Phenytoin has been widely prescribed as a broad-spectrum anticonvulsant for the prevention and treatment of seizure disorders. Several of its pharmacological characteristics (i.e., narrow therapeutic range, slow absorption, and saturable metabolism) have been frequently implicated in clinically significant drug interactions (Nation et al., 1990; Monaco and Cicolin, 1999). p-Hydroxylation is the major metabolic pathway of phenytoin, and forms 5-[(p-hydroxyphenyl)-5-phenylhydantoin (HPPH), which accounts for 90% of all metabolites in humans (Browne and LeDuc, 1995). This conversion is catalyzed mainly by CYP2C9, although CYP2C19 also plays a role (Baipai et al., 1996; Giancarlo et al., 2001).

Some authors have observed that TCAs inhibit phenytoin elimination, with a consequent risk for toxic phenomena. Imipramine and nortriptyline have been reported to increase plasma phenytoin levels in patients with epilepsy (Richens and Houghton, 1975; Perucca and Richens, 1977). However, the mechanism of this interaction has not been addressed.

Even though TCAs are commonly coadministered with a wide variety of other drugs, little is known about their inhibitory effects on P450 isoforms. Therefore, it is difficult to predict the P450 isoform(s) involved in the interaction between TCAs and phenytoin, a known substrate of CYP2C9 and CYP2C19 (Bajpai et al., 1996; Giancarlo et al., 2001).

Even though CYP2C9 is the main isoform involved in the formation of HPPH, a primary metabolite of phenytoin, this isoform does not appear to be inhibited by TCAs. There is indirect evidence that the increase in plasma phenytoin produced by TCAs does not involve inhibition of CYP2C9; for instance, omeprazole, cimetidine, and ticlopidine have been reported to increase plasma phenytoin concentrations (Levy, 1995; Rindone and Bryan, 1996), but it has not been shown that any of these drugs inhibit CYP2C9-catalyzed 7-hydroxylation in vitro or affect S-warfarin clearance in vivo (Andersson et al., 1990; Niopas et al., 1991). Furthermore, since no interaction is observed when TCAs are coadministered with two well known CYP2C9 substrates, mephenytoin and moclobemide (Zimmer et al., 1990; Baumann et al., 1992), TCAs appear not to strongly inhibit the metabolism of CYP2C9 substrates. None of these reports explain how TCAs increase the plasma concentration of phenytoin in epileptics (Richens and Houghton, 1975; Perucca and Richens, 1977).

Therefore, in this study, we assessed the potential of TCAs to inhibit different P450 isoforms in vitro, to examine the mechanism of the drug interaction between TCAs and phenytoin. First, we evaluated
whether the TCAs—amitriptyline, nortriptyline, imipramine, and desipramine—inhibited phenytoin \( p \)-hydroxylation in microsomal incubations in vitro. Then, we used incubation studies of human liver microsomes and cDNA-expressed P450s to determine the inhibitory potential of TCAs on P450 isomeric-specific metabolic pathways.

### Materials and Methods

#### Chemicals and Reagents

5,5-Diphenylhydantoin (phenytoin) and \( \beta \)-HPPH were purchased from Sigma-Aldrich (St. Louis, MO). 1-Hydroxymidazolam, \( \beta \)-warfarin, 7-hydroxywarfarin, \( \beta \)-mephenytoin, dextromethorphan, and 4\(^{-}\)-hydroxymephenytoin were obtained from Ultrafine Chemical Co. (Manchester, UK). Dextromethorphan, ketoconazole, tricyclic antidepressants (imipramine, desipramine, amitriptyline, and nortriptyline), omeprazole, sulfaphenazole, chlorozoxazone, furafylline, NADP, NADPH, EDTA, MgCl\(_2\), G-6-P, and G-6-PDH were obtained from Sigma-Aldrich, Acetonitrile and methanol were acquired from Fisher Scientific Co. (Pittsburgh, PA). Midazolam was kindly provided by Roche Korea Co. (Seoul, Korea). All other reagents and chemicals used were of analytical or HPLC grade.

#### Human Liver Microsomes and cDNA-Expressed P450s

Microsomes were prepared from human liver tissue (HL-10, 14, 15, 19, and 20) that was obtained from patients undergoing partial hepatectomy for removal of metastatic tumor at the Department of General Surgery, Busan Paik Hospital (Busan, Korea). The tissues were nontumor-bearing parenchyma and confirmed to be histopathologically normal. Tissue obtained from patients who had taken any known P450 inhibitors or inducers in the week before the surgical operation was not used. The use of human liver tissue was approved by the Institutional Review Board of the hospital. Microsomes were prepared by differential centrifugation of liver homogenate as described previously (Ko et al., 1997). The resulting microsomal pellets were resuspended at a final protein concentration of 10 mg/ml in 100 mM phosphate buffer (pH 7.4) by the manufacturer.

#### Incubation Studies

From the preliminary studies, the optimum conditions for microsomal incubation were determined in the linear range for HPPH formation from phenytoin, expressed as the quantity of HPPH formed per unit of protein concentration and time. In all experiments, phenytoin was dissolved and diluted serially in methanol. The solvent was subsequently removed by evaporation to dryness, under reduced pressure in 1.5-ml polypropylene tubes, with an AES2010 SpeedVac (Savant Instruments Inc., Holbrook, NY). The phenytoin was then reconstituted in 50 mM phosphate buffer (pH 7.4). The incubation mixtures containing either 25 \( \mu \)l of microsomes (10 mg/ml of stock) or 25 \( \mu \)l of cDNA-expressed P450s (diluted to 200 pmol/ml with buffer, pH 7.4) and phenytoin reconstituted in phosphate buffer were prewarmed for 5 min at 37°C. Reactions were initiated by adding the NADPH-regenerating system (including 1.3 mM NADP, 3.3 mM G-6-P, 3.3 mM MgCl\(_2\), and 1.0 U/ml G-6-PDH), and the reaction mixtures (final volume, 250 \( \mu \)l) were incubated for 60 min at 37°C. Reactions were stopped by placing the incubation tubes on ice and adding 100 \( \mu \)l of 10% ice-cold perchloric acid. After addition of 20 \( \mu \)l (10 \( \mu \)M) of chlorozoxazone as an internal standard, the mixtures were centrifuged at 14,000 rpm for 5 min at 4°C, and aliquots of supernatant were injected onto an HPLC system.

#### Analytical Procedures

The concentrations of HPPH and internal standard were measured by HPLC. The system consisted of a Gilson 307 pump, an 118 UV detector, a 234 Autoinjector (Gilson Co., Villiers Le Bel, France), and a \( \mu \)-Bondapak C\(_{18}\) column (3.9 mm i.d. \( \times \) 30 cm) packed with 10-\( \mu \)m particles at ambient temperature. Acetonitrile, 0.05% phosphoric acid, and 50 mM potassium phosphate (33:47:20, v/v, pH 3.2) constituted the mobile phase; the flow rate was 1.0 ml/min. The Unipoint analysis system (Gilson Co.) was used to calculate the HPPH concentration from the peak area ratios. Chromatograms were obtained with UV detection at a wavelength of 210 nm. Under these conditions, peaks of HPPH, internal standard, and phenytoin appeared at 6.0, 10.0, and 12.5 min, respectively. Most of the TCAs and their metabolites did not interfere with the HPPH peak, but since the desipramine peak partially overlapped the HPPH peak, we changed the mobile phase to acetonitrile, 0.05% phosphoric acid, and 50 mM potassium phosphate (30:47:23, v/v, pH 3.2) when desipramine was a test compound.

### Inhibition Studies on Phenytoin \( p \)-Hydroxylation

The inhibitory effects of TCAs and known P450 isomeric-selective inhibitors on phenytoin \( p \)-hydroxylation were compared to determine the P450 isoform(s) responsible for the interaction between TCAs and phenytoin. The formation rate of HPPH from phenytoin (final concentration, 25 \( \mu \)M) was determined from mixtures incubated in the absence or presence of TCAs and known P450 isomeric-selective inhibitors, furafylline for CYP1A2 (Sesardic et al., 1990), sulfaphenazole for CYP2C9 (Baldwin et al., 1995), omeprazole for CYP2C19 (Ko et al., 1997), quinidine for CYPD2B6 (Brolly et al., 1989), and ketoconazole for CYP3A4 (Baldwin et al., 1995). Quercetin was tested as a potential inhibitor of CYP2C8 (Desai et al., 1998). Since amitriptyline and imipramine showed remarkable inhibition of HPPH formation from phenytoin, detailed inhibition studies were conducted after coincubation of various combinations of phenytoin concentration (final, 10, 25, 50, and 100 \( \mu \)M) and a TCA (concentration range of 1–100 \( \mu \)M). We also incubated 25 \( \mu \)M of phenytoin and TCAs (10 and 50 \( \mu \)M) with 20 pmol of cDNA-expressed CYP2C9 or CYP2C19, to determine which specific P450 isoform-catalyzed phenytoin \( p \)-hydroxylation was inhibited by the TCAs.

### Inhibition Studies on P450 Isomeric-Specific Substrates

The inhibitory effects of the TCAs on each P450 isoform were evaluated by human liver microsomal incubations, using probe drugs specific for each P450 isoform. The apparent kinetic parameters for phenytoin \( p \)-hydroxylation by CYP2C9 and CYP2C19- and 3A4-mediated metabolism were determined from microsomal incubations, using probe drugs specific for each P450 isoform. The reaction probes used were phenacetin O-demethylation for CYP1A2 (Tasaneeyakul et al., 1993), \( \beta \)-warfarin 7-hydroxylation for CYP2C9 (Rettie et al., 1992), \( \beta \)-mephenytoin 4\(^{-}\)-hydroxylation for CYP2C19 (Hoth et al., 1993), dextromethorphan O-demethylation for CYP2D6 (Brolly et al., 1989), and midazolam \( 1 \)-hydroxylation for CYP3A4 (Thummel et al., 1994). The incubation conditions and analytical assays for the activity of these isoforms were similar to the method previously described (Thummel et al., 1994; Shin et al., 1999).

#### Data Analysis

Results were expressed as mean ± SD of estimates obtained from the three different liver microsomes with duplicated experiments. The apparent kinetic parameters for phenytoin \( p \)-hydroxylation \( (K_m\) and \( V_{\max} \)) and inhibitory potential \( (IC_{50}\) and \( K_i \)) were initially estimated by graphical methods (Lineweaver-Burk plot, Dixon plot, and secondary Lineweaver-Burk plot) but ultimately determined by nonlinear least square regression analysis from the best enzyme kinetic model and enzyme inhibition model (Segel, 1975) using WinNolin software (Scientific Consulting Inc., Apex, NC).

### Results

#### Characterization of Phenytoin \( p \)-Hydroxylation

Under our experimental conditions, the five human liver microsomal preparations produced sufficient \( p \)-hydroxy metabolite from phenytoin, and showed a 2- and 3-fold variance of \( K_m\) (range, 14.4 to 30.7 \( \mu \)M) and \( V_{\max} \) (range, 9.0 to 26.6 nmol/min/mg of protein) for phenytoin \( p \)-hydroxylation (Table 1). HPPH formation was completely abolished by 5 \( \mu \)M sulfaphenazole, and partly inhibited by 10 \( \mu \)M omeprazole, but not by furafylline, quercetin, quinidine, or ketoconazole (Fig. 1). These results indicate that our experimental conditions were appropriate for the subsequent inhibition studies on CYP2C9- and CYP2C19-catalyzed phenytoin \( p \)-hydroxylation. In addition, we also confirmed that cDNA-expressed CYP2C9 and CYP2C19 produced HPPH metabolite at a velocity of 31.1 ± 5.6 and 31.8 ± 3.1 pmol/min/mg of protein for phenytoin \( p \)-hydroxylation (Table 1). HPPH formation was de-
The inhibitory effects of TCAs on specific P450 isoform-catalyzed phenytoin ⁶-hydroxylation were evaluated, none inhibited the HPPH formation catalyzed by cDNA-expressed CYP2C9 (Fig. 4). However, HPPH formation catalyzed by cDNA-expressed CYP2C19 was strongly inhibited by all of the TCAs.

**Inhibitory Effects of TCAs on P450 Isoform-Specific Substrates.** Among the P450 isoforms tested, CYP2D6-catalyzed dextromethorphan O-demethylation was the most strongly and competitively inhibited by all of the TCAs (Fig. 5D); the dextrorphan formation rate was decreased to 17 to 30% of control activity at the highest concentrations tested (100 μM). With this enzyme, secondary amine TCAs were more potent inhibitors than tertiary amine TCAs.

TCAs also inhibited CYP2C19-catalyzed S-mephenytoin ⁴'-hydroxylation (Fig. 5A). In contrast to the results for CYP2D6, nortriptyline and imipramine showed remarkable inhibition of CYP2C19-catalyzed S-mephenytoin ⁴'-hydroxylation, whereas nor- triptyline and desipramine showed minimal inhibition. The estimated IC₅₀ values were 59.1 ± 7.0 and 111.6 ± 8.0 μM for amitriptyline and imipramine, respectively, whereas those for nortriptyline and

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**TABLE 1**

<table>
<thead>
<tr>
<th>Microsome No.</th>
<th>Kinetic Parameters of HPPH Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vₘₐₓ (nmol/min/mg protein)</td>
</tr>
<tr>
<td>HL-10</td>
<td>3.5</td>
</tr>
<tr>
<td>HL-14</td>
<td>20.0</td>
</tr>
<tr>
<td>HL-15</td>
<td>18.7</td>
</tr>
<tr>
<td>HL-19</td>
<td>9.0</td>
</tr>
<tr>
<td>HL-20</td>
<td>26.6</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>17.6 ± 6.7</td>
</tr>
</tbody>
</table>

Cₘₐₓ, intrinsic clearance.

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**Fig. 2.** Inhibition of HPPH formation from phenytoin (25 μM) by TCAs in human liver microsomal incubations.

Data indicate mean ± S.D. of percentage of control activity estimated from three different human liver microsomal preparations (HL-10, 14, and 19). The symbols used are amitriptyline (○), imipramine (●), nortriptyline (▲), and desipramine (▼).

**Fig. 3.** Representative Dixon plots for the inhibition by amitriptyline (A) and imipramine (B) of the formation of HPPH from phenytoin in human liver microsomal incubations (HL-14).

The human liver microsomes were incubated with 10 (○), 25 (●), 50 (▲), and 100 μM (▼) of phenytoin in the absence or presence of TCAs as described under Materials and Methods.

When the inhibitory effects of TCAs on specific P450 isoform-catalyzed phenytoin ⁶-hydroxylation, a competitive inhibition model was best-fitted to the data of both amitriptyline and imipramine (estimated Kᵢ values of 5.2 ± 3.5 μM and 15.5 ± 8.1 μM, respectively, Fig. 3). However, the secondary amine TCAs, nortriptyline and desipramine, produced only weak inhibition—less than 15% reduction in activity—of phenytoin ⁶-hydroxylation, even at the highest concentrations tested (100 μM, Fig. 2).

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**Fig. 1.** Comparison of the inhibitory potential of TCAs (each 50 μM) on phenytoin ⁶-hydroxylation with those of known CYP-selective inhibitors, including furafylline (CYP1A2, 10 μM), quercetin (CYP2C8, 20 μM), sulfaphenazole (CYP2C9, 5 μM), omeprazole (CYP2C19, 10 μM), quinidine (CYP2D6, 1 μM), and ketoconazole (CYP3A4, 1 μM), in human liver microsomal incubations (HL-14, 15, and 20).

Each value indicates mean ± S.D. of percentage of remaining activity relative to control HPPH formation rate (9.7 ± 0.7 nmol/min/mg of protein at 25 μM phenytoin).
**Fig. 4.** Inhibitory effects of TCAs on HPPH formation from phenytoin (25 μM) in incubations of cDNA-expressed CYP2C9 (A) and CYP2C19 (B), respectively. CYP2C19-catalyzed HPPH formation was competitively abolished by 50 μM of amitriptyline and nortriptyline (B). Data are averages of duplicate determinations.

**Fig. 5.** Effect of TCAs on CYP2C19-catalyzed S-mephenytoin 4′-hydroxylation (A), CYP1A2-catalyzed phenacetin O-deethylation (B), CYP2C9-catalyzed S-warfarin 7-hydroxylation (C), CYP2D6-catalyzed dextromethorphan O-demethylation (D), and CYP3A4-catalyzed midazolam 1-hydroxylation (E) in human liver microsomal incubations. The symbols for the TCAs used are amitriptyline (○), imipramine (○), nortriptyline (▲), and desipramine (▼). Each data point indicates the average value obtained from three different liver microsomal preparations (HL-10, 19, 20).
desipramine were 286 and 732 μM, respectively. Inhibition by amitriptyline and imipramine of CYP2C19-catalyzed S-mephenytoin 4'-hydroxylation was best-fitted to a competitive inhibition model with mean estimated $K_i$ values of 37.7 ± 7.6 and 56.8 ± 16.8 μM (Fig. 6).

CYP1A2-catalyzed phenacetin O-deethylation, CYP2C9-catalyzed S-warfarin 7-hydroxylation and CYP3A4-catalyzed midazolam 1-hydroxylation were only negligibly inhibited, if at all, by any of the TCAs tested in the microsomal incubations [Fig. 5 (B, C, and E)].

Discussion

TCAs have been used extensively in the treatment of depression, and their metabolic pathways and associated enzymes are well known. All TCAs are converted to 2- or 10-hydroxy metabolites by CYP2D6 and their metabolic pathways and associated enzymes are well known. S-warfarin 7-hydroxylation and CYP3A4-catalyzed midazolam 1-hydroxylation has been demonstrated strong inhibition of CYP2D6-catalyzed dextromethorphan hydroxylation. However, different P450 isoforms has not been extensively described. Our study demonstrated strong inhibition of CYP2D6-catalyzed dextromethorphan O-demethylation by all of the TCAs examined; estimated $K_i$ values of the TCAs were ranked as follows: amitriptyline (31.0 μM) > imipramine (28.6 μM) > desipramine (12.5 μM) > nortriptyline (7.9 μM). This hierarchy suggests that of the four TCAs tested, nortriptyline is the most potent CYP2D6 inhibitor. These results are comparable to the inhibitory potential of other antidepressants, such as venlafaxine (Otton et al., 1994) and nefazodone (Schmider et al., 1996), against CYP2D6 activity in vitro.

In this study, TCAs also competitively inhibited CYP2C19-catalyzed S-mephenytoin 4'-hydroxylation. However, the inhibitory potential of tertiary and secondary amine TCAs differed. The tertiary amine TCAs, amitriptyline and imipramine, showed remarkable inhibition of CYP2C19, with estimated $K_i$ values of 37.7 and 56.8 μM, respectively. In contrast, the secondary amine TCAs, nortriptyline and desipramine, produced only minimal inhibition. These results seem reasonable, when we consider that nortriptyline and desipramine are competitive substrates with phenytoin metabolism due to CYP2C9 mutation such as CYP2C9*3 (Ile359 Leu) allele. In one study, mean phenytoin $V_{max}$ was 42% lower in heterozygous CYP2C9*3 subjects than in subjects with wild-type CYP2C9*1 alleles (Mamiya et al., 1998). Since the allele frequency of CYP2C9*3 is 1 to 2% in Asians and 6 to 10% in Caucasians (Yoon et al., 2001), it will not be unusual to observe interactions between TCAs and phenytoin.

Although CYP2C9 is mainly responsible for phenytoin $p$-hydroxylation (Bajpai et al., 1996; Giancarlo et al., 2001), it seems not to be involved in the inhibitory interactions with TCAs in phenytoin metabolism. In our microsomal incubation studies, the TCAs failed to inhibit CYP2C9-catalyzed S-warfarin 7-hydroxylation, CYP1A2-catalyzed phenacetin O-deethylation, or CYP3A4-catalyzed midazolam 1-hydroxylation. Moreover, while all of the TCAs tested strongly inhibited phenytoin $p$-hydroxylation catalyzed by cDNA-expressed CYP2C19, none inhibited the formation of HPPH catalyzed by cDNA-expressed CYP2C19, none inhibited the formation of HPHH catalyzed by cDNA-expressed CYP2C9. Tertiary amine TCAs also showed remarkable inhibition of CYP2C19-catalyzed S-mephenytoin 4'-hydroxylation in microsomal incubations. Together, these data suggest that CYP2C19-catalyzed phenytoin $p$-hydroxylation is the metabolic pathway responsible for the interaction between TCAs and phenytoin. Several other drugs (i.e., ticlopidine, omeprazole, and cinetidine) are also known to interact with phenytoin through CYP2C19 inhibition, not by CYP2C9 inhibition (Levy, 1995; Rindone and Bryan, 1996).

Since CYP2C9 is an enzyme that shows genetic polymorphism, inhibition of CYP2C19-catalyzed phenytoin $p$-hydroxylation by TCAs is more likely to be significant in patients who are deficient in phenytoin metabolism due to CYP2C9 mutation such as CYP2C9*3 (He^{359}Leu) allele. In one study, mean phenytoin $V_{max}$ was 42% lower in heterozygous CYP2C9*3 subjects than in subjects with wild-type CYP2C9*1 alleles (Mamiya et al., 1998). Since the allele frequency of CYP2C9*3 is 1 to 2% in Asians and 6 to 10% in Caucasians (Yoon et al., 2001), it will not be unusual to observe interactions between TCAs and phenytoin in patients with the CYP2C9*3 allele.
As expected, CYP2D6 inhibition does not seem to be implicated in the inhibitory interactions of TCAs with phenytoin. All of the TCAs in this study strongly inhibited CYP2D6-catalyzed dextromethorphan O-demethylation. However, quinidine, a CYP2D6-selective inhibitor (Brolly et al., 1998), had no effect on phenytoin p-hydroxylation, and cDNA-expressed CYP2D6 failed to produce HPPH, which is consistent with previous reports (Komatsu et al., 2000; Giancarlo et al., 2001).

In conclusion, our results demonstrate that TCAs have moderate to strong inhibitory potential on CYP2C19 as well as CYP2D6 in human liver microsomes. They also suggest that the drug interaction between TCAs and phenytoin is caused by their inhibition of CYP2C19-catalyzed phenytoin p-hydroxylation.

References


