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The contributions of cytochrome P450 3A4 and 3A5 to the metabolism of the phosphodiesterase type 5 inhibitors (PDE5Is) sildenafil, udenafil, and vardenafil

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Running title page: The contribution of CYP3A to the metabolism of PDE5Is

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ABBREVIATIONS:

P450: cytochrome P450; HLM: human liver microsomes; LC/MS/MS: liquid chromatography-tandem mass spectrometry; PDE5Is: phosphodiesterase type 5 inhibitors

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Abstract

The role of the genetically polymorphic CYP3A5 in the metabolism of CYP3A substrates is unclear. We investigated the contributions of the CYP3A4 and CYP3A5 isoforms to the metabolism of the phosphodiesterase type 5 inhibitors (PDE5Is) sildenafil, udenafil, and vardenafil. *In vitro* incubation studies of sildenafil *N*-demethylation, udenafil *N*-dealkylation, and vardenafil *N*-deethylation 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 (Cl_{int} = V_{max}/apparent K_m) of the rCYP3A5 isoform for vardenafil *N*-deethylation 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 as well as CYP3A4 plays a significant role in the metabolism of PDE5Is. The genetic polymorphism of CYP3A5 might contribute to inter-individual 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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Introduction

Sildenafil, udenafil, and vardenafil are potent, selective inhibitors of cyclic guanosine monophosphate (cGMP)-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 oral treatment of erectile dysfunction. Previous studies have reported large inter-individual variability in the pharmacokinetic disposition of sildenafil, udenafil, and vardenafil (Klotz et al., 2001; Rajagopalan et al., 2003; Shim et al., 2003; Bischoff, 2004; Gupta et al., 2005; Mehrotra et al., 2007). Vardenafil showed the highest inter-individual variation among three PDE5Is, with a 14-fold variability among subjects receiving 20 mg of vardenafil (Klotz et al., 2001; Rajagopalan et al., 2003). The mechanisms of these large inter-individual variations *in vivo* have not been elucidated.

Sildenafil, udenafil, and vardenafil undergo *N*-dealkylation in the liver and intestines, and cytochrome P450 (CYP) 3A is primarily involved in their metabolism (Ji et al., 2004; Kivisto et al., 2004; Mehrotra et al., 2007). CYP3A is most abundant human hepatic P450 and is involved in the metabolism of approximately 50% of commonly administered drugs (Evans and Relling, 1999). In adults, CYP3A4 and CYP3A5 are predominant among the four known isoforms (CYP3A4, CYP3A5, CYP3A7, and CYP3A43) in the liver and intestine (Nelson et al., 1996). CYP3A5 is similar to CYP3A4 with regard to sequence and substrate selectivity (Kuehl et al., 2001). There are 84% amino acid sequence similarity and overlapping substrate specificities between CYP3A4 and CYP3A5 (Aoyama et al., 1989; Wrighton and Stevens, 1992). However, some differences have been reported in enzymatic properties of CYP3A4 and CYP3A5, including substrate specificity and inhibition (Gibbs et al., 1999; Cook et al., 2002; Patki et al., 2003; Emoto and Iwasaki, 2006).

Unlike CYP3A4, CYP3A5 is polymorphically expressed in human liver (Koch et al., 2002; Xie et al., 2004). The rate of CYP3A5 is 6–68% of the entire hepatic CYP3A content

(Wrighton et al., 1990). The frequency of CYP3A5 is higher in the livers of African-Americans than in those of Caucasians (Kuehl et al., 2001). Several genetic variants have been described for CYP3A5; the most common, the *CYP3A5*3* allele, causes the loss of CYP3A5 activity. The *CYP3A5*3* genotype reportedly affects the disposition of some drugs and influences the plasma concentrations of simvastatin and alprazolam (Kivisto et al., 2004; Park et al., 2006; Kim et al., 2007).

The importance of the CYP3A enzyme in the metabolism of sildenafil, udenafil, and vardenafil has been demonstrated *in vitro* (Hyland et al., 2001; Bischoff, 2004; Ji et al., 2004). However, until now, the relative contributions of CYP3A4 and CYP3A5 were 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.

Materials and Methods

Chemicals and reagents. Udenafil and *N*-desalkyludenafil were kindly supplied by the Dong-A Pharmaceutical Company (Yongin, Korea). Midazolam, 1′-hydroxymidazolam, sildenafil, *N*-desmethylsildenafil, vardenafil, and *N*-desethylvardenafil were purchased from Toronto Research Chemicals (North York, 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 over-expressing CYP3A4 or CYP3A5, were purchased from BD Gentest (Woburn, MA); the recombinant human CYP3A4 and CYP3A5 isoforms in supersomes are co-expressed with human P450 reductase and cytochrome b_5. The manufacturer supplied information regarding protein concentration and P450 content. All chemicals and solvents were of the highest grade commercially available.

Metabolism of sildenafil, udenafil, and vardenafil by HLMs or recombinant P450 isoforms. The optimal conditions for microsomal incubation were determined within the linear range for the formation of *N*-desalkyl metabolites of PDE5Is. For all experiments, sildenafil, udenafil, and vardenafil 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-bp DNA fragment that contains the *CYP3A5*3* allele was amplified with the specific primers 5′-CATGACTTAGTAGACAGATGA-3′ and 5′-GGTCCAAACAGGGAAGAAATA-3′, and the PCR product was digested with SspI. The

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presence of the *CYP3A5*3* allele was detected as fragments of 168 bp and 125 bp, whereas the wild-type allele gave fragments of 148 bp, 125 bp, and 20 bp. Various concentrations of PDE5Is (0 to 500 μM) were pre-incubated for 5 min at 37°C with the recombinant CYP3A enzymes or HLMs. The reaction was initiated by adding a NADPH-regenerating system (3.3 mM glucose-6-phosphate, 1.3 mM NADP, 3.3 mM $MgCl_2$, 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 20,000 × *g* 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 of midazolam at 37°C in a shaking water bath. The reactions were terminated by placing the incubation tubes on ice, adding 80 μL of acetonitrile after 15 min. The mixtures were centrifuged, and aliquots of the supernatant were injected into a LC/MS/MS system.

Analysis of the PDE5I metabolites *N*-desmethylsildenafil, *N*-desalkyludenafil, and *N*-desethylvardenafil. A tandem quadrupole mass spectrometer (QTrap 4000 LC/MS/MS, Applied Biosystems, Foster City, CA) coupled with an Agilent 1100 series HPLC system (Agilent, Wilmington, DE) was used to quantify the *N*-desalkyl metabolites. The separation was performed on an Atlantis® HILIC silica column (2.1 mm i.d. × 50 mm, 3 μm, Waters, 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*-desmethylsildenafil (collision energy, 53 eV), 406.18→364.2 for *N*-desalkyludenafil (collision energy, 39 eV), and 460.9→151.2 for *N*-desethylvardenafil (collision energy, 71 eV). The peak areas for all components were automatically integrated using Analyst software (version 1.4). The lower limits of quantification for *N*-desmethylsildenafil, *N*-desalkyludenafil, 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 × 30mm 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→203 for 1′-hydroxymidazolam (collision energy, 23 eV).

Data analysis. The results are expressed as means ± S.D. of estimates 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 [$V = V_{max} \times S/(K_m + S\ (1 + S/K_s))$]. 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 ($Cl_{int} = V_{max}$/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 standard errors of the parameter estimates.

Statistical analysis. The data are expressed as means ± SD 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 might be involved in the *N*-dealkylation of PDE5Is (Keating and Scott, 2003). Previous studies have demonstrated that PDE5I pharmacokinetics show large inter-individual 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 inter-individual 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 to 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 vincristine (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 as well as 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 P450) 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 (Busan, Korea). 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 (in pmol/min/mg protein: for sildenafil *N*-demethylation, 2.1 ± 1.2 vs. 1.0 ± 0.8; for vardenafil *N*-deethylation, 9.4 ± 4.0 vs. 4.3 ± 3.5; and for udenafil *N*-dealkylation, 0.7 ± 0.4 vs. 0.4 ± 0.2). These data indicated that CYP3A5 genetic polymorphism could be an important factor affecting inter-individual 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 inter-individual variation of vardenafil pharmacokinetics (about 70% variation for C_{max} and AUC values).

In conclusion, this study demonstrates that CYP3A5 as well as CYP3A4 is 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.

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Footnotes

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Legends for Figures

Fig. 1. The metabolic pathway of sildenafil, udenafil, and vardenafil in human liver microsomes.

Fig. 2. The kinetics of metabolite formation from sildenafil, udenafil, and vardenafil by the recombinant human CYP3A4 (A) and CYP3A5 isoforms (B). Various concentrations of substrate (0–500 μM) were incubated with recombinant human CYP3A and an NADPH-generating system at 37°C for 10 min. The velocity (pmol/min/pmol P450) versus PDE5I concentration was fitted to a substrate inhibition equation (sildenafil and vardenafil) or Hill equation (udenafil) (see *Data analysis* under *Materials and Methods*). The corresponding Eadie–Hofstee plot (velocity vs. velocity/substrate concentration) is shown in the inset. Each point represents the average of triplicate incubations.

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

Fig. 4. Mean rates for *N*-desalkyl metabolite formation from sildenafil (A), udenafil (B), and vardenafil (C) in human liver microsome (HLM) preparations with high CYP3A5 expression (n=9) and with low CYP3A5 expression (n=6). The nine HLMs that were heterozygous for the *CYP3A5*3* allele (*CYP3A5*1/*3*) had higher mean metabolite formation activity than the six that were homozygous for *CYP3A5*3* (*CYP3A5*3/*3*). Differences with $p < 0.05$ were considered statistically significant

Table 1

Mean enzyme kinetic parameters of the formation of *N*-desalkyl metabolites from vardenafil, sildenafil, and udenafil from the rCYP3A4 and rCYP3A5 isoforms.

Parameters	Vardenafil		Sildenafil		Udenafil	
	Substrate inhibition model: $V = V_{max} \times S / (K_m + S \times (1 + S / K_s))$				*Sigmoidal model:* $V = V_{max} \times S^n / (K_m + S^n)$	
	CYP3A4	CYP3A5	CYP3A4	CYP3A5	CYP3A4	CYP3A5
V_{max} (pmol/min/pmol P450)[a]	1.51 ± 0.07	1.77 ± 0.15	1.00 ± 0.15	1.38 ± 0.06	3.91 ± 0.55	1.07 ± 0.13
K_m (μM)[b]	7.8 ± 1.1	2.9 ± 0.9	15.0 ± 5.4	14.7 ± 1.6	531.9 ± 246.3	216.4 ± 72.3
K_s (μM)[c]	1595.6 ± 489.9	611.4 ± 237.8	548.5 ± 266.9	485.3 ± 68.0		
n[d]					0.57 ± 0.03	0.77 ± 0.07
Cl_{int} (μl/min/pmol P450)[e]	0.19	0.60	0.07	0.09	0.0074	0.0049

[a] V_{max}, the maximum velocity

[b] K_m, the substrate concentration at which the reaction velocity is 50% of V_{max}

[c] K_s, the substrate inhibition constant

[d] n, Hill coefficient

[e] Cl_{int}, intrinsic clearance (= V_{max}/K_m)

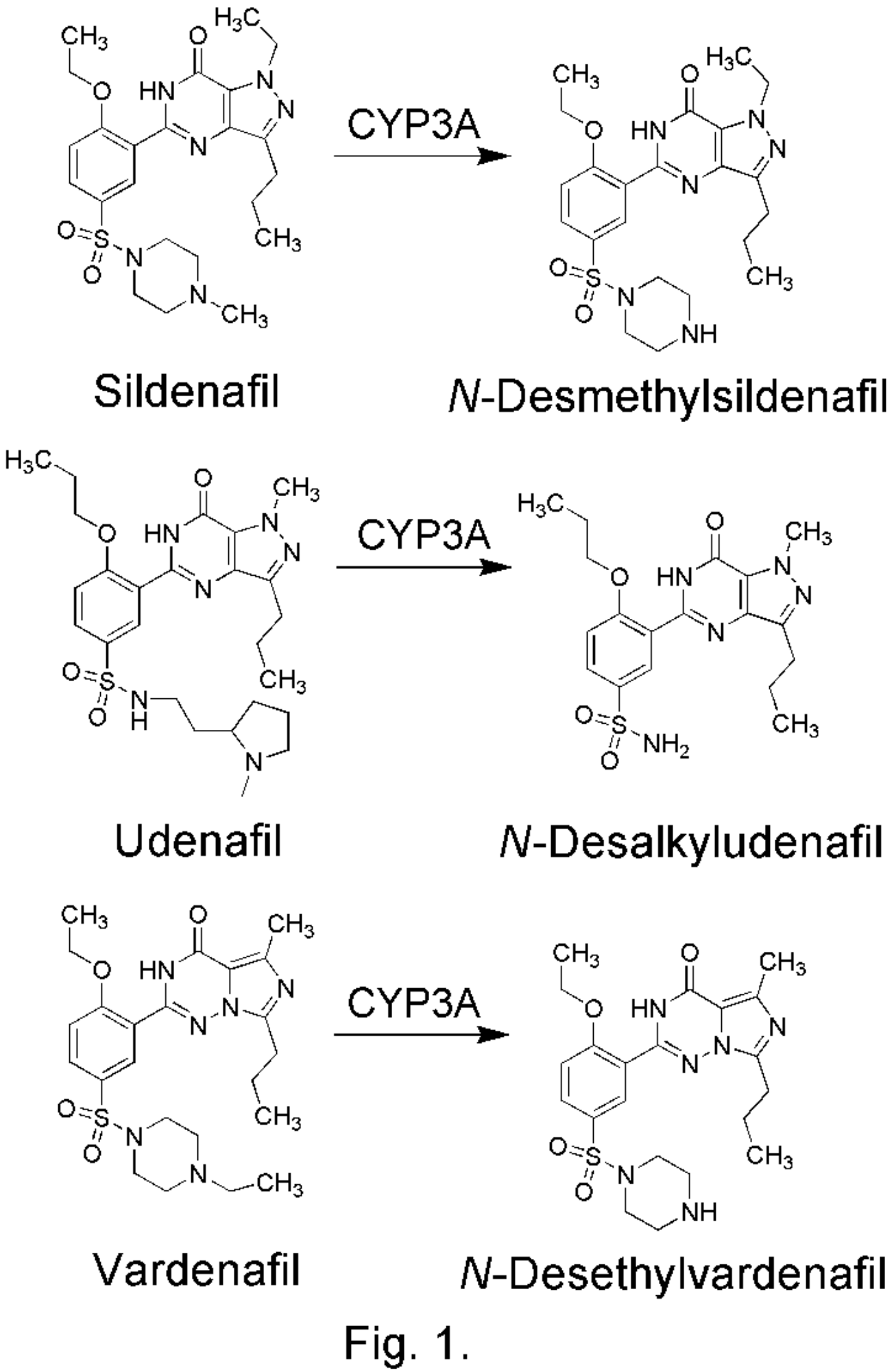
CYP3A
Sildenafil
N-Desmethylsildenafil
CYP3A
Udenafil
N-Desalkyludenafil
CYP3A
Vardenafil
N-Desethylvardenafil

Fig. 1.

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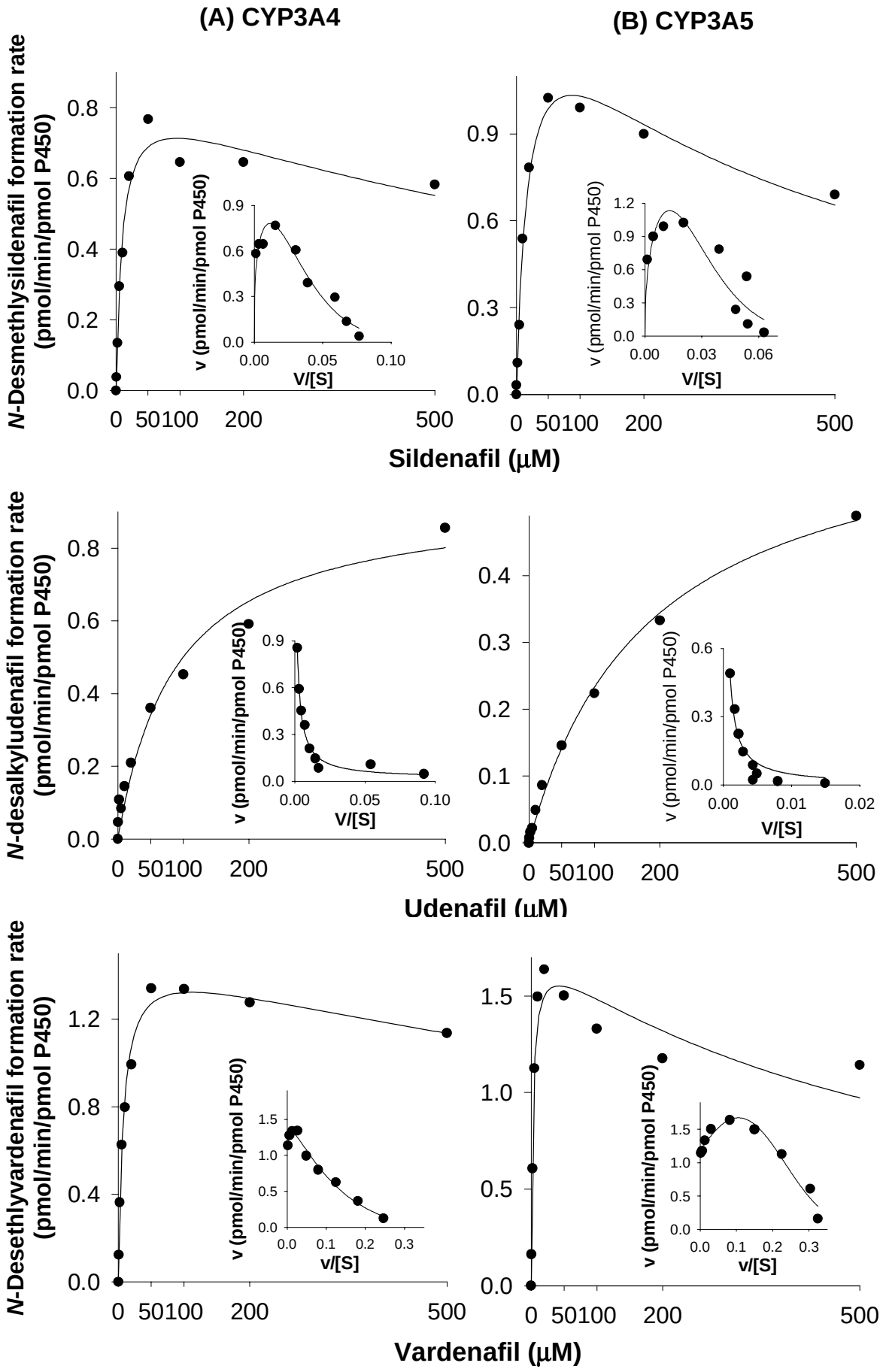

Fig. 2.

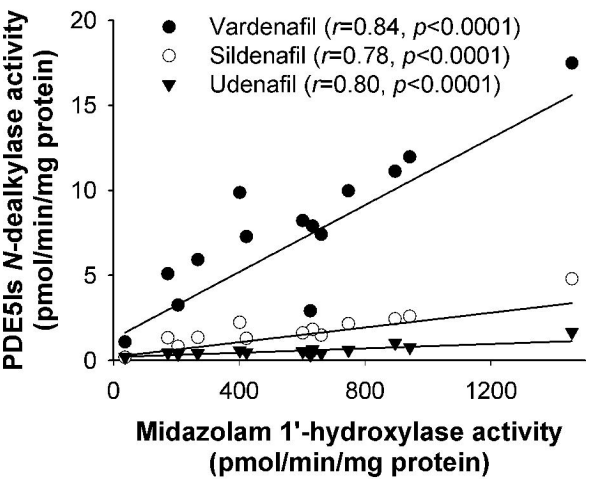
PDE5Is N-dealkylase activity
(pmol/min/mg protein)
20
15
10
5
0
0
400
800
1200
Midazolam 1'-hydroxylase activity
(pmol/min/mg protein)
Vardenafil (r=0.84, p<0.0001)
Sildenafil (r=0.78, p<0.0001)
Udenafil (r=0.80, p<0.0001)

Fig. 3.

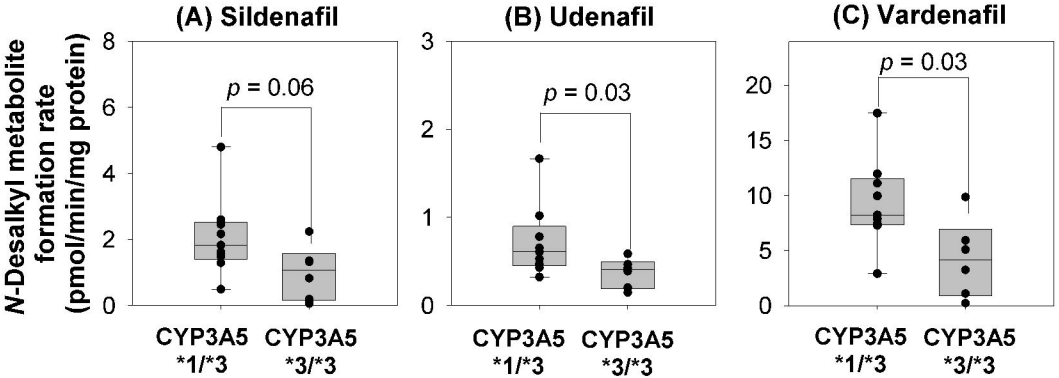
(A) Sildenafil
(B) Udenafil
(C) Vardenafil
N-Desalkyl metabolite formation rate (pmol/min/mg protein)
p = 0.06
p = 0.03
p = 0.03
0
2
4
6
8
0
1
2
3
0
5
10
15
20
CYP3A5 *1/*3
CYP3A5 *3/*3
CYP3A5 *1/*3
CYP3A5 *3/*3
CYP3A5 *1/*3
CYP3A5 *3/*3

Fig. 4.