The Contributions of Cytochromes P450 3A4 and 3A5 to the Metabolism of the Phosphodiesterase Type 5 Inhibitors Sildenafil, Udenafil, and Vardenafil

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Sildenafil, udenafil, and vardenafil are potent, selective inhibitors of cyclic GMP-specific phosphodiesterase type 5 (PDE5) in the smooth muscle cells lining blood vessels, especially in the corpus cavernosum of the penis. These drugs are used for effective p.o. treatment of erectile dysfunction. Previous studies have reported large interindividual variation in vivo have not been elucidated. Rajagopalan et al., 2003). The mechanisms of these large interindividual variation was unknown. In the present study, we evaluated the relative contributions of human CYP3A4 and CYP3A5 to the metabolism of three PDE5Is: sildenafil, udenafil, and vardenafil. In vitro incubation studies of sildenafil and vardenafil were conducted using recombinant CYP3A enzymes and 15 human liver microsome (HLM) preparations with predetermined CYP3A5 genotypes. Recombinant CYP3A4 and CYP3A5 both produced N-desalkyl metabolites of sildenafil, udenafil, and vardenafil. The catalytic efficiency ($C_{\text{int}} = V_{\text{max}}/K_{\text{m}}$) of the rCYP3A5 isoform for vardenafil N-dealkylation was about 3.2-fold that of rCYP3A4, whereas the intrinsic clearance rates for N-dealkylation of both sildenafil and udenafil were similar between rCYP3A5 and rCYP3A4. The metabolite formation activity was higher in HLMs heterozygous for the CYP3A5*3 allele ($n = 9$) than in HLMs homozygous for CYP3A5*3 ($n = 6$). These findings suggest that CYP3A5 and CYP3A4 play a significant role in the metabolism of PDE5Is. The genetic polymorphism of CYP3A5 may contribute to interindividual variability in the disposition of PDE5Is, especially vardenafil. Further in vivo studies are needed to confirm the effects of CYP3A5 genotypes on the pharmacokinetics of PDE5Is.

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Materials and Methods

Chemicals and Reagents. Udenafil and N-desalkyludenafil were supplied by the Dong-A Pharmaceutical Company (Yongin, Korea). Midazolam, 1'-hydroxymidazolam, sildenafil, N-desmethysildenafil, vardenafil, and N-des-
tribution of CYP3A to the metabolism of PDE5Is

Ethylvardenafil were purchased from Toronto Research Chemicals (North York, ON, Canada). Single-donor human liver microsomes (HLMs) were obtained from the tissue bank of Pharmacogenomics Research Center, Inje University (Busan, Korea). Microsomes derived from baculovirus-infected insect cells, transfected with human P450 cDNA and overexpressing CYP3A4 or CYP3A5, were purchased from BD Gentest (Woburn, MA); the recombinant human CYP3A4 and CYP3A5 isoforms in supersones are coexpressed with human P450 reductase and cytochrome b5. The manufacturer supplied information regarding protein concentration and P450 content. All the chemicals and solvents were of the highest grade commercially available.

**Metabolism of Sildenafil, Udenafil, and Vardenaf in by HLMs or Recombinant P480 Isoforms.** The optimal conditions for microsomal incubation were determined within the linear range for the formation of N-desalkyl metabolites of PDE5Is. For all the experiments, sildenafil, udenafil, and vardenaf in were dissolved and serially diluted with methanol to the required concentrations, such that the final concentration of organic solvent did not exceed 0.5% in the final incubation mixture. The incubation mixture contained either 20 μl of a recombinant CYP3A (diluted to 20 pmol/ml in 100 mM phosphate buffer, pH 7.4) or 5 μl of HLMs (5 mg protein/ml). Fifteen different HLM preparations, each with a predetermined CYP3A5 genotype, were examined. The CYP3A5*3 allele was detected using a previously published method (Roy et al., 2005). The 293-base pair (bp) DNA fragment that contains the CYP3A5*3 allele was amplified with the specific primers 5’-CATGACTTGTAGACAGACATGA-3’ and 5’-GGTCCCAAACAGGGAAAGATAA-3’, and the polymerase chain reaction product was digested with SphI. The presence of the CYP3A5*3 allele was detected as fragments of 168 and 125 bp, whereas the wild-type allele gave fragments of 148, 125, and 20 bp. Various concentrations of PDE5Is (0–500 μM) were preincubated for 5 min at 37°C with the recombinant CYP3A enzymes or HLMs. The reaction was initiated by adding an NADPH-regenerating system (3.3 mM glucose 6-phosphate, 1.3 mM NADP, 3.3 mM MgCl2, and 1.0 unit/ml glucose-6-phosphate dehydrogenase), and the mixture (final volume, 200 μl) was further incubated for 10 min at 37°C in a shaking water bath. The reaction was terminated by placing the incubation tubes on ice and immediately adding 80 μl of acetonitrile. The mixtures were centrifuged at 20,000g for 5 min at 4°C, and aliquots of the supernatant were injected into a liquid chromatography/tandem mass spectrometry (LC/MS/MS) system.

CYP3A-catalyzed midazolam 1’-hydroxylase activity in HLMs was assayed by the method previously described (Kim et al., 2005). In brief, the incubation mixtures contained 0.25 mg/ml microsomal protein, 0.1 M phosphate buffer, pH 7.4, 1 mM NADPH, and 5 mM midazolam at 37°C in a shaking water bath. The reaction was terminated by placing the incubation tubes on ice and adding 80 μl of acetonitrile after 15 min. The mixtures were centrifuged, and aliquots of the supernatant were injected into an LC/MS/MS system.

**Analysis of the PDE5I Metabolites N-Desethylsildenafil, N-Desalkyludenafil, and N-Desethylvardenaf in.** A tandem quadrupole mass spectrometer (QTrap 4000 LC/MS/MS, Applied Biosystems, Foster City, CA) coupled with an Agilent 1100 series high-performance liquid chromatography system (Agilent, Wilmington, DE) was used to quantify the N-desethyl metabolites. The separation was performed on an Atlantis HILIC silica column (2.1 mm i.d. × 50 mm, 3 μm, Waters, Milford, MA) using a mobile phase of acetonitrile and water (40:60, v/v) at a flow rate of 0.2 ml/min. To identify the metabolites, mass spectra were recorded by electrospray ionization with a positive mode. The turbo ion spray interface was operated at 5500 V and 500°C. The operating conditions, optimized by flow injection of an analyte, were as follows: nebulizing gas flow, 40 psi, and curtain gas flow 15 psi. Quadrupoles Q1 and Q3 were set on unit resolution. Multiple reaction-monitoring mode using specific precursor/product ion transition was used for the quantification. The ions were detected by monitoring the transitions of m/z 460.9→283.2 for N-desethyl-sildenafil (collision energy, 53 eV), 406.18→364.2 for N-desalkyludenaf in (collision energy, 39 eV), and 460.9→151.2 for N-desethylvardenaf in (collision energy, 71 eV). The peak areas for all the components were automatically integrated using Analyst software (version 1.4, Applied Biosystems). The lower limits of quantification for N-desethylsildenafil, N-desalkyludenaf in,

**FIG. 1.** The metabolic pathway of sildenafil, udenaf in, and vardenaf in in human liver microsomes.

**TABLE 1**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vardenaf in</th>
<th>Sildenafil</th>
<th>Udenaf in</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V&lt;sub&gt;max&lt;/sub&gt; (pmol/min/pmol P450)</strong></td>
<td>1.51 ± 0.07</td>
<td>1.77 ± 0.15</td>
<td>1.38 ± 0.06</td>
</tr>
<tr>
<td><strong>K&lt;sub&gt;m&lt;/sub&gt; (μM)</strong></td>
<td>7.8 ± 1.1</td>
<td>2.9 ± 0.9</td>
<td>14.7 ± 1.6</td>
</tr>
<tr>
<td><strong>K&lt;sub&gt;c&lt;/sub&gt; (μM)</strong></td>
<td>1595.6 ± 489.9</td>
<td>611.4 ± 237.8</td>
<td>485.3 ± 68.0</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>0.19</td>
<td>0.60</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Cl&lt;sub&gt;int&lt;/sub&gt; (μl/min/pmol P450)</strong></td>
<td></td>
<td></td>
<td>0.57 ± 0.03</td>
</tr>
</tbody>
</table>

**V<sub>max</sub>** maximum velocity; **K<sub>m</sub>** substrate concentration at which the reaction velocity is 50% of **V<sub>max</sub>;** **K<sub>c</sub>** substrate inhibition constant; **n**, Hill coefficient; **Cl<sub>int</sub>**, intrinsic clearance (V<sub>int</sub>/K<sub>c</sub>).
and N-desethylvardenafil were 1, 1, and 2 nM, respectively. The interassay precision for the analytes was less than 15%.

1-Hydroxymidazolam was identified by the method previously described (Kim et al., 2005). The separation for the 1-hydroxymidazolam was performed on Luna C18 column (2.0 × 30 mm i.d., 5-µm particle size, Phenomenex, Torrance, CA) using a mobile phase of acetonitrile and water (10:90, v/v) at a flow rate of 0.2 ml/min. The ions were detected by monitoring the transitions of \( m/z = 342 \) for 1-hydroxymidazolam (collision energy, 23 eV).

**Data Analysis.** The results are expressed as mean ± S.D. obtained from each recombinant P450 in triplicate experiments. The apparent kinetic parameters of PDE5I biotransformation (\( K_m \) and \( V_{max} \)) were determined by fitting the Hill equation \( V = V_{max} \times S^n / (K_m + S^n) \) or uncompetitive substrate inhibition equation \( V = V_{max} \times S / [K_m + (1 + S/K_s)] \). The calculated parameters were the maximum rate of formation (\( V_{max} \)), Michaelis-Menten constant (apparent \( K_m \)), noncompetitive substrate inhibition constant (\( K_s \)), intrinsic clearance (\( C_l_{in} = V_{max} / apparent \ K_m \)), and Hill coefficient (\( n \)). Calculations were performed using WinNonlin software (Pharsight, Mountain View, CA). In the incubation study using recombinant P450s, each model was fitted to the unweighted data for the formation rate of the metabolite to estimate the enzyme kinetic parameters. The best-fit models were selected based on the dispersion of residuals and S.E. of the parameter estimates.

**Statistical Analysis.** The data are expressed as mean ± S.D. unless otherwise indicated, and differences at \( p < 0.05 \) were considered significant. Statistical comparisons of the metabolic rate between heterozygous CYP3A5*3 livers and homozygous CYP3A5*3 livers were made using the Wilcoxon rank-sum test. Statistical analyses were performed with the SAS statistical software package (version 9.1.3, SAS Institute, Cary, NC).

**Results and Discussion**

An intronic mutation in the CYP3A5 gene (CYP3A5*3) explains in large part the polymorphic pattern of enzyme expression found...
in human liver and intestine (Lin et al., 2002); however, its significance in the overall hepatic CYP3A activity is disputed (Williams et al., 2002; Huang et al., 2004). CYP3A4 has been reported to play an important role in the metabolism of PDE5Is such as sildenafil, udenafil, and vardenafil (Warrington et al., 2000; Keating and Scott, 2003; Ji et al., 2004; Mehrotra et al., 2007). In addition, members of the CYP3A subfamily other than CYP3A4 may be involved in the N-dealkylation of PDE5Is (Keating and Scott, 2003). Previous studies have shown that PDE5Is pharmacokinetics show large interindividual variability (Klotz et al., 2001; Rajagopalan et al., 2003; Shim et al., 2003; Bischoff, 2004; Gupta et al., 2005; Mehrotra et al., 2007). However, the in vivo mechanisms of these large interindividual variations have not been elucidated. In the present study, we provided evidence that sildenafil, udenafil, and vardenafil are metabolized by not only CYP3A4 but also CYP3A5, which is a genetic polymorphic enzyme. We also observed that the metabolic activities of sildenafil, udenafil, and vardenafil may be affected by CYP3A5 genetic polymorphism.

Recombinant CYP3A5 metabolized vardenafil with a V_{max} comparable with that of CYP3A4 but with a catalytic efficiency (V_{max}/K_m) that was about 300% that of CYP3A4 (Table 1). This further supports the growing recognition that the specific activity of CYP3A5 is higher than that of CYP3A4 for certain substrates, including tacrolimus (Kamdem et al., 2005) and vinorelbine (Dennison et al., 2007). Both the CYP3A4 and CYP3A5 isoforms showed similar catalytic activity for the N-dealkylation of sildenafil and udenafil (Table 1; Fig. 2). These findings suggest that sildenafil, udenafil, and vardenafil are substrates of CYP3A5 and CYP3A4 and that both CYP3A4 and CYP3A5 contribute to the metabolism of sildenafil, udenafil, and vardenafil in HLMs. The role of CYP3A in the N-dealkylation of PDE5Is was in agreement with the good correlation observed between PDE5I N-dealkylation activity and midazolam 1'-hydroxylation in the liver samples investigated (Fig. 3). The latter assay is widely used as a probe of CYP3A activity (Kim et al., 2005).

Under our experimental conditions, the metabolism of sildenafil and vardenafil by rCYP3A enzymes exhibited substrate inhibition (Warrington et al., 2000), whereas the metabolism of udenafil showed biphasic saturation kinetics (Fig. 2). Evidence for atypical enzyme kinetics of CYP3A enzymes has been reported by other researchers (Schrag and Wienkers, 2001; Williams et al., 2002; Huang et al., 2004); thus, our findings of biphasic saturation for udenafil and substrate inhibition for sildenafil and vardenafil were not unexpected. The product formation kinetics for sildenafil N-demethylation and udenafil N-dealkylation that were observed in this study were similar to those previously reported (Warrington et al., 2000; Ji et al., 2004), and the intrinsic clearance rate observed for rCYP3A4 (0.07 μl/min/pmol) was similar to a previously reported rate (0.08 μl/min/pmol P450) (Warrington et al., 2000).

We then investigated the variability of the N-dealkylation of PDE5Is in a bank of 15 HLMs obtained from the PharmacoGenomics Research Center. For the heterozygous CYP3A5*3 (CYP3A5*1/*3) HLMs, the rate of PDE5I metabolism in 10 μM substrate concentration was higher with high CYP3A5 expression than with low CYP3A5 expression (Fig. 4). The nine HLMs heterozygous for the CYP3A5*3 allele had higher mean metabolite formation activity compared with the six homozygous CYP3A5*3 HLMs (for sildenafil N-demethylation, 2.1 ± 1.2 versus 1.0 ± 0.8 pmol/min/mg protein; for udenafil N-deethylation, 9.4 ± 4.0 versus 4.3 ± 3.5 pmol/min/mg protein; and for udenafil N-dealkylation, 0.7 ± 0.4 versus 0.4 ± 0.2 pmol/min/mg protein). These data indicated that CYP3A5 genetic polymorphism could be an important factor affecting interindividual variation in the disposition of sildenafil, udenafil, and vardenafil in humans. Among the three PDE5Is tested, vardenafil was metabolized to a greater extent by CYP3A5 than by CYP3A4. The present results support the findings of previous in vivo pharmacokinetic studies of vardenafil (Klotz et al., 2001; Rajagopalan et al., 2003; Shim et al., 2003; Bischoff, 2004; Gupta et al., 2005; Mehrotra et al., 2007), which showed large interindividual variation of vardenafil pharmacokinetics (about 70% variation for C_{max} and area under the curve values).

In conclusion, this study shows that CYP3A5 and CYP3A4 are responsible for the formation of the N-dealkylation metabolites of sildenafil, udenafil, and vardenafil. Our data also suggest that the CYP3A5*3 genotype may be a substantial factor influencing the plasma concentrations of the PDE5Is tested. In addition, the present data may be useful in understanding the in vivo pharmacokinetics and drug interactions of the three PDE5Is. Further in vivo pharmacogenetic investigations of these PDE5Is remain to be conducted.

**FIG. 3.** Correlation analysis between CYP3A-mediated midazolam 1'-hydroxylase activity and the rate of formation of N-desmethylsildenafil, N-desalkyldenafil, and N-desethylvardenafil from sildenafil, udenafil, and vardenafil in 15 different human liver microsome preparations, respectively.
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