.

<sup>a</sup> Estimated by the UV absorbance at 270 nm.
olites because of 16 mass unit increment compared with E2101. M1, one of the most abundant microsomal metabolites, was dissociated under CID in the triple quadrupole or the ion trap MS to the product ions at m/z 194, 222, 229, 241, and 392 (Fig. 3). These characteristic product ions suggested that the metabolite was the hydroxylated E2101 at the moiety of fluorophenethyl piperidine. The product ions of the metabolite at m/z 194 and 222 were likely the counterparts of E2101 at m/z 178 and 206, respectively. The product ions at m/z 176 and 164, however, were possibly secondary. The abundant ion at m/z 392, apparently formed by the H$_2$O elimination from the MH$^+$ ion, of the metabolite at m/z 194 and 222 were likely the counterparts of E2101 at m/z 178 and 206, respectively. The product ions at m/z 176 and 164, however, were possibly secondary. The abundant ion at m/z 392, apparently formed by the H$_2$O elimination from the MH$^+$ ion,

Fig. 1. E2101 metabolic profiles in reconstituted in vitro human enzyme systems.

The metabolic profiles were monitored by the UV absorbance at 270 nm. The final protein concentration was 2 mg/ml in the reaction mixtures containing the subcellular fractions and 50 pmol/ml in the mixtures containing the recombinant proteins. The other reaction conditions were described under Experimental Procedures.

Fig. 2. E2101 MS (A) and MS/MS spectrum (B).

The spectra were generated by an online MS/MS (API 2000) after the compound was eluted from the HPLC column. The MS/MS product ion spectrum was generated after the fragmentation of the quasi-molecular ions of E2101 (MH$^+$) applying the collision energy. The detail LC/MS and LC/MS/MS conditions were described under Experimental Procedures.

Fig. 3. Product ion spectra of metabolite M1 formed in the reaction mixture containing HLM; the MS$^2$ spectrum by the ion trap MS (A) and the MS/MS spectrum by the MS/MS (B).

The product ion spectra were generated after the CID fragmentation of the quasi-molecular ions (MH$^+$) of M1 eluted from HPLC column. The in vitro metabolic reaction and LC/MS (ion trap) and LC/MS/MS condition were described under Experimental Procedures.
would further suggest the position of hydroxyl group was aliphatic rather than aromatic. Therefore, it would be reasonable to assign this metabolite as monohydroxylated E2101 with the hydroxyl group at one of the two carbons between the piperidinyl and the fluorophenyl group, preferably at the 9-carbon to the piperidinyl nitrogen. In contrast, the other major hydroxylated metabolite (M2) produced identical MS/MS product ions at m/z 206, 178, and 123, as E2101 (Fig. 4B). Therefore, the hydroxyl group of the metabolite was not likely at the fluorophenethyl piperidinyl moiety, which was further supported by the MS2 spectrum produced by the ion trap mass spectrometer (Fig. 4A). The formation of product ions at m/z 351 and 379 in the ion trap MS indicated that the site of the hydroxyl group should be at the fluoroephenthyl piperidinyl moiety, which was further supported by the MS2 spectrum produced by the ion trap mass spectrometer (Fig. 4A). The formation of product ions at m/z 351 and 379 in the ion trap MS indicated that the site of the hydroxyl group should be at the indolyl acetamide, likely either on the indolyl ring or the carbon between the indolyl and amido group. However, the actual position of the M2 hydroxyl group could not be clarified although the lack of the MS2 product ion at m/z 392 did imply that the hydroxyl group was aromatic rather than aliphatic. E2101 could be also hydroxylated at more than one site, evidenced by the detection of M3. M3 was one of the major, if not the only, diol metabolite detected because of the increment of 32 atomic mass units of the MH+ ion (m/z 426) as compared with that of E2101 (Fig. 5A). M3, similar to M2 and E2101, produced the MS2 product ions at m/z 206, 178, and 123, thus possessed the intact E2101 fluorophenethyl piperidinyl moiety (Fig. 5B). Interestingly, the contrast between the abundant product ion at m/z 408 and the lack of the product ion at m/z 390 indicated the possible elimination of one, but not likely two, H2O molecule from the MH+ ion of M3 during the CID fragmentations, thus suggesting

![Figure 4](image4.png)

**Fig. 4.** Product ion spectra of metabolite M2 formed in the reaction mixture containing HLM: the M2 spectrum by the ion trap MS (A) and the MS/MS spectrum by the MS/MS (B).

The product ion spectra were generated after the CID fragmentation of the quasi-molecular ions (MH+1) of MS2 eluted from HPLC column. The in vitro metabolic reaction and LC/MS (ion trap) and LC/MS/MS condition were described under Experimental Procedures.

![Figure 5](image5.png)

**Fig. 5.** MS and MS2 spectrum of metabolite M3 formed in reaction mixture containing HLM: the MS spectrum (A) and the MS2 spectrum (B).

The spectra were generated by an online ion trap MS after M3 was eluted from the HPLC column. The MS2 product ion spectrum was generated after the CID fragmentation of the quasi-molecular ions (MH+1) of M3. The detail LC/MS (ion trap) condition was described under Experimental Procedures.

the possible coexistence of both aliphatic and aromatic hydroxyl group. Therefore, the sites where the hydroxyl groups attached would likely be in the indolyl ring and between the indolyl and amido group. N-Dealkylated metabolite of E2101 (M4), in addition, was also detected. The structural elucidation for the dealkylated metabolite was straightforward, particularly when the nitrogen rule was applied (McLafferty and Turecek, 1993). The MH+ ion at m/z 272 (Fig. 6A) indicated that M4 was a cleavage product of E2101, likely a dealkylated metabolite. The even m/z number of the MH+1 ion of M4 would suggest that the metabolite possessed an odd number of nitrogen atoms, as the case of that of E2101 (Fig. 2). Therefore, the metabolite should have either one or three nitrogen atoms, and such a requirement would be fulfilled only if M4 was formed by the dealkylation at the piperidinyl nitrogen (Figs. 6B and 7). Several minor E2101 metabolites including the N-demethylated metabolite, detected only by LC/MS, were not presented and discussed. The major metabolic pathways of E2101 are proposed in Fig. 8.

**Metabolic enzyme identification.** The metabolic enzymes were identified by detecting the formation of metabolites in reconstituted enzyme systems. The rate of NADPH-dependent metabolism was found to be faster in the reaction mixture containing pooled HLM than that containing pooled HLS9 (Fig. 1). Therefore, the major metabolic enzymes of E2101 would be microsomal oxidases, or hepatic P450s. The responsible P450 forms for E2101 metabolism were further determined using a panel of recombinant human P450s including CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. In the presence of approximately 50 μM E2101, CYP2C19, CYP2D6, and CYP3A4, among the major P450 forms tested, metabolized E2101 (Fig. 1). The formation of the major E2101 metabolites was P450 form-dependent (Table 1). CYP3A4 produced the broadest
The spectra were generated by an online ion trap MS after M4 was eluted from the HPLC column. The MS² product ion spectrum was generated after the CID fragmentation of the quasi-molecular ion (MH⁺) of M4. The detail LC/MS (ion trap) condition was described under Experimental Procedures.

The spectra of metabolites including M1-M4, similar to that generated by HLM or HLS9 preparations. However, CYP2C19 and CYP2D6 produced rather distinctive metabolite profiles. CYP2D6 preferably converted E2101 to M3, and M2 to a much less extent, whereas CYP2C19 converted E2101 to form all the major metabolites but M3. Apparently, the formation of the hydroxylated metabolite M2 was not metabolizing P450 form-specific.

P450 Inhibition. A panel of P450 substrate assays was applied to determine the P450 form-specific inhibition as described under Experimental Procedures. The quantifications were based on the standard calibration curves. The correlation coefficient (r²) for each calibration curve was at least 0.990. The experimental quality was also ensured by quality control samples. As shown in Table 2, no inhibitory effect of E2101 at 10 or 50 μM on CYP1A2, CYP2C9, CYP2E1, and CYP3A4 activity was detected, as assessed by R-warfarin 6- (CYP1A2) and 10-hydroxylation (CYP3A4), S-warfarin 7-hydroxylation (CYP2C9), and chlorzoxazone 6-hydroxylation (CYP2E1). However, the activities of CYP2C19-mediated S-mephénytoin 4'-hydroxylation and CYP2D6-mediated bufuralol 1'-hydroxylation were significantly reduced at the presence of E2101 in a concentration-dependent manner.

The apparent inhibition constants (Kᵢ) of CYP2C19 and CYP2D6 were determined using the microsomal preparations containing cDNA-expressed recombinant P450 proteins. The apparent Kᵢ values were first estimated by the Dixon plots. The estimated Kᵢ varied between 25 to 45 μM for CYP2C19 (Fig. 9A), and approximately 20 μM for CYP2D6 (Fig. 9B). The apparent Kᵢ values were also determined by SNLR analysis using the common reversible inhibition models of Michaelis-Menten kinetics including the competitive, uncompetitive, noncompetitive, and mixed inhibition model. The competitive inhibition model was selected to determine the Kᵢ because of the best curve fitting (r² > 0.97 for CYP2C19 inhibition; r² > 0.99 for CYP2D6 inhibition). In consistence with the Dixon plots, the apparent Kᵢ values determined by SNLR were 48 μM for CYP2C19 inhibition and 15 μM for CYP2D6 inhibition. Both the Dixon plots and SNLR analyses suggested that the inhibitions of CYP2C19 and 2D6 by E2101 were primarily, if not fully, competitive.

P450 Induction. P450 induction was evaluated applying primary culture of human hepatocytes from three donors (two smokers and one nonsmoker). At the time of the reception of the hepatocyte cultures, the confluence of the cells from the nonsmoker was slightly denser than those from the smokers. Moreover, the cell density tended to reduce slightly although marked morphologic change was not observed among the control cells and those exposed to E2101 during the treatment. Therefore, it may not be suitable to compare the protein expression among the cells from different donors. With the exception of the monoclonal anti-CYP2D6 antibodies, the polyclonal primary antibodies used for immunochemical detection would cross-react with the members in the same P450 subfamilies. Therefore, the enzymes determined were CYP1A1/2, CYP2C8/9/19, CYP2D6, and CYP3A4/5. As shown in Fig. 10, the cells responded to the inductions of CYP1A1/2, CYP3A4/5, and CYP2C8/9/19 as demonstrated by the elevated expressions of these proteins in the hepatocytes after the exposure to TCDD and rifampicin. However, the expression of CYP1A1/2, CYP2C8/9/19, CYP2D6, or CYP3A4/5 did not increase in the cells exposed to E2101 at 10 or 50 μM for 72 h. Therefore, E2101 did not appear to induce the expression of these P450 forms.

Discussion

The advance in molecular biology during the last decade has led us to determine the metabolic roles of human P450s and to uncover the
possible mechanism of the clinical drug-drug interactions (Fang and Gorrod, 1999; Malaty and Kuper, 1999; Venkatakrishnan et al., 2000). Today it has been acknowledged that the majority of drug-drug interactions were due to the interactions between the therapeutic agents and their metabolic enzymes, especially P450s. Therefore, metabolic interaction studies of new drug entities in vitro for predicting potential risk of drug-drug interaction before the clinical trials are becoming useful.

E2101, similar to some of the 5-HT1/2 antagonistic amine derivatives, was metabolized by CYP2C19, CYP2D6, and CYP3A4 to a series of oxidative metabolites, including the mono- and dihydroxylated, and N-dealkylated metabolites. Based on the MS/MS spectral analyses, the structures of the major microsomal metabolites were proposed. Without synthetic standards, the MS/MS spectra may not be sufficient for the identification of the exact sites of the hydroxyl groups for M1–M3. However, the potential mechanism of P450 catalytic reactions may assist, at least to some extent, the structural interpretation of these metabolites. For example, the hydroxylation to form M1 could possibly take place at the positions other than the proposed, especially the α-carbon next to the piperidinyl nitrogen since the CID fragmentations would possibly produce the same MS/MS spectra as shown in Fig. 3. However, the formation of such metabolite would not be feasible based on well known P450-mediated oxidative dealkylations. If the hydroxylation did take place at this putative site, the metabolite would exist rather as the more stable N-dealkylated metabolite M4.

Although exemplified only for the structural elucidation of the N-dealkylated metabolite M4, the nitrogen rule was indeed applicable for the interpretation of all of the MS spectra shown (Figs. 2–6). Noticeably, the nitrogen rule in ESI-MS/MS would be the reverse of what originally applied in GC/MS due to the preferred formation of quasi-molecular ion (or quasi-product ion). For example, as shown in Fig. 2, the MH+ ion of the odd nitrogen atoms containing E2101 exhibited an even m/z number (394). In addition, two of the proposed MS/MS product ions of E2101 containing the even nitrogen atoms (0 and 2) exhibited odd m/z numbers (123 and 229), and the other product ions containing the odd nitrogen atom (1) exhibited even m/z numbers (178 and 206). Therefore, such a useful tool might be applied more often for the interpretation of ESI-MS/MS spectra because the formation of odd-electron ions or radical ions is usually unstable, thus rare in ESI-MS/MS.

The potential contribution of individual E2101-metabolizing P450 forms would not be accurately estimated without knowing the kinetic parameters. However, the metabolic profile of E2101 generated by recombinant CYP3A4, the most abundant human hepatic
P450 form (Shimada et al., 1994), was similar to that observed in the reconstituted system containing HLM (Fig. 1). This metabolic profile in HLM was virtually concentration-independent as determined using 10, 50, and 200 μM E2101 (data not shown). Moreover, at 50 μM E2101 concentration, 16 μM CYP3A4 inhibitor ketoconazole inhibited at least 75% of the formation of all major metabolites (M1–M4), whereas 1.6 μM CYP2D6 inhibitor quinine inhibited approximately 40% of the formation of M3 but M2 to a much less extent in the reaction mixture containing HLM. The concentrations of the inhibitors used were at least several-fold higher than their respective $K_i$ values (Bourrie et al., 1996; Fogelman et al., 1999), thus they should be able to inhibit the majority, if not all, of these P450 form-specific activities in HLM. Therefore, the metabolic profile in HLM appeared to be largely controlled by the hepatic CYP3A4 activity, and CYP3A4 is likely the one responsible for the hepatic metabolism of E2101, with the contribution of CYP2D6, especially for the formation of M3.

The structural requirements to be the substrates (or inhibitors) of CYP2D6 are probably the best defined among the major metabolic P450 forms. E2101 containing basic nitrogens, and two or more hydrophobic regions, is structurally similar to some of the well known CYP2D6 substrates (Ekins et al., 1999). However, such structural requirements for CYP2C19 or CYP3A4 could not be depicted due to the limited structure-activity relationship studies reported for CYP2C19 (Lock et al., 1998; Ekins et al., 2001) or the diverse substrate selectivity of CYP3A4 (Smith et al., 1997). The lack of CYP3A4 inhibition by E2101 as determined by CYP3A4-mediated R-warfarin 10-hydroxylation may also indicate atypical enzyme kinetics for CYP3A4-mediated reactions including possibly two or more different substrate (or inhibitor) binding sites (Wang et al., 2000; Oda and Kharasch, 2001; Shou et al., 2001). Therefore, our data would not exclude the possibility of the E2101 alteration of CYP3A4-mediated metabolism other than R-warfarin, which may require further studies to clarify. On the other hand, based on Michaelis-Menten enzyme kinetics, the apparent $K_i$ values for CYP2C19 and CYP2D6 inhibition were estimated by both Dixon plots and SNLR analyses in this study. The values generated by these two methods were quite consistent. Dixon plots, providing the visual indication of the enzyme inhibition mechanism, were used for the initial estimation of the $K_i$ values, which were confirmed using SNLR method (Kakkar et al., 1999). The data obtained from a single experiment were simultaneously analyzed by both methods, and the consistent results would be thus expected reliable for the inhibition of CYP2C19 and CYP2D6 detected.

The induction of the members in the subfamilies of CYP1A, CYP2C, and CYP3A, and as well as CYP2D6 at protein level was not detected in the primary human hepatocytes after E2101 exposure. CYP1A enzymes are responsible for the bioactivation of a variety of polycyclic aromatic hydrocarbons (Liu et al., 2001), whereas CYP3A4 is the most common drug-metabolizing P450 form (Shimada et al., 1994). In addition, the effect of E2101 on the expression of the minor but inducible CYP2C19, and constitutive CYP2D6, was also determined for the elimination of possible feedback from enzyme inhibition. Instead of the possible induction, the expression of some P450s appeared to be slightly suppressed in cells exposed to 50 μM E2101. However, such a weak suppression by E2101 at higher concentration may need to be confirmed since Western blots were at most

### TABLE 2

<table>
<thead>
<tr>
<th>E2101</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP2E1</th>
<th>CYP3A4</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>0</td>
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<td>0.81 ± 0.01</td>
<td>49 ± 5</td>
<td>22 ± 0.9</td>
<td>193 ± 45</td>
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<td>44 ± 3</td>
<td>7.4 ± 2.5**</td>
<td>142 ± 25</td>
<td>9.7 ± 1.7</td>
</tr>
<tr>
<td>50</td>
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<td>0.91 ± 0.05</td>
<td>33 ± 4*</td>
<td>4.2 ± 0.4**</td>
<td>185 ± 27</td>
<td>12.5 ± 1.1</td>
</tr>
</tbody>
</table>

* Significant difference from the controls (0.01 < p < 0.05).
** Highly significant difference from the controls (p < 0.01).

$K_i$ values (Bourrie et al., 1996; Fogelman et al., 1999).

$N$ = 4.

Fig. 10. Effect of E2101 on P450 expression in primary human hepatocytes after 72-h exposure.

The protein expression was determined immunochemically, and anti-P450 antibodies, and chemiluminescent reagents were used for the detection. Cells treated with TCDD (0.4 μM) were used to serve as the positive control for CYP1A induction, and cells treated with rifampicin (50 μM) were used to serve as the positive control for CYP3A, and probably CYP2C19, induction. The detail experimental condition was described under Experimental Procedures.
semiquantitative, which may not be applied appropriately to interpret subtle differences. Nevertheless, the lack of induction of the major E2101-metabolizing P450 forms in primary culture of human hepatocytes suggests that the potential drug-drug interaction in clinic due to P450 induction would be unlikely.

In summary, the drug-drug interaction between E2101, a potential muscle relaxant, and human P450s was characterized in vitro. E2101 was metabolized by CYP2C19, CYP2D6, and CYP3A4, and CYP3A4 to form a series of phase I oxidative metabolites including mono- and dihydroxylation, and N-dealkylated metabolites. The structures of these metabolites were elucidated based on the MS/MS spectral analyses. E2101 moderately inhibited the activities of CYP2C19 and CYP2D6 in a competitive manner but did not induce the expression of any E2101-metabolizing P450 or CYP1A enzymes in human primary hepatocytes. Therefore, although the understanding of in vitro interaction between E2101 and P450s would be beneficial for the clinical trials, in particular for patients taking coadministered medications that are metabolized by CYP2C19 and/or CYP2D6, a low risk of drug-drug interaction in clinic would be anticipated.

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References