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Multiple Novel Nonsynonymous *CYP2B6* Gene Polymorphisms in Caucasians: Demonstration of Phenotypic Null Alleles

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Summary

The human microsomal cytochrome P450, CYP2B6, is involved in the biotransformation of several clinically important drugs. By complete sequence analysis of the human CYP2B6 gene coding regions in selected Caucasian DNA samples we identified the five novel missense mutations 62A>T (Q21L in exon 1), 136A>G (M46V in exon 1), 12820G>A (G99E in exon 2), 13076G>A (R140Q in exon 3), and 21388T>A (I391N in exon 8). The recently described but functionally uncharacterized variant 13072A>G (K139E) was also observed. Haplotype analysis indicated the presence of at least six novel alleles which code for the protein variants CYP2B6.10 (Q21L, R22C), CYP2B6.11 (M46V), CYP 2B6.12 (G99E), CYP2B6.13 (K139E, Q172H, K262R), CYP2B6.14 (R140Q), and CYP2B6.15 (I391N). Heterologous expression in COS-1 cells revealed comparable levels of CYP2B6 apoprotein and bupropion hydroxylase activity for CYP2B6.1 (wild type) and CYP2B6.10, whereas all other variants exhibited reduced expression and/or function. The three amino acid changes M46V, G99E, and I391N resulted in almost unmeasurable (M46V) or undetectable (G99E, I391N) enzyme activity, despite the presence of residual protein. The K139E change lead to completely abolished protein expression and consequently no function was detected. Expression in insect cells by recombinant baculoviruses confirmed these results and demonstrated the virtual absence of incorporated heme in these protein variants. The collective allele frequency of the four very low or null activity variants M46V, G99E, K139E, and I391N was 2.6 % in a Caucasian study population. These data provide further insight into the genetic variability of *CYP2B6* and demonstrate the existence of phenotypic null alleles in this gene.

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Introduction

The Cytochromes P450 are a multigene family of heme-thiolate monooxygenases which catalyse the oxidative biotransformation of a wide variety of xenobiotics and endobiotics (Nelson et al., 1996). In humans, about ten isozymes of families CYP1, CYP2 and CYP3 catalyze 70 to 80% of the entire phase I drug metabolism (Ingelman-Sundberg 2004). Liver expression levels and function of most of these P450s are extremely variable both inter- and intraindividually, thereby leading to differences in drug biotransformation, a major cause for unpredictable drug effects (Daly 2003). CYP2B6 is the orthologous human form to the rodent phenobarbital-inducible P450s and is inducible by phenobarbital and several other drugs, involving the key transcription factors CAR and PXR (Wang and Negishi 2003). In liver the enzyme makes a contribution of about three to five percent to the total microsomal P450 pool (Gervot et al., 1999; Lang et al., 2001). At lower levels it is also found in extrahepatic tissues including intestine, kidney, lung (Gervot et al., 1999), skin (Yengi et al., 2003) and the brain (Miksys et al., 2003). Clinically used drug substrates of CYP2B6 include the prodrug cyclophosphamide (Roy et al., 1999), the antimalarial artemisinine (Svensson and Ashton 1999), the anaesthetics ketamine (Yanagihara et al., 2001) and propofol (Court et al., 2001), and the HIV-1 reverse transcriptase inhibitors nevirapine and efavirenz (Erickson et al., 1999; Ward et al., 2003). Furthermore, the antidepressant and anti-smoking agent bupropion is one of the most specific substrates of CYP2B6 and is therefore a preferred *in vitro* probe drug for the enzyme (Hesse et al., 2000; Faucette et al., 2000). Additional substrates known to be in part metabolized by CYP2B6 include stimulants like ecstasy (MDMA; Kreth et al., 2000) and nicotine (Yamazaki et al., 1999), the antioestrogen tamoxifen (Crewe et al., 2002; Coller et al., 2002), and others (Ekins and Wrighton, 1999). Recently, several highly potent and specific inhibitors have been described, including the anticancer agent thioTEPA (Rae et al.,

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2002), 17-α-ethynylestradiol (Kent et al., 2002), and the thrombolytic, clopidogrel (Richter et al., 2003).

The first systematic search for sequence polymorphisms in the *CYP2B6* gene, which is located together with the pseudogene *CYP2B7P* on chromosome 19q13.2, resulted in the identification of nine single nucleotide polymorphisms, five of which cause amino acid substitutions (Lang et al., 2001). In Caucasians, these SNPs were found at variant frequencies of up to 30% whereas in Japanese they were generally less frequent (Hiratsuka et al., 2002). Phenotype-genotype correlation analysis in human liver samples showed that a 1459C>T change (R487C) in exon 9 was associated with an 8-fold lower mean protein expression level in Cys487 homozygotes compared to wild type, whereas the other variants were associated with more moderate changes in expression and function (Lang et al., 2001). In contrast, a Q172H variant, more frequently found among Japanese compared to Caucasians, had increased 7-ethoxycoumarin O-deethylase activity *in vitro* (Ariyoshi et al., 2001). The related variant CYP2B6.6 (Q172H, K262R) was found to be associated with increased 4-hydroxylation of cyclophosphamide in human liver (Chang et al., 1993) and variants with the K262R change had in common an increased V_{max} and V_{max}/K_m with 7-ethoxy-4-trifluoromethylcoumarin as substrate (Jinno et al., 2003). Numerous additional SNPs were recently reported within the 5'-flanking sequence and within introns, including a 15631G>T change that appears to trigger formation of a nonfunctional splice variant, and a novel amino acid variant (K139E; allele **8*) not further characterized (Lamba et al., 2003). Clinical studies have so far been scarce. It was suggested that smokers with the 1459C>T (R487C) variant may be more vulnerable to abstinence symptoms and relapse following treatment with bupropion as a smoking cessation agent (Lerman et al., 2002). In a recent *in vivo* study on bupropion pharmacokinetics, increased total clearance via allele **4* (K262R) was found

whereas other alleles were not different from wild type (Kirchheiner et al., 2003). It should be noted, however, that no homozygotes for the low-expressor variant Cys487 participated in that study.

Taken together, these findings indicate that *CYP2B6* polymorphisms can influence expression and function of this enzyme, but only moderate effects on metabolism, pharmacokinetics and therapeutic efficacy of drugs can be expected for the presently known polymorphisms. Remarkably, in all previous studies, no null alleles, resulting in completely nonfunctional gene product, have been found. In this study, we have continued our efforts to identify *CYP2B6* variant alleles. Here we describe six novel alleles with nonsynonymous amino acid changes. Detailed assessment of the functional properties of the corresponding recombinant enzymes allowed us to characterize some of these variants as phenotypic null mutants.

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Materials and methods

Chemicals and general methods

Cell culture media, supplements and other products for the cultivation of insect cells and COS-1 cells were received from Gibco Invitrogen (Karlsruhe, Germany). *Spodoptera frugiperda* (*Sf9*) cells were obtained from American Type Culture Collection (Rockville, MD,USA) and *Trichoplusia ni* 5B1-4 (*Tn5*) from Invitrogen Corp (San Diego, CA, USA). Oligonucleotides were from MWG Biotech AG (Ebersberg, Germany). Restriction enzymes were purchased at Roche Applied Sciences (Mannheim, Germany) or New England Biolabs GmbH (Frankfurt, Germany). T4 DNA-ligase was from Roche, Thermoscript RT-PCR System from Gibco Invitrogen (Karlsruhe, Germany), Taq polymerase from Qiagen (Hilden, Germany) and PCR dNTPs from Eppendorf (Wesseling-Berzdorf, Germany). Heme and EDTA were obtained from Fluka (Sigma-Aldrich Laborchemikalien, Seelze, Germany). All other reagents and solvents were obtained from commercial sources in analytical grade. DNA concentrations were determined with the GeneQuant photometer (Amersham Biosciences Europe, Freiburg, Germany). Protein concentrations were determined using the BioRad protein assay (BioRad Laboratories GmbH, Munich, Germany) with bovine serum albumine as standard.

DNA and liver samples

Anonymized blood samples were obtained from healthy individuals of German origin, based upon medical history, clinical investigations and routine laboratory parameters, who had been selected for participating in phase-1 clinical trials. All individuals gave written informed consent to genetic testing. Leukocyte DNA was isolated from blood samples by standard methods using the Qiamp system (Qiagen, Hilden, Germany) on a Qiagen 9604 robot (Qiagen DNA Blood Midi Prep). Human liver tissue was obtained as nontumourous tissue surrounding

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surgically removed liver tumours or metastases or material surgically resected for other reasons and liver microsomes were prepared as described (Lang et al. 2001). The use of liver samples was approved by the ethics committees of the Medical Faculties of the Charité, Humboldt-University Berlin, and of the University of Tübingen, and written informed consent was obtained from each patient.

PCR Amplifications and DNA sequencing

Specific intronic PCR primer pairs used in the *CYP2B6* mutation screen were designed using *CYP2B6* genomic sequences retrieved from GenBank (AC023172.1). Exon-intron boundaries of the *CYP2B6* gene were defined by comparing genomic sequences with the mRNA sequence (M29874.1; Yamano et al. 1989). PCR reactions were conducted in a reaction volume of 50 µl with 20-30 ng of genomic DNA, 10x PCR buffer (Qiagen, Hilden, Germany), 200 µM dNTPs, 10 pmol of each primer and 1unit Taq polymerase (Qiagen, Hilden, Germany). PCR fragments were generated in a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: initial denaturation step of 2 min at 94°C followed by 34 cycles of denaturation 94°C for 45 sec, annealing for 45 sec at 62°C and extending for 1 min at 72°C. The initial denaturation temperature for exons 2 and 7 was 96°C for 3 min, followed by 34 cycles of denaturation at 96°C for 45sec, annealing at 62°C for 45sec, and extension for 1 min at 72°C. Informations about oligonucleotide primers used for PCR are listed in table 1. Amplicons were purified by using QiaQuick 96 PCR purification kits (Qiagen, Hilden, Germany) on a Qiagen 9604 robot and directly sequenced on PE ABI 3700 DNA Analysers (ABI, Weiterstadt, Germany) using BigDye RR Terminator Cycle Sequencing kit (Applied Biosystems). Sequence analyses were performed using the PHRED/PHRAPPOLYPHRED/consed software package (University of Washington, Seattle, WA,USA) and inspected for deviations from the wild type sequence (AC023172.1).

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Construction of Expression Vectors

Wild-type CYP2B6 cDNA was obtained by reverse transcription of total RNA of a previously genotyped human liver sample followed by a two step amplification using first specific CYP2B6 primers (CYP2B6_cFE1, CYP2B6_cRE9; Table1) and then nested primers to introduce a *Bam*HI site in front of the start codon (CYP2B6_cF1Bam) and an *Xba*I site at the 3'-end (CYP2B6_cR9Xba). After cloning into pGEMTeasy (Promega, Mannheim, Germany) the sequence was completely confirmed. The cDNA was cloned into pFASTBac1 (BAC-TO-BAC® Baculovirus Expression System, Gibco Invitrogen, Karlsruhe, Germany) resulting in plasmid pIKAT15. Mutations were introduced in pIKAT15 using appropriate oligonucleotides (Table 2) and the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). To construct the *10 variant, a fragment spanning exon 1 to 4 was directly amplified from cDNA of liver L006 (primer CYP2B6_cF1Bam and CYP2B6_cRE4, Table 1), cloned into pGEMTeasy vector, cut with *Bam*HI and *Sal*I and combined with the wild type CYP2B6 *Sal*I to *Xba*I fragment of pIKAT15 in pCMV4 vector. All mutants were confirmed by sequencing the complete cDNA region using IRD800 labelled oligonucleotides (Table 1). *E.coli* MAX Efficiency® DH10BAC™ competent cells (Invitrogen Life Technologies, Karlsruhe, Germany) were transformed with the pFASTBAC1 derived vectors to produce bacmid-DNA by transposition for 5h at 37°C. Bacmid DNA was prepared and stored at -20°C in aliquots until use. Bacmid preparations were controlled on a 0.6 % agarose gel and by a *CYP2B6* specific PCR reaction. For expression in COS-1 cells, a 1.4 kb *Bam*HI/*Xba*I fragment containing the CYP2B6 cDNA from pIKAT15 and variant derivatives were ligated into *Bgl*II/*Xba*I-digested pCMV4 expression vector and cloned in *E.coli* One Shot TOP10F' competent cells (Invitrogen Life Technologies, Karlsruhe,Germany) selected on LB

agarplates with ampicillin (100µg/ml). DNA for transfection experiments was isolated from liquid cultures (100 ml) using Midi-Plasmid Isolation Kit (Qiagen, Hilden, Germany).
For haplotype determination in samples L006 and L135, PCR-fragments of cDNA spanning exons 1 to 4 (~600 bp; primer CYP2B6_cF1Bam and CYP2B6_cRE4, Table 1) were cloned in pGEMTeasy-vector and about 20 individual clones were completely sequenced in both directions using IRD800 labelled M13 universal and reverse primers.

Expression in COS-1 cells

COS-1 cells were cultivated in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum and penicillin/streptomycin at 37°C under 5 % CO_2 atmosphere. On the day before transfection, $3*10^4$ COS-1 cells per cm² were seeded into 100 mm dishes. Lipofectamine plus or Lipofectamine 2000 (Invitrogen Life Technologies, Karlsruhe, Germany) was used to transfect the pCMV4 derivatives according to the instructions of the manufacturer. The cells were incubated for 45 h at 37°C, washed once with cold PBS and then scraped off the plate. The cells were centrifuged and resuspended in 0.1M potassium phosphate buffer (pH 7.4) containing 250 mM sucrose. For the isolation of microsomal protein the cells were disrupted using Lysing Matrix D and a FastPrep System (both: Q-Biogene, Heidelberg, Germany) two times level 6 for 10 sec and separated by differential centrifugation steps at 10,000 *g* and 100,000 *g* in a TLA 55 rotor (Beckman Optima Max-E ultracentrifuge, Beckmann Coulter GmbH, Krefeld, Germany). Microsomes were resuspended in 0.1 M postassium phosphate (pH7.4) supplemented with 10% glycerol. Transfection efficiency was determined by cotransfection with pCMVβ control plasmid (Clontech BD Biosciences, Heidelberg, Germany). Relative activity of ß-galactosidase was determined using Galacto-Light™ (Tropix Inc., PE Biosystems, Massachusetts) on a Auto-Lumat plus (Berthold technologies, Bad Wildbad, Germany).

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Insect cell culture, infection, and preparation of Tn5 membranes

Serum-free cultivated Sf9 cells were transfected using Cellfectin reagent according to the instruction manual (BAC-TO-BAC® Baculovirus Expression System, Gibco Invitrogen, Karlsruhe, Germany). Baculoviruses were amplified for several rounds resulting in a titer of $\approx 10^7$ pfu/ml determined via plaque assay. For expression, Tn5 cells were infected using a MOI of 5 ($5*10^8$ pfu / 10^8 cells) in SF900 medium containing hemin (8 µM) and cultivated in a rotary shaker water bath (Infors GmbH, Einsbach, Germany) at 27°C for 3 days at 100 rpm. The infection process was monitored by counting the cells and recording their size distribution using a cell counter (Casy 1, Schärfe System GmbH, Reutlingen, Germany). The cells were harvested and microsomes were prepared using ultrasonic disruption with 50% duty cycle, 3 times for 5 sec (Bandelin Sonopuls HD200 / KE76, Bandelin electronic, Berlin, Germany) in 10 mM Tris/Cl (pH 7.5), 10 mM NaCl, and 0.5 mM Pefabloc protease inhibitor (Roth, Karlsruhe, Germany) followed by differential centrifugation steps at 10,000 g and 100,000 g. The microsomal pellet was finally homogenized in 3 ml of 0.1 M sodium phosphate buffer (pH 7.4) and 20 % glycerol (w/v) per 10^8 cells using a dounce homogenizator (S) and frozen in aliquots at -80°C. The content of CYP2B6 holoprotein in the microsomal fractions was quantitated by recording reduced CO-difference spectra according to standard methodology.

Immunoblotting

CYP2B6 apoprotein was quantified using Western blotting with recombinant CYP2B6 lymphoblast microsomes (BD Gentest Corp., Woburn, MA, USA) as standard protein. Samples were separated on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. The transfer was controlled by Ponceau S staining. Immunodetection of CYP2B6

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was done with human CYP2B6-selective monoclonal antibody mab2B6 (BD Gentest Corp., Woburn, MA, USA). Bound antibody was quantified using peroxidase-conjugated goat secondary anti-mouse antibody (Oncogene Merck Biosciences, Bad Soden, Germany). The baculovirus gp64 envelope protein was detected using anti-baculovirus envelope gp64 protein (eBioscience, THP Medical Products Vertriebs GmbH, Wien, Austria)
Immunoblots were developed using Supersignal WestDura chemiluminescence substrate (Pierce, St.Augustin, Germany). Chemoluminescence was measured with a Fuji LAS-1000 CCD-camera (Raytest, Straubenhardt, Germany) and results were analysed using AIDA 2.31 software (Raytest).

Enzyme assay and kinetic analysis of CYP2B6 variants

Bupropion hydroxylase activity was determined in COS-1 cell extracts essentially as recently described for liver microsomes (Richter et al 2004). In brief, 50 µg of protein was incubated in a final volume of 0.25 ml. After equilibrating the reaction mixture at 37°C for 3 min, the reactions were started by adding 25 µl of 10-fold concentrated NADPH-regenerating system (final concentrations, 5 mM $MgCl_2$, 4 mM glucose 6-phosphate, 0.5 mM $NADP^+$ and 4 U/ml glucose 6-phosphate dehydrogenase) and terminated after 15 min at 37°C. Reconstitution of insect cell-expressed CYP2B6 was performed by preincubation of cell membranes corresponding to 5 pmol spectrally quantitated P450 together with 5 pmol of purified rat NADPH:cytochrome P450 oxidoreductase in 0.25 ml of 0.1 M sodium phosphate buffer, pH 7.4, at 37°C for 10 min. In the case of spectrally inactive variants, 125 µg of protein were used per assay. The reactions were then started and terminated as described above. Substrate concentration was generally 1 mM except for Michaelis-Menten kinetics in COS-1 cells, where a concentration range between 1 and 1600 µM was used. Hydroxybupropion was determined by LC-MS as described (Richter et al 2004). In the case of extremely low

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activities, background levels of product contaminating the substrate were subtracted. All incubations were performed in duplicate. Enzyme kinetic data were analyzed using the program GraphPadPrism v3.0 (GraphPad Software Inc., San Diego, CA).

Nomenclature, statistics, and structural analyses

Allele designations were made in accordance with the Human CYPallele Nomenclature Committee (web-site http://www.imm.ki.se/CYPalleles/criteria.htm). Numbering was based on the cDNA with the full length cDNA sequence published by Yamano *et al.* (1989) defined as the wild-type (*CYP2B6*1*). Genomic sequence numbers are available at the CYPallele nomenclature web-site. Statistical analysis of pair comparisons was performed using nonparametric methods (Mann-Whitney *U*-test) due to low sample numbers and therefore unknown data distribution. Multiple comparisons were performed by one-way analysis of variance and post-hoc testing using the program GraphPad InStat v3.00 (GraphPad Software Inc., San Diego, CA). All statistical tests were performed two-tailed and statistical significance was defined as $P<0.05$. Protein sequence alignment (accession number humanCYP2B6: P20813) was done using AlignX (Vector NTI Suite 7, InforMax, USA). Prediction of tolerability of amino acid exchanges at all positions was calculated using the SIFT software (http://blocks/fhrc.org/sift/SIFT.html; Ng & Henikoff, 2003). Alignments at mutated positons were done using the PolyPhen tool (http://www.bork.embl-heidelberg.de/PolyPhen; Ramensky et al., 2002). Secondary structure prediction was performed using SSpro (http://www.igb.uci.edu/tools/scratch/; Pollastri et al., 2002) and substrate recognition sites were taken from to Domanski et al. (1999) and Williams et al (2000).

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Results

Mutation screening

Complete sequence analysis of the CYP2B6 gene in all nine coding exons and their exon/intron boundaries in a selection of 12 DNA samples from previously phenotyped and genotyped human livers and in 55 samples from a random Caucasian population revealed the five novel missense mutations, 62A>T (Q21L in exon 1), 136A>G (M46V in exon 1), 12820G>A (G99E in exon 2), 13076G>A (R140Q in exon 3), and 21388T>A (I391N in exon 8). In addition, we detected the recently described variation 13072A>G (K139E, CYP2B6*8; Lamba et al., 2003). Furthermore, 13 novel intronic sequence variations and one within the 3' untranslated region were observed (Table 3). Individual frequencies were calculated for each variant position based on data obtained from the sequenced samples and an additional 189 liver DNA samples which were screened for the novel variants through exons 1, 2, 3, and 8. The nonsynonymous mutations were observed only in heterozygous form at frequencies of up to about 1%. Thus, according to general understanding, I391N in exon 8 and K139E in exon 3 can be considered as SNPs with a frequency of ~1%, whereas the others should be regarded as rare variants. It is however possible that some of these variants are more frequent in other ethnic groups. The five missense and four silent exonic mutations described previously (Lang et al. 2001) were also found and confirmed to be present at frequencies similar to those reported earlier (data not shown).

Haplotype analysis

Figure 1 shows the haplotypes of the 12 completely sequenced liver samples and of four Caucasian samples with novel amino acid changes. Analysis of the sequence constellations allowed the definition of new distinct alleles with one, two or three amino acid changes (Table 4). The 62A>T change (Q21L) was found in two samples also heterozygous for R22C

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and the silent SNP 12740G>C (Lang et al 2001). To clarify whether the two amino acid changes occur on the same allele, we amplified a cDNA fragment from L006 RNA by RT-PCR. Sequence analysis of subcloned PCR products revealed only triple mutants, thus defining allele *CYP2B6*10* as shown in Table 4. The change 136A>G (M46V), also located in exon 1, was observed in three different constellations (samples B15, L 135, and L180; Fig. 1). Using RNA of samples L 135 we investigated whether M46V was linked to R22C. Because sequence analysis of subcloned PCR fragments revealed only single mutants, M46V alone defines the novel allele *CYP2B6*11*. The large intron between exon 1 and exon 2 precluded a molecular haplotype analysis of samples B15 and L180, which had additional amino acid changes in exons 4+5 and 9, respectively (Fig. 1). Two variant forms **11A* and **11B* could be further distinguished based on the intron 5 SNP 18273G>A. The nonconservative change 12820G>A (G99E) in exon 2 was observed in one sequenced liver sample heterozygous for R22C and homozygous for 18273G>A in intron 5. We tentatively designated *CYP2B6*12* as the allele without R22C. Mutant 13072A>G (K139E), recently reported as single amino acid change in *CYP2B6*8* (Lamba et al., 2003) was found in heterozygous form in four samples (Fig. 1). Three of these were also homozygous for Q172H and K262R and for two SNPs in introns 5 and 8, thus defining the novel allele *CYP2B6*13A*. Allele *CYP2B6*13B* is characterized by the additional intron 3 SNP 15582C>T (Table 4). Another amino acid change in exon 3, R140Q (13076G>A), was found in heterozygous constellation together with R22C, Q172H, and K262R and with homozygous 18273G>A. Because molecular haplotyping could not be performed, definition of allele *CYP2B6*14* also remains tentative. Finally, the exon 8 mutation I391N (21388T>A) was observed five times heterozygously. Four samples were homozygous wild type at all polymorphic amino acid positions, thus clearly establishing *CYP2B6*15* as novel allele with the single amino acid exchange I391N. With respect to intronic changes, the two variants **15A* and **15B* could be

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distinguished (Fig. 1 and Table 4). With these novel allele definitions (Table 4), all but one of the 16 samples of figure 1 could be assigned a consistent genotype. Figure 1 contains further informations on silent and intronic sequence changes which could not be assigned to particular alleles with certainty.

Recombinant Expression

To study the effects of these mutations on expression and function of CYP2B6 we generated mutant *CYP2B6* cDNAs by site directe mutagenesis and expressed them in two different heterologous expression systems, transiently transfected mammalian COS-1 cells and insect cells. Compared to the wild type cDNA, which expressed on average 40 pmoles of CYP2B6 apoptotein per mg of microsomal COS-1 cell protein, variants CYP2B6.10 and CYP2B6.14 were expressed at slightly higher and lower levels, respectively. These differences were however not significant. By contrast, variants CYP2B6.11, CYP2B6.12, and CYP2B6.15 expressed much less protein than the wild type and variant CYP2B6.8 was completely undetectable (Fig. 2, Table 5). These results were consistently obtained in at least three independent experiments for each variant. Measurement of bupropion hydroxylase activity in COS-1 cell microsomes revealed values of 65 µM for Km and 215 pmol/mg/min for Vmax of the the wild type protein, in good agreement with previous reports (Hesse et al., 2000; Faucette et al., 2000). Whereas variant CYP2B6.10 had functional properties very similar to the wild type, CYP2B6.14 had about a two-fold increased Km and almost 80 % reduced Vmax, resulting in an intrinsic clearance (Vmax/Km) of 12.5% compared to wild type. No 1-hydroxylated product was detected with variant M46V up to 200 µM bupropion. At higher substrate concentrations, some product was formed, corresponding to less than 2 % of wild type activity, but these residual activities did not follow Michaelis-Menten kinetics and no

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kinetic parameters could thus be determined. Variants G99E, K139E and I391N had no detectable enzyme activity at all.

Expression in insect cells by recombinant baculoviruses was performed to further characterize P450 variants spectroscopically and functionally. At the apoprotein level, expression characteristics in Tn5 insect cells differed to some extent from that in COS-1 cells (Fig. 3A). Variants CYP2B6.11 and CYP2B6.12 were expressed at relatively higher levels than in COS-1 cells, although still at lower than wild type levels. No expression was observed for variant CYP2B6.15, in contrast to COS-1 cells, whereas variant CYP2B6.8 showed the same result of no expression in both expression systems. The observation that nonsynonymous amino acid changes lead to complete absence of protein expression was surprising. We therefore wanted to exclude the possibility that the recombinant baculoviruses had become non-infectious. For this purpose we analyzed expression of the baculovirus gp64 envelope protein by using a monoclonal antibody. As shown in Fig. 3A, gp64 was expressed at similar levels in all recombinants, demonstrating successful infection of insect cells. Enzyme activities determined with rat NADPH:P450 oxidoreductase-reconstituted insect cell membranes at a ratio of P450:oxidoreductase of 1:1 confirmed the results seen with COS-1 cells. Compared to the wild type, which produced 1-hydroxybupropion at a turnover of $2.73*min^{-1}$, variants M46V and R140Q had only 3 % and 18.0 % activity, respectively, whereas variants G99E, K139E and I391N were completely inactive (Fig. 3A). Analysis of the reduced CO-difference spectra (Fig. 3B) with membrane fractions of infected cells revealed that CYP2B6.14 (R140Q) was comparable to wild type. CYP2B6.11 (M46V) had drastically reduced heme content and an absorption maximum above 450 nm. It can however not be excluded that this residual spectral activity was due to background because it was similar to those of the nonexpressing variant CYP2B6.8 (K139E) and to control spectra recorded from uninfected

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cells cultured under identical conditions. The spectra of the expressed variants CYP2B6.12 (G99E) and CYP2B6.15 (I391N) were also not distinguishable from background.

Analysis of liver expression

The CYP2B6 variants described herein were only found in heterozygous condition in our collection of human liver samples (Fig. 1). Figure 4 shows the amount of CYP2B6 apoprotein determined by Western blotting in liver microsomes and the corresponding bupropion hydroxylase activities, in comparison to a subset of livers with wild type sequence at all polymorphic amino acid positions (N=28). Due to the low sample numbers in the individual genotype groups, we statistically compared the group of samples with one deficient allele (Fig. 4B) with the wild type group. The two samples with one **10* allele were omitted from this analysis, because CYP2B6.10 was functionally similar to the wild type. Collectively, both protein expression and enzyme activity were significantly different between the wild type and the mutant group. The mean expression level of the 10 mutant samples was 9.6 pmol apoprotein per mg, compared to 22.5 pmol/mg in the wild type subgroup (P=0.0023; Mann-Whitney test). Bupropion (50 µM) hydroxylase activity was even more reduced in the mutant group (68.3 versus 262.4 pmol/mg*min; P=0.0002), in agreement with the findings of low enzyme activities of these mutants in the recombinant system. Thus, although only present in heterozygous condition, there was a drastic effect of these mutations on expression and function of hepatic CYP2B6.

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Discussion

In this study we have identified five novel nonsynonymous variants of the human *CYP2B6* gene. By using site-directed mutagenesis and recombinant expression in mammalian COS-1 cells and in baculovirus-infected insect cells, we found that the two amino acid changes, M46V, G99E, and I391N, results in substantially decreased expression of the corresponding proteins with virtually undetectable enzyme activity. We also observed the previously reported K139E variant (Lamba et al., 2004) and showed that this mutation completely extinguishes the formation of any detectable CYP2B6 protein. The phenotypic data characterize at least the three variants G99E, K139E, and I391N as *bona fide* functional null mutations, i.e., if expression in liver is similar to that in the two heterologous expression systems used, these three variants (and presumably also M46V) will not be able to make a measurable contribution to in vivo metabolism. Furthermore, two additional novel nonsynonymous changes, Q21L and R140N, were shown to result in functionally normal and substantially but not completely deficient protein variants, respectively.

The most N-terminally observed amino acid changes Q21L and R22C were found to occur together on allele **10*. Neither recombinant expression nor analysis of liver microsomes indicated significant functional consequences of these amino acid changes (see also Lang et al., 2001; Jinno et al., 2003). Position 22 is assumed to be buried within the lipid bilayer or just emerging from it (Williams et al., 2000). Several positively charged amino acids found at the C-terminal end of the transmembrane domain of microsomal P450s are believed to function as a halt transfer signal (Sakaguchi et al., 1987). Both Gln21 and Arg22 are not well conserved (Fig. 5) and may thus not be very critical in this context, probably because of the redundancy of the signal which may also include Arg29 and Lys30.

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The 136A>G change (M46V) in exon 1 was observed with a frequency of 0.6 % (Table 3). In COS-1 cells, the corresponding protein variant CYP2B6.11 was expressed at less than 20 % compared to wild type and the residually expressed protein was enzymatically almost completely deficient. This was in agreement with the measurements in liver microsomes. Whereas the mean apoprotein content of the two samples with *CYP2B6*1/*11B* and *CYP2B6*5/*11A* genotype was only slightly lower than the mean wild type level, the corresponding enzyme activities were reduced to about 50 %, in agreement with the assumption that only one of the two alleles was active. Structurally, position 46 maps to a putative membrane attachment region located between the CYP2-typical proline-rich sequence (amino acids 32 to 39) and the first helix A (Kusano et al., 2001; Williams et al., 2000). It is not immediately comprehensible how the conservative change M46V could affect expression and function so dramatically. Using SIFT (sorting intolerant from tolerant; Ng and Henikoff, 2002), a computer program based on evolutionary comparisons that predicts whether an amino acid change may be functionally tolerable or not, the M46V change was predicted to be tolerable, based on the occurrence of Val at the homologous position of several other P450 sequences, including the entire CYP2D subfamily (Fig. 5). From spectral analysis of the insect cell-expressed protein it can not be excluded that the expressed variant contains some residual thiolate-bound heme. It is therefore possible that the M46V variant shows enzyme activity with other substrates.

Variant 12820G>A in exon 2, resulting in the nonconservative amino acid change Gly99Glu, was observed once in heterozygous form, i.e. the mutation frequency was only 0.2 % (Table 3). It could not be excluded with certainty that G99E is linked to R22C (*CYP2B6*2*), because this sample was heterozygous for 64C>T (Fig. 1). The G99E variant protein was expressed at less than 10 % compared to the wild type in COS-1 cells (Fig. 2) and less than 1 % of residual

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bupropion hydroxylase activity was detected. Although higher expression was achieved in insect cells, neither a CO difference spectrum nor any reconstituted enzyme activity was measurable, demonstrating that this amino acid change is not compatible with correct folding and heme incorporation. This was in agreement with the measurements in liver microsomes, which revealed a very low content of both, protein and activity. Structurally, position 99 is located within the loop between helices B and C, directly preceding the substrate recognition sequence SRS1, which includes amino acids Met103, Phe107, and Phe108 (Domanski et al., 1999). Because the position corresponding to Gly99 is highly confirmed in all known P450 sequences, SIFT predicted G99E to be intolerable (Fig.5).

SNP 13072A>G in exon 3 was recently reported to occur as single amino acid change K139E in allele *CYP2B6*8* (Lamba et al., 2003). The authors did not determine functional properties of this variant, but they noted that SIFT predicts this change to affect function. We observed this variant four times, resulting in an overall frequency of 0.8 % (Table 3). At least three of the samples were homozygous for the common double amino acid change Q172H and K262R, which proofs existence of the novel allele **13* harbouring all three amino acid changes, whereas the fourth sample was heterozygous and thus either **1/*13* or **6/*8* (Fig. 1). Thus, the triple mutant allele may be the more frequent variant carrying this mutation. No protein at all was obtained by heterologous expression. Although we did not explicitly express the triple mutant, it is highly unlikely that it will be functional. This is supported by measurements in microsomes of three livers with a **13* allele, all of which had much lower activities than homozygous wild types, although their genotypes included either a **1* or a **6* functional allele (Figs 1 and 4). Structurally, position 139 is located within a short loop between helices C and D with Lys139 being highly conserved within the CYP2 family (Fig. 5).

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Directly adjacent to K139E we found the amino acid change R140Q in one liver sample with very low bupropion hydroxylase activity. Because this sample was heterozygous at several positions including R22C, Q172H, and K262R in addition to 13076G>A in exon 3, it must be assumed that some of these amino acid changes are linked to each other. Although an allele with three changes R22C, Q172H, and K262R has so far not been described, this would be one possibility. Alternatively, either R22C or Q172H/K262R may be linked to R140Q. The precise structure of allele **14* thus remains undetermined for the moment, but certainly R140Q represents its key mutation. SIFT predicts this change to be functionally tolerable because rat CYP2B3 and CYP2E1 from several species also contain Gln (Fig. 5). In both expression systems, the R140Q variant could be well expressed and displayed a normal CO-difference spectrum, but only about 20 % enzyme activity compared to wild type. Interestingly, Arg140 has been identified by homology modeling as one of the positively charged amino acids on the P450 surface that constitute putative contact sites for the interaction with the oxidoreductase (Lewis et al., 1999). This would explain why the spectroscopically normal enzyme has a lower enzyme activity than the wild type.

The most frequent of the novel mutations was I391N (21388T>A), which was observed five times, repesenting a total frequency of 1 % (Table 3). The results from the heterologous expression studies established this variant as a further phenotypic null mutant. No activity was measurable in both expression systems, although some residual protein was expressed in COS-1 cells. Liver microsomes carrying this mutation in a heterozygous condition had also significantly lower protein content and activity than the wild type group. Position 391 is not well conserved among P450s of different species and Asn391 occurs on several human P450s (Fig. 5). Nevertheless, I391N was predicted by SIFT to affect function. Structurally, this

position lies between helix K and the heme binding region, but does not seem to participate in any known critical function. The mechanism by which this change completely prevents formation of an active enzyme remains thus unknown.

In conclusion , we have characterized four novel *CYP2B6* alleles as phenotypic null alleles. To our knowledge, this is the first demonstration of null mutations in the *CYP2B6* gene. According to current knowledge, most but not all human drug metabolizing P450 genes appear to have phenotypic null alleles in the population. The best investigated examples are the polymorphic *CYP2D6* and *CYP2C19* genes, for which homozygous carriers appear in the population at relatively high frequencies of about 7 to 10 % and 3 to 5 %, respectively (Meyer and Zanger 1997). Null alleles have also been reported for *CYP2A6* and for *CYP1B1* (CYPallele website at www.imm.ki.se/cypalleles/). In the latter case, null mutations lead to glaucoma whereas in the other cases, pharmacokinetic rather than clinical phenotypes are associated with the homozygous null situation. Collectively, we observed a total of 13 samples with *CYP2B6* null or extremely low activity alleles in heterozygous condition corresponding to 5.2 % carriers in the normal Caucasian population (Table 3). According to Hardy-Weinberg law, only one in 1500 individuals would be expected to be homozygous or compound heterozygous (i.e. carrying two different null alleles). This frequency is too low to become apparent given the usual size of clinical studies. However, our microsomal studies strongly suggest that heterozygous carriers may have significantly lower enzyme activity compared to homozygous wild type carriers. It remains to be investigated for which substrates and under which conditions genotyping for these alleles could be clinically useful.

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Legends to Figures

Figure 1: *CYP2B6* Haplotypes and inferred genotypes

CYP2B6 was completely sequenced through all exons and exon-intron boundaries in 4 blood samples (B) and 12 liver samples (L). Variant positions are shown on top with genomic DNA base numbers and the corresponding exon (e) or intron (i). Nonsynonymous changes are emphasized by shaded background. Sequence changes compared to the wild type are indicated by hatched (heterozygous) or black (homozygous) fields. Inferred genotype are based on haplotype analysis and on other known alleles (http://www.imm.ki.se/CYPalleles/CYP2B6).

Figure 2: Expression analysis of CYP2B6 variants in the microsomal fraction of transfected COS-1 cells

COS-1 cells were transiently transfected with cDNA vectors encoding CYP2B6.1 (wild type) or the indicated variants. Transfection efficiency was controlled by cotransfection with pCMVβ plasmid DNA and quantification of β-galactosidase activity in transfected cell homogenates. Upper panel: microsomal proteins (2.5 to 4.5 µg, normalized to relative β-galactosidase units) prepared from transfected COS-1 cells were separated by SDS-PAGE and CYP2B6 apoprotein was quantitated by Western blotting using a monoclonal antibody against CYP2B6. Microsomes from lymphoblast cells stably transfected with CYP2B6.1 were used as standard protein (range 0.125 pmol to 2 pmol). The value obtained for CYP2B6.1 was 40 pmol of CYP2B6 per mg of total protein. At least three independent expression experiments were performed for each variant, of which one representative blot is shown. The lower panel shows the corresponding bupropion hydroxylase activities determined at 1.6 mM substrate concentration.

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Figure 3: Analysis of *CYP2B6* expression in baculovirus-infected insect cells.

A: Tn5 insect cells were infected with recombinant baculoviruses encoding the indicated CYP2B6 variants. Upper panel: insect cells were cultured in the presence of hemin and 10 µg cell membranes were analyzed by SDS-PAGE and Western blotting using a monoclonal antibody against CYP2B6. Microsomes from lymphoblast cells stably transfected with CYP2B6.1 (wild type) were used as standard protein (range 0.25 pmol to 2 pmol). The blot was probed with a second monoclonal antibody against baculovirus envelope protein gp64 as a positive control for infection. Three analyses were performed, of which one representative blot is shown. The lower panel shows the corresponding bupropion hydroxylase activities determined at 1.6 mM substrate concentration after equimolar reconstitution of Tn5 membranes with rat NADPH:P450 oxidoreductase.

B: Reduced CO-difference spectra of Tn5 cell membranes expressing the indicated CYP2B6 variants. Membrane fractions were prepared from infected Tn5 cell homogenates (control: uninfected cells cultured under identical conditions) by ultracentrifugation. The amount of membrane protein analyzed in each case was 1 mg.

Figure 4: Phenotypic analysis of CYP2B6 variants in a human liver bank

CYP2B6 apoprotein was determined by quantitative Western blotting (A) and bupropion hydroxylase activity was determined by an LC-MS assay (B) in microsomes prepared from human livers selected for *CYP2B6* genotypes containing novel amino acid variants in comparison to a subgroup of 28 samples with homozygous wild type (wt) genotype. Mean and standard deviation are shown for the homozygous wild type group and individual values are shown for the mutant samples.

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Figure 5: Comparison of partial amino acid sequences of human CYP2B6 and other P450s. Various CYP2B orthologs from other species as well as human drug metabolizing P450s were chosen for comparison. The alignment. was calculated based on ClustalW algorithm using AlignX of the VectorNTI program. Polymorphic amino acid changes are indicated on top of each block. Identical, conserved or similar residues are highlighted as indicated.

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Table 1: Oligonucleotides used for CYP2B6 amplification and sequencing

Fragment	Primer	Sequence 5´- 3´	Length	Product size (bp)
Exon 1	CYP2B6_E1f1	GGGATAGGCATCAGGTCACTGG	22	431
	CYP2B6_E1r1	TTCCCCAAGTACCAAGGCAAGA	22	
Exon 2	CYP2B6_E2f1	GCTTTACTGGCCCAACCAGA	20	518
	CYP2B6_E2r1	GGAGGTGTGGGTTGGTTGTA	20	
Exon 3	CYP2B6_E3f1	GGTGCATCAGGGAAGGGAGTAT	22	450
	CYP2B6_E3r1	TCTGTGTTTCTCTCCTGCCCATC	23	
Exon 4	CYP2B6_E4f	CTCGGTCTGCCCATCTATAAAC	22	520
	CYP2B6_E4r	CATGTCTGCGTTTCTTCCAAGG	22	
Exon 5	CYP2B6_E5f	CTCTCTCCCTGTGACCTGCTA	21	496
	CYP2B6_E5r	CTCCCTCTGTCTTTCATTCTGTC	23	
Exon 6	CYP2B6_E6f1	GCGGTTGGATGTGTAGAGGACA	22	440
	CYP2B6_E6r1	TGGTTGGAATCTAGCCCAGAGC	22	
Exon 7	CYP2B6_E7f	CCACCCACCTCAACCTCCAA	20	443
	CYP2B6_E7r	TCCAACCCTCCACACACTCC	20	
Exon 8	CYP2B6_E8f	TGGAGTGTGTGGAGGGTTGG	20	493
	CYP2B6_E8r	TACAGTGGTGCCCAACAGAT	20	
Exon 9	CYP2B6_E9f	TCTGGTTTCAGAGCAGCTTCC	21	510
	CYP2B6_E9r	CTTGCAATGTGACCTCAGAC	20	
Exon 1-9	CYP2B6_cFE1	ATGGAACTCAGCGTCCTCCTCTTC	21	1476
	CYP2B6_cRE9	TCAGCGGGGCAGGAAGCGGATCTG	24	
	CYP2B6_cF1 Bam	GCGGATCCATGGAACTCAGCGTCC	24	1493
	CYP2B6_cR9 Xba	GCTCTAGACTCAGCGGGGCAGGAAG	25	
Exon 1-4	CYP2B6_cF1 Bam	GCGGATCCATGGAACTCAGCGTCC	24	635
	CYP2B6_cRE4	GATGAGTGAAAAAGTCTGGT	20	

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Table 2: Sequences of oligonucleotides (31-mers) used for mutagenesis

SNP[1]	Primer	Primer sequence 5´-3´
136A>G	2B6_Ex1_M_2	GGAAACCTTCTGCAGGTGGATAGAAGAGGCC
12820G>A	2B6_Ex2_M_4	CCTTCTCTGGCCGGGAAAAAATCGCCATGGT
13072A>G	2B6_Ex3_M_5	GACTTCGGGATGGGAGAGCGGAGTGTGGAGG
13076G>A	2B6_Ex3_M_6	TCGGGATGGGAAAGCAGAGTGTGGAGGAGCG
21388T>A	2B6_Ex8_M_7	CAGAAGTATTTCTCAACCTGAGCACTGCTCT

[1] Position based on *CYP2B6* genomic DNA numbering (mutated base underlined)

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Table 3: SNPs and their frequencies in Caucasians

SNP[1] cDNA genomic position	Sequence context 5´> 3´ (wt)	Protein	Alleles[2] n / total	frequency %
c.62A>T (e 1) g.62A>T	CTCCTGGTTCaGCGCCACCCT	Q21L	2 / 510	0.4
c.136A>G (e 1) g.136A>G	CTTCTGCAGaTGGATAGAA	M46V	3 / 510	0.6
c.172-100G>A (i 1) g.12.596G>A	GGATGTTGGGgAGGGGCTAAT		1 / 500	0.2
c.296G>A (e 2) g.12.820G>A	TCTGGCCGGGgAAAAATCGCC	G99E	1 / 498	0.2
c.334+60A>T (i 2) g.12.917A>T	GAAGGGAGTaTATGGGAGG		52 / 488	10.7
c.415A>G (e 3) g.13.072A>G	GGGATGGGAaAGCGGAGTG	K139E	4 / 496	0.8
c.419G>A (e 3) g.13.076G>A	ATGGGAAAGCgGAGTGTGGAG	R140Q	1 / 496	0.2
c.484+91G>A (i 3) g.13.232G>A	GGTATATAAGgGCACAGACAG		1 / 496	0.2
c.485-17C>T (i 3) g.15.582C>T	TGTCCTTGAcCTGCTGCTT		41 / 110	37.3
c.646-17C>T (i 4) g.17.897C>T	ACCTCACCAcCCCTTCTTT		2 / 504	0.4
c.822+183G>A (i 5) g.18.273G>A	GTGAAAGGAgGGAGAAAAT		164 / 496	33.1
c.823-85G>A (i 5) g.18.604G>A	TAGGCCAACgGAGGGCAGC		4 / 100	4
c.1152+23A>G (i 7) g.21.203A>G	GAACCCCATaGCCCTCCTG		6 / 108	5.6
c.1153-6C>T (i 7) g.21.363C>T	GTGATCCTCcCTCAGGACA		1 / 506	2
c.1172T>A (e 8) g.21.388T>A	TATTTCTCAtCCTGAGCAC	I391N	5 / 504	1
c.1294+53C>T (i 8) g.21.563C>T	GTACTATCCcCAACTTGA		131 / 500	26.2
c.1294+153A>G (i 8) g.21.663A>G	TACAACCCTaTAAGGAGGC		5 / 500	1
c.1294+177T>G (i 8) g.21.687T>G	AAGAAGATtACATTCCC		5 / 500	1
c.1295-100A>G (i 8)[3] g.25.241A>G	CTTATGCAAaTCTGTTGCA		6 / 106	5.7
c.1476+26G>A (3'UTR) g.25.548G>A	GGGTCAAAGgATTCCAGGG		2 / 110	1.8

[1]Position based on cDNA numbering (Yamano et al., 1989) and genomic DNA (CYPallele nomenclature website)

[2]Number n of observations / total number of successfully sequenced alleles

[3] Identical to NCBI SNP ID rs3211368

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Table 4: Allele designations for novel variants of the human *CYP2B6* gene

Allele[1]	Protein	Genetic variants[2]	Amino Acid Changes
*CYP2B6*10*	CYP2B6.10	**62A>T**, **64G>T**, 12740G>T	Q21L, R22C
*CYP2B6*11A*	CYP2B6.11	**136A>G**	M46V
*CYP2B6*11B*	CYP2B6.11	**136A>G**, 18273G>A	M46V
*CYP2B6*12*[3]	CYP2B6.12	**12820G>A**, 18273G>A	G99E
*CYP2B6*13A*	CYP2B6.13	**13072A>G**, **15631G>T**, **18053A>G** 18273G>A, 21563C>T	K139E, Q172H, K262R
*CYP2B6*13B*	CYP2B6.13	**13072A>G**, 15582C>T , **15631G>T**, **18053A>G**, 18273G>A, 21563C>T	K139E, Q172H, K262R
*CYP2B6*14*[3]	CYP2B6.14	**13076G>A**, 18273G>A	R140Q
*CYP2B6*15A*	CYP2B6.15	15582C>T , **21388T>A**	I391N
*CYP2B6*15B*	CYP2B6.15	15582C>T, 18273 G>A, **21388T>A**	I391N

[1]CYPallele Nomenclature Homepage: http://www.imm.ki.se/CYPalleles/CYP2B6

[2]nonsynonymous changes in bold type

[3]tentative assignment

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Table 5: Characteristics of CYP2B6 variants expressed in COS-1 cells[1]

Variant	Apoprotein % of CYP2B6.1	Km µM bupropion	Vmax pmol/mg/min	Vmax/Km % of CYP2B6.1
CYP2B6.1 wild type	100	65	215	100
CYP2B6.8 K139E	0	n.d.	n.d.	n.d.
CYP2B6.10 Q21L, R22C	120.3	72	192	80.6
CYP2B6.11 M46V	16.2	n.a.	~4[2]	~ 2[2]
CYP2B6.12 G99E	7.5	n.d.	n.d.	n.d.
CYP2B6.14 R140Q	82.5	117	48.7	12.5
CYP2B6.15 I391N	18.1	n.d.	n.d.	n.d.

[1] all values are means of two independent experiments

[2] at 1.6 mM substrate concentration

n.d., not detectable after correction for background due to ~4.4 ppm of contaminating product in bupropion substrate

n.a.: not applicable due to linear relationship

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Table 6: Characteristics of liver samples expressing novel CYP2B6 variants

Variants (N)	Genotype	CYP2B6 apoprotein	bupropion hydroxylase activity
CYP2B6.1 (28) wild type	*1 / *1	22.5 ± 16.1	262 ± 295
Q21L, R22C (1)	*1 /*10	6.98	46.5
Q21L, R22C, R487C (1)	*5 / *10	14.62	280
R22C, M46V (1)	*2 / *11	21.1	79.5
M46V, R487C (1)	*5 / *11	18.1	182.4
R22C, G99E (1)	*2 / *12	1.55	49.5
K139E, Q172H, K262R (1)	*1 / *13	19.05	136.2
K139E, 172HH, 262RR (2)	*6 / *13	1.45, 10.61	29.5, 23.7
R22C, R140Q, Q172H, K262R (1)	*14 / n.d.	12.63	39.4
I391N (3)	*1 / *15	3.9 ± 0.6	47.4 ± 2.3

n.d. genotype not unequivocally definable

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Figure 1

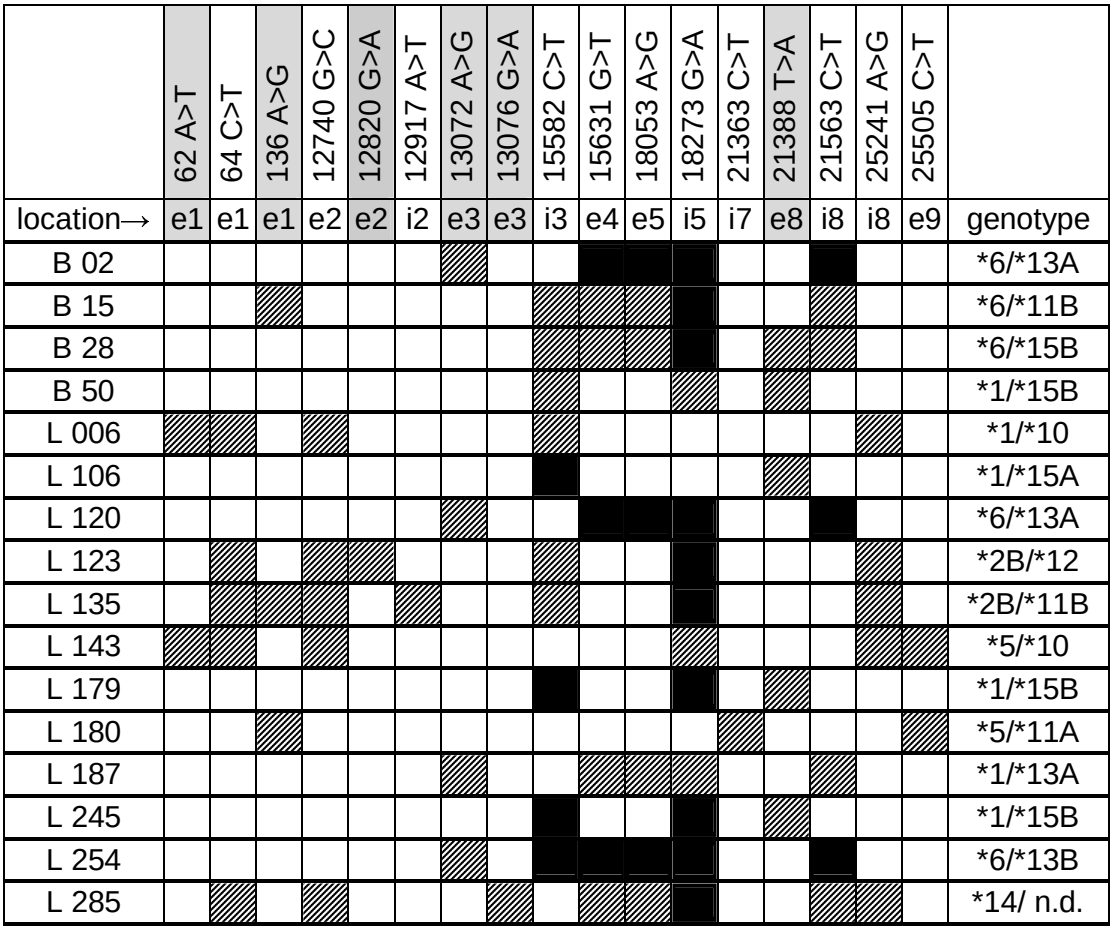

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Figure 2

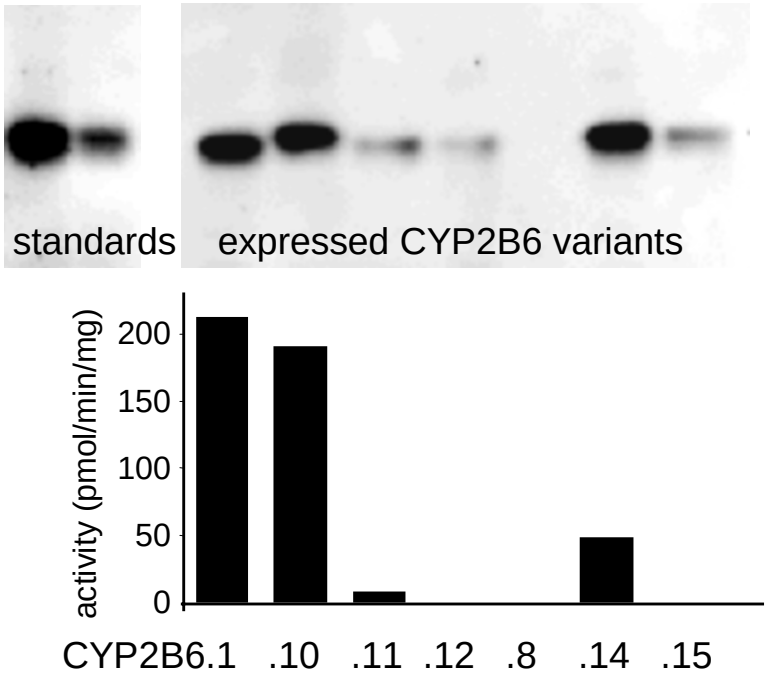

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Figure 3A

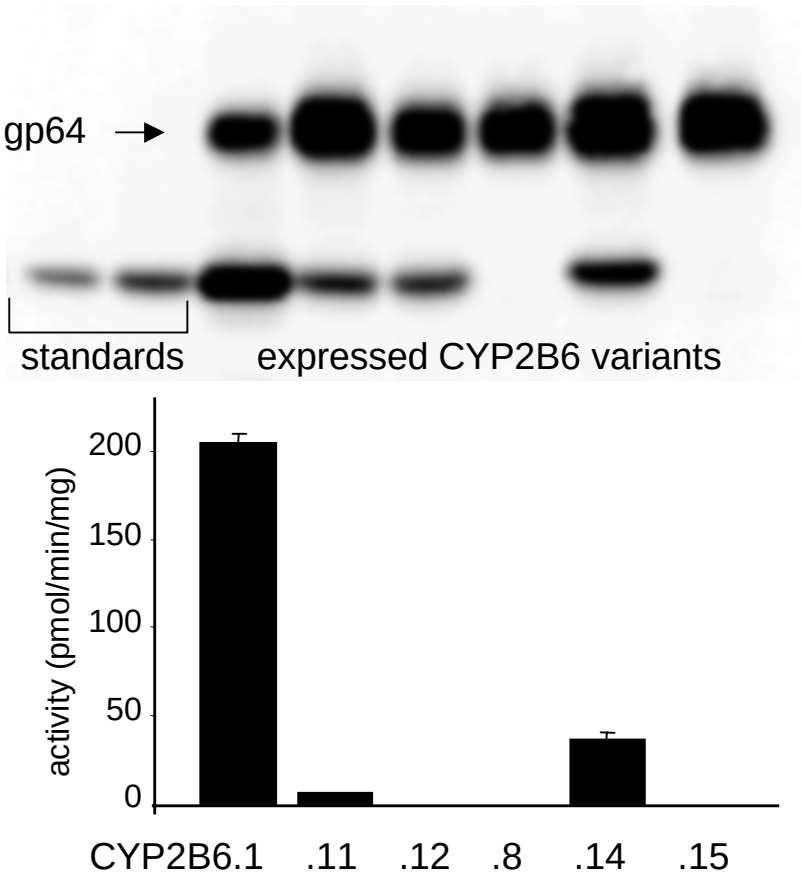

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Figure 3B

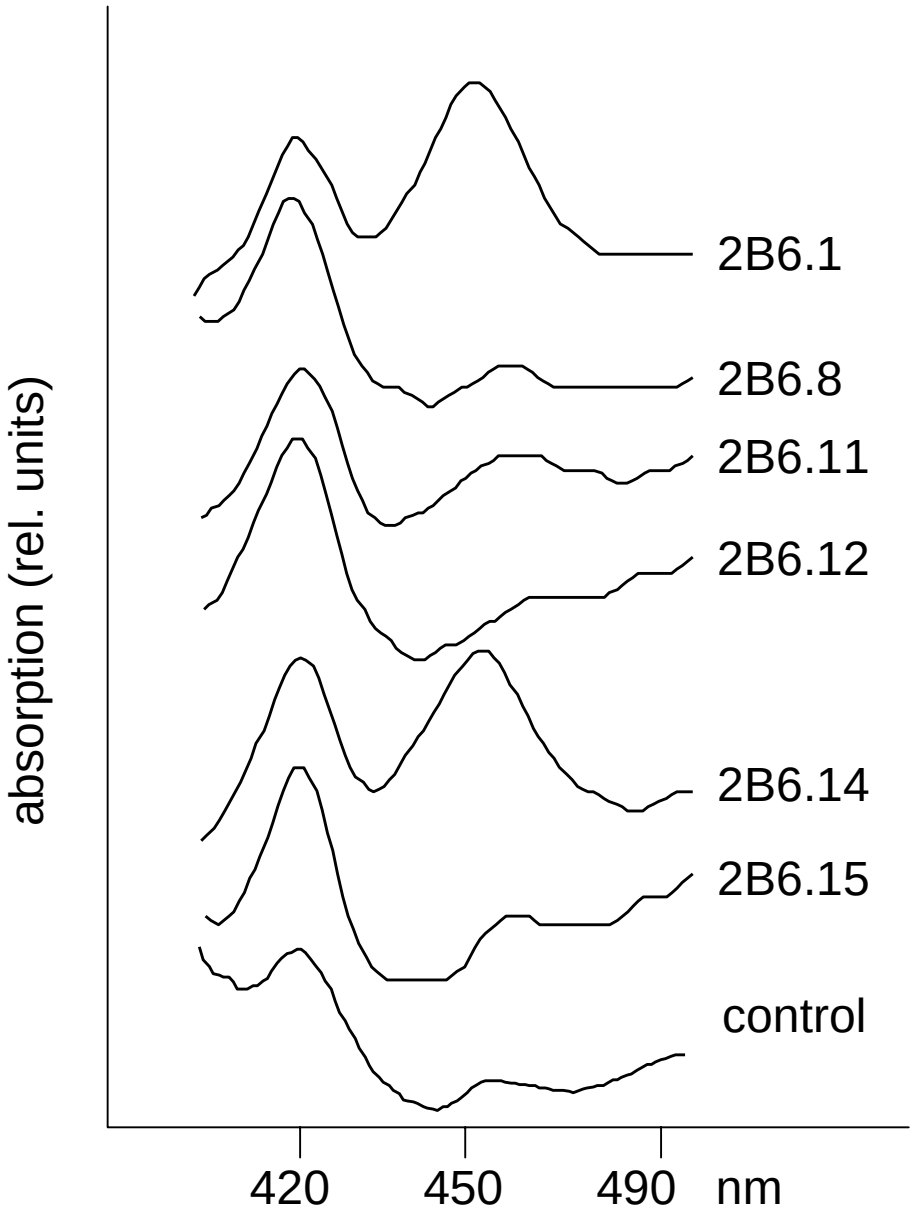

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Figure 4

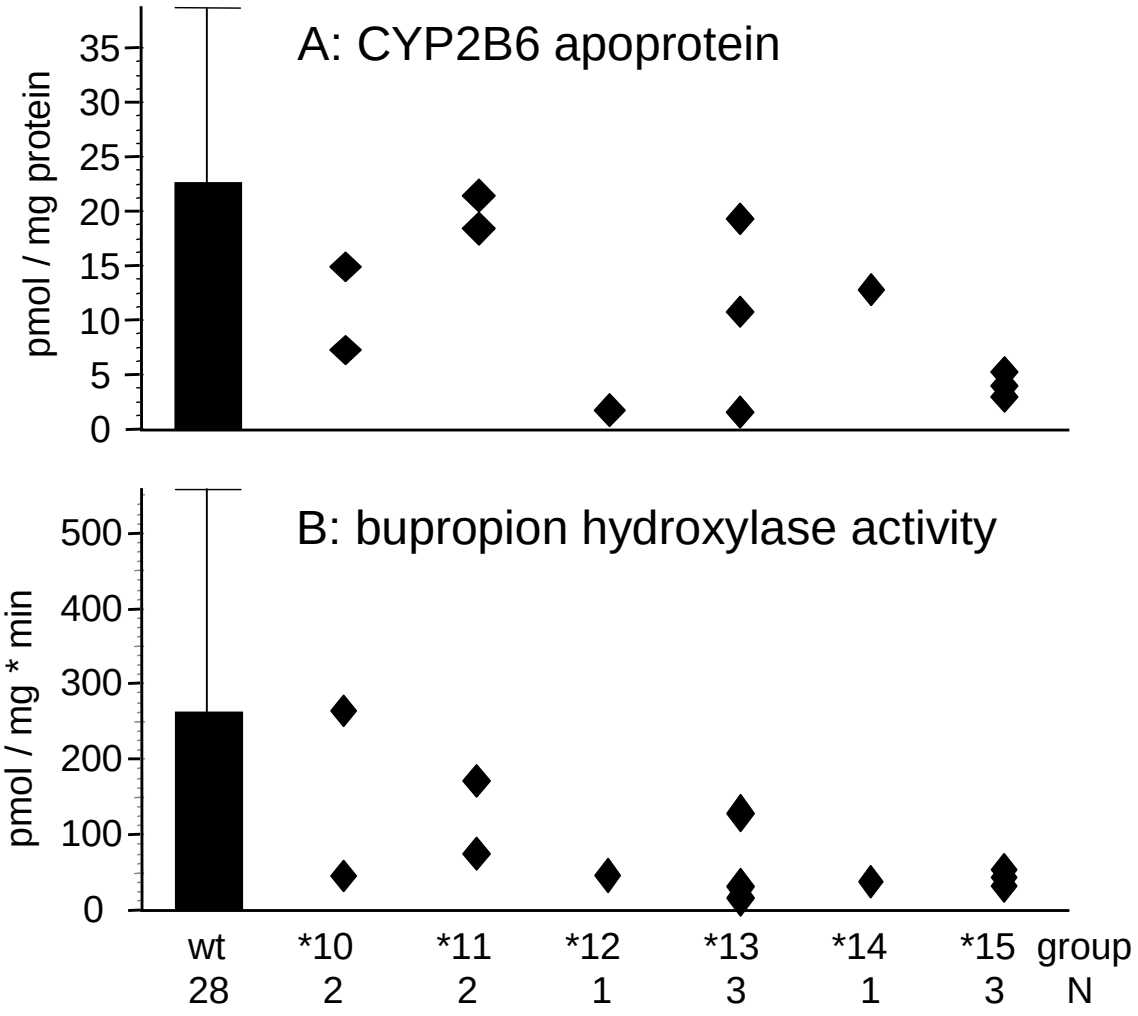

Figure 5