IDENTIFICATION AND PHENOTYPE CHARACTERIZATION OF TWO *CYP3A* HAPLOTYPES CAUSING DIFFERENT ENZYMATIC CAPACITY IN FETAL LIVERS

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ABSTRACT

Background

The fetal liver CYP3A enzymes metabolize potentially toxic and teratogenic substrates and drugs in addition to endogenous hormones and differentiation factors. CYP3A7 is the most abundant CYP in human liver during fetal stages and first months of postnatal age and shows a large interindividual variability of unknown molecular basis.

Methods

A new variant gene (*CYP3A7*2*), which carries a mutation in exon 11 of *CYP3A7* causing a T409R substitution, was identified by direct sequencing. Genotype analysis was performed using PCR followed by restriction enzyme analysis. CYP3A7.2 activity was assessed in heterologous expression systems and human fetal liver microsomes.

Results

The frequency of *CYP3A7*2* was 8, 17, 28 and 62% in Caucasians, Saudi Arabians, Chinese and Africans, respectively. Using human HEK293 cells no significant differences in expression between CYP3A7.1 and CYP3A7.2 were found and fetal livers homozygous for *CYP3A7*2* had similar or higher CYP3A7 protein contents than *CYP3A7*1* livers. Kinetic studies showed that CYP3A7.2 was a functional enzyme with a significantly higher k_{cat} as compared to CYP3A7.1 ($P<0.05$). Interestingly, fetal livers that expressed CYP3A7.2 also expressed CYP3A5 protein and we found a linkage disequilibrium between the *CYP3A7*2* and *CYP3A5*1* alleles, that was subjected to interethnic differences. Determination of alprazolam 1-hydroxylation rate revealed that CYP3A5 plays a significant role in the metabolism of CYP3A substrates in fetal liver.

Conclusion

We have identified two different CYP3A phenotypes in fetal liver: one which is the result of a *CYP3A7*1/CYP3A5*3* haplotype causing CYP3A7.1 but no CYP3A5 expression, and another with higher detoxification capacity, inherent in the *CYP3A7*2/CYP3A5*1* haplotype, where CYP3A5 as well as a more active form of CYP3A7 are expressed.

INTRODUCTION

The cytochrome P450 (CYP) is a large superfamily of enzymes that catalyse the biotransformation of endogenous substrates and xenobiotics. Genes in families 1, 2 and 3 encode the most relevant enzymes catalysing the biotransformation of xenobiotics, including a wide variety of drugs and toxicants, and have a large impact on disease treatment [1]. Polymorphic CYP variants are of importance for individual susceptibility to adverse drug reactions or lack of therapeutic effect of drugs at ordinary dosages. The CYP3As are the most abundant P450s in human liver and small intestine and their activity is made up by the expression of CYP3A4, CYP3A5 and CYP3A7, whereas the low expression levels of CYP3A43 and improper folding in mammalian systems, rule out any significant contribution of this enzyme to CYP3A activity [2, 3]. The expression of CYP3A enzymes is regulated in a developmental manner [4-6]. CYP3A4, which is involved in the metabolism of 50% of currently used therapeutic drugs, has insignificant or a very low expression in the fetus but comprises 30 to 50% of total hepatic and intestinal P450 in adults [7, 8]. CYP3A5 expression starts at fetal stage and continues through adulthood [6, 9], but a frequent single nucleotide polymorphism (SNP) within intron 3 of *CYP3A5* (6986G>A, *CYP3A5*3* allele) results in absence of CYP3A5 protein [10]. Mainly individuals with at least one *CYP3A5*1* allele have correctly spliced *CYP3A5* mRNA and functional protein. CYP3A7 is the major CYP expressed in fetal liver, where it accounts for 50% of the total CYP content [11]. Its expression starts after 50-60 days of gestation and continues up to 6 months of postnatal age [5, 6]. In adults, CYP3A7 expression is much lower, although some individuals have low but significant *CYP3A7* gene expression in liver and intestine [12].

CYP3A7 shares substrate specificity with CYP3A4 and CYP3A5, but has unique activity towards many xenobiotics and endogenous substrates. Thus, it plays an important role in the metabolism of key steroids in the adrenals and gonads, such as dehydroepiandrosterone and

testosterone, and also in that of potentially toxic and teratogenic endogenous substrates such as retinoic acid [13-16]. In addition, CYP3A7 is involved in the metabolic clearance of many xenobiotics that reach the fetus when taken during pregnancy such as therapeutic drugs for women with chronic medical conditions, for example heart disease, seizure disorders, coagulopathies or AIDS, and substances of abuse as cocaine, cannabinoids and methadone [17-20].

Because of the important roles of CYP3A7, the substantial interindividual expression differences found in fetal livers [9] is expected to have a significant impact in interindividual differences in embryotoxicity and teratogenicity. Polymorphism in *CYP3A7* could explain the expression variability of CYP3A7, however, no enzyme variants have been described so far. We here identify a novel SNP in *CYP3A7* coding region that results in the replacement of Thr^{409} with Arg, altering the enzyme kinetics. The corresponding allele, *CYP3A7*2*, (approved by the Human CYP allele nomenclature committee, http://www.imm.ki.se/CYPalleles) is in linkage disequilibrium with *CYP3A5*1* and, therefore, with functional CYP3A5 enzyme in the liver, allowing the identification of a high and a low CYP3A activity haplotypes in the fetus.

MATERIALS AND METHODS

Materials. δ-Aminolevulinic acid, isopropyl β-D-thiogalactoside (IPTG), glucose, galactose and NADPH were purchased from Sigma-Aldrich. Protease Inhibitor Cocktail Tablets were from Roche. Dehydroepiandrosterone (DHEA) [1,2,6,7-^{3}H(N)] (specific activity 74Ci/mmol) was purchased from Perkin Elmer. P450-Glo^{TM} CYP3A7 Assay was purchased from Promega. Lipofectamine-2000, Minimum Essential Medium, FBS, streptomycin, penicillin, Non Essential Aminoacids and Sodium Pyruvate were from Invitrogen. The expression vectors pCWori+, pCW´/NPR and pCW´/3A4#17 were kindly provided by Dr. Elisabeth Gillam (University of Queensland, Australia). Alprazolam, 4-OH alprazolam and 1-OH alprazolam were a gift from Leif Bertilsson (Karolinska Institutet, Sweden).

Sequencing of CYP3A7 and genotyping. The *CYP3A7*2* allele was detected after PCR amplification of CYP3A7 cDNA from fetal liver (see http://www.imm.ki.se/CYPalleles for allele nomencalture. *CYP3A7*1* GenBank accession number: NM_000765.2). The DNA fragment containing *CYP3A7*2* was amplified using 5´-ATCTCATCCCAAACTTGGCCG-3´ forward and 5´-TCAGGCTCCACTTACGGTCT-3´ reverse primers, was purified using Wizard PCR Kit (Stratagene), sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems). The SNP detected was confirmed at the genomic level by direct sequencing.

*CYP3A7*2* genotyping was performed by restriction fragment length polymorphism (RFLP). A 385-bp region covering parts of exon 11 and intron 11 of *CYP3A7* was amplified by PCR using 5´-CACCTATGATACTGTGCTACAGT -3´ forward and 5´-TGTGGTTTGTAAAGTGTGACAATG-3´ reverse primer. Ten µl of this PCR fragment were incubated with *GsuI* over night and separated by electrophoresis in an agarose gel. *CYP3A7*1*

allele resulted in the 385 bp undigested PCR fragment while *CYP3A7*2* allele resulted in two DNA fragments of 177 and 208-bp. *CYP3A5*1* and *CYP3A5*3* genotypes were assessed by allele-specific PCR as previously described [21]. *CYP3A7*1C* was analyzed as previously described [12]. To determine whether *CYP3A7*2* could be in linkage disequilibrium with other polymorphisms in *CYP3A7* coding region, *CYP3A7* full coding region was amplified with 5´-CAGGAAAGCTCCACACACAC-3´ forward and 5´-TGGGGCACAGCTTTCTTAAA-3´ reverse primers, using the cDNA from 8 fetal and 19 adult Caucasian livers. The PCR fragments were purified and sequenced as described above.

Subjects. Allelic frequencies were determined using genomic DNA from unrelated individuals from different populations. One hundred and one volunteers from Zaragoza area in the north of Spain [22], 47 Swedish [21, 23], 96 Chinese individuals [24], 70 Saudi Arabians [25] and 90 Tanzanians (kindly provided by Dr. Rajaa A. Mirghani, Karolinska University Hospital, Stockholm, Sweden) were included. The genomic DNA from Swedish liver samples was extracted using the QIAamp Tissue Kit (Qiagen). The present study was approved by the ethical committee at Karolinska Institutet.

Heterologous expression of CYP3A7*1 and CYP3A7*2. Full length *CYP3A7*1* and *CYP3A7*2* cDNAs were PCR amplified using Elongase (Invitrogene) and reverse transcribed human liver RNA, from individuals homozygous for the different alleles. The cDNAs were cloned in appropriate expression vectors and the inserted sequences were analyzed by ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and ABI PRISM 377 DNA sequencer (Applied Biosystems), to rule out PCR artefacts in the cloned sequences. Quantification of expressed P450 holo-enzyme was performed by carbon monoxide binding spectral analyses [26].

For heterologous expression in *Escherichia Coli,* the PCR was carried out with a forward primer which introduced a *Nde*I site at the 5´end: 5´-AAGGCATATGGATCTCATCCCAAACTTGGCCG - 3´ and a reverse primer containing a *Xba*I site at the 5´end: 5´- CTTATCTAGATCAGGCTCCACTTACGGTCT- 3´. After *Nde*I/*Xba*I digestion of the CYP3A7 cDNAs, DNA fragments were cloned into pCWori+ expression vector digested with the same restriction enzymes. *BamHI/Xba*I fragments from these plasmids were subcloned into pCW´/hNPR, that contained human NADPH P450 reductase [27], to generate a bicistronic vector. To achieve high levels of P450s expression, the DNA sequence corresponding to the N-terminal part of the protein was modified by digestion of the CYP3A7 bicistronic vectors with *Nde*I/*Stu*I, DNA purification to eliminate the small DNA fragments liberated and ligation of the *Nde*I/*Stu*I fragment from pCW´/3A4#17 [28]. The final bicistronic expression vectors contained pCW´/3A4#17 sequence until *Stu*I site followed by the sequence of *CYP3A7*1* or *CYP3A7*2* from amino acids 83 to 503. P450 expression was achieved as previously described [29], with IPTG induction for 24 h at 30ºC and using a bacteria strain, Rosetta (DE3) (Novagen), which contains a chloramphenicol-resistant plasmid that provides tRNAs that are rare in *E. Coli.* Bacteria fractioning was performed as previously described [27].

For heterologous expression in *Saccaromyce Cerevisae,* CYP3A7 cDNAs were amplified by PCR with a forward primer which introduced three A residues in front of the translation start to increase the expression efficiency [30] and a *BamH*I site at the 5´end: 5´-AAGGGGATCCAAAATGGATC TCATCCCAAACTTGGCCG- 3´ and a reverse primer containing a *Kpn*I site at the 5´ end: 5´-CTTAGGTACCTCAGGCTCCACTTACGGTCT-3´. After double-digestion of the amplified DNA with *BamH*I/*Kpn*I, fragments were cloned into the pYeDP60 expression vector [31] generating

the plasmids V60-*3A7*1* and V60-*3A7*2*, respectively. The *S. cerevisiae* strain W(R) engineered to over-express the yeast cytochrome P450 reductase [32, 33] was used to express *CYP3A7*1* and *CYP3A7*2*, essentially as described elsewhere [32, 34]. In brief, the yeast was transformed with the pYeDP60-derived expression vectors and grown to high density with glucose as the main energy source; thereafter, galactose was added to induce expression. After expression, the cells were harvested, mechanically disrupted [34] and microsomes containing the recombinant enzyme isolated by differential centrifugation (20,000 x *g* 10 min plus 100,000 x *g* 60 min).

For expression in the adenovirus 5 transformed Human Embryonic Kidney cell line 293 (HEK293; CRL-1573, ATCC), the V60-*CYP3A7*1* and V60-*CYP3A7*2* plasmids described above were digested with *BamH*I/*Kpn*I and the insert was subcloned into pCMV4 expression vector [35] digested with *Bgl*II/*Kpn*I. The resulting expression vectors: pCMV4-*CYP3A7*1* and pCMV4-*CYP3A7*2* were transfected using Lipofectamine-2000 into HEK293 cells. After transfection the cells were maintained in Minimum Essential Medium containing 10% FBS, 100µg/ml streptomycin, 100U/ml penicillin and non essential amino acids and sodium pyruvate. Sixty hours after transfection the cells were harvested and homogenized in a glass Dounce homogenizator in 10 mM Tris HCl pH 7.4 containing 0.25 M sucrose, 1 mM EDTA and Protease Inhibitor Cocktail Tablets. This suspension was loaded on top of 10 mM Tris HCl pH 7.4, 0.5 M sucrose and centrifuged at 900 x g for 10 min. The supernatant and interface was transferred to a new tube containing 10 mM Tris HCl pH 7.4, 0.5 M sucrose and centrifuged at 9,000 x g for 10 min. The supernatant was transferred to a new tube and microsomes were pelleted by 1h centrifugation at 100,000 x g.

Liver tissue. Fetal human liver pieces were obtained from legal abortions on socio-medical indications. Gestational ages of the fetal livers are listed in Table II. All the samples were frozen in liquid nitrogen and stored at –70ºC until subcellular fractionation and isolation of RNA and genomic DNA. The study was approved by the Ethical committee at Karolinska Institutet, Huddinge, Stockholm.

Subcellular fractionation of human liver. Human fetal liver samples were homogenised in a glass homogenizer in 4 volumes of ice cold 50 mM Tris HCl, pH 7.4 containing 0.25 M sucrose and protease inhibitors. The resulting homogenate was centrifuged for 20 min at 10,000 x g at 4ºC, the supernatant was collected and protein concentration was measured according to Bradford [36]. When the amount of human fetal material permitted, microsomes were obtained by centrifugation at 10,000 x g for 20 min at 4ºC, followed by centrifugation at 100,000 x g for 1 h at 4ºC. The pellet was washed and resuspended in 0.1 M phosphate-buffered saline, pH 7.4 with 10% glycerol and protease inhibitors. Protein content was measured by Bradford [36].

Immunoquantitation of CYP3A5 and CYP3A7. Proteins from fetal livers and from heterologous systems used to express CYP3A7, were separated by 8.5% SDS-polyacrylamide gel electrophoresis using the Mini-PROTEAN II electrophoresis cell (Bio-Rad, Sundbyberg, Sweden) and transferred to Hybond-C nitrocellulose membranes (Amersham Biosciences). The membranes were blocked and then incubated with primary antibodies A234, A254 or A235 (Gentest), following the manufacturer´s instructions. A234 recognizes CYP3A4-7, A254 recognizes CYP3A4-5-7 and A235 recognizes CYP3A5. After washing, the membranes were incubated with the corresponding horseradish peroxide-conjugated secondary antibody and the proteins were visualized using SuperSignal West Pico Chemiluminescence method (Pierce Chemical, Rockford, IL) and scanned using LAS-1000 (Fujifilm, Dusseldorf, Germany). For the CYP protein

quantification different amounts of standards were loaded in the gel: between 0.10 and 0.73 pmols of CYP3A5 and between 0.22 and 1.32 pmols of CYP3A7. The limit of detection under the conditions used were 6 pmols/ mg protein and 15 pmols/ mg protein for CYP3A5 and CYP3A7, respectively. The relative intensity of each band as determined by Image Gauge V3.6 and the CYP3A5 and CYP3A7 content for each sample was determined from standard curves. A microsomal sample quantified with respect to the apo-CYP3A7 content determined by peptide specific antibody and peptide conjugated lysozyme as standard was kindly provided by Robert J. Edwards, Imperial College London, UK, and Sarah C. Sim, Karolinska Institutet, Sweden (S. C. Sim *et al.* manuscript in preparation) and used as reference for CYP3A7 immunoquantitation. Human liver microsomes in which CYP3A5 expression had been precisely quantified previously [21] were used as standards for CYP3A5 immunoquantitation. Fifteen µg of protein of human adult and fetal liver microsomes were used for CYP3A5 quantification with the antibody A235 (Gentest), 1 µg of human fetal liver microsomes was used for CYP3A7 quantification with the antibody A234 (Gentest).

Assays for CYP activitites. Measurements of DHEA activity were performed in a final volume of 0.5 ml with 100 mM of potassium phosphate buffer pH 7.4 containing 15 nM of [^{3}H]DHEA and DHEA to get final concentrations between 0.2 and 100 µM and 200 to 300 µg or 5 to 50 µg of microsomal protein from HEK293 cells and fetal liver, respectively. The reaction mixtures were pre-incubated at 37ºC for 5 min before the reactions were started by the addition of NADPH to a final concentration of 2 mM. The reactions were carried out at 37ºC for 15-30 min, within the linear range of formation of metabolites. The reactions were stopped by addition of 5 ml of dichloromethane and the extraction of the metabolites was carried out by vortexing for 20 min, 10 min centrifugation at 5000 x g and separation and drying of the organic phase under a stream of

N_2 gas. Dried extracts were dissolved in 100 µl of mobile phase and analyzed by high-performance liquid chromatography (HPLC). A 5µm 4 x 250 mm LiChrospher-100 RP-18 column (Merk) using a 64:36 methanol:H_2O (v/v) isocratic mobile phase was used to separate the hydroxylated products. Radioactive peaks were detected by an in-line Berthold Radioactivity Monitor LB506C-1, a Berthold LB5035 pump was used for the scintillation liquid at a 1.5 ml/min flow rate (Quicksafe Flow 2, Zinsser Analytic).

For the kinetic studies, two independent HEK293 transfections and microsomes preparations for each *CYP3A7* allele were used. The mean of each independent preparation was used to calculate a mean value and to generate a Michaelis-Menten plot using GraphPad Prism version 4.00 for Windows with a nonlinear regression fit to a hyperbola equation.

Measurements of Luciferin 6´ benzyl ether (Luciferin BE) debenzylation were performed as recommended by the manufacturer in the P450-Glo^{TM} CYP3A7 Assay. The reaction volume was 50 µl, the concentration of Luciferin BE 150 µM and the reactions were incubated at 37ºC for 15 min, when the formation of metabolites was linear. Reactions were stopped by addition of Luciferin Detection Reagent and the luminescence was recorded after incubation for 20 min at room temperature. For kinetic analysis, one HEK293 microsomal preparation was used in triplicates.

Alprazolam incubations were performed in 1 ml reaction mixtures (50 mM Tris HCl pH 7.4, alprazolam 500 µM and NADPH 2mM) containing 200µg of fetal liver microsomal protein. Methanol concentration was 0.25%. Reaction mixtures were pre-incubated at 37ºC for 4 min, then NADPH was added and the reactions were terminated after 30 min with the addition of 40 µl of 100 µM caffeine (internal standard), 1 ml of 1 M borate buffer pH 9.0 and 4.5 ml dichloromethane. The incubations were vortexed, centrifuged for 5 min at 5,000 x g and the lower organic phase was separated and evaporated under N_2 atmosphere. The residues were dissolved in

the HPLC mobile phase (100 mM phosphate buffer pH 6.0 containing acetonitrile 35% v/v) and immediately injected into a HPLC column (5µm 4 x 250 mm LiChrospher-100 RP-18 column) at a flow rate of 1 ml/ min. The formation of metabolites was detected by monitoring absorption at 214 nm. The amount of metabolites formed was estimated from standard curves of 1-OH alprazolam and 4-OH alprazolam.

Statistical analysis. Data were analyzed using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical analysis was done by Mann-Whitney test or Student's *t*-test. Results are expressed, as mean ± SD. Differences were considered significant when *P* values were less than .05.

RESULTS

*Identification and allelic frequency of CYP3A7*2*

A C to G change in exon 11 of *CYP3A7* (*CYP3A7*2*) that causes the amino acid substitution T409R was detected by direct sequencing of a full length *CYP3A7* cDNA derived from human fetal liver RNA. This SNP was confirmed in genomic DNA and a RFLP assay was set up for genotyping. Genomic DNA from unrelated individuals from different ethnic groups was used to determine *CYP3A7*2* allele frequency. *CYP3A7*2* had a higher occurrence than the wild-type allele in Africans (*CYP3A7*2*, q = 0.62). The frequencies were considerably lower in Chinese (q = 0.28), Saudi Arabians (q = 0.17) and Caucasians (q = 0.08), as shown in Table I. These results show that *CYP3A7*2* is a frequent allele with high interethnic differences in its distribution.

To determine whether *CYP3A7*2* could be in linkage disequilibrium with other polymorphisms in *CYP3A7* coding region, we sequenced the full coding region of *CYP3A7* using the cDNA from 27 Caucasian livers. Only *CYP3A7*2* was identified, indicating that polymorphisms in *CYP3A7* coding region are rare, suggesting an important role of CYP3A7 in the metabolism of endogenous compounds.

It has been shown that some adult individuals express CYP3A7 in liver and gastrointestinal tract due to a replacement of part of *CYP3A7* proximal promoter by the corresponding region of *CYP3A4* containing the PXR element and yielding the *CYP3A7*1C* allele (q= 0.03 Caucasians, q= 0.06 African Americans) [10, 12]. In order to determine whether *CYP3A7*2* is associated with *CYP3A7*1C*, 44 Swedish individuals were genotyped for *CYP3A7*1C*. We found two subjects heterozygous for *CYP3A7*1C*, one of them was heterozygous for *CYP3A7*2* and the other was homozygous for *CYP3A7*1*. To further investigate whether CY*P3A7*2* could be in linkage disequilibrium with *CYP3A7*1C*, 73 Chinese and 50 Saudi Arabians were genotyped. However, none of them carried the *CYP3A7*1C* allele.

Characterization of CYP3A7*2 expression in heterologous systems

Thr409 of CYP3A7.1, which is replaced by Arg in CYP3A7.2, is located within the ERR triad, where the Glu (E362) and first Arg (R365) derive from a highly conserved motif in the K-helix, and the distal Arg (R418) derives from the meander region. The requirement of the ERR triad for the retention of the heme in a functional orientation, has been demonstrated in bacterial P450s of known structures [37, 38]. SNPs in *CYP3A4* that cause amino acid changes in this region, T363M and P416L, and to a lower extent L373F, result in a decreased bacterial expression of the CYP3A4 enzyme [39]. In order to determine whether CYP3A7.2 was expressed at lower levels than CYP3A7.1, suitable *CYP3A7*1* and *CYP3A7*2* expression vectors were introduced into *E. coli* (Rosetta), *S. cerevisae* (W(R)) and HEK293 cells. The resulting CYP3A7 expression was determined by Western blotting. Two different antibodies were used, one recognizing CYP3A4-5-7 (Gentest A254, monoclonal) and another recognizing CYP3A4-7 (Gentest A234, polyclonal), and both gave similar results. As shown in Figure 1, the CYP3A7.2 protein content was significantly lower than wild-type CYP3A7.1 in *E. coli* (Rosetta), and *S. cereviase* W(R) (40 and 65 %, respectively, $P<0.05$), suggesting a lower stability of CYP3A7.2. However, in microsomes from human HEK293 cells the CYP3A7.2 content was not significantly different from CYP3A7.1 content (85 %), suggesting that the amino acid substitution T409R would not cause important differences in expression and stability in hepatocytes. The capacity of the different heterologous systems to produce CYP3A7 protein is shown in Figure 1.

To investigate whether the T409R substitution of CYP3A7.2 could alter the folding capacity, membranes and microsomes isolated from the heterologous expressions in bacteria and yeast were investigated for the amount of holo-CYP3A7 by measuring the CO-reduced difference spectra. As

shown in Figure 2, CYP3A7.2 was successfully expressed as a native, correctly folded enzyme. It has previously been shown that the expression levels of CYP3A7.1 achieved in heterologous systems are low, usually resulting in preparations with large quantities of inactive apo-enzyme together with some active holo-enzyme [28]. We found approximately 20-fold higher amounts of CYP3A7.1 apo-enzyme than holo-enzyme in bacteria and 3-fold higher amounts of the apo-enzyme in yeast. No difference in these ratios was found between CYP3A7.1 and CYP3A7.2 in the bacteria and yeast (data not shown).

Expression of CYP3A7.2 and CYP3A5 in human fetal liver

To further investigate CYP3A7.2 expression and stability, we used fetal human liver samples that were identified as homozygous for *CYP3A7*1* or *CYP3A7*2* (see Table II). When the small amount available of some fetal liver samples did not allow to isolate microsomes, 10,000 x g centrifugation supernatants were used for Western blotting. The CYP3A7 content in fetal liver was determined using a CYP3A4-7 antibody (A234, Genetest). This antibody does not recognize CYP3A5 and CYP3A4 protein content, in fetal livers of 94-168 days of gestational age, is negligible when compared to CYP3A7 [9]. We used real-time PCR to confirm this, and found that, in all of the fetal samples used in this study, *CYP3A4* mRNA content was insignificant when compared to *CYP3A7* mRNA content (in average, 1000-fold lower, data not shown). This indicates that, in these Western blots, the signal of the A234 antibody corresponds to CYP3A7. Figure 3A shows that the CYP3A7.2 content was higher or similar to that of CYP3A7.1 in fetal liver, indicating that the CYP3A7.2 variant does not have a decreased stability in fetal hepatocytes. Interestingly, when a CYP3A5 specific antibody was used for detection (A235, Gentest) only the two fetal livers homozygous for the *CYP3A7*2* allele showed CYP3A5 expression (Fig. 3B). In order to quantify the amounts of CYP3A7 and CYP3A5 protein we used

standard samples in which the amount of CYP3A5 and CYP3A7 had been precisely estimated (CYP3A5 [21] and CYP3A7 as described in Materials and Methods). The CYP3A7 protein content measured in microsomes from 4 fetal livers varied from 218 to 518 pmol per mg of microsomal protein. CYP3A7.1 and CYP3A7.2 protein contents were not statistically different. With respect to CYP3A5, the two fetal livers with detectable CYP3A5 expression had 44 and 45 pmol of CYP3A5 per mg of microsomal protein.

CYP3A7*2 is in linkage disequilibrium with CYP3A5*1

As mentioned, the fetal livers with *CYP3A7*2* alleles (FL61 and FL65) expressed CYP3A5 protein. Functional CYP3A5 protein expression requires mostly at least one *CYP3A5*1* allele [10] and both FL61 and FL65 were identified as homozygous for *CYP3A5 *1* (Table II), suggesting a linkage disequilibrium between *CYP3A7*2* and *CYP3A5*1*. This was further investigated by genotyping for the *CYP3A5 *1* and *CYP3A5*3* alleles in the ethnic groups used to determine the frequency of the *CYP3A7*2* allele. Table I shows that the frequency of the *CYP3A5*1* allele is similar to that of *CYP3A7*2* in Spanish, Swedish and Chinese populations. In contrast, Saudi Arabians had a higher frequency of *CYP3A7*2* (q=0.17) than *CYP3A5*1* (q=0.09) and Africans had a higher frequency of *CYP3A5*1* (q=0.79) than of *CYP3A7*2* (q=0.62). These results indicate a linkage disequilibrium between the two polymorphisms studied with important interethnic differences. Thus, in Swedish and Chinese populations a strong linkage disequilibrium was observed, whereas a lower extent of linkage was found in Spanish, Africans and Saudi Arabian populations. In general, most Caucasians, Saudi Arabians and Chinese express CYP3A7.1 but have no CYP3A5 protein, whereas most Africans have CYP3A7.2 and express the CYP3A5 protein.

Functional characterization of CYP3A7.1 and CYP3A7.2

DHEA is a CYP3A-specific substrate and, among the three main CYP3A enzymes, CYP3A7 is the dominant DHEA 16α hydroxylase. In CYP3A7 catalyzed reactions, 16α-OH DHEA comprises more than 95% of the total metabolites and only to a very small extent 7β-OH DHEA is produced [6]. We used the DHEA hydroxylation assay to compare the catalytic activities of CYP3A7.1 and CYP3A7.2. HEK293 cells, transfected with *CYP3A7*1* or *CYP3A7*2* expression vectors or with the pCMV4 empty vector as a control, were homogenized, microsomes isolated and the CYP3A7 content quantified. The microsomal fractions were used to measure DHEA hydroxylase activity as described in Materials and Methods using DHEA concentrations between 0.02 μM and 100 μM. The kinetic studies showed that replacement of Thr^{409} with Arg produced a statistically significant 1.5-fold increase in k_{cat} for16α-hydroxylation of DHEA (k_{cat} = 0.035 ± 0.002 s^{-1} for CYP3A7.1 and 0.052 ± 0.002 s^{-1} for CYP3A7.2, P<0.05) indicating CYP3A7.2 to be a more efficient enzyme in this reaction (Fig. 4). At DHEA concentrations near physiological conditions (16 nM) the DHEA hydroxylation catalysed by CYP3A7.2 was 1.4-fold higher than that of CYP3A7.1. In agreement with this, use of another CYP3A7-specific substrate, Luciferin BE between 50 and 200 μM, revealed a moderate increase in the rate of debenzylation (1.3-fold) catalysed by CYP3A7.2 (data not shown).

Fetal liver metabolism of DHEA and Luciferin BE

To further investigate the catalytic capacity of CYP3A7.2, incubations of fetal liver microsomes from subjects homozygous for *CYP3A7*1* or *CYP3A7*2* were carried out with DHEA and luciferin BE. Incubations at saturating concentrations (50 μM) of DHEA showed a reaction rate between 2.0 and 3.5 nmol of hydroxylated metabolite min^{-1} mg^{-1} microsomal protein. The contribution of CYP3A5 (only present in the CYP3A7.2 livers) to the DHEA hydroxylation in

fetal liver has previously been show to be negligible [6]. As shown in Figure 5A, the fetal livers FL61 and FL65, which express CYP3A7.2 and contain similar amounts of CYP3A7 as FL55, metabolised DHEA at significantly higher rate than FL55, which expresses CYP3A7.1. The DHEA hydroxylation rate of the CYP3A7.2 fetal livers at saturating concentrations of the substrate (50 µM) was 1.6- and 1.8-fold higher than that of the CYP3A7.1 liver (FL61, $P<0.05$ and FL 65, $P<0.001$). This is in agreement with the difference in k_{cat} calculated with heterologously expressed enzymes (1.5-fold increase). Similarly, at physiological concentrations of DHEA (16 nM), a 1.6- and 2.0- fold more rapid DHEA hydroxylations were found for FL61 and FL65 when compared to FL55 ($P<0.01$ and $P<0.001$, data not shown).

CYP3A7 produces a 35-fold higher amount of debenzylated luminescent product from Luciferin BE than CYP3A5, and CYP3A4 a 20-fold higher rate of product formation than CYP3A5, in all cases under the same incubation conditions (Manufacturer´s information). Therefore, this substrate can be used to monitor CYP3A7 activity in fetal liver, without interference of CYP3A5. Similarly to the results obtained with DHEA, liver microsomes from FL61 and FL65 had a 1.9- and 2.7-fold significantly higher rate of Luciferin BE debenzylation activity than those from FL55 (Fig. 5B).

Alprazolam metabolism to 4-OH and 1-OH alprazolam is catalysed by CYP3A enzymes, CYP3A5 being more active than CYP3A4 and CYP3A7 [40]. When we used heterologously expressed P450s, we found that alprazolam 1- and 4-hydroxylation were 60- and 4-fold higher, respectively, for CYP3A5 as compared to CYP3A7.1, at a 500 µM alprazolam concentration (data not shown). In addition, a moderately increased activity for CYP3A7.2 in 1- and 4-hydroxylations when compared to CYP3A7.1 was found (data not shown). When alprazolam was incubated with the fetal liver microsomes a significant increase in 4-OH alprazolam production was detected in

the *CYP3A7*2/CYPA5*1* livers as compared to the *CYP3A7*1/CYPA5*3* liver (2.0- and 2.8-fold, $P<0.001$ and $P<0.01$, respectively) (Fig. 5C), similar to that found with DHEA and Luciferin BE. However, 1-hydroxylation of alprazolam was much higher for FL61 and FL65 (18.0- and 18.4-fold, $P<0.0001$ and $P<0.001$, respectively) when compared to liver microsomes from FL55 (Fig. 5D). This is in agreement with a major contribution of CYP3A5 to alprazolam 1-hydroxylation.

DISCUSSION

CYP3A7 accounts for more than 50% of total CYP content in fetal liver [11] and plays an important role in the metabolism of xenobiotics that the fetus is exposed to as well as endogenous substrates. This enzyme and CYP3A5 are apparently the major fetal CYP enzymes active in the metabolism of drugs and other xenobiotics. Therefore, variant *CYP3A* haplotypes can significantly influence the pharmacokinetics, carcinogenicity and metabolism of exogenous as well as of endogenous compounds in the fetal liver. In addition, CYP3A7 is also expressed in the liver and intestines of some adults and in the adrenal gland, prostate and thymus [41], tissues where it could play an important role. In this study we report a novel *CYP3A7* allele, present at various frequencies in different populations, that encodes a functional CYP3A7 variant enzyme (CYP3A7.2) that is associated to the protein expression of CYP3A5 in fetal livers, yielding phenotypes of high and low CYP3A metabolic activity.

The frequency of the novel *CYP3A7*2* allele differs between populations (Table I), from 8 % in Caucasians to the highest frequency of 62 % found in Africa. In addition, we found a linkage disequilibrium between *CYP3A7*2* and *CYP3A5*1*. The association between these two polymorphisms, which are separated by 36 kb, is also subjected to interethnic differences. There is a high linkage in Chinese and Swedish populations, and lower in Spanish, Africans and Saudi Arabians (Table I). The most frequent haplotype in Caucasians was identified as *CYP3A7*1/CYP3A5*3* whereas *CYP3A7*2/CYP3A5*1* was the most common haplotype in Africans. These haplotypes result in different CYP3A phenotypes: individuals expressing CYP3A7.1 but with defective CYP3A5 expression and individuals that express both CYP3A7.2 and CYP3A5. In addition, we also found some evidence suggesting a linkage disequilibrium between *CYP3A7*2* and *CYP3A7*1C*, which is associated with CYP3A7 expression in adult liver and intestine [12].

The Thr[409], that is changed to Arg in CYP3A7.2, is conserved among human CYP3As and it is located within a region (ERR triad) that is necessary for the bacterial holo-enzyme stability [37, 38]. Human HEK293 cells showed no major differences between CYP3A7.1 and CYP3A7.2 expression levels and suggested a normal stability of the CYP3A7.2 enzyme. *E. coli* and *S. cerevisiae* cells had however a reduced expression of CYP3A7.2 enzyme, compared to the wild-type CYP3A7.1, but this does not necessarily indicate a factual instability of the CYP3A7.2 enzyme because different heterologous expression systems have various capabilities for correct folding (Fig. 1). The functional expression of CYP3A7.2 *in vivo* is strongly supported by experiments using microsomes from fetal livers. Thus, using a panel of 7 human fetal livers, we found that livers homozygous for *CYP3A7*2* contained similar or higher amounts of CYP3A7 protein than *CYP3A7*1* livers (Fig. 3). The CYP3A7 content of 4 livers varied between 218 to 518 pmols mg^{-1} microsomal protein. These quantities are similar to those estimated by Stevens *et al.* [6] (311 pmol mg^{-1} protein, in the same age group) and higher than the 100 pmol mg^{-1} value estimated by Shimada *et al.* [13, 42]. In our study the CYP3A5 protein content of the two *CYP3A5*1* homozygous livers was 44 and 45 pmols CYP3A5 mg^{-1} microsomal protein, values that are similar to those found in CYP3A5*1/*3 heterozygous adult livers [21] and are in accordance with a previous study that showed that the content of fetal liver CYP3A5 was generally independent of age [6]. In the same study, the maximum CYP3A5 content, using 115 livers with detectable CYP3A5 protein, was estimated to be 25 pmol mg^{-1} microsomal protein. The difference between this value and our calculated CYP3A5 content could be caused by the different standards used. In the current study, the standards consisted of carrier protein-coupled peptides used by Westlind-Johnsson *et al*. (2003) while Stevens *et al.* (2003) used baculovirus expressed CYP3A5. In any case, CYP3A5 levels were considerably lower than those of CYP3A7, which are in turn comparable in fetal liver to those of CYP3A4 in adult liver [21].

CYP3A7.2 formed a spectrally active holo-enzyme (Fig. 2) and the catalytic activity of the enzyme was slightly different using two CYP3A7-specific substrates. Kinetic analysis with DHEA showed that CYP3A7.2 had a 1.5-fold significantly higher k_{cat} than CYP3A7.1, when the two variants were expressed in HEK293 cells. Consistently, fetal livers expressing CYP3A7.2 had an increased metabolism of the CYP3A7-specific substrates DHEA and Luciferin BE when compared to livers expressing CYP3A7.1 (Fig. 5A-B). Interestingly, we found a 18-fold increase in 1-hydroxylation of alprazolam in the fetal livers with *CYP3A7*2/CYP3A5*1* haplotype when compared to those with *CYP3A7*1/CYP3A5*3* haplotype (Fig. 5D). The reason for the large differences found in alprazolam 1-hydroxylation is the major contribution of CYP3A5 to this reaction. In adult liver, due to the fact that CYP3A5 catalyses the same reactions as CYP3A4 [40], the contribution of CYP3A5 to total CYP3A metabolism is mainly determined by its relative expression to CYP3A4. In fetal liver the amount of CYP3A5 is lower than that of CYP3A7, but there are major differences in substrate specificity between the two enzymes (i.e. 16α-DHEA hydroxylation and alprazolam 1-hydroxylation). Thus, CYP3A5 can have a significant contribution to fetal CYP3A metabolism for CYP3A5-specific substrates such as alprazolam.

In conclusion, we have identified a new polymorphism that results in a CYP3A7-variant enzyme (CYP3A7.2) which has a moderately increased catalytic activity and that is in linkage disequilibrium with *CYP3A5*1.* This allelic linkage results in haplotypes that are causing different CYP3A phenotypes during the fetal stage and the first months after birth and they exhibit important interethnic differences in their distribution (Table I). The two most common phenotypes are: (i) individuals in which CYP3A7.1 is the only CYP3A enzyme present in the liver and corresponds to the frequent *CYP3A7*1/CYP3A5*3* Caucasian haplotype and (ii) individuals that have a *CYP3A7*2/CYP3A5*1* haplotype, most frequent in Africa, who express two catalytically

different CYP3A enzymes, CYP3A7.2 and CYP3A5, that will provide more active and versatile biotransformation pathways for drugs and other xenobiotics as well as of endogenous compounds.

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LEGENDS FOR FIGURES

Fig 1. Stability of the CYP3A7.2 protein expressed in *E. coli*, *S. cerevisae* and HEK293 cells with reference to that of CYP3A7.1. CYP3A7 was expressed in *E. coli*-Rosetta, *S. cerevisae*- W(R) and human HEK293 cells. The expression of CYP3A7.2 was immunoquantified as described in Materials and Methods, and compared to that obtained for CYP3A7.1 in parallel preparations. The amount of CYP3A7.2 protein was expressed relatively to the amount of CYP3A7.1 protein. These results were obtained from four independent experiments in *E. coli* and HEK293 cells and five experiments in *S. cerevisae* cells and are expressed as the mean ± SD. In the lower panel the amounts of CYP3A7.1 versus the total protein in the preparations are recorded (pmol P450 μg^{-1} protein). CYP3A7.2 expression was significantly different from CYP3A7.1 expression in *E. coli* and *S. cerevisae* cells (Mann-Whitney test, $*P<0.05$).

Fig 2. CO difference spectra of heterologously expressed CYP3A7.1 and CYP3A7.2. The shown spectra was obtained in *E. coli*-BL21(DE3) strain, basically as described in Materials and Methods. Bacteria was transformed with expression vectors for CYP3A7.1, CYP3A7.2 or empty control vector and grown at 30ºC. After inducing CYP expression with IPTG for 24 h, membranes were isolated and used to determine the reduced CO difference spectra at 400-500 nm. Between 1 and 1.5 mg of membrane protein were used for the spectra.

Fig 3. CYP3A7 and CYP3A5 protein levels in human fetal livers. Proteins were separated by electrophoresis, transferred to a nitrocellulose membrane and the CYP3A7 and CYP3A5 enzymes detected with antibodies recognizing CYP3A4-7 (A234-Gentest) and CYP3A5 (A235-Gentest). Forty µg of 10,000 x g supernatant protein from 7 different human fetal livers together with yeast-expressed CYP3A7 and CYP3A5, were used for electrophoresis, blotting and detection of

CYP3A7 (A) and CYP3A5 (B) proteins. *CYP3A7* and *CYP3A5* genotypes of the livers are indicated. The amount of CYP3A7 (C) and CYP3A5 (D) were quantified in microsomal preparations from 4 fetal livers using suitable standards, as described in Materials and Methods. At least two independent Western blotting experiments were carried out. The values shown in the figure are the mean of at least two independent experiments. Means ± SD: (C) 218 ± 34, 434 ± 70, 518 ± 41 and 425 ± 13 pmol CYP3A7 mg^{-1} microsomal protein for FL37, FL55, FL61 and FL65, respectively; (D) 45 ± 5 and 44 ± 2 pmol CYP3A5 mg^{-1} microsomal protein for FL61 and FL65, respectively.

Fig 4. Kinetic analysis of DHEA hydroxylase activity for the CYP3A7.2 enzyme versus the wild-type CYP3A7.1. The two proteins were expressed in HEK293 cells as described in Materials and Methods and DHEA hydroxylase activity was measured at substrate concentrations varying from 0.2 to 100 µM. Open circles correspond to CYP3A7.2 and the closed circles to CYP3A7.1. The values shown are the mean of two independent HEK293 transfections and microsomal preparations. K_m and k_{cat} for the two enzymes were calculated by non-linear fitting. K_m = 2.6 ± 0.6 µM and k_{cat} = 0.035 ± 0.002 s^{-1} for CYP3A7.1 and K_m = 3.1 ± 0.5 µM and k_{cat} = 0.052 ± 0.002 s^{-1} for CYP3A7.2.

Fig. 5. Rate of DHEA hydroxylation, Luciferin BE debenzylation, and alprazolam 4- and 1-hydroxylation by CYP3A7.1 or CYP3A7.2 from human fetal livers. Human fetal liver microsomes were prepared from genotyped livers that belonged to haplotypes *CYP3A7*1/CYP3A5*3* (FL55) and *CYP3A7*2/CYP3A5*1* (FL61 and FL65) and incubated with 50 µM of DHEA (A) 150 µM of Luciferin BE (B) or 500 µM alprazolam (C and D), as described in Materials and Methods. Each column represents the mean ± SD of 4 (DHEA and Luciferin BE)

and 3 (alprazolam) independent experiments. Statistical differences in metabolization rate were analysed by means of paired Student's *t*-test * P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, FL55 compared to FL61 and FL65.

Table I

Allele frequencies of *CYP3A7*2* and *CYP3A5*1* in different ethnic groups and linkage disequilibrium between them. The frequencies of the haplotypes are given for each population.

Ethnic Group	**No. Chromosomes Analysed**	**Allele Freq.** [a] ***CYP3A7*2***	**Allele Freq. *CYP3A5*1***	**Freq. *CYP3A7*1/ CYP3A5*3***	**Freq. *CYP3A7*1/ CYP3A5*1***	**Freq. *CYP3A7*2/ CYP3A5*3***	**Freq. *CYP3A7*2/ CYP3A5*1***
Caucasians - Spanish	202	0.08	0.09	0.89	0.03	0.02	0.06
- Swedish	94	0.08	0.07	0.92	0	0.01	0.07
Chinese	192	0.28	0.27	0.72	0	0.01	0.27
Saudi Arabians	140	0.17	0.09	0.80	0.03	0.12	0.05
Africans - Tanzania	180	0.62	0.79	0.20	0.17	0.01	0.62

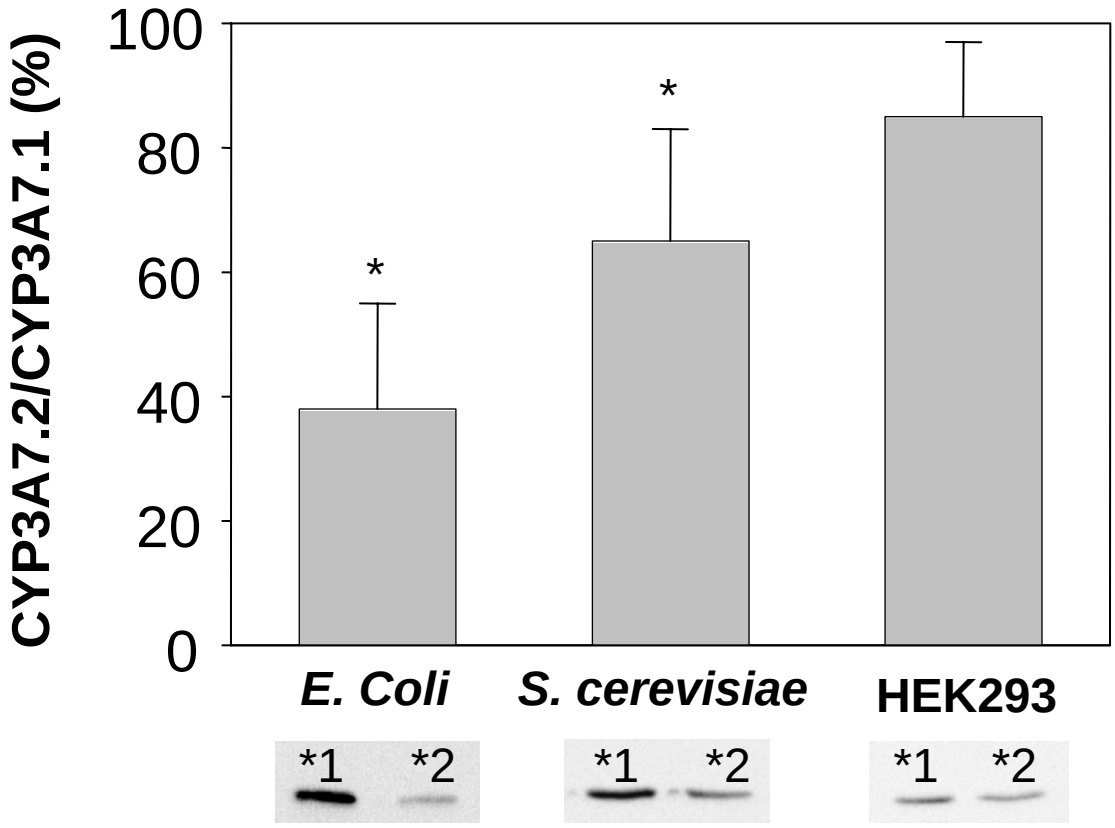
[a]The frequency of the allele was calculated using the formula: frequency = [2*(number of people homozygous for the allele) + (number of heterozygous people)]/ [2*(total number of people)]. *CYP3A7*1* GenBank accession number: NM_000765.2.

Table II

CYP3A7 and *CYP3A5* genotypes of the fetal livers investigated.

Fetal Liver	**Gestational Age (weeks)**	***CYP3A7* Genotype**	***CYP3A5* Genotype**
FLU	Unknown	*1/*1	*3/*3
FL24	15	*1/*1	*3/*3
FL26	15	*1/*1	*3/*3
FL37	9.5	*1/*1	*3/*3
FL55	12	*1/*1	*3/*3
FL61	14	*2/*2	*1/*1
FL65	21	*2/*2	*1/*1

Figure 1



CYP3A7.1 pmols/µg prot	1.82	0.07	0.04

Figure 2

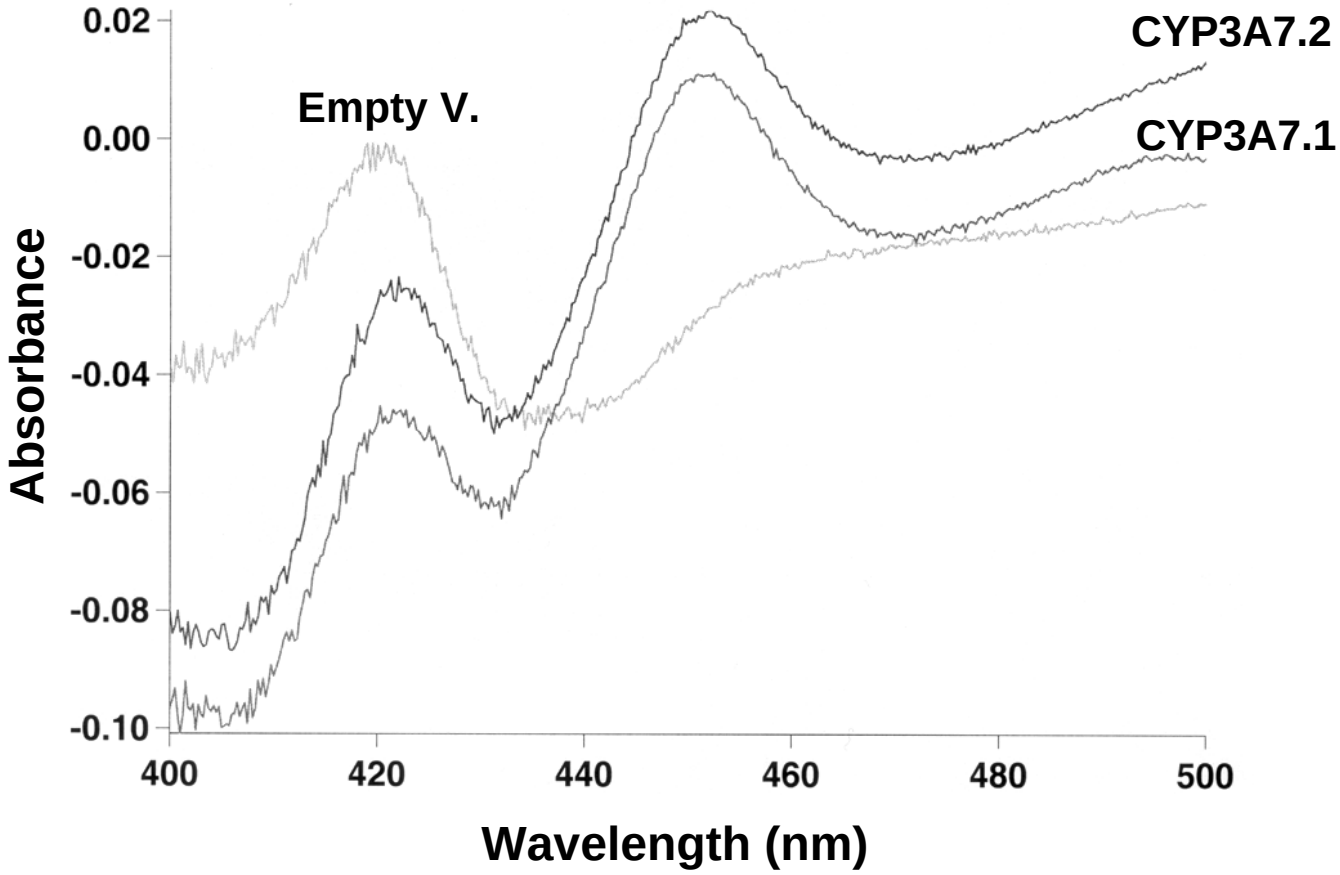

0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
Absorbance
400
420
440
460
480
500
Wavelength (nm)
Empty V.
CYP3A7.2
CYP3A7.1

Figure 3

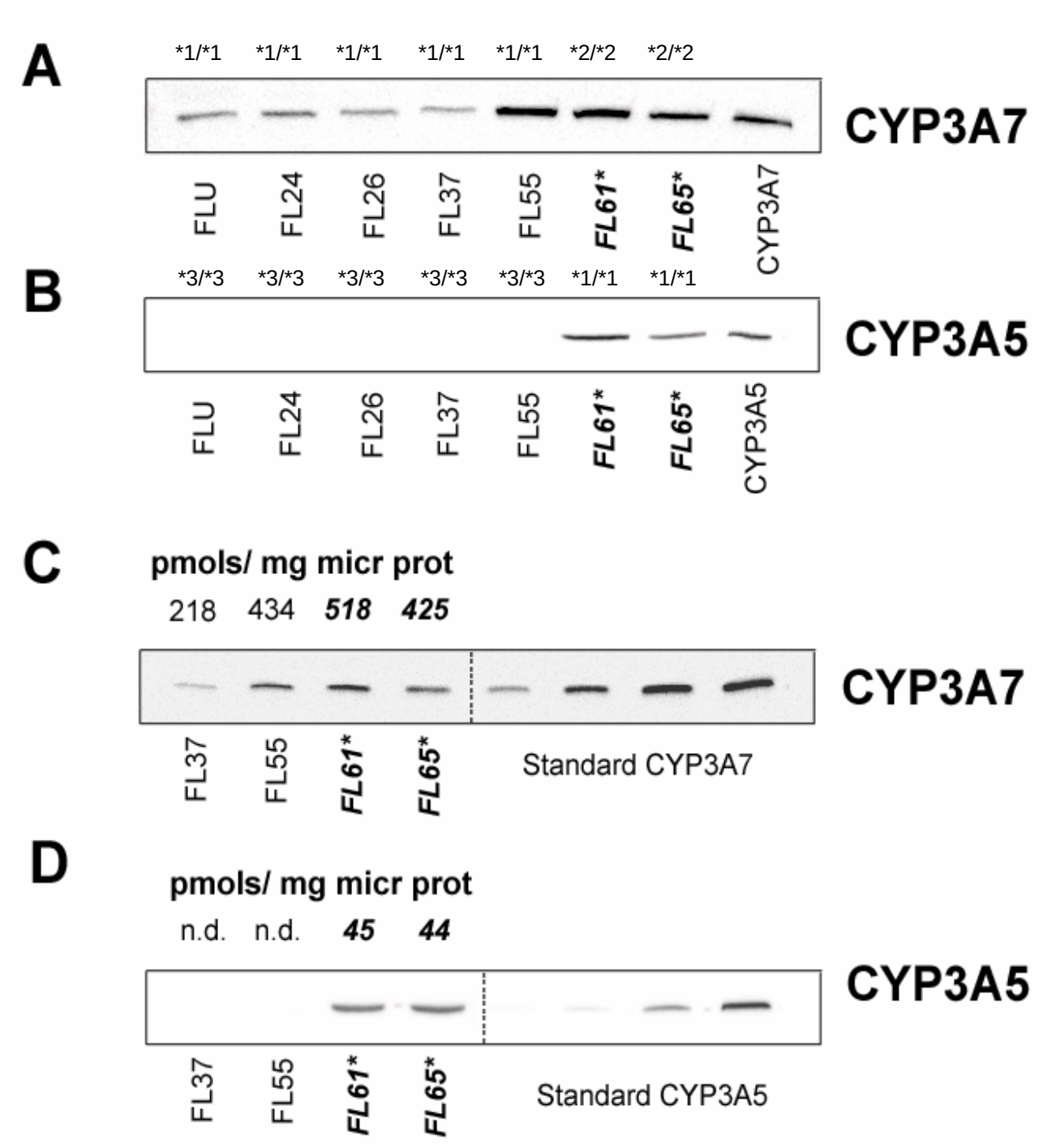

A
*1/*1 *1/*1 *1/*1 *1/*1 *1/*1 *2/*2 *2/*2
CYP3A7
FLU FL24 FL26 FL37 FL55 FL61* FL65* CYP3A7
B
*3/*3 *3/*3 *3/*3 *3/*3 *3/*3 *1/*1 *1/*1
CYP3A5
FLU FL24 FL26 FL37 FL55 FL61* FL65* CYP3A5
C
pmols/ mg micr prot
218 434 518 425
CYP3A7
FL37 FL55 FL61* FL65* Standard CYP3A7
D
pmols/ mg micr prot
n.d. n.d. 45 44
CYP3A5
FL37 FL55 FL61* FL65* Standard CYP3A5

Figure 4

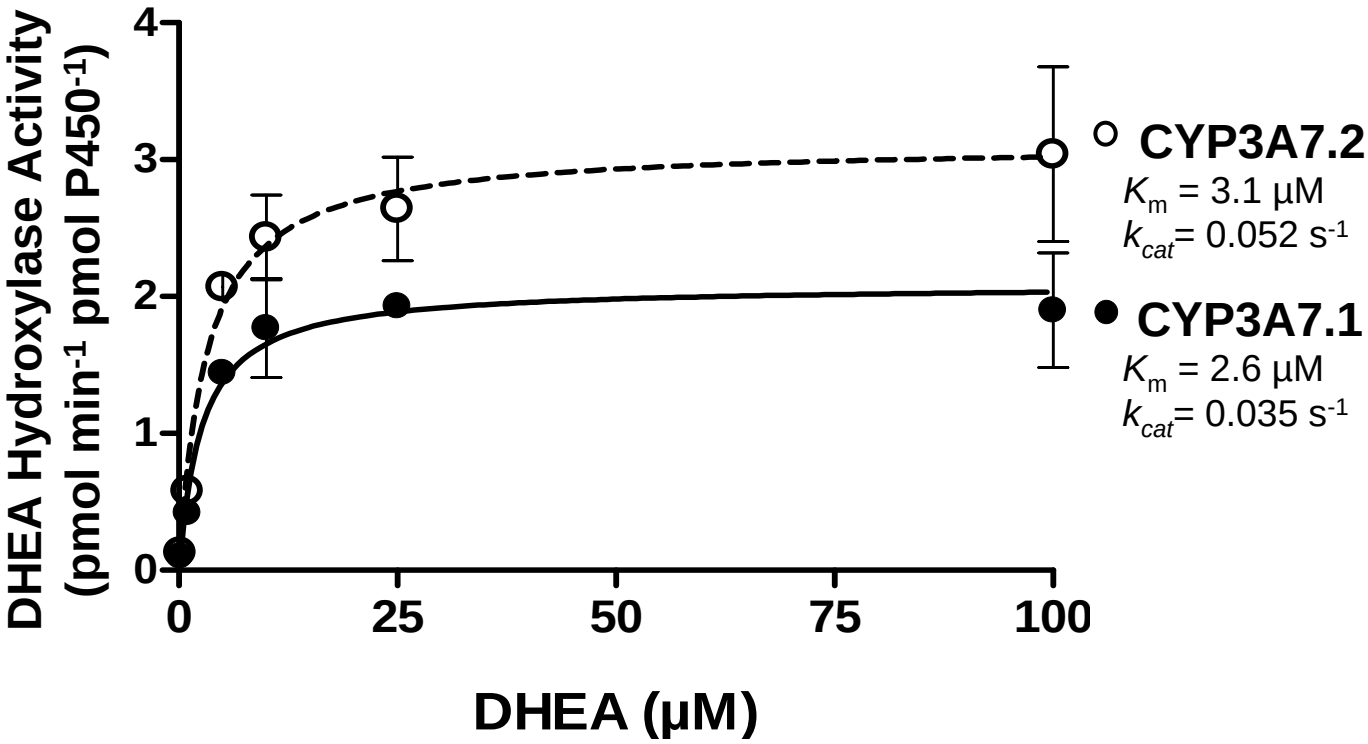
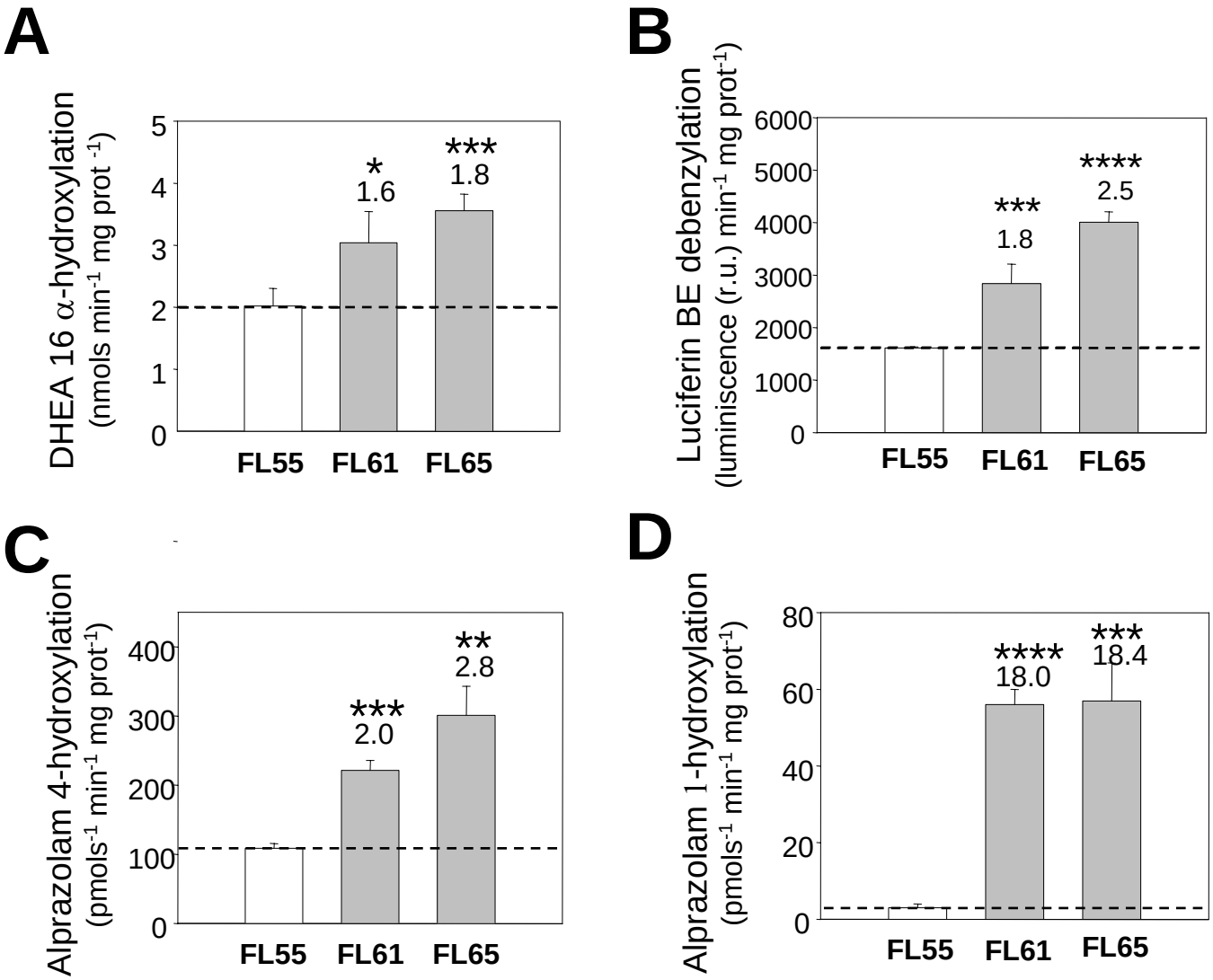

DHEA Hydroxylase Activity
(pmol min-1 pmol P450-1)
4
3
2
1
0
0
25
50
75
100
DHEA (μM)
CYP3A7.2
Km = 3.1 μM
kcat= 0.052 s-1
CYP3A7.1
Km = 2.6 μM
kcat= 0.035 s-1

Figure 5


A
DHEA 16 α-hydroxylation
(nmols min-1 mg prot -1)
5
4
3
2
1
0
*
1.6
***
1.8
FL55
FL61
FL65
B
Luciferin BE debenzylation
(luminiscence (r.u.) min-1 mg prot-1)
6000
5000
4000
3000
2000
1000
0
***
1.8
****
2.5
FL55
FL61
FL65
C
Alprazolam 4-hydroxylation
(pmols-1 min-1 mg prot-1)
400
300
200
100
0
***
2.0
**
2.8
FL55
FL61
FL65
D
Alprazolam 1-hydroxylation
(pmols-1 min-1 mg prot-1)
80
60
40
20
0
****
18.0
***
18.4
FL55
FL61
FL65