Published in final edited form as:

Drug Metab Pharmacokinet. 2012 ; 27(6): 663–667.

Comparison of CYP3A4 and CYP3A5: the effects of cytochrome b5 and NADPH-cytochrome P450 reductase on testosterone hydroxylation activities

Su-Jun Lee¹ and Joyce A. Goldstein²

¹Department of Pharmacology and Pharmacogenomics Research Center, Inje University College of Medicine, Inje University, 633-165 Gaegum-dong, Jin-gu, Busan 614-735, Korea.

²Human Metabolism Section, Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA

Abstract

CYP3A4 and CYP3A5 require cytochrome b5 (b5) and NADPH-cytochrome P450 oxidoreductase (CPR) for optimum metabolism, but little is known about the specific requirements for b5 and CPR to produce optimal activities for these enzymes. The metabolism of testosterone (TT) by CYP3A4 and CYP3A5 were analyzed by various combinations of b5 and CPR using a fixed amount of recombinant P450 which had been purified from an *E. coli* expression system. CYP3A4 and CYP3A5 required 4 and 8-fold more of CPR than of the P450s, respectively, for optimal activity. The requirement of b5 for optimal activity showed the same pattern for both CYP3A4 and CYP3A5, exhibiting a gradual stimulation of the activity reaching a maximum at 16 fold more b5 than P450. Although CYP3A4 exhibited higher activities than the CYP3A5 in all combinations, both enzymes exhibited the same dependency profile for b5 and CPR. Therefore, the stronger activity of CYP3A4 compared to CYP3A5 appears to be intrinsic to the CYP3A4 protein itself and not to different requirements for b5 and CPR. Since the relative amounts of b5 and CPR are important in the maintenance of CYP3A4 and CYP3A5 activities, different levels of these proteins in vitro and in vivo may cause altered metabolism of their substrates or misinterpretation of enzyme properties.

Keywords

CYP3A4; CYP3A5; cytochrome b5; NADPH-cytochrome P450 oxidoreductase; testosterone; reconstitution

Introduction

CYP3A4 and CYP3A5 are highly expressed in human liver and are the two major drug metabolizing enzymes in the CYP3A subfamily in adults, accounting for approximately 50% of clinical drug metabolism.¹⁾ CYP3A4 comprises around 30-50% of the total P450 in liver and CYP3A5, a highly polymorphic enzyme, has been reported to range from undetectable amounts to amounts comparable to those of CYP3A4 in human liver.^{2,3)} In assessing CYP3A4 and CYP3A5 metabolism in vitro, reconstitution with an optimal amount

Corresponding author: Su-Jun Lee, PhD. Department of Pharmacology and Pharmacogenomics Research Center, Inje University College of Medicine, Inje University, 633-165 Gaegum-Dong, Jin-Gu, Busan 614-735, Korea. 2sujun@inje.ac.kr Tel: +82-51-890-8665; Fax: +82-51-895-6555.

of cytochrome P450 reductase (CPR) and cytochrome b5 (b5) has been a difficult problem in comparing kinetic profiles or attempting to maximize activity for more accurate kinetic values. In addition, multiple studies suggested that the effect of b5 were highly dependent on the P450 isoforms and substrates.^{4, 5)} Studies using an equal protein concentration of microsomes for the kinetic comparison of CYP3A4 and CYP3A5 may yield inaccurate data due to the polymorphic characters of both genes, and because the levels of b5 and CPR in the microsomes are not consistent. It is generally accepted that CYP3A4 has a higher activity than CYP3A5 for most CYP3A substrates.^{6,7)} However, there have been few comparison studies using these two enzymes together in respect to the amount of b5 and CPR required for optimal conditions. Inadequate amounts of these proteins in the reconstitution can cause lower activity than that of the true potential, of the two CYPs resulting in the underestimation of the enzyme activity. Catalytic discrepancy of these two enzymes may also come from the differing affinities for b5 and CPR. Therefore, the dependency of these two CYP3A4 and CYP3A5 enzymes on b5 and CPR was examined using testosterone (TT), a prototype CYP3A substrate.

Materials and Methods

Materials

DH5 α *Escherichia coli* competent cells, isopropyl β -D-thiogalactopyranoside (IPTG), and antibiotics were purchased from Invitrogen (Boston, MA, USA). Ni-NTA affinity columns were purchased from Qiagen (Valencia, CA, USA). Imidazole was purchased from Calbiochem (La Jolla, CA, USA). Wild-type proteins of human NADPH-cytochrome P450 reductase and cytochrome b5 were obtained from Oxford Biomedical Research (Oxford, MI, USA). Testosterone, reduced β -nicotinamide adenine dinucleotide phosphate (NADPH), δ -aminolevulinic acid (ALA), sodium cholate, L- α -dilauroyl-sn-glycero-3-phosphocholine, L- α -dioleoyl-sn-glycero-3-phosphocholine, bovine brain phosphatidylserine, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, and lysozyme were purchased from Sigma Chemical Co. (St Louis, MO, USA). 2 β , 6 β -, and 16 β -hydroxytestosterone were from Steraloids (Wilton, NH, USA). ¹⁴C-Testosterone was purchased from Invitrogen (Boston, MA, USA). Restriction enzymes, T4 DNA ligase, and T4 polynucleonucleotide kinase were obtained from New England Biolabs Inc. (Beverly, MA, USA). Oligonucleotide primers were obtained from Sigma Genosys Woodlands, TX, USA). Polymerase chain reactions (PCR) were performed by using a proofreading Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). All other chemicals and organic solvents for high performance liquid chromatography (HPLC) were of the highest grade from commercial sources.

Expression and purification of CYP3A4 and CYP3A5 proteins

Both CYP3A4 and CYP3A5 proteins were prepared using the same culture conditions and the purification steps to minimize inter-experimental variations. Briefly, CYP3A4 (NM_017460.5) and CYP3A5 (NM_000777.3) cDNAs were confirmed as wild-type cDNAs by direct full DNA sequencing. For both CYP3A4 and CYP3A5 proteins, the first eight amino acids in the N terminus of the protein were modified to MALLLAVF as described for the 17- α hydroxylase⁸⁾ and a 5XHis tag was added to the C-terminal region. Primers for the N- and C-terminal modifications were described previously.^{9,10,11)} The modified cDNA was cloned into the pCW vector via NdeI and HindIII sites. Expression of CYP3A4 and CYP3A5 proteins were induced by 0.5 mM IPTG and 0.5 mM δ -ALA, and incubation proceeded at 22°C for 96 h. To minimize inter-experimental variations in expression and purification, CYP3A4 and CYP3A5 constructs were expressed simultaneously, solubilized by sonication with detergent, and purified under the same conditions, twice, as previously described.^{10,11)} Two independent sets of purified recombinant P450s were used to verify results. To increase the purity and the recovery of P450, solubilized membrane extract was

purified on Ni-NTA affinity columns using a histidine tag. The eluted P450 was dialyzed for 48 h in dialysis buffer (100 mM potassium phosphate, pH 7.4, and 20% glycerol), and P450 content was measured on a DW-2000/OLIS spectrophotometer.

Reconstitution and Enzyme Assays

Enzyme reconstitution conditions were tested under various conditions using expressed CYP3A4 and CYP3A5 proteins with TT as a substrate. Enzyme activity was reconstituted with incubation mixtures containing 5 pmol of P450 (determined spectrally), variable amount of CPR and b5, lipid mix (2 μ g/reaction, a 1:1:1 mixture of L- α -dilauroylsn-glycero-3-phosphocholine, L- α -dioleoyl-sn-glycero-3-phosphocholine, and bovine brain phosphatidylserine), sodium cholate (0.05 μ mol/reaction), TT (200 μ M), and MgCl₂ (30 mM in final) in a total reaction volume of 0.1 ml in 50 mM potassium buffer, pH 7.7. The concentration of TT used was 200 μ M in all of the reactions, since this concentration was shown to be saturating substrate without enzyme inhibition in the previous study.¹¹⁾ The lipid mixture gave higher catalytic activity than dioleoylphosphatidyl choline (DOPC).¹²⁾ All components were constant except the CPR and b5 in the comparative studies of CYP3A4 and CYP3A5 reactions. All the assays were performed in triplicate. The reaction mixture was pre-incubated at 37°C for 3 min, and the reaction initiated by the addition of NADPH (1 mM), incubated at 37°C for 10 min, and stopped with 0.5 volumes of methanol. The reaction mixture was centrifuged at 13,000 r.p.m. for 20 min and the supernatant solution was analyzed by HPLC. Testosterone metabolites were measured as previously described.¹¹⁾ There was no catalytic activity in the absence of NADPH. All metabolites were quantified based on the molar concentration calculated from the standard calibration curve.¹³⁾

Analysis of Enzyme activities and statistics

To control inter-prep variations, two independently purified proteins for each P450 were examined first by P450 spectrum and assayed activities using TT as a substrate in triplicate reactions. A pair of two independently purified CYP3A4 and CYP3A5 proteins exhibited similar activities and therefore one of two preps was used for full comparative analysis as described in the Materials and Methods. Values represent the means $\pm$ S.D. of triplicate reactions.

Results and Discussion

CYP3A4 and CYP3A5 were expressed in *E. coli* DH5 α competent cells as previously described.^{9,10,11)} After expression, P450 was purified by Ni-NTA column utilizing a histidine tag to increase the purity. To avoid inter-experimental variations in expression and purification, CYP3A4 and CYP3A5 were simultaneously expressed and purified under the same conditions. A second set of CYP3A4 and CYP3A5 proteins were expressed and prepared under the same methods as the first set. The first and second sets of purified CYP3A4 and CYP3A5 were compared and confirmed to have the similar pattern of 6 β -hydroxy-TT activity and a clear P450 peak with no P420 peak (data not shown), and then the first set of CYP3A4 and CYP3A5 was used for detailed comparison studies as follows. The assay of TT metabolism using HPLC was described earlier.¹¹⁾ The amount of P450 was 5 pmol per reaction with different amount of CPR and b5 in the 0.1 ml reaction volume.

Both CYP3A4 and CYP3A5 showed a similar pattern of dependency of CPR in the titration of CPR amount (**Fig 1, A and B**). The ratio of 1:1, P450 to CPR, showed 6 β -hydroxy-TT at 18.74 ± 0.1 and 5.45 ± 1.9 nmol /min/nmol P450, in CYP3A4 and CYP3A5, respectively. The ratio of 1: 2 showed 6 β -hydroxy-TT at 28.6 ± 5 and 8.5 ± 0.9 nmol in the same condition as above. Instead of a gradual increase in activity with the increased amount of

CPR, the use of four fold more CPR than P450 usage sharply increased the 6 β -hydroxy-TT activity at 77.0 ± 6.6 (3 fold increase for CYP3A4) and 32.3 ± 6.3 (4-fold increase for CYP3A5) nmol/min/nmol P450 compared to the two-fold CPR amount in both CYP3A4 and CYP3A5 (**Fig. 1**). This sharp increase in activity agrees well with the positive cooperative stimulation of CYP3A activity for steroid substrates.^{14,15} The cooperative stimulation of CYP3A4 and CYP3A5 appear to be strongly influenced by the levels of CPR, more so than by b5. CYP3A4 and CYP3A5 required a similar amount of CPR, 4- to 8-fold more than P450, for the optimal activity. CYP3A4 protein showed approximately 2-3 fold higher activity than that of CYP3A5 in every point of b5 concentrations in 6 β (**Fig 2**) and 2 β hydroxylation of testosterone (data not shown). Since the optimal amount of CPR for CYP3A4 and CYP3A5 was 8-fold and 4-fold higher than P450, respectively, b5 titration was conducted with the fixed amount of CPR in each reaction (**Fig 2A and B**). The requirement of b5 for optimal activity showed the same pattern in both CYP3A4 and CYP3A5, exhibiting a gradual stimulation of the activity until an 8 fold increase than that of CYP3A protein amount was achieved. There was no significant increase in the presence (P450: b5 = 1: 1) or absence of b5 in the CYP3A4 reconstitution (1.5-fold increase), but a 3.6-fold difference in CYP3A5 reconstitution between the presence (P450: b5 = 1:1) or absence of b5, suggesting that CYP3A5 is more dependent upon b5 for optimal activity than that of CYP3A4. Again, there was a 2.7-fold difference in the TT metabolism between catalytic activity without b5 and with an optimal amount b5 in the CYP3A4 reconstitution. However, CYP3A5 exhibited a 10.5-fold difference in the catalytic activity between catalytic activity without b5 and with an optimal amount of b5. Excessive amounts of CPR (P450: CPR = 1: >32) inhibited both CYP3A4 and CYP3A5 activities (Fig 1A, B). However, the effects of the excessive amount of b5 (P450: CPR = 1: >32) on CYP3A4 and CYP3A5 metabolism did not produce the same patterns as those of CPR (Fig 2A, B). An excessive amount of b5 (P450: b5 = 1: 64) in CYP3A5 reaction was more inhibitory than that of CYP3A4 when compared to the optimal activity conditions for each enzyme. In the limited reaction volume, the larger size of CPR than b5 may affect the reconstitution of enzyme reaction differently. Or the aggregation may be adversely affected at high concentrations of b5 and CPR. Further studies may be needed to understand the differences in the catalytic activities between CYP3A4 and CYP3A5 in case of the excessive amounts of b5 (P450: b5 =1: 64).

Cytochrome b5, an ubiquitous heme protein with a mass of 17 kDa, has been reported to stimulate P450 catalysis by increasing the electron delivery to P450,^{16,17,18} or to induce a conformational change in P450 which affects P450 catalysis,^{19, 20, 21} or to inhibit P450 activity.^{22, 23} The present study confirms that b5 has a stimulation effect on both CYP3A4 and CYP3A5 catalysis in the metabolism of TT. CYP3A4 and CYP3A5 required the similar amount of b5, an 8-16 fold increased presence, for optimal activity. There are growing amounts of information about genetic polymorphisms in the b5 gene, resulting in altered expression of b5 proteins or its decreased function.^{24,25} Although the roles of b5 in P450 reactions are different depending on substrates, its polymorphic expression would affect the metabolism of drugs and endogenous substrates.

As a summary, there is no particular difference between two CYPs in the affinity pattern for b5 and CPR. This may suggest that higher activity of CYP3A4 comes from its protein sequence differences compared to that of CYP3A5 rather than the differences of the interaction with b5 and CPR. This study describes that the optimal reconstitution among CYP3A4/5, CPR, and b5 in *in vitro* assays and emphasizes the important role of b5 in the catalytic activity. Depending on the research labs, most of comparative P450 studies use different reconstitution systems that include commercially obtained P450s or microsomes, different mixtures of b5 and CPR, and even unknown information on b5, lipid composition, and CPR in the reactions. We believe that the present study is the first comparative study of

CYP3A4 and CYP3A5 activity using quantitatively controlled reaction components of the same purified-P450s, lipid mixture, b5, and CPR. This information will be helpful in the construction of optimal reconstitution system with the heterologously expressed and purified CYP3A4 and CYP3A5 enzymes in the metabolism analysis of CYP3A substrates.

Acknowledgments

This study was supported by the Intramural Research Program of NIH, National Institute of Environmental Health Sciences under NIH intramural project number Z01ES02124 and by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. R13-2007-023-00000-0).

References

1. Rendic S. Summary of information on human CYP enzymes: human P450 metabolism data. *Drug Metab. Rev.* 2002; 34:83–448. [PubMed: 11996015]
2. Lin YS, Dowling AL, Quigley SD, Farin FM, Zhang J, Lamba J, Schuetz EG, Thummel KE. Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism. *Mol. Pharmacol.* 2002; 62:162–72. [PubMed: 12065767]
3. Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Venkataramanan R, Strom S, Thummel K, Boguski MS, Schuetz E. Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat. Genet.* 2001; 27:383–91. [PubMed: 11279519]
4. Vatsis KP, Theoharides AD, Kupfer D, Coon MJ. Hydroxylation of prostaglandins by inducible isozymes of rabbit liver microsomal cytochrome P-450. Participation of cytochrome b5. *J. Biol. Chem.* 1982; 257:11221–9. [PubMed: 6889595]
5. Yamazaki H, Nakamura M, Komatsu T, Ohyama K, Hatanaka N, Asahi S, Shimada N, Guengerich FP, Shimada T, Nakajima M, Yokoi T. Roles of NADPH-P450 reductase and apo- and holo-cytochrome b5 on xenobiotic oxidations catalyzed by 12 recombinant human cytochrome P450s expressed in membranes of *Escherichia coli*. *Protein. Expr. Purif.* 2002; 24:329–37. [PubMed: 11922748]
6. Williams JA, Ring BJ, Cantrell VE, Jones DR, Eckstein J, Ruterbories K, Hamman MA, Hall SD, Wrighton SA. Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. *Drug Metab. Dispos.* 2002; 30:883–91. [PubMed: 12124305]
7. Thummel KE, Wilkinson GR. In vitro and in vivo drug interactions involving human CYP3A. *Annu. Rev. Pharmacol. Toxicol.* 1998; 38:389–430. [PubMed: 9597161]
8. Barnes HJ, Arlotto MP, Waterman MR. Expression and enzymatic activity of recombinant cytochrome P450 17 α -hydroxylase in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 1991; 88:5597–601. [PubMed: 1829523]
9. Lee SJ, van der Heiden IP, Goldstein JA, van Schaik RH. A new CYP3A5 variant, CYP3A5*11, is shown to be defective in nifedipine metabolism in a recombinant cDNA expression system. *Drug Metab. Dispos.* 2007; 35:67–71. [PubMed: 17035598]
10. Lee SJ, Bell DA, Coulter SJ, Ghanayem B, Goldstein JA. Recombinant CYP3A4*17 is defective in metabolizing the hypertensive drug nifedipine, and the CYP3A4*17 allele may occur on the same chromosome as CYP3A5*3, representing a new putative defective CYP3A haplotype. *J. Pharmacol. Exp. Ther.* 2005; 313:302–9. [PubMed: 15634941]
11. Lee SJ, Usmani KA, Chanas B, Ghanayem B, Xi T, Hodgson E, Mohrenweiser HW, Goldstein JA. Genetic findings and functional studies of human CYP3A5 single nucleotide polymorphisms in different ethnic groups. *Pharmacogenetics.* 2003; 13:461–72. [PubMed: 12893984]
12. Imaoka S, Imai Y, Shimada T, Funae Y. Role of phospholipids in reconstituted cytochrome P450 3A form and mechanism of their activation of catalytic activity. *Biochemistry.* 1992; 31:6063–6069. [PubMed: 1627548]
13. Purdon MP, Lehman-McKeeman LD. Improved high-performance liquid chromatographic procedure for the separation and quantification of hydroxytestosterone metabolites. *J. Pharmacol. Toxicol. Methods.* 1997; 37:67–73. [PubMed: 9174981]

14. Patki KC, Von Moltke LL, Greenblatt DJ. In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes p450: role of cyp3a4 and cyp3a5. *Drug Metab. Dispos.* 2003; 31:938–44. [PubMed: 12814972]
15. Houston JB, Kenworthy KE. In vitro-in vivo scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. *Drug Metab Dispos.* 2000; 28:246–54. [PubMed: 10681367]
16. Schenkman JB, Jansson I. Interactions between cytochrome P450 and cytochrome b5. *Drug Metab. Rev.* 1999; 31:351–64. [PubMed: 10335440]
17. Guryev OL, Gilep AA, Usanov SA, Estabrook RW. Interaction of apo-cytochrome b5 with cytochromes P4503A4 and P45017A: relevance of heme transfer reactions. *Biochemistry.* 2001; 40:5018–31. [PubMed: 11305918]
18. Yamazaki H, Shimada T, Martin MV, Guengerich FP. Stimulation of cytochrome P450 reactions by apo-cytochrome b5: evidence against transfer of heme from cytochrome P450 3A4 to apo-cytochrome b5 or heme oxygenase. *J. Biol. Chem.* 2001; 276:30885–91. [PubMed: 11413149]
19. Locuson CW, Wienkers LC, Jones JP, Tracy TS. CYP2C9 protein interactions with cytochrome b(5): effects on the coupling of catalysis. *Drug Metab Dispos.* 2007; 35:1174–81. [PubMed: 17446262]
20. Yamada M, Ohta Y, Bachmanova GI, Nishimoto Y, Archakov AI, Kawato S. Dynamic interactions of rabbit liver cytochromes P450IA2 and P450IIB4 with cytochrome b5 and NADPH-cytochrome P450 reductase in proteoliposomes. *Biochemistry.* 1995; 34:10113–9. [PubMed: 7640265]
21. Kumar S, Davydov DR, Halpert JR. Role of cytochrome B5 in modulating peroxide-supported cyp3a4 activity: evidence for a conformational transition and cytochrome P450 heterogeneity. *Drug Metab. Dispos.* 2005; 33:1131–6. [PubMed: 15870379]
22. Reed JR, Hollenberg PF. Comparison of substrate metabolism by cytochromes P450 2B1, 2B4, and 2B6: relationship of heme spin state, catalysis, and the effects of cytochrome b5. *J. Inorg. Biochem.* 2003; 93:152–60. [PubMed: 12576277]
23. Yamaori S, Yamazaki H, Suzuki A, Yamada A, Tani H, Kamidate T, Fujita K, Kamataki T. Effects of cytochrome b(5) on drug oxidation activities of human cytochrome P450 (CYP) 3As: similarity of CYP3A5 with CYP3A4 but not CYP3A7. *Biochem. Pharmacol.* 2003; 66:2333–40. [PubMed: 14637191]
24. Kok RC, Timmerman MA, Wolffenbuttel KP, Drop SL, de Jong FH. Isolated 17,20-lyase deficiency due to the cytochrome b5 mutation W27X. *J. Clin. Endocrinol. Metab.* 2010; 95:994–9. [PubMed: 20080843]
25. Sacco JC, Trepanier LA. Cytochrome b5 and NADH cytochrome b5 reductase: genotype-phenotype correlations for hydroxylamine reduction. *Pharmacogenet. Genomics.* 2010; 20:26–37. [PubMed: 19997042]

Figure 1. Comparison of the metabolic profiles of 6β-hydroxylation of testosterone by CYP3A4 (A) and CYP3A5 (B) with different combinations of P450, CPR, and b5

The reactions were performed using 5 pmol of spectrally determined P450, an amount of b5 2-fold higher than the P450, 200 μ M TT, and various fold amounts of CPR. Detailed methods are indicated under the *Materials and Methods*. The fold ratio was described in the bottom. All values are the means of triplicate experiments $\pm$ SD.

Figure 2. Effects of b5 on the metabolism of testosterone by CYP3A4 (A) and CYP3A5 (B) in the various reconstitutions

The reactions were performed using 5 pmol of spectrally determined P450, 200 μ M TT fixed amounts of CPR (8-fold more CPR than CYP3A4 and 4-fold more CPR than CYP3A5), and varying fold amounts of b5. Detailed methods are indicated under the *Materials and Methods*. The fold ratio was described in the bottom. All values are the means of triplicate experiments $\pm$ SD, except for the last 2 reactions (containing 32-and 64 fold more b5 than P450) which represent the average of duplicate determinations.