Published in final edited form as:

Pharmacogenet Genomics. 2010 October ; 20(10): 611–618. doi:10.1097/FPC.0b013e32833e0cb5.

Substrate-specific modulation of CYP3A4 activity by genetic variants of cytochrome P450 oxidoreductase (POR)

Vishal Agrawal^a, Ji Ha Choi^{b,c}, Kathleen M. Giacomini^b, and Walter L. Miller^a

^a Department of Pediatrics, University of California, San Francisco, California, USA

^b Department of Biopharmaceutical Sciences, University of California, San Francisco, California, USA

Abstract

Objectives—CYP3A4 receives electrons from P450 oxidoreductase (POR) to metabolize about 50% of clinically used drugs. There is substantial inter-individual variation in CYP3A4 catalytic activity that is not explained by CYP3A4 genetic variants. CYP3A4 is flexible and distensible, permitting it to accommodate substrates varying in shape and size. To elucidate mechanisms of variability in CYP3A4 catalysis, we examined the effects of genetic variants of POR, and explored the possibility that substrate-induced conformational changes in CYP3A4 differentially affect the ability of POR variants to support catalysis.

Methods—We expressed human CYP3A4 and four POR variants (Q153R, A287P, R457H, A503V) in bacteria, reconstituted them in vitro and measured the Michaelis constant and maximum velocity with testosterone, midazolam, quinidine and erythromycin as substrates.

Results—POR A287P and R457H had low activity with all substrates; Q153R had 76–94% of wild type (WT) activity with midazolam and erythromycin, but 129–150% activity with testosterone and quinidine. The A503V polymorphism reduced CYP3A4 activity to 61–77% of wild type with testosterone and midazolam, but had nearly wild type activity with quinidine and erythromycin.

Conclusion—POR variants affect CYP3A4 activities. The impact of a POR variant on catalysis by CYP3A4 is substrate-specific, probably due to substrate-induced conformational changes in CYP3A4.

Keywords

Cytochrome P450; drug metabolism; pharmacogenetics; enzymology; electron transfer; protein-protein interaction

INTRODUCTION

P450 oxidoreductase (POR), a 79 kDa multidomain, diflavin, microsomal protein, is the electron donor for several oxygenase enzymes, including squalene epoxidase [1], 7-dehydrocholesterol reductase [2], heme oxygenase [3] and microsomal cytochrome P450 (CYP) monooxygenases [4]. The FAD moiety of POR accepts a pair of electrons from NADPH, eliciting a conformational change that permits the electrons to transfer to the FMN domain; POR then returns to its initial conformation and donates the electrons sequentially to the

Correspondence to Professor Walter L. Miller, MD, Department of Pediatrics, HSE-1401, University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0978, USA, Tel: +1 415 476 2598; fax: +1 415 476 6286; wmlab@ucsf.edu.

^cPresent address: Department of Pharmacology, School of Medicine, Ewha Womans University, Seoul, Korea

recipient enzyme [5,6]. Some microsomal P450 enzymes catalyze two or three sequential reactions, requiring two or three pairs of electrons donated by a corresponding number of NADPH molecules. The central role of POR in mammalian biology is illustrated by the embryonic lethality of POR-knockout mice [7,8]. By contrast, liver-specific POR-knockouts are morphologically and reproductively normal, indicating normally functioning extrahepatic P450 enzymes, but have minimal capacity to metabolize drugs [9,10].

Since the first description in 2004 [11], at least 22 human POR mutations have been described in ~90 patients [11–16], and 18 more have been found in sequencing surveys [17,18]. Most POR-deficient individuals have a severe bony dysplasia called Antley-Bixler Syndrome and defects in steroid hormone biosynthesis due to defective function of P450c17 (17 α -hydroxylase/17,20 lyase; CYP17A1), P450c21 (21-hydroxylase, CYP21A2) and P450aro (aromatase, CYP19). Two recurrent mutations accounting for most disease: A287P predominates among patients of European ancestry and R457H predominates among Japanese patients [19]. The human POR gene is highly polymorphic: 3.1 single nucleotide polymorphisms per kilobase were found among the 1682 alleles from 842 healthy people [17]. The most common polymorphism causes the amino acid sequence variant A503V, found on 28% of human alleles, varying from 19% in African Americans to 37% in Chinese Americans [17]. The alleles encoding the enzymes studied here are Q153R, POR*13; A287P, POR*5; R457H, POR*2; A503V, POR*28 (see <http://www.cypalleles.ki.se/por.htm>) [20].

Hepatic microsomal P450 enzymes require POR and metabolize more than 80% of clinically used drugs [21]. CYP3A4, accounts for about half of hepatic, P450-mediated drug metabolism [22]. CYP3A4 metabolizes drugs having a broad array of sizes and structures because it is flexible and distensible. Crystallography shows that human CYP3A4 has the same fold as other P450 enzymes, but that its substrate-binding pocket is distensible with a volume of ~520 Å³ in the absence of substrate or in association with metyropone (212 Da) or progesterone (318 Da) [23], but expands to ~2000 Å³ when binding erythromycin (734 Da) [24]. Whether such substrate-induced conformational changes in CYP3A4 affect electron donation from POR is unknown.

There is considerable inter-individual variation in CYP3A4-mediated drug metabolism [25]. While such variations might be genetic [26], as they are for CYP2D6 [27], known CYP3A4 polymorphism have allele frequencies of < 1%, and hence cannot account for all variation seen in CYP3A4-mediated drug metabolism [28,29]. Thus we investigated whether genetic variation in POR could account for some variation in CYP3A4-mediated drug metabolism, whether such genetic variations affect CYP3A4 in a substrate-specific fashion, and whether substrate-induced conformational changes in CYP3A4 affect the interaction of CYP3A4 with POR and its isoforms.

METHODS

Reagents and Proteins

Midazolam was from BD Gentest (San Jose, CA); [¹⁴C]testosterone, 53.0 mCi/mmol, was from GE Healthcare (Piscataway, NJ); testosterone, quinidine, erythromycin and other reagents were purchased from Sigma (St. Louis, MO).

Wild type human (WT) POR and its variants were expressed as the N-27 protein in *E. coli* using vectors created by PCR-based site-directed mutagenesis as described [17,19]. *E. coli* membranes containing POR were isolated and the amount of POR in each membrane fraction was quantitated by western blotting against a standard curve of purified WT POR as described [30]. Human cytochrome b₅ was expressed in *E. coli* and purified as described [19]. Human CYP3A4 was expressed from the cDNA in pCWoRI [31], in *E. coli* JM109. *E. coli* membranes

containing CYP3A4 were isolated, and the bound CYP3A4 was quantitated by CO-induced difference spectra using a molar extinction coefficient of 91 mM^{-1} [32].

Assays with Testosterone

Metabolism of [^{14}C]testosterone to 6β -hydroxytestosterone was measured by thin layer chromatography (TLC). CYP3A4 (6 pmol) was combined with 30 pmol of WT or mutant POR, 12 pmol of cytochrome b_5 and 20 μg of 'lipid mix' containing equi-molar amounts of DLPC (1,2-didodecanoyl-sn-glycero-3-phosphocholine), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and DLPS (1,2-dilauroyl-sn-glycerol-3-phosphoserine) [33] in a buffer A [50 mmol/l HEPES (pH 7.4), 50 nmol sodium cholate, 3 mmol/l reduced glutathione, 30 mmol/l MgCl_2] and 25, 40, 60, 100, 160 or 250 $\mu\text{mol/l}$ testosterone mixed with 50,000 cpm of [^{14}C] testosterone, in a final reaction volume of 200 μl . The reaction was started by adding 30 μl of 'NADPH regeneration mix' (3.3 mmol/l glucose-6-phosphate, 1 mmol/l NADP, 0.8 U/ml glucose-6-phosphate dehydrogenase), incubated at 37°C for 1 h and stopped by adding 0.8 ml ethyl acetate. Steroids were extracted in ethyl acetate, dried under a stream of nitrogen, redissolved in 20 μl ethyl acetate, spotted on a 250 μm silica gel TLC plate (Whatman) and developed with methylene chloride:acetone (4:1) [34]. Testosterone and 6β -hydroxytestosterone on TLC plates were quantitated by phosphorimaging, and are represented as nmol of 6β -hydroxytestosterone formed per pmol P450 per min.

Assays with Midazolam

Conversion of midazolam to its 1-hydroxy and 4-hydroxy products was determined by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). CYP3A4 (4 pmol) was combined with 20 pmol of POR, 8 pmol of cytochrome b_5 and 20 μg of 'lipid mix' and 2, 5, 10, 20, 40, 70, 100, 200 or 400 $\mu\text{mol/l}$ midazolam in a total reaction volume of 200 μl using buffer A. The reaction was started by adding 30 μl of 'NADPH regeneration mix', incubated at 37°C for 30 min and stopped by adding 200 μl of acetonitrile. Samples were centrifuged, 75 μl of supernatant was mixed with 75 μl of the internal standard (propranolol, 400 ng/ml in acetonitrile), vortexed briefly and 30 μl of each sample was injected directly into the liquid chromatography system (Shimadzu Corporation, Tokyo, Japan) and analyzed in a MDS Sciex API 4000 MS/MS system equipped with a Turbo Ionspray interface, binary pump and SIL-20AC autosampler (Shimadzu). A CAPCELL PAK C_{18} column (4.6 mm x 75 mm; 3 μm particle size; Shiseido Corporation, Tokyo, Japan) was equilibrated with the mobile phase (5% acetonitrile, 95% 5 mmol/l ammonium acetate) at 0.8 ml/min prior to analysis. The LC profile was: 0 to 0.5 min, 5% acetonitrile; 0.5 to 3.5 min, linear gradient of 5% to 90% acetonitrile; 3.5 to 9.0 min, 5% acetonitrile. The MS/MS was operated in electrospray positive ionization mode. For midazolam, 1-hydroxymidazolam, 4-hydroxymidazolam, and propranolol, the precursor-to-product ion reactions monitored were: mass-to-charge (m/z) ratios $326.1 \rightarrow 291.1$, $342.0 \rightarrow 203.0$, $342.0 \rightarrow 325.0$, and $259.9 \rightarrow 56.2$ respectively [35]. The retention times for midazolam, 1-hydroxymidazolam, 4-hydroxymidazolam, and propranolol were 3.72, 3.92, 3.53, and 3.46 min. Inter-assay variability was less than 10%.

Assays with Quinidine

Concentrations of quinidine and 3-hydroxyquinidine were determined by LC-MS/MS. CYP3A4 (20 pmol) was mixed with 100 pmol of POR, 40 pmol of cytochrome b_5 , 20 μg of 'lipid mix' and 40, 60, 100, 150, 200, 300, 400 or 500 $\mu\text{mol/l}$ of quinidine in a total reaction volume of 200 μl in buffer A. The reaction was started by adding 30 μl of 'NADPH regeneration mix', incubated at 37°C for 2 h and stopped by adding 100 μl of 0.6 M ice-cold sodium borate. Quinidine and 3-hydroxyquinidine were extracted twice with 1 ml methylene chloride:isopropanol (4:1), dried under a stream of nitrogen [36], dissolved with 150 μl of the internal standard (propranolol, 400 ng/ml in acetonitrile), vortexed briefly and 20 μl of each

sample was injected directly into the LC-MS/MS system described above. The CAPCELL PAK C₁₈ column was equilibrated with the mobile phase (5% acetonitrile, 95% 5 mmol/l ammonium acetate) at 0.8 ml/min prior to analysis. The LC profile was: 0 to 0.5 min, 5% acetonitrile; 0.5 to 2.5 min, linear gradient of 5% to 90% acetonitrile; 2.5 to 9.0 min, 5% acetonitrile. The MS/MS was operated in electrospray positive mode. For quinidine, 3-hydroxyquinidine, and propranolol, the precursor-to-product ion reactions monitored were: *m/z* ratios 325.1→78.9, 341.1→176.0, and 259.9→56.2, respectively [37]. The retention times for quinidine, 3-hydroxyquinidine, and propranolol were 2.92, 2.83, and 3.02 min. Interassay variability was less than 10%.

Assays with Erythromycin

Concentrations of erythromycin and N-demethyl-erythromycin were determined by LC-MS/MS. CYP3A4 (20 pmol) was mixed with 100 pmol of POR, 40 pmole cytochrome b₅, 20 µg of 'lipid mix' and 20, 40, 60, 100, 150, 200, 300, 400 or 500 µmol/l of erythromycin in a total reaction volume of 200 µl in buffer A. The reaction was started by adding 30 µl of 'NADPH regeneration mix', incubated at 37 °C for 2 h, and stopped by adding 200 µl of acetonitrile; 75 µl samples were mixed with 75 µl of internal standard (propranolol, 400 ng/ml in acetonitrile), vortexed briefly and 15 µl of each sample was injected directly into the LC-MS/MS system described above. The CAPCELL PAK C₁₈ column was equilibrated with the mobile phase (5% acetonitrile, 95% 5 mM ammonium acetate) at 0.8 ml/min prior to analysis. The LC profile was as described for quinidine metabolism. The MS/MS was operated in electrospray positive mode. For erythromycin, N-demethyl-erythromycin, and propranolol, the precursor-to-product ion reactions monitored were: *m/z* ratios 734.6→158.1, 750.0→592.0, and 259.9→56.2, respectively. The retention times for erythromycin, N-demethyl-erythromycin, and propranolol were 2.99, 3.10, and 3.02 min. Interassay variability was less than 10%.

Kinetics

Kinetic analyses were done using GraphPad Prism 3 (GraphPad Software, San Diego, CA). The Michaelis constant (*K_m*) and maximum velocity (*V_{max}*) of each reaction were calculated using Lineweaver-Burk plots. *K_m* is given as µmol/l of substrate and *V_{max}* is given as nmol product/pmol CYP3A4/min for testosterone and midazolam, and as pmol product/pmol of CYP3A4/min for quinidine and erythromycin. *V_{max}* and *K_m* values are rounded to three significant digits and are reported as mean ± SEM from at least three independent experiments each performed in duplicate. Catalytic efficiency is expressed as *V_{max}*/*K_m*; data for POR variants are compared to WT.

RESULTS

Experimental Design and Characterization of proteins

Individual POR variants exhibit dramatically different activities to transfer electrons to cytochrome c, steroidogenic P450c17, P450c21 [17,19,38], and drug metabolizing CYP1A2 and CYP2C19 [30]. Because CYP3A4 undergoes dramatic substrate-induced conformational changes [24], we hypothesized that POR variant activities with a single P450 will differ with the substrate being metabolized. Therefore, we used substrates of different sizes and belonging to different chemical classes (Fig 1A). CYP3A4 expressed in *E. coli* membranes was quantitated by CO-difference spectra (Fig. 1B). CYP3A4-mediated metabolism of each substrate was assessed with electron donation from NADPH from five forms of POR: wild-type (WT); the common disease-causing mutants A287P and R457H [19]; the A503V polymorphism found on ~28% of human alleles [17], and Q153R, which exhibits diminished function with some P450 enzymes [19], but increased function with others [30]. POR isoforms were expressed lacking 27 N-terminal residues, facilitating expression of stable proteins without affecting membrane-binding or activity [19,30,39,40]. POR in *E.coli* membranes was

quantitated by western blotting in comparison to a standard curve of purified wild-type POR [30]. Bacterial membranes containing POR were mixed with membranes containing CYP3A4 in a lipid/detergent environment that yields active mono-oxygenase system [33]. The molar ratio of POR:P450 was set at 5:1 so that the amount of POR was not limiting. Velocities were calculated over a range of substrate concentrations, V_{max} and K_m were calculated by Lineweaver-Burk analysis, and the ratio of V_{max} to K_m was used as an index of catalytic efficiency. The activities of POR variants are expressed as a percentage of the activity of WT POR, set at 100%.

Metabolism of Test Compounds

Testosterone, a 288 Da steroid, is essential for male sexual development and reproduction; CYP3A4 is the principal enzyme disposing of testosterone by catalyzing its 6 β -hydroxylation. The FDA recommends assessing testosterone metabolism in studies of CYP3A4-catalyzed drug disposition

(<http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm081177.htm#inVitro>). POR R457H supported minimal activity, so that kinetic parameters could not be calculated (Table 1). POR A287P had 17% of WT activity (Table 1; Fig. 2A); A287P also has reduced activity with P450c17 [11,12,19] and with CYP1A2 and CYP2C19 [30], but has normal WT activity with CYP19A1 [41]. Q153R, identified as a disease-causing mutant with loss of function in cytochrome c and P450c17 assays [19] but with increased activity with CYP1A2 and CYP2C19 [30], had 129% of WT activity (Table 1; Fig. 2A). The A503V polymorphism, which has WT activity with CYP1A2 and CYP2C19 [30] and ~60% of WT activity with P450c17 [19] had 77% of WT activity for 6 β -hydroxylation of testosterone (Table 1; Fig. 2A). This may partially offset the expected reduction in testosterone biosynthesis predicted by the impairment of P450c17 activity.

Midazolam is a 326 Da benzodiazepine widely used in anaesthesia. There is significant inter-individual variation in the metabolism of midazolam among people homozygous for A503V [42]. CYP3A4 metabolizes midazolam to its 1-hydroxy and 4-hydroxy derivatives in about a 5:1 ratio; the FDA recommends studies of midazolam 1-hydroxylation in studies of CYP3A4. POR variants affected the metabolism of midazolam to varying extents (Table 1; Figs. 2B and 2C). R457H had minimal activity, so that kinetic parameters could not be calculated. A287P had 17% and 14% of WT activity for 1-hydroxylation and 4-hydroxylation of midazolam, respectively (Table 2). The activity of Q153R was 94% for 1-hydroxylation and 92% for 4-hydroxylation. A503V reduced 1-hydroxylation to 61% and 4-hydroxylation to 74% of WT levels.

Quinidine, a 324 Da antimalarial compound with two fused rings, is 3-hydroxylated by CYP3A4. The activities of POR variants on CYP3A4 activity with quinidine as substrate were similar to those with midazolam (Table 1; Fig. 2D). POR mutants A287P and R457H supported very low activity, 3% and 1%; Q153R had 150% of WT activity, and A503V had of 89% of WT activity.

CYP3A4 catalyzes N-demethylation of erythromycin, a 734 Da macrolide antibiotic containing a 14-member lactone ring. Crystallography shows that erythromycin binding dramatically expands the substrate-binding pocket of CYP3A4 from ~520 Å³ [23,43] to ~2000 Å³ [24]. Thus use of erythromycin directly tests the hypothesis that substrate-induced conformational changes in CYP3A4 will affect the efficiency of electron transfer from POR. As when testosterone was used as substrate, POR mutants A287P and R457H supported minimal activity and kinetic parameters could not be calculated (Table 1; Fig. 2E). Q153R, which had WT activity with midazolam and slightly increased activity with testosterone and quinidine showed only 76% of WT activity for erythromycin-N-demethylation. The A503V polymorphism, which modestly impaired the metabolism of testosterone and midazolam, had 97% of WT

activity with erythromycin. Thus the ability of different POR sequence variants to support the catalytic activity of CYP3A4 varied with the test substrate. Both the size of the substrate, as evidenced by erythromycin, and its chemical structure, as evidenced by midazolam, appear to be important.

DISCUSSION

Adverse drug reactions cost about \$100 billion annually and cause more than 100,000 deaths in USA [21]. Intra-individual variations in drug metabolism contribute to such adverse outcomes. Extensive work has addressed genetic variations in drug-metabolizing enzymes [22], hepatic drug transporters [44–46] and other factors [47,48]. The discovery that severe mutations in POR are compatible with human life [11] and that the human POR gene is highly polymorphic [17] suggested that POR might also contribute to inter-individual variations in drug metabolism. The common A503V polymorphism lies in the FAD binding domain of POR [5,19], and despite its conservative nature, reduces activity the 17 α -hydroxylase activity of P450c17 to 68% and its 17,20-lyase to 58% of WT [19], but has normal activity with P450c21 [38], and with CYP1A2 and CYP2C19 [30]. Assessing whether A503V and other POR variants affect the activity of CYP3A4 entailed another variable, substrate-induced CYP3A4 conformational changes, as crystallographic studies show that CYP3A4 can increase the volume of the substrate binding pocket up to 4-fold when binding large substrates such as erythromycin [24].

The effects of the POR variants on CYP3A4 generally followed their activities in other assays, with some notable exceptions. As in studies assessing POR activity with assays based on cytochrome c, P450c17 [11,19], CYP1A2 and CYP2C19 [30], R457H had no measureable activity to support CYP3A4 metabolism of testosterone, midazolam or quinidine, and only 1% of WT activity to support CYP3A4 metabolism of erythromycin. Models of human POR [19, 49], based on crystal structures of rat POR [5,50], show that R457 participates directly in binding the FAD moiety. The R457H mutation should interfere with FAD binding, thus preventing electron transport from NADPH to the FMN moiety. Consistent with this, Japanese patients homozygous for R457H have an especially severe form of Antley-Bixler syndrome [16]. A molar excess of flavin cofactor can compensate for defective cofactor-binding by some POR mutants [40,51,52]. A287P, which is common among Caucasian patients, has 20–40% of WT activity in assays based on P450c17 [11,19], undetectable activity with CYP1A2 and CYP2C19 [30], but normal activity with CYP19A1 [41] (Table 2). A287P had 14–17% of WT activity to support CYP3A4 with testosterone and midazolam, but minimal activity with quinidine or erythromycin. Q153R is a rare disease-causing mutation that retains 9–11% of WT activity with cytochrome c and 27–31% of WT activity with P450c17 [19], but showed a gain of function with CYP1A2 (144%) and CYP2C19 (284%) [30]. Q153R is located in the POR domain that binds FMN and interacts with the P450 through charge-charge interactions; the specific charged residues of POR that interact with the P450 appear to vary depending on the P450 receiving the electrons. Q153R changes the charge distribution, resulting in different behaviors with different P450 enzymes. The A503V polymorphism reduced the activity of CYP3A4 with testosterone to 79% and with midazolam to 66%, but it supported nearly normal activity with quinidine and erythromycin as substrates. Our study represents the first observation that the effects of specific POR mutants can vary with the substrate being metabolized as well as with the P450 enzyme. This appears to result from substrate-induced conformational changes in CYP3A4. It is not known whether this phenomenon will be seen with other P450 enzymes.

A503V is a likely contributor to pharmacogenetic variation in drug metabolism. Our data indicate that it can reduce CYP3A4 activity with some substrates, similar to its reduced activity with P450c17. In contrast to our enzymatic data, a clinical study found that A503V increases

metabolism of midazolam (presumably by CYP3A4) 1.6 fold [42]. However, these subjects were also receiving methadone and clozapines, which are CYP3A4 substrates that may modify CYP3A4 activity, and other CYP3A4 inhibitors were present in about 25% of the patients [42]. Clinical studies with healthy people are needed to determine the role of A503V on the pharmacogenetic variation in drug metabolism.

Acknowledgments

The authors thank Dr. F. P Guengerich, Vanderbilt University, for the CYP3A4 expression vector and Ms Izabella Damm for excellent technical assistance. This work was supported by NIH grant R01 GM073020 to W.L.M.

References

1. Ono T, Bloch K. Solubilization and partial characterization of rat liver squalene epoxidase. *J Biol Chem* 1975;250:1571–1579. [PubMed: 234459]
2. Nishino H, Ishibashi T. Evidence for requirement of NADPH-cytochrome P450 oxidoreductase in the microsomal NADPH-sterol Delta7-reductase system. *Arch Biochem Biophys* 2000;374:293–298. [PubMed: 10666310]
3. Wilks A, Black SM, Miller WL, Ortiz de Montellano PR. Expression and characterization of truncated human heme oxygenase (hHO-1) and a fusion protein of hHO-1 with human cytochrome P450 reductase. *Biochemistry* 1995;34:4421–4427. [PubMed: 7703255]
4. Vermilion JL, Ballou DP, Massey V, Coon MJ. Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome P-450 reductase. *J Biol Chem* 1981;256:266–277. [PubMed: 6778861]
5. Wang M, Roberts DL, Paschke R, Shea TM, Masters BS, Kim JJ. Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. *Proc Natl Acad Sci U S A* 1997;94:8411–8416. [PubMed: 9237990]
6. Ellis J, Gutierrez A, Barsukov IL, Huang WC, Grossmann JG, Roberts GC. Domain motion in cytochrome P450 reductase: conformational equilibria revealed by NMR and small-angle x-ray scattering. *J Biol Chem* 2009;284:36628–36637. [PubMed: 19858215]
7. Shen AL, O'Leary KA, Kasper CB. Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. *J Biol Chem* 2002;277:6536–6541. [PubMed: 11742006]
8. Otto DM, Henderson CJ, Carrie D, Davey M, Gundersen TE, Blomhoff R, et al. Identification of novel roles of the cytochrome p450 system in early embryogenesis: effects on vasculogenesis and retinoic Acid homeostasis. *Mol Cell Biol* 2003;23:6103–6116. [PubMed: 12917333]
9. Henderson CJ, Otto DM, Carrie D, Magnuson MA, McLaren AW, Rosewell I, et al. Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. *J Biol Chem* 2003;278:13480–13486. [PubMed: 12566435]
10. Gu J, Weng Y, Zhang QY, Cui H, Behr M, Wu L, et al. Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. *J Biol Chem* 2003;278:25895–25901. [PubMed: 12697746]
11. Fluck CE, Tajima T, Pandey AV, Arlt W, Okuhara K, Verge CF, et al. Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley-Bixler syndrome. *Nat Genet* 2004;36:228–230. [PubMed: 14758361]
12. Arlt W, Walker EA, Draper N, Ivison HE, Ride JP, Hammer F, et al. Congenital adrenal hyperplasia caused by mutant P450 oxidoreductase and human androgen synthesis: analytical study. *The Lancet* 2004;363:2128.
13. Adachi M, Asakura Y, Matsuo M, Yamamoto T, Hanaki K, Arlt W. POR R457H is a global founder mutation causing Antley-Bixler syndrome with autosomal recessive trait. *American Journal Of Medical Genetics Part A* 2006;140A:633–635. [PubMed: 16470797]

14. Sahakitrungruang T, Huang N, Tee MK, Agrawal V, Russell WE, Crock P, et al. Clinical, Genetic, and Enzymatic Characterization of P450 Oxidoreductase Deficiency in Four Patients. *J Clin Endocrinol Metab* 2009;94:4992–5000. [PubMed: 19837910]
15. Homma K, Hasegawa T, Nagai T, Adachi M, Horikawa R, Fujiwara I, et al. Urine Steroid Hormone Profile Analysis in Cytochrome P450 Oxidoreductase Deficiency: Implication for the Backdoor Pathway to Dihydrotestosterone. *J Clin Endocrinol Metab* 2006;91:2643–2649. [PubMed: 16608896]
16. Fukami M, Nishimura G, Homma K, Nagai T, Hanaki K, Uematsu A, et al. Cytochrome P450 oxidoreductase deficiency: identification and characterization of biallelic mutations and genotype-phenotype correlations in 35 Japanese patients. *J Clin Endocrinol Metab* 2009;94:1723–1731. [PubMed: 19258400]
17. Huang N, Agrawal V, Giacomini KM, Miller WL. Genetics of P450 oxidoreductase: sequence variation in 842 individuals of four ethnicities and activities of 15 missense mutations. *Proc Natl Acad Sci U S A* 2008;105:1733–1738. [PubMed: 18230729]
18. Hart SN, Wang S, Nakamoto K, Wesselman C, Li Y, Zhong XB. Genetic polymorphisms in cytochrome P450 oxidoreductase influence microsomal P450-catalyzed drug metabolism. *Pharmacogenetics And Genomics* 2008;18:11–24. [PubMed: 18216718]
19. Huang N, Pandey AV, Agrawal V, Reardon W, Lapunzina PD, Mowat D, et al. Diversity and function of mutations in p450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis. *Am J Hum Genet* 2005;76:729–749. [PubMed: 15793702]
20. Sim SC, Miller WL, Zhong XB, Arlt W, Ogata T, Ding X, et al. Nomenclature for alleles of the cytochrome P450 oxidoreductase gene. *Pharmacogenet Genomics* 2009;19:565–566. [PubMed: 19535965]
21. Ingelman-Sundberg M. Pharmacogenetics of cytochrome P450 and its applications in drug therapy: the past, present and future. *Trends Pharmacol Sci* 2004;25:193–200. [PubMed: 15063083]
22. Evans WE, Relling MV. Pharmacogenomics: translating functional genomics into rational therapeutics. *Science* 1999;286:487–491. [PubMed: 10521338]
23. Williams PA, Cosme J, Vinkovic DM, Ward A, Angove HC, Day PJ, et al. Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. *Science* 2004;305:683–686. [PubMed: 15256616]
24. Ekroos M, Sjogren T. Structural basis for ligand promiscuity in cytochrome P450 3A4. *Proc Natl Acad Sci U S A* 2006;103:13682–13687. [PubMed: 16954191]
25. Schellens JHM, Soons PA, Breimer DD. Lack of bimodality in nifedipine plasma kinetics in a large population of healthy subjects. *Biochemical Pharmacology* 1988;37:2507. [PubMed: 3390213]
26. Ozdemir V, Kalow W, Tang BK, Paterson AD, Walker SE, Endrenyi L, et al. Evaluation of the genetic component of variability in CYP3A4 activity: a repeated drug administration method. *Pharmacogenetics* 2000;10:373–388. [PubMed: 10898107]
27. Zhou SF. Polymorphism of human cytochrome P450 2D6 and its clinical significance: Part I. *Clin Pharmacokinet* 2009;48:689–723. [PubMed: 19817501]
28. Hart SN, Zhong XB. P450 oxidoreductase: genetic polymorphisms and implications for drug metabolism and toxicity. *Expert Opin Drug Metab Toxicol* 2008;4:439–452. [PubMed: 18433346]
29. Ingelman-Sundberg M, Sim SC, Gomez A, Rodriguez-Antona C. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoeypigenetic and clinical aspects. *Pharmacol Ther* 2007;116:496–526. [PubMed: 18001838]
30. Agrawal V, Huang N, Miller WL. Pharmacogenetics of P450 oxidoreductase: effect of sequence variants on activities of CYP1A2 and CYP2C19. *Pharmacogenet Genomics* 2008;18:569–576. [PubMed: 18551037]
31. Gillam EMJ, Baba T, Kim BR, Ohmori S, Guengerich FP. Expression of Modified Human Cytochrome P450 3A4 in *Escherichia coli* and Purification and Reconstitution of the Enzyme. *Archives of Biochemistry and Biophysics* 1993;305:123. [PubMed: 8342945]
32. Omura T, Sato R. The Carbon Monoxide-Binding Pigment Of Liver Microsomes. I. Evidence For Its Hemoprotein Nature. *J Biol Chem* 1964;239:2370–2378. [PubMed: 14209971]
33. Miwa GT, Lu AY. The association of cytochrome P-450 and NADPH-cytochrome P-450 reductase in phospholipid membranes. *Arch Biochem Biophys* 1984;234:161–166. [PubMed: 6435533]

34. Waxman DJ, Attisano C, Guengerich FP, Lapenson DP. Human liver microsomal steroid metabolism: identification of the major microsomal steroid hormone 6 beta-hydroxylase cytochrome P-450 enzyme. *Arch Biochem Biophys* 1988;263:424–436. [PubMed: 3259858]
35. Yu KS, Cho JY, Jang IJ, Hong KS, Chung JY, Kim JR, et al. Effect of the CYP3A5 genotype on the pharmacokinetics of intravenous midazolam during inhibited and induced metabolic states. *Clin Pharmacol Ther* 2004;76:104–112. [PubMed: 15289787]
36. Guengerich FP, Muller-Enoch D, Blair IA. Oxidation of quinidine by human liver cytochrome P-450. *Mol Pharmacol* 1986;30:287–295. [PubMed: 3748010]
37. Smalley J, Kadiyala P, Xin B, Balimane P, Olah T. Development of an on-line extraction turbulent flow chromatography tandem mass spectrometry method for cassette analysis of Caco-2 cell based bi-directional assay samples. *J Chromatogr B Analyt Technol Biomed Life Sci* 2006;830:270–277.
38. Gomes LG, Huang N, Agrawal V, Mendonca BB, Bachega TA, Miller WL. The Common P450 Oxidoreductase Variant A503V Is Not a Modifier Gene for 21-Hydroxylase Deficiency. *J Clin Endocrinol Metab* 2008;93:2913–2916. [PubMed: 18397975]
39. Dierks EA, Davis SC, Ortiz de Montellano PR. Glu-320 and Asp-323 are determinants of the CYP4A1 hydroxylation regiospecificity and resistance to inactivation by 1-aminobenzotriazole. *Biochemistry* 1998;37:1839–1847. [PubMed: 9485309]
40. Nicolo C, Fluck CE, Mullis PE, Pandey AV. Restoration of mutant cytochrome P450 reductase activity by external flavin. *Mol Cell Endocrinol* 2010;321:245–252. [PubMed: 20188793]
41. Pandey AV, Kempna P, Hofer G, Mullis PE, Fluck CE. Modulation of human CYP19A1 activity by mutant NADPH P450 oxidoreductase. *Molecular Endocrinology* 2007;21:2579–2595. [PubMed: 17595315]
42. Oneda B, Crettol S, Sirot EJ, Bochud M, Ansermot N, Eap CB. The P450 oxidoreductase genotype is associated with CYP3A activity in vivo as measured by the midazolam phenotyping test. *Pharmacogenet Genomics*. 2009
43. Yano JK, Wester MR, Schoch GA, Griffin KJ, Stout CD, Johnson EF. The Structure of Human Microsomal Cytochrome P450 3A4 Determined by X-ray Crystallography to 2.05-Å Resolution. *J Biol Chem* 2004;279:38091–38094. [PubMed: 15258162]
44. Wilke RA, Lin DW, Roden DM, Watkins PB, Flockhart D, Zineh I, et al. Identifying genetic risk factors for serious adverse drug reactions: current progress and challenges. *Nat Rev Drug Discov* 2007;6:904–916. [PubMed: 17971785]
45. Dresser MJ, Leabman MK, Giacomini KM. Transporters involved in the elimination of drugs in the kidney: organic anion transporters and organic cation transporters. *J Pharm Sci* 2001;90:397–421. [PubMed: 11170032]
46. Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest* 2007;117:1422–1431. [PubMed: 17476361]
47. Weinshilboum R. Inheritance and drug response. *N Engl J Med* 2003;348:529–537. [PubMed: 12571261]
48. Evans WE, McLeod HL. Pharmacogenomics--drug disposition, drug targets, and side effects. *N Engl J Med* 2003;348:538–549. [PubMed: 12571262]
49. Fluck CE, Mullis PE, Pandey AV. Modeling of human P450 oxidoreductase structure by in silico mutagenesis and MD simulation. *Mol Cell Endocrinol* 2009;313:17–22. [PubMed: 19744540]
50. Hubbard PA, Shen AL, Paschke R, Kasper CB, Kim JJ. NADPH-cytochrome P450 oxidoreductase. Structural basis for hydride and electron transfer. *J Biol Chem* 2001;276:29163–29170. [PubMed: 11371558]
51. Marohnic CC, Panda SP, Martasek P, Masters BS. Diminished FAD binding in the Y459H and V492E Antley-Bixler syndrome mutants of human cytochrome P450 reductase. *Journal Of Biological Chemistry* 2006;281:35975–35982. [PubMed: 16998238]
52. Marohnic CC, Panda SP, McCammon K, Rueff J, Masters BS, Kranendonk M. Human cytochrome P450 oxidoreductase deficiency caused by the Y181D mutation: molecular consequences and rescue of defect. *Drug Metab Dispos* 2010;38:332–340. [PubMed: 19884324]

A **B** **FIGURE 1. Components studied**

A. Chemical structures of the CYP3A4 substrates used. **B.** Difference spectrum (Fe²⁺-CO vs Fe²⁺) of the human CYP3A4 preparation in *E. coli* membranes.

FIGURE 2. Lineweaver-Burk plots of CYP3A4 catalysis

In all panels, support by WT POR is indicated by circles and solid lines; Q153R with squares and dotted lines; A287P with triangles and dashed lines; and A503V with diamonds and dash-dot lines. Each point represents the mean of three experiments, each performed in duplicate. Catalysis could not be measured with R457H POR. **A.** 6 β -hydroxylation of testosterone. **B.** 1-hydroxylation of midazolam. **C.** 4-hydroxylation of midazolam. **D.** 3-hydroxylation of quinidine. **E.** N-demethylation of erythromycin.

Table 1

Catalytic activity of CYP3A4 supported by WT and mutant P450 oxidoreductase

Mutation	Vmax ± SEM	Km ± SEM	Vmax/Km	% WT
6β-hydroxylation of testosterone				
WT	2.74 ± 0.26	112 ± 5.78	0.024	100
Q153R	2.53 ± 0.54	80.4 ± 3.53	0.031	129
A287P	1.88 ± 0.57	464 ± 165	0.004	17
R457H	NC	NC	--	--
A503V	3.15 ± 0.39	168 ± 21.0	0.019	77
1-hydroxylation of midazolam				
WT	1.16 ± 0.17	13.7 ± 1.63	0.085	100
Q153R	0.80 ± 0.15	10.1 ± 2.19	0.079	94
A287P	0.32 ± 0.10	21.9 ± 8.88	0.015	17
R457H	NC	NC	--	--
A503V	0.88 ± 0.11	17.2 ± 4.08	0.051	61
4-hydroxylation of midazolam				
WT	4.60 ± 0.36	103 ± 30.0	0.045	100
Q153R	3.82 ± 1.94	93.0 ± 54.0	0.041	92
A287P	1.75 ± 1.00	277 ± 124.0	0.006	14
R457H	NC	NC	--	--
A503V	3.63 ± 1.07	110 ± 35.8	0.033	74
3-hydroxylation of quinidine				
WT	108 ± 25.5	327 ± 24.8	0.330	100
Q153R	170 ± 5.91	343 ± 3.17	0.496	150
A287P	3.61 ± 1.29	335 ± 8.98	0.011	3
R457H	0.91 ± 0.17	216 ± 46.3	0.004	1
A503V	99.7 ± 14.0	339 ± 19.0	0.294	89
N-demethylation of erythromycin				
WT	7.75 ± 0.87	205 ± 1.70	0.038	100
Q153R	9.55 ± 2.43	329 ± 22.5	0.029	76
A287P	NC	NC	--	--
R457H	NC	NC	--	--
A503V	9.71 ± 3.84	262 ± 82.7	0.037	97

NC = not calculatable

Table 2

Comparison of the activities of POR variants in various assays

CYP	Drug Metabolism						Steroidogenesis		
	3A4						1A2	2C19	17
Reaction	6 β -OH T	1-OH M	4-OH M	3-OH Q	N-deMe E	EOMCC	EOMCC	17 α -OH	17, 20 lyase
WT	100	100	100	100	100	100	100	100	100
Q153R	129	94	92	150	76	144	284	31	27
A287P	17	17	14	3	nil	nil	nil	40	21
R457H	nil	nil	nil	1	nil	nil	nil	3	nil
A503V	77	61	74	89	97	85	113	68	58
									aromatase
									100
									ND
									104
									1
									ND

All data are V_{max}/K_m, shown as a percentage of the wild-type control, set at 100%; ND, not done. The reactions assayed are: 6 β -hydroxylation of testosterone (6 β -OH T), 1-hydroxylation (1-OH M) and 4-hydroxylation (4-OH M) of midazolam, 3-hydroxylation of quinine (3-OH Q), and N-demethylation of erythromycin (N-deMe E) by CYP3A4 (Table 1–4 of this work); activation of fluorogenic EOMCC by CYP1A2 and CYP2C19 (from [30]); 17 α -hydroxylation of progesterone (17 α OH) and 17,20 lyase cleavage of 17OH-pregnenolone by CYP17 (from [19]); aromatization of androstenedione to estrone by CYP19 (from [41]).