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Estimation of drug-metabolizing capacity by CYP-genotyping and CYP-expression

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CYP: cytochrome P450
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
SNP: single nucleotide polymorphism

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ABSTRACT

Many undesired side-effects or therapeutic failures of drugs are the results of differences or changes in drug-metabolism, primarily depending on the levels and activities of cytochrome P450 (CYP) enzymes. In order to assess whether CYP-expression profiles can reflect the hepatic drug-metabolism, we compared CYP mRNA levels in the liver or in peripheral leukocytes with the corresponding hepatic CYP-activities. A preliminary CYP-genotyping for the most frequent polymorphisms in Caucasian populations (*CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2C19*3, CYP2D6*3, CYP2D6*4, CYP2D6*6* and *CYP3A5*3*) was carried out before CYP-phenotyping, excluding the donors with non-functional alleles of *CYP2C9, CYP2C19* and *CYP2D6*, and those with functional *CYP3A5*1* allele from a correlation analysis. The hepatic mRNA levels of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 displayed a strong association with CYP-activities in the liver, whereas the expression of CYP1A2, CYP2C9, CYP2C19 and CYP3A4 in leukocytes was proven to reflect the hepatic activities of these CYP species. The leukocytes were found to be inappropriate cells for the assessment of hepatic CYP2B6 and CYP2D6 activities. Combining the results of CYP-genotyping and CYP-phenotyping analyses, patients' drug-metabolizing capacities can be estimated by the CYP-expression in the liver and also in leukocytes, with some limitations. Patients' genetic and non-genetic variations in CYP-status can guide the appropriate selection of drugs and the optimal dose, minimizing the risk of harmful side-effects and ensuring a successful outcome of drug therapy.

Introduction

The lack of therapeutic effect of drugs or the appearance of undesired side-effects, resulting in adverse events for patients, is partly caused by differences or changes in drug-metabolism. Of significant importance in inter-individual differences is the genetic variability of drug-metabolizing enzymes, causing reduced or even no enzyme activity. As an effect, the drug-metabolizing capacity of a patient can be weaker ('poor metabolizer') compared to other members ('intermediate or extensive metabolizer') of the population (Ingelman-Sundberg, 2001). An individual with poor drug-metabolism capacity can live a normal life until he/she is treated with a drug metabolized by an enzyme with reduced or no activity. Patients with poor drug-metabolizing capacities produce significantly higher blood levels of certain drugs, causing more severe and frequent side-effects (Brockmöller et al., 2000; Wilke et al., 2005).

The principal organ of drug-metabolism is the liver; however, every tissue has some ability to metabolize xenobiotics. Although the drug-metabolizing activities of the gut wall, kidneys, lungs or even the brain can contribute to the overall biotransformation to some extent, a patient's drug-metabolizing capacity can be roughly estimated from the hepatic metabolism. Therapeutic failure or drug toxicity is strongly influenced by hepatic drug-metabolism, primarily depending on the levels and activities of the cytochromes P450 (CYP). The enzymes belonging to the CYP1-3 families play a central role in the biotransformation of various drugs to more polar compounds, which are readily excreted (Lewis, 2004; Monostory and Pascussi, 2008). One of the most important reasons for inter-individual variations in drug-metabolism is the genetic polymorphism of *CYP* genes. Some *CYP* genes (*CYP2C9, CYP2C19, CYP2D6, CYP3A5*) are highly polymorphic, resulting in enzyme variants with reduced or even no activity (Solus et al., 2004). The genetically determined variance in CYP enzyme activities is transiently modulated by environmental (nutrition, co-medication) or internal factors (age, hormonal status, liver function, diseases), leading to different drug-

metabolism phenotypes (Monostory and Pascussi, 2008). Individuals with defective *CYP* alleles display permanent poor drug-metabolism, whereas those who have wild type *CYP* genes may become transient poor metabolizers. This means that the CYP-phenotype and drug-metabolizing capacity dynamically change in the course of external and internal influences, adapting to everyday chemical exposure. A transient decline in drug-metabolism capacity may arise due to a decrease in physical and health conditions, or the consumption of certain drugs or citruses. In contrast, an extensive metabolism can occur upon St. John's wort tea consumption, or during treatment with steroids or rifampicin (Monostory and Pascussi, 2008).

By recognizing individual differences in drug efficacy and toxicity, personalized drug therapy adjusted to a patient's drug-metabolizing capacity can help to avoid the potential side-effects of drugs. Tailored medication as a part of modern medical practices requires reliable diagnostic tools for the identification of inactivating mutations or the lack of functional CYP enzymes. Recently, pharmacogenetic services for the estimation of drug-metabolizing capacity have expanded worldwide (Brockmöller et al., 2000; Wilke et al., 2005); however, these test services offer to determine non-functional CYP enzymes only by CYP-genotyping, and do not provide information about the drug-metabolizing capacity of patients who do not have CYP mutations. CYP-phenotyping can add a novel element to the available diagnostics, and a combination of CYP-genotyping and CYP-phenotyping enables a more accurate qualification of a patient's drug-metabolism. In contrast to the already-existing assays, this multi-step diagnostic system qualifies the patient's drug-metabolizing capacity, and suggests a more rational drug therapy that is adjusted to the results. The complex diagnostic system provides an opportunity to predict CYP enzyme deficiency or an extremely reduced/increased CYP-expression that identifies the limitations of drug therapy. With an estimation of the patient's drug-metabolizing capacity, a modification of the drug therapy in a rational,

individually adjusted way can lower the incidences of adverse drug reactions. The quality of a patient's life can eventually be improved if the diminished drug-metabolism capacity is recognized in time and an individually adjusted therapy is applied.

The goal of the present work is to introduce a global approach by combining CYP-genotyping and CYP-phenotyping tools to estimate patients' drug-metabolizing capacity. To approach this goal, i) the first task is to screen the defective *CYP* alleles that result in a clinically significant reduction in CYP-activities; ii) then to estimate the current CYP expression in those subjects who have wild-type *CYP* genes. An additional aim is to decipher the correlation between the drug-metabolizing capacity of the liver and the CYP-expression in leukocytes (peripheral blood). If the CYP-status of leukocytes can inform us about the drug-metabolizing capacity of the liver, the qualification of a patient's drug-metabolizing capacity will have predictive power regarding future medication. A prospective investigation of the CYP-status allows a prediction of potential 'poor (or extensive) metabolizer' phenotypes and facilitates an improvement of the individual therapy, leading to the optimization of drug choice and/or dosage for a more effective therapy, to avoid serious adverse effects, and to decrease medical costs.

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Materials and methods

Liver and blood samples. The drug-metabolizing capacity of human livers not selected for transplantation (n=146) or of liver tissues remaining after reduced-size transplantation (n=18) was determined by CYP-genotyping and CYP-phenotyping approaches. In parallel, samples of peripheral blood were also taken from all the donors. The male/female ratio of the donors was 91/73, their age ranged between 3 and 74 years (43.4±14.46 years), and the cause of death included intracranial bleeding (65.2%) and cerebral contusion (34.8%). The livers were retrieved from hemodynamically stable brain-death donors with a normal liver function. The livers were perfused and stored in HTK (Fresenius AG, Bad Homburg v.d.H., Germany). The use of human tissues for scientific research was approved by the Hungarian Committee of Science and Research Ethics. All experimental activities were carried out under the regulation of Act CLIV of 1997 on Health and the decree 23/2002 of the Minister of Health of Hungary.

The microsomes and total RNA were isolated from the liver samples. The liver tissues were homogenized in Tris-HCl buffer (0.1 M pH 7.4) containing 1 mM EDTA and 154 mM KCl. The hepatic microsomal fraction was prepared by differential centrifugation (van der Hoeven and Coon, 1974). All the procedures of preparation were performed at 0-4°C. The protein content of the hepatic microsomes was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard. About 50 mg of liver tissues were homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA), and the total RNA was extracted according to the manufacturer's instructions. The RNA was precipitated using ethanol and stored at -80˚C for further analyses.

Leukocytes were isolated from 0.5 ml of blood samples using red blood cell lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany), and were suspended either in 0.2 ml of phosphate-buffered saline for DNA extraction or in 1 ml of TRIzol reagent for isolation

of the total RNA. The genomic DNA was extracted using the high pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany). The total RNA was isolated from leukocytes in a similar way to the hepatic RNA extraction. The purity and the concentration of the DNA and RNA samples were determined with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington DE).

In order to determine the effect of the period from liver sampling to RNA extraction, and from blood taking to leukocyte isolation and RNA extraction, *CYP* gene expressions were compared among the RNA preparations extracted immediately after the liver sampling and blood taking from three donors, and after storage for 4h, 8h and 24h at 4°C. No effect of storage for 4h on the CYP mRNA levels was observed; however, some degradation of the CYP mRNAs occurred in samples stored for 8h and 24h. Thus, the time between liver tissue and blood delivery, and storage until leukocyte isolation and RNA extraction was limited to 4h.

Positive control samples with various CYP-genotypes were kind gifts from Matthias Schwab, Ulrich Zanger (Dr Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany), and from Julia Kirchheiner (University of Ulm, Ulm, Germany).

CYP enzyme assays. Published methods were followed to determine the CYP-selective enzyme activities: phenacetin O-dealkylation for CYP1A2 (Butler et al., 1989), mephenytoin N-demethylation for CYP2B6 (Heyn et al., 1996), tolbutamide 4-hydroxylation for CYP2C9 (Miners and Birkett, 1996), mephenytoin 4'-hydroxylation for CYP2C19 (Srivastava et al., 1991), dextromethorphan O-demethylation for CYP2D6 (Kronbach et al., 1987), and nifedipine oxidation (Guengerich et al., 1986), midazolam 1'- and 4-hydroxylation (Kronbach et al., 1989) for CYP3A4/5. The incubation mixture contained a NADPH-generating system (1 mM NADPH, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$ and 2 units/ml glucose 6-phosphate dehydrogenase), human liver microsomes and various substrates

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selective for CYP isoforms (phenacetin for CYP1A2, tolbutamide for CYP2C9, mephenytoin for CYP2B6 and CYP2C19, dextromethorphan for CYP2D6, or nifedipine and midazolam for CYP3A4/5). The amount of microsomal protein used in the enzymatic reactions was 0.8 mg/ml, except for the phenacetin O-dealkylation (1 mg/ml). The microsomal CYP enzyme reactions were linear in the 10–30 minute incubation period. The enzyme reactions were terminated by the addition of ice-cold methanol. HPLC analyses were performed according to published methods (Guengerich et al., 1986; Kronbach et al., 1987 and 1989; Butler et al., 1989; Srivastava et al., 1991; Miners and Birkett, 1996; Heyn et al., 1996). All the measurements were performed in duplicate with <5% inter- and intra-day precision.

Quantitative real time-PCR. RNA (3 μg) was reverse transcribed into single-stranded cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules CA), and then real-time PCR with human cDNA was performed using FastStart Taq DNA polymerase (LightCycler 480 Probes Master, Roche Diagnostics GmbH, Mannheim, Germany) and UPL probes for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Roche Diagnostics GmbH, Mannheim, Germany). The sequences of primers and probes used for the real-time PCR analyses are shown in Table 1. The quantity of target RNA relative to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined. The CYP mRNA levels were quantified by real-time PCR measurements in the liver tissues and leukocytes from all donors.

CYP-genotyping with TaqMan probes. Hydrolysis SNP (single nucleotide polymorphism) analysis for *CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2C19*3, CYP2D6*3, CYP2D6*4, CYP2D6*6* and *CYP3A5*3* was performed by PCR with TaqMan probes (BioSearch Technologies Novato, CA) using the CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc.). Allelic discrimination was based on the design of two TaqMan probes, specific for the wild-type allele and the mutant allele labelled with different

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fluorescent tags (FAM, CalFlourGold540, CalRed610 or Quasar670). Primers and probes (Table 1) were designed based on the reference SNP sequences in the NCBI reference assembly. The real-time PCR was carried out with 80 ng genomic DNA using FastStart Taq DNA polymerase (LightCycler 480 Probes Master, Roche, or iQ Supermix, Bio-Rad Laboratories Inc., Hercules CA). The CYP-genotypes were distinguished by post-PCR allelic discrimination plotting the relative fluorescence values for wild-type and mutant alleles. The allelic content of each sample was determined by a multicomponent algorithm, yielding three allelic clusters representing the CYP-genotypic constituent: homozygous wild type, homozygous mutant type and heterozygous genotype. To confirm the results of the CYP-genotyping, a sequence analysis was also performed. 100 ng of DNA were amplified using the primers designed for the hydrolysis SNP analysis and the iQ Supermix. The PCR products were sequenced directly in an ABI PRISM 3100 Genetic Analyzer by the Sequencing Service of Biomi Ltd. (Gödöllő, Hungary).

Data analysis. The hepatic CYP enzyme activities were determined individually in each donor, and the frequency distributions of the CYP-activities were recorded for 164 donors. Three categories for each CYP-activity, low, medium and high, were statistically distinguished by calculating the quartiles of the CYP-activity distributions. The cut-off values between the categories were set to the 1st and the 3rd quartiles of the donors. The quartiles were chosen over standard deviation values as the CYP-activity distributions were skewed. The low, medium and high activity categories were used to describe the drug-metabolizer phenotypes, as poor, intermediate and extensive metabolizers, respectively.

The gene expressions of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 were also determined in both the liver and the peripheral leukocytes. The correlation between the hepatic CYP-activities and the relative CYP mRNA levels in the liver or in the leukocytes was estimated. The donors with homozygous mutant or heterozygous genotypes

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were excluded from the correlation analysis for all the CYPs, except for CYP3A4. For the evaluation of the CYP3A4 activity-mRNA correlation, the donors carrying *CYP3A5*1* allele were excluded from the analysis. The correlation among the CYP-activities and the CYP gene expression levels was quantified, and the correlation coefficients (r_S) and 95% confidence intervals were calculated using the Spearman approach (GraphPad InStat version 3.05, San Diego, CA). A strong correlation between the gene expression and the hepatic CYP-activities was considered if the probability value (P) was under 0.0001.

Results

Variations in CYP enzyme activities. Drug-metabolism in the liver can be roughly estimated by the activities of the most relevant drug-metabolizing CYP enzymes, thus we determined the catalytic activities of various CYPs in hepatic microsomal fractions of 164 Hungarian (Caucasian) cadaveric donors. CYP-selective substrates that are metabolized by a single CYP isoenzyme to produce a given metabolite were used to measure the CYP-activities. Figure 1 shows the frequency distributions of the CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A activities in the Hungarian liver donors. The hepatic CYP-activities of different donors showed a wide variation, as was also reported by several authors (Transon et al., 1996; Blanco et al., 2000; Shu et al., 2001). The inter-individual variations of the CYP-activities towards selective marker substrates were wide ranged from 80- to 750-fold variations for CYP2B6 and CYP3A4, respectively. In several cases the activities ranged from non-detectable to rather high values for CYP1A2, CYP2C9, CYP2C19 and CYP2D6 (Table 2). The CYP-activity values did not show a Gaussian distribution, rather they exhibited a skewed distribution. As a result, the median±QD values were calculated. The 1st and 3rd quartiles were considered to be the cut-off values between the categories of low, medium and high activities, characterizing poor, intermediate and extensive metabolizer phenotypes, respectively.

CYP-expression in liver. Assays of CYP enzyme activities require a large amount of liver tissue. This can be considered as a significant drawback when testing the drug-metabolizing capacity from liver needle-biopsies, where the available tissue is limited. Real-time PCR techniques can measure the CYP-expressions in small liver samples. These techniques may provide a useful method for an assessment of the liver's drug-metabolizing capacity, if the hepatic CYP mRNA levels reflect the hepatic CYP-activities. The total RNA was isolated, and the expression levels of *CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6*

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and *CYP3A4* genes were determined in the same liver samples in which the CYP-activities were measured. The primers for each CYP mRNA assay were designed in two consecutive exons separated by an intron on the corresponding genomic DNA; thus the primer pairs amplified the cDNA generated from the CYP mRNA, and did not yield products on any possible contamination with genomic DNA. All the CYP mRNA species investigated were detectable in liver tissues, but at varying levels (Table 3). Variations in the hepatic CYP mRNAs (ranging from 20- to 2000-fold) were more or less similar to the variations in the CYP-activities. The CYP3A4 mRNA displayed the highest inter-individual variation with the three-magnitude difference between the highest and lowest levels of hepatic expression; whereas the CYP2D6 expression exhibited the lowest variation (20-fold) between donors.

Correlation between CYP enzyme activities and CYP-expression in liver tissues. A relationship between the hepatic CYP mRNA levels and enzyme activities has been reported by other investigators (Sumida et al., 1999; Rodriguez-Antona et al., 2001). A correlation between the CYP-selective activities and CYP gene expressions, both determined in liver samples of Hungarian donors (n=117-164), was estimated. Although the expression of various CYPs would theoretically reflect the drug-metabolizing capacity of the liver, genetic polymorphisms of CYPs can give rise to perpetually reduced or even extensive metabolism. Several SNPs frequently occuring in the Caucasian population were determined in donors in parallel with CYP-phenotyping by CYP-activities and CYP mRNA levels.

The hepatic CYP1A2 and CYP2B6 mRNA levels correlated well with the activities of phenacetin O-dealkylation and mephenytoin N-demethylation, respectively (Fig. 2 and Table 4). A somewhat weaker correlation was found between the activities and mRNA levels of CYP2C9, CYP2C19, CYP2D6 and CYP3A4, if all donors were included in the correlation analysis irrespective of their CYP-genotypes (results of the analysis are not shown). The most common polymorphisms of the *CYP2C9* gene in Caucasian populations, *CYP2C9*2*

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(430C>T) and *CYP2C9*3* (1075A>C) alleles, produce enzymes with reduced function. The prevalence of the allelic variants for *CYP2C9* was found to be 7.9% for *CYP2C9*2* and 7.0% for *CYP2C9*3*. The donors with mutated *CYP2C9* gene were excluded from the correlation analysis, and homozygous wild types were analyzed. A strong association between the hepatic CYP2C9 mRNA levels and tolbutamide 4-hydroxylation activity (r_S=0.9255) was displayed in donors with the *CYP2C9*1/*1* genotype (Fig. 2 and Table 4). Mutations in the *CYP2C19* gene, resulting in non-functional *CYP2C19* alleles, *CYP2C19*2* (681G>A) and *CYP2C19*3* (636G>A) were also determined in liver donors. The *CYP2C19*2* allelic variant was detected with a frequency of 18.3% in the liver donors, whereas the *CYP2C19*3* allele was not observed at all. Excluding the donors carrying the *CYP2C19*2* allele from the correlation analysis, the hepatic mephenytoin 4-hydroxylation activity of the donors with the *CYP2C19*1/*1* genotype significantly correlated with the CYP2C19 mRNA levels in the liver (r_S=0.8808). A deficiency of the *CYP2D6* gene, resulting in *CYP2D6*3* (2549delA) and *CYP2D6*4* (1846G>A) alleles, is associated with the lack of enzyme activity, whereas the *CYP2D6*6* mutation (1795delT) leads to a lack of enzyme protein and consequently to a lack of CYP2D6 activity. The prevalence of the *CYP2D6*4* allelic variants was relatively high (17.4%), while the occurrence of both *CYP2D6*3* and *CYP2D6*6* was found to be 0.89%. A correlation analysis was carried out with the donors having exclusively the wild-type *CYP2D6* gene, and a strong association was found between the hepatic CYP2D6 mRNA levels and the dextromethorphan O-demethylase activities (r_S=0.9130).

CYP3A4 forms the bulk of the hepatic CYP3A protein and activity (about 95% of the CYP3A pool); however, the other members, primarily CYP3A5, can also contribute to the metabolism of CYP3A substrates in an adult liver. The *CYP3A5*3* mutation (6986A>G in intron 3) results in a splicing defect, leading to a lack of the CYP3A5 enzyme. The estimated *CYP3A5*3* allele frequency is more than 90% in the Caucasian population. Those individuals

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who have a functional CYP3A5 enzyme (with *CYP3A5*1/*1* and *CYP3A5*1/*3* genotypes) metabolize some CYP3A substrates (e.g., tacrolimus, cyclosporine A, nifedipine, midazolam) more rapidly than CYP3A5 non-expressors. Some 89.5% of the Hungarian donors did not express functional CYP3A5, carrying the *CYP3A5*3/*3* genotype. We did not find the homozygous wild genotype (*CYP3A5*1/*1*) among the donors investigated, but the functional *CYP3A5*1* allele was detected in donors with the heterozygous genotype. The frequency of wild-type (*CYP3A5*1*) allele was found to be 5.5% in the liver donors. Excluding the donors with the *CYP3A5*1/*3* genotype from the correlation analysis, a strong correlation was displayed between the hepatic CYP3A4 expression and all three CYP3A activities, nifedipine oxidation, midazolam 1'- and 4-hydroxylation (Fig. 2 and Table 4).

CYP-expression in leukocytes. Although liver biopsies can be available, it is risky and impractical to obtain specimens from the liver in patients. The major advantage would reside be in easily accessible biological samples, e.g., peripheral blood, providing information on drug-metabolizing capacity. Mature human erythrocytes, the main cellular components of blood, are anucleate cells; thus, they are not capable of active RNA synthesis. However, Kabanova et al. (2009) provided strong evidence that red blood cells have substantial RNA content. This fact supports the assumption of nucleus-independent protein synthesis, and an obvious lack of the transcriptional regulation of gene expression in mature erythrocytes. Consequently, the mRNA levels of various genes indicate the current regulatory effects of environmental and internal factors before the moment of nucleus discarding, and do not display a prompt transcriptional response to transient modulation during the 120-day lifespan in circulation. Leukocytes are nucleated cells displaying active RNA synthesis; thus, they were chosen as the target cells of the CYP-status assays. Leukocytes were isolated from peripheral blood samples, and the expression levels of *CYP* genes in leukocytes were determined in the same donors whose hepatic CYP-activities and CYP mRNA levels were

also measured. The CYP expression profiles of the leukocytes showed some similarities to the liver; however, the expression levels displayed significant differences. All the CYP mRNAs that were detected in liver tissues were also expressed in leukocytes, in contrast to those observed in other studies. Koch et al. (2002) could not detect mRNAs of the CYP3A subfamily, whereas Furukawa et al. (2004) could not display the expression of CYP2C9, CYP2C19, CYP2D6 and CYP3A4 in peripheral blood cells. Artefactual causes due to inappropriate sample collection and storage, inefficient RNA isolation procedure or inadequate primer/probe design might have led to their negative results. Koch et al. (2002) isolated the total RNA from lymphocytes using the RNeasy kit (Qiagen GmbH, Hilden, Germany), whereas Furukawa et al. (2004) used the Qiagen miniprep kit for RNA extraction from leukocytes. Both kits offer an easy isolation procedure and produce high-purity RNA samples, although they are efficient in the isolation of transcripts expressed in relatively large amounts. According to our experience, the isolation of CYP mRNAs from leukocytes using these Qiagen kits seemed to be inefficient, whereas TRIzol reagent (Invitrogen) or TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) was found to be appropriate for the extraction of the CYP mRNAs present in relatively small amounts.

In our study, the relative expression of various CYPs was generally 10^2–10^4-fold higher in liver tissues than in leukocytes, except for CYP2B6 (Table 3). The expression of the *CYP2B6* gene in leukocytes was just 20-fold lower than in liver tissues. High inter-individual variations of CYP-expression were also detected in leukocytes, similar to the liver tissues. The largest variations (10^4–10^5-fold) between individuals were found in the expression of CYP2B6, CYP2D6 and CYP3A4 in leukocytes, whereas CYP1A2 and CYP2C9 mRNA levels displayed only 285- and 445-fold variations, respectively.

Correlation between CYP enzyme activities in the liver and CYP expression in leukocytes. Taking blood from patients and isolating leukocytes is a simple way to obtain

biological material that may be assumed to provide sufficient information on the hepatic drug-metabolizing capacity. Our aim was to study the relationship between CYP enzyme activities in the liver and the expression of *CYP* genes in leukocytes collected simultaneously. If Spearman's correlation coefficient was higher than 0.7, we defined the CYP expression levels in leukocytes and the corresponding hepatic CYP activities as closely associated.

The CYP1A2 mRNA levels in leukocytes significantly correlated with the activity of phenacetin O-dealkylation in the liver (Fig. 3, Table 4). Thus we can conclude that the CYP1A2 expression in leukocytes reflects the hepatic CYP1A2 activity. The donors carrying mutated *CYP2C9* alleles (*CYP2C9*2* or *CYP2C9*3*) were excluded from correlation analysis. The strong association between CYP2C9 mRNA levels in leukocytes and tolbutamide 4-hydroxylation activity in liver was also displayed in donors with the *CYP2C9*1/*1* genotype. The expression levels of CYP2C19 in leukocytes were closely associated with mephenytoin 4-hydroxylation in the liver of the donors carrying the *CYP2C19*1/*1* genotype. In conclusion, the leukocytes isolated from subjects carrying wild-type *CYP2C9* or *CYP2C19*, reflect hepatic CYP2C9 and CYP2C19 activities. Furthermore, all three CYP3A activities in hepatic microsomes displayed a close correlation with CYP3A4 mRNA levels in leukocytes, if we excluded the donors with *CYP3A5*1* alleles from the correlation analysis. On the other hand, no association could be observed for the expression of CYP2B6 or CYP2D6 in leukocytes and hepatic enzyme activities, mephenytoin N-demethylation and dextromethorphan O-demethylation, respectively (r_S=-0.1824, P=0.1525 for CYP2B6 and r_S=0.07087, P=0.5905 for CYP2D6).

Cut-off values for distinguishing poor, intermediate and extensive metabolizers. The drug-metabolizing capacity of the liver tissues was qualified according to the frequency distributions of hepatic CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 activities in 164 Hungarian donors. The 1st and 3rd quartiles determined the cut-off values

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between the categories of low, medium and high CYP-activities, characterizing poor, intermediate and extensive metabolizer phenotypes, respectively (Table 2). The expression of these CYPs in the liver exhibited a strong correlation with hepatic CYP-activities; therefore, an estimation of a patient's drug-metabolizing capacity can be carried out on the basis of the mRNA levels in a liver biopsy sample. The optimal cut-off values for mRNA levels of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 in the liver were set on the basis of the cut-off values for hepatic CYP-activities, allowing a distinction between poor, intermediate and extensive metabolizers (Table 5). The mRNA levels of CYP2B6 and CYP2D6 in the leukocytes did not correlate with the respective CYP-activities in the liver, and consequently the leukocytes cannot serve as appropriate cells for the assessment of hepatic CYP2B6 and CYP2D6 activities. However, the expression of CYP1A2, CYP2C9, CYP2C19 and CYP3A4 in leukocytes was proven to reflect the respective CYP-activities in the liver, and thus the hepatic activities of these CYP enzymes were suggested to be qualified by the leukocyte mRNA levels of these CYP species. The cut-off values for the mRNAs levels of CYP1A2, CYP2C9, CYP2C19 and CYP3A4 in leukocytes were also established on the basis of the cut-off values for the hepatic CYP-activities, allowing a distinction between poor, intermediate and extensive metabolizers (Table 5).

Discussion

The personalized medication of modern therapy requires reliable diagnostic tools to estimate a patient's drug-metabolizing capacity. Although the assessment of overall drug-metabolism is difficult to establish, much information can be obtained by employing some simplification: i) the enzymes involved in the biotransformation of drugs are primarily located in the liver; ii) the majority of drugs are metabolized by CYP enzymes; iii) about 90% of drugs in clinical practice undergoing oxidative biotransformation involve at least a partial metabolism by one or more enzymes belonging to the CYP1-3 family (e.g., CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3As) (Chen et al., 2011). Thus, drug-metabolizing capacity can be roughly assessed through an integrative analysis of the current hepatic expression of CYP enzymes and the genomic identification of defective *CYP* alleles. The qualification of the patient's drug-metabolizing status together with personalized medication can contribute to the improvement of drug therapy, resulting in increasing drug-efficacy and a decreasing risk of adverse drug events. Liver biopsies are generally not available from patients; however, information on drug-metabolizing capacity obtained from leukocytes would be of clinical interest, if the CYP mRNA levels in leukocytes reflect the hepatic CYP-activities. Nevertheless, a series of questions arises: i) does the expression of drug-metabolizing *CYP* genes in leukocytes reflect hepatic CYP-activities; ii) is the regulation of CYP-expression in leukocytes similar to that of the liver; and iii) can we obtain information on hepatic drug-metabolizing capacity from leukocytes. To answer these questions, the present study was designed to investigate CYP mRNA levels in leukocytes collected simultaneously with liver tissues, and to study the correlation between CYP-expression and hepatic CYP-activities.

The basic methods for estimating drug-metabolizing capacity are a determination of the catalytic activities of individual CYPs by CYP-selective activity probes (Yuan et al.,

2002). Although these techniques are reliable in hepatic microsomes, the catalytic analyses require a relatively large amount of liver tissues, which is a serious drawback for human studies. CYP enzyme assays require relatively large amounts of microsomal proteins (0.2-0.25 mg), which in practise cannot be obtained from leukocytes, and thus the correlation between liver and blood activities cannot be analyzed. The levels of mRNAs as the prerequisite for enzyme proteins and enzyme activities can be determined in small amounts of biological material. The CYP mRNA contents of the liver or leukocytes may be assumed to correlate with hepatic CYP-activities. If the correlation is high, the activity levels can be estimated by measuring the CYP mRNA contents. We determined the hepatic activities of the six most relevant drug-metabolizing CYPs (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4) and the mRNA levels in the liver and leukocytes of 164 cadaveric donors.

The relationships between the hepatic mRNA levels and the CYP-activities were established to find evidence for *CYP* gene-expression that could provide accurate information about respective CYP-activities. A strong correlation (r_s>0.87) was displayed between CYP1A2 mRNA and phenacetin O-dealkylation, suggesting that the hepatic expression of the *CYP1A2* gene reflects the CYP1A2 activities in the liver. A similar conclusion was drawn from the results of Rodriguez-Antona et al. (2001) and of George et al. (1995), obtaining a potential association between hepatic CYP1A2 mRNA levels and 7-methoxyresorufin O-demethylation activity or CYP1A2 protein content. We also found a close relationship between the hepatic mRNA levels and the mephenytoin N-demethylation of CYP2B6 (r_s=0.89). However, Rodriguez-Antona et al. (2001) reported a weaker correlation between the hepatic CYP2B6 mRNA levels and the benzoxyresorufin O-debenzylation activities of 12 human liver samples (r=0.52). The fact that the benzoxyresorufin O-debenzylation is

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catalyzed by both CYP2B6 and CYP3A4 (Niwa et al., 2003) could account for the lower correlation coefficient found in the CYP2B6 by Rodriguez-Antona et al. (2001).

CYP2C9, CYP2C19, CYP2D6 and CYP3A4 showed a somewhat weaker correlation, if the hepatic mRNA levels and activities were compared for all the donors. Genetic polymorphisms, producing less active CYP enzymes or even null activities, are responsible for the fact that some liver tissues displayed relatively high mRNA levels, but low activities of CYP2C9, CYP2C19, or CYP2D6. Therefore, a preliminary CYP-genotyping study, detecting the most frequent CYP-polymorphisms in Caucasian populations was carried out before the estimation of the relationship between the hepatic CYP mRNA levels and activities. The donors carrying mutant *CYP* alleles (heterozygous or homozygous for *CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2C19*3, CYP2D6*3, CYP2D6*4, CYP2D6*6*) were excluded from the correlation analyses. The high correlation coefficients (r_s>0.88) indicated that the hepatic expression of *CYP2C9*, *CYP2C19* and *CYP2D6* genes is appropriate for an estimation of the respective CYP-activities. George et al. (1995) also reported a significant, but not close correlation between CYP2C9 mRNA and enzyme protein which was due to the poor selectivity of the CYP2C antibody, recognizing not only CYP2C9, but several other CYP2Cs. Rodriguez-Antona et al. (2001) described the lack of correlation between the CYP2C9 mRNA levels and diclofenac 4'-hydroxylation; however, they did not take allelic variants producing non-functional enzymes into account. They found a lower correlation between the hepatic CYP2D6 mRNA levels and dextromethorphan O-demethylation than we observed, most likely because of neglecting the *CYP2D6* polymorphic alleles.

For CYP3A, the liver tissues of some donors showed relatively high activities, but low CYP3A4 expression. The weak correlation was assumed to be due to CYP3A5 polymorphism, since the functional CYP3A5 enzyme as the product of *CYP3A5*1* allele also catalyzes the metabolism of the CYP3A substrates to some extent. The relatively rare

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*CYP3A5*1* allele (5-10% of Caucasian population) and eventually the functional CYP3A5 enzyme can contribute to the overall metabolism of CYP3A substrates, such as nifedipine, midazolam, cyclosporine, tacrolimus, erythromycin, carbamazepine, or lidocaine (Patki et al., 2003; Dai et al., 2006; Huang et al., 2004). Thus, we excluded the donors with *CYP3A5*1* allele from the correlation analysis, which resulted in a much stronger correlation between the hepatic CYP3A4 mRNAs and the CYP3A activities (r_s>0.8). In previous studies by Sumida et al. (1999) and by Rodriguez-Antona et al. (2001), a relatively high correlation between the CYP3A4 mRNA amounts and the testosterone 6β-hydroxylation was observed. Hepatic CYP3A4 expression also seemed to be related to the normalized plasma concentrations (plasma level/dose*weight) of the CYP3A substrates, cyclosporine or tacrolimus in liver transplants (Thörn et al., 2004).

Hepatic CYP-activities are considered to best characterize a patient's drug-metabolism, although CYP mRNA levels in leukocytes may provide a tool to estimate the drug-metabolizing capacity of the liver. Information about intra-individual correlations between hepatic CYP-activities and blood mRNA levels is virtually non-existent. Although several efforts have been undertaken to establish CYP mRNA levels in liver tissues and in blood (Finnström et al., 2001; Nowakowski et al., 2002; Koch et al., 2002; Furukawa et al., 2004; Lee et al., 2010), a comprehensive analysis of the relationship between hepatic CYP-activities and CYP mRNA levels in leukocytes has not been reported. Finnström et al. (2001) demonstrated no correlation of CYP1A2 and CYP3A4 expression in blood with mRNA levels of CYP1A2 and CYP3A4 in the liver, which is due to the RNA extraction from whole-blood samples. Leukocytes can be assumed, but red blood cells cannot be expected to reflect the hepatic CYP expression, since peripheral red blood cells are in different maturation and turnover status, displaying different transcriptional responses to environmental and internal factors. No association was observed between the CYP1A2 mRNA levels in the liver and in

the leukocytes by Furukawa et al. (2004); furthermore, CYP2C9, CYP2C19 or CYP3A4 mRNAs were undetectable in the leukocytes. The yield rate of CYP mRNAs from the leukocytes using the Qiagen miniprep kit or a less sensitive analytical method could be the limitations for the detection of CYP-expression in leukocytes. Lee et al. (2010) reported a poor correlation of CYP3A4 mRNA levels between the liver and leukocytes, although the limited number of samples (n=5) cannot provide strong evidence for a correlation. We demonstrated that the mRNA levels of CYP1A2, CYP2C9, CYP2C19 and CYP3A4 in leukocytes correlated strongly with the respective CYP-activities in the liver, allowing a good estimation of the hepatic drug-metabolizing activities of these CYPs. Preliminary CYP-genotyping for frequent mutations in *CYP* genes was necessary to discard the subjects carrying polymorphic *CYP* alleles, which can be transcribed, but are not associated with enzyme activities. The leukocyte CYP-expression of the donors with homozygous wild genotypes for CYP2C9 and CYP2C19, and with homozygous mutant genotypes for CYP3A5 strongly reflected the hepatic activities of CYP2C9, CYP2C19 and CYP3A. It should be noted that the mRNA levels of CYP2B6 and CYP2D6 in leukocytes did not display any relationship to the respective hepatic CYP-activities. Although polymorphic *CYP2D6* alleles can provide information on CYP2D6 poor metabolism, the current CYP2D6 activities in the liver cannot be estimated from the CYP2D6 mRNA levels in leukocytes.

The present work indicates that hepatic CYP-activities can be estimated by combining CYP-genotyping and CYP-phenotyping analysis of liver biopsy samples or leukocytes. CYP-genotyping analysis for frequent mutations in *CYP* genes is suggested to carry out first. CYP-genotyping determines the permanent poor metabolism, since defective *CYP* alleles produce enzymes with reduced activity or even non-functional enzymes. CYP-phenotyping analysis of liver samples for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 provides information about the current hepatic CYP-activities in those subjects who do not carry

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mutations in *CYP2C9, CYP2C19* and *CYP2D6* genes and have *CYP3A5*3/*3* genotype. Peripheral blood is a more easily accessible biological sample; therefore, a CYP-phenotyping analysis of leukocytes can be preferred to liver tissues. CYP-phenotyping for CYP1A2, CYP2C9, CYP2C19 and CYP3A4 in leukocytes provides information on the current hepatic CYP-activities in those subjects who carry homozygous wild genotypes of *CYP2C9* and *CYP2C19* or homozygous mutant genotype of *CYP3A5* (Fig. 4). In conclusion, a patient's drug-metabolizing capacity can be qualified by CYP-genotyping and CYP-phenotyping in liver samples and also in leukocytes with some limitations. The profile of a patient's genetic and non-genetic variations in drug-metabolism can guide the selection of drugs and the optimal dose that can minimize harmful side effects and ensure a more successful outcome. Tailored medication will eventually contribute to the improvement of quality of patients' lives.

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Authorship Contributions

Participated in research design: Kóbori, Monostory

Conducted experiments: Temesvári, Kóbori, Paulik, Sárváry, Monostory

Contributed new reagents or analytical tools: Temesvári, Paulik, Monostory

Perform data analysis: Belic, Monostory

Wrote or contributed to the writing of the manuscript: Monostory

References

Blanco JG, Harrison PL, Evans WE, and Relling MV (2000) Human cytochrome P450 maximal activities in pediatric versus adult liver. *Drug Metab Dispos* **28:** 379-382.

Brockmöller J, Kirchheiner J, Meisel C, and Roots I (2000) Pharmacogenetic diagnostics of cytochrome P450 polymorphisms in clinical drug development and in drug treatment. *Pharmacogenomics* **1:** 125-151.

Butler MA, Iwasaki M, Guengerich FP, and Kadlubar FF (1989) Human cytochrome P-450$_{PA}$ (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc Natl Acad Sci USA* **86**: 7696-7700.

Chen Q, Zhang T, Wang JF, and Wie DQ (2011) Advances in human cytochrome P450 and personalized medicine. *Curr Drug Metab* **12:** 436-444.

Dai Y, Hebert MF, Isoherranen N, Davis CL, Marsh C, Shen DD, and Thummel KE (2006) Effect of CYP3A5 polymorphism on tacrolimus metabolic clearance in vitro. *Drug Metab Dispos* **34**: 836-847.

Finnström N, Thörn M, Lööf L, and Rane A (2001) Independent patterns of cytochrome P450 gene expression in liver and blood in patients with suspected liver disease. *Eur J Clin Pharmacol* **57**: 403-409.

Furukawa M, Nishimura M, Ogino D, Chiba R, Ikai I, Ueda N, Naito S, Kuribayashi S, Moustafa MA, Uchida T, Sawada H, Kamataki T, Funae Y, and Fukumoto M (2004) Cytochrome P450 gene expression levels in peripheral blood mononuclear cells in comparison with the liver. *Cancer Sci* **95**: 520-529.

George J, Liddle C, Murray M, Byth K, and Farrell GC (1995) Pre-translational regulation of cytochrome P450 genes is responsible for disease-specific changes of individual P450 enzymes among patients with cirrhosis. *Biochem Pharmacol* **49**: 873-881.

Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T, and Waxman DJ (1986) Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J Biol Chem* **261**: 5051-5060.

Heyn H, White RB, and Stevens JC (1996) Catalytic role of cytochrome P4502B6 in the N-demethylation of S-mephenytoin. *Drug Metab Dispos* **24**: 948-954.

van der Hoeven TA, and Coon MJ (1974) Preparation and properties of partially purified cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from rabbit liver microsomes. *J Biol Chem* **249:** 6302-6310.

Huang W, Lin YS, McConn DJ, Calamia JC, Totah RA, Isoherranen N, Glodowski M, and Thummel KE (2004) Evidence of significant contribution from CYP3A5 to hepatic drug metabolism. *Drug Metab Dispos* **32**: 1434-1445.

Ingelman-Sundberg M (2001) Pharmacogenetics: an opportunity for a safer and more efficient pharmacotherapy. *J Intern Med* **250:** 186-200.

Kabanova S, Kleinbongard P, Volkmer J, Andree B, Kelm M, and Jax TW (2009) Gene expression analysis of human red blood cells. *Int J Med Sci* **6:** 156-159.

Koch I, Weil R, Wolbold R, Brockmöller J, Hustert E, Burk O, Nuessler A, Neuhaus P, Eichelbaum M, Zanger U, and Wojnowski L (2002) Interindividual variability and tissue-specificity in the expression of cytochrome P450 3A mRNA. *Drug Metab Dispos* **30**: 1108-1114.

Kronbach T, Mathys D, Gut J, Catin T, and Meyer UA (1987) High-performance liquid chromatographic assays for bufuralol 1'-hydroxylase, debrisoquine 4-hydroxylase, and dextromethorphan O-demethylase in microsomes and purified cytochrome P-450 isozymes of human liver. *Anal Biochem* **162**: 24-32.

Kronbach T, Mathys D, Umeno M, Gonzalez FJ, and Meyer U (1989) Oxidation of midazolam and triazolam by human liver cytochrome P450IIIA4. *Mol Pharmacol* **36**: 89-96.

Lee CY, Lai TY, Wu YM, Hu RH, Lee PH, Hsu LC, and Tsai MK (2010) Gene expression of P-glycoprotein and cytochrome P450 3A4 in peripheral blood mononuclear cells and correlation with expression in liver. *Transplant Proc* **42**: 834-836.

Lewis DFV (2004) 57 varieties: the human cytochromes P450. *Pharmacogenomics* **5:** 305-318.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with Folin phenol reagent. *J Biol Chem* **193:** 265-275.

Miners JO, and Birkett DJ (1996) Use of tolbutamide as a substrate probe for human hepatic cytochrome P450 2C9. *Methods in Enzymol* **272**: 139-145.

Monostory K, and Pascussi J-M (2008) Regulation of drug-metabolizing human cytochrome P450s. *Acta Chim Slo* **55:** 20-37.

Niwa T, Shiraga T, Yamasaki S, Ishibashi K, Ohno Y, and Kagayama A (2003) In vitro activation of 7-benzyloxyresorufin O-debenzylation and nifedipine oxidation in human liver microsomes. *Xenobiotica* **33**: 717-729.

Nowakowski-Gashaw I, Mrozikiewicz PM, Roots I, and Brockmöller J (2002) Rapid quantification of CYP3A4 expression in human leukocytes by real-time reverse transcription-PCR. *Clin Chem* **48:** 366-370.

Patki KC, von Moltke LL, and Greenblatt DJ (2003) In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes p450: role of CYP3A4 and CYP3A5. *Drug Metab Dispos* **31**: 938-944.

Rodriguez-Antona C, Donato MT, Pareja E, Gomez-Lechon MJ, and Castell JV (2001) Cytochrome P-450 mRNA expression in human liver and its relationship with enzyme activity. *Arch Biochem Biophys* **393**: 308-315.

Shu Y, Cheng Z-N, Liu Z-Q, Wang L-S, Zhu B, Huang S-L, Ou-Yang D-S, and Zhou H-H (2001) Interindividual variations in levels and activities of cytochrome P-450 in liver microsomes of Chinese subjects. *Acta Pharmacol Sin* **22:** 283-288.

Solus JF, Arietta BJ, Harris JR, Sexton DP, Steward JQ, McMunn C, Ihrie P, Mehall JM, and Edwards EP (2004) Genetic variation in eleven phase I drug metabolism genes in an ethnically diverse population. *Pharmacogenomics* **5:** 895-931.

Srivastava PK, Yun C-H, Beaune PH, Ged C, and Guengerich FP (1991) Separation of human liver microsomal tolbutamide hydroxylase and (S)-mephenytoin 4'-hydroxylase cytochrome P-450 enzymes. *Mol Pharmacol* **40**: 69-79.

Sumida A, Kinoshita K, Fukuda T, Matsuda H, Yamamoto I, Inaba T, and Azuma J (1999) Relationship between mRNA levels quantified by reverse transcription-competitive PCR and metabolic activity of CYP3A4 and CYP2E1 in human liver. *Biochem Biophys Res Commun* **262:** 499-503.

Thörn M, Lundgren S, Herlenius G, Ericzon BG, Lööf L, and Rane A (2004) Gene expression of cytochromes P450 in liver transplants over time. *Eur J Clin Pharmacol* **60**: 413-420.

Transon C, Lecoeur S, Leemann T, Beaunne P, and Dayer P (1996) Interindividual variability in catalytic activity and immunreactivity of three major human liver cytochrome P450 isozymes. *Eur J Clin Pharmacol* **51:** 79-85.

Wilke RA, Musana AK, and Weber WW (2005) Cytochrome P450 gene-based drug prescribing and factors impacting translation into routine clinical practice. *Person Med* **2:** 213-224.

Yuan R, Madani S, Wei XX, Reynolds K, and Huang SM (2002) Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. *Drug Metab Dispos* **30:** 1311-1319.

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Footnotes

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Legends for figures

Fig. 1. Frequency distribution of CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A activities in Hungarian liver donors (n=164).

Fig. 2. Correlation of relative CYP gene expression and corresponding CYP-activities in the liver. If Spearman's correlation coefficient (r_S) was higher than 0.7, CYP expression levels and the corresponding hepatic CYP-activities were defined as closely associated.

Fig. 3. Correlation of relative CYP gene expression in leukocytes and corresponding CYP-activities in the liver. If Spearman's correlation coefficient (r_S) was higher than 0.7, CYP expression levels in leukocytes and the corresponding hepatic CYP-activities were defined as closely associated.

Fig. **4.** Estimation procedure for a patient's drug-metabolizing capacity in leukocytes isolated from peripheral blood.

Table 1: Sequences of PCR primers and probes for CYP-phenotyping and CYP-genotyping

Primer	Sequence	Probe	Sequence
For CYP-phenotyping			
CYP1A2 forward	5’-ACAACCCTGCCAATCTCAAG-3’	CYP1A2	FAM-5’-CTGCCTCT-3’-BHQ
reverse	5’-GGGAACAGACTGGGACAATG-3’		
CYP2B6 forward	5’-AAAGCGGAGTGTGGAGGA-3’	CYP2B6	FAM-5’-AGGAGGAG-3’-BHQ
reverse	5’-AAGGTGGGGTCCATGAGG-3’		
CYP2C9 forward	5’-GTGCACGAGGTCCAGAGATAC-3’	CYP2C9	FAM-5’-CTTCTCCC-3’-BHQ
reverse	5’-CAGGGAAATTAATATGGTTGTGC-3’		
CYP2C19 forward	5’-TGAAGGTGGAAATTTTAAGAAAAGTAA-3’	CYP2C19	FAM-5’-CAGCAGGA-3’-BHQ
reverse	5’-CCCTCTCCCACACAAATCC-3’		
CYP2D6 forward	5’-TTCCTCAGGCTGCTGGAC-3’	CYP2D6	FAM-5’-AGGAGGAG-3’-BHQ
reverse	5’-CGCTGGGATATGCAGGAG-3’		
CYP3A4 forward	5’-CATGGACTTTTTAAGAAGCTTGG-3’	CYP3A4	FAM-5’-CTCTGCCT-3’-BHQ
reverse	5’-TTCCATGTCAAACATACAAAAGC-3’		
GAPDH forward	5’-AGCCACATCGCTCAGACAC-3’	GAPDH	FAM-5’-TGGGGAAG-3’-BHQ
reverse	5’-GCCCAATACGACCAAATCC-3’		

Primer	Sequence	Probe	Sequence
For CYP-genotyping			
CYP2C9*2 forward	5’-AGCAATGGAAAGAAATGGAAG-3’	CYP2C9*2 wild	FAM-CTCTTGAACAC**G**GTCCTC-BHQ1
reverse	5’-TAAGGTCAGTGATATGGAGTAGG-3’	mutant	CalRed610-CTCTTGAACAC**A**GTCCTC-BHQ2
CYP2C9*3 forward	5’-GCAAGACAGGAGCCACATG-3’	CYP2C9*3 wild	CalFluorGold540-CGAGGTCCAGAGATAC**A**TTGAC-BHQ1
reverse	5’-AGGAGAAACAAACTTACCTTGG-3’	mutant	Quasar670-CGAGGTCCAGAGATAC**C**TTGAC-BHQ2
CYP2C19*2 forward	5’-CTTAGATATGCAATAATTTTCCCAC-3’	CYP2C19*2 wild	CalGold540-TGATTATTTCCC**G**GGAACCCATAAC-BHQ1
reverse	5’-GAAGCAATCAATAAAGTCCCGA-3’	mutant	Quasar670-TGATTATTTCCC**A**GGAACCCATAAC-BHQ2
CYP2C19*3 forward	5’-AGATCAGCAATTTCTTAACTTGATG-3’	CYP2C19*3 wild	FAM-ACCCCCTG**G**ATCCAGG-BHQ1
reverse	5’-TGTACTTCAGGGCTTGGTC-3’	mutant	CalRed610-ACCCCCTG**A**ATCCAGG-BHQ2
CYP2D6*3 forward	5’-TGGCAAGGTCCTACGC-3’	CYP2D6*3 wild	FAM-CAC**A**GGATGACCTGGGACC-BHQ1
reverse	5’-TCCATCTCTGCCAGGAAG-3’	mutant	CalRed610-CACGGATGACCTGGGACC-BHQ2
CYP2D6*4 forward	5’-GGCGACCCCTTACC-3’	CYP2D6*4 wild	CalRed610-CCCCA**G**GACGCCC-BHQ2
reverse	5’-GATCACGTTGCTCACG-3’	mutant	FAM-CCCCA**A**GACGCCC-BHQ1
CYP2D6*6 forward	5’-TCTCCGTGTCCACCTTG-3’	CYP2D6*6 wild	FAM-GCTGGAGCAG**T**GGGTGAC-BHQ1
reverse	5’-GCGAAGGCGGCACA-3’	mutant	CalRed610-GCTGGAGCAGGGGTGAC-BHQ2
CYP3A5*3 forward	5’-GAGAGTGGCATAGGAGATACC-3’	CYP3A5*3 wild	FAM-TTTGTCTTTCA**A**TATCTCTTCCCTGT-BHQ1
reverse	5’-TGTACGACACACAGCAACC-3’	mutant	CalRed610-TTTGTCTTTCA**G**TATCTCTTCCCTGT-BHQ2

FAM, CalRed610, CalFluorGold540, Quasar670: fluorescent labelling, BHQ: black hole quencher

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Table 2: Characterization of human liver tissues (n=164) for selective substrates of CYP enzymes. The values are expressed as pmol product/mg microsomal protein*min.

CYP	**Enzyme activity** median±QD	**Min.**	**Max.**	**Cut-off values**	
				PM-IM 1st quartile	**IM-EM** 3rd quartile
CYP1A2					
phenacetin O-dealkylation	155.9±110.75	0	1107.1	60.2	281.7
CYP2B6					
Mephenytoin N-demethylation	35.7±22.20	6.47	538.3	22.3	66.7
CYP2C9					
tolbutamide 4-hydroxylation	218.2±100.55	0	1056.0	104.4	305.6
CYP2C19					
mephenytoin 4'-hydroxylation	25.2±19.15	0	342.9	11.9	50.2
CYP2D6					
Dextromethorphan O-demethylation	261.0±162.0	0	1461.0	140.6	464.6
CYP3A4/5					
nifedipine oxidation	414.2±272..25	3.79	2861.5	231.8	776.3
midazolam 4-hydroxylation	175.2±141.25	11.9	1180.0	101.1	383.6
midazolam 1'-hydroxylation	107.7±77.4	3.42	630.7	72.8	227.6

PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer phenotypes

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Table 3: CYP mRNA levels relative to GADPH mRNA in liver tissues and in leukocytes of the donors. The values are the mRNA ratio*10^{-3}

CYP	**CYP mRNA levels** median±QD	**Min.**	**Max.**
CYP1A2 - liver	89.2±55.15	3.92	678.3
leukocytes	0.419±0.5026	0.0168	4.78
CYP2B6 - liver	57.91±42.80	10.9	607.1
leukocytes	3.26±4.643	0.00454	166.1
CYP2C9 - liver	453.8±237.32	30.4	1853.2
leukocytes	0.0178±0.01031	0.00022	0.0982
CYP2C19 - liver	179.9±130.14	10.17	1635.8
leukocytes	0.0057±0.0056	0.000048	0.0761
CYP2D6 - liver	219.7±132.86	67.92	1274.6
leukocytes	5.68±40.63	0.148	16 056
CYP3A4 - liver	484.7±406.8	2.31	4316.9
leukocytes	0.0362±0.0184	0.000009	0.218

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Table 4: Relationship between CYP enzyme activities and CYP mRNA levels in liver and in leukocytes. Spearman's correlation coefficients (r_S), 95% confidence intervals (95% CI) and probability values (P) are calculated.

CYP activity		**CYP mRNA**	
		in liver	**in leukocytes**
CYP1A2		CYP1A2 mRNA	
phenacetin O-dealkylation	r_S	0.8780	0.8896
	95% CI	0.8058 – 0.9245	0.7868 – 0.9443
	P	< 0.0001	< 0.0001
CYP2B6		CYP2B6 mRNA	
mephenytoin	r_S	0.8900	-0.1824
N-demethylation	95% CI	0.8342 – 0.9278	-04178 – 0.07595
	P	< 0.0001	0.1525
CYP2C9		CYP2C9 mRNA	
tolbutamide 4-hydroxylation	r_S	0.9255	0.9179
	95% CI	0.8858 – 0.9518	0.8703 – 0.9485
	P	< 0.0001	< 0.0001
CYP2C19		CYP2C19 mRNA	
mephenytoin	r_S	0.8808	0.8018
4'-hydroxylation	95% CI	0.8160 – 0.9237	0.6792 – 0.8679
	P	< 0.0001	< 0.0001
CYP2D6		CYP2D6 mRNA	
dextromethorphan	r_S	0.9130	0.07087
O-demethylation	95% CI	0.8532 – 0.9491	-0.1939 – 0.3260
	P	< 0.0001	0.5905
CYP3A		CYP3A4 mRNA	
nifedipine oxidation	r_S	0.8984	0.9475
	95% CI	0.8436 – 0.9346	0.9145 – 0.9680
	P	< 0.0001	< 0.0001
CYP3A		CYP3A4 mRNA	
midazolam 1'-hydroxylation	r_S	0.8112	0.7718
	95% CI	0.7165 – 0.8765	0.6484 – 0.8557
	P	< 0.0001	< 0.0001
CYP3A		CYP3A4 mRNA	
midazolam 4-hydroxylation	r_S	0.8001	0.8078
	95% CI	0.7005 – 0.8783	0.7005 – 0.8794
	P	< 0.0001	< 0.0001

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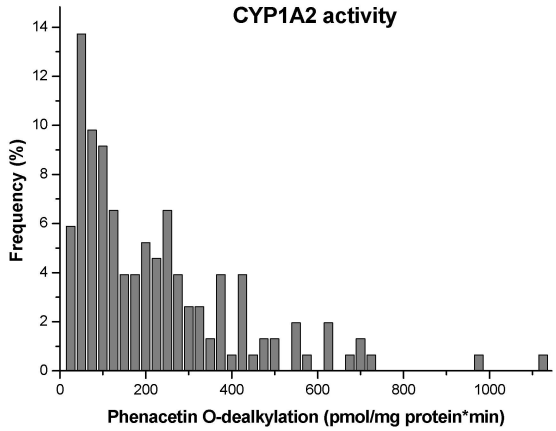
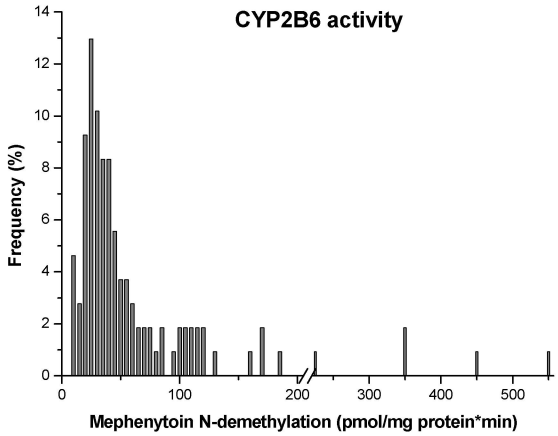
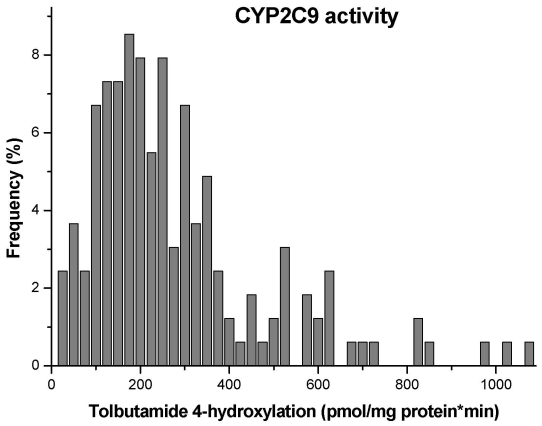
Table 5: Cut-off values for CYP mRNA levels relative to GADPH mRNA in liver tissues and in leukocytes distinguishing poor (PM), intermediate (IM) and extensive (EM) metabolizers.

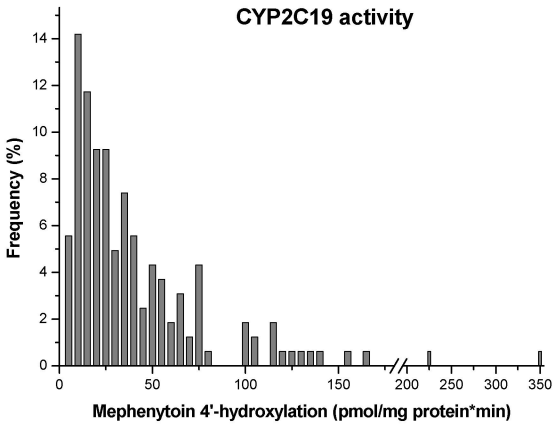
CYP	**Cut-off values**	
	PM-IM	**IM-EM**
CYP1A2 - liver	$4.5*10^{-2}$	$1.5*10^{-1}$
leukocytes	10^{-4}	$2*10^{-3}$
CYP2B6 - liver	$3.5*10^{-2}$	$1.5*10^{-1}$
leukocytes	-	-
CYP2C9 - liver	$1.5*10^{-1}$	$7*10^{-1}$
leukocytes	$5*10^{-6}$	$2.5*10^{-5}$
CYP2C19 - liver	$8*10^{-2}$	$3.5*10^{-1}$
leukocytes	10^{-6}	10^{-5}
CYP2D6 - liver	$1.5*10^{-1}$	$5*10^{-1}$
leukocytes	-	-
CYP3A4 - liver	$2.5*10^{-1}$	1.5
leukocytes	10^{-6}	10^{-4}

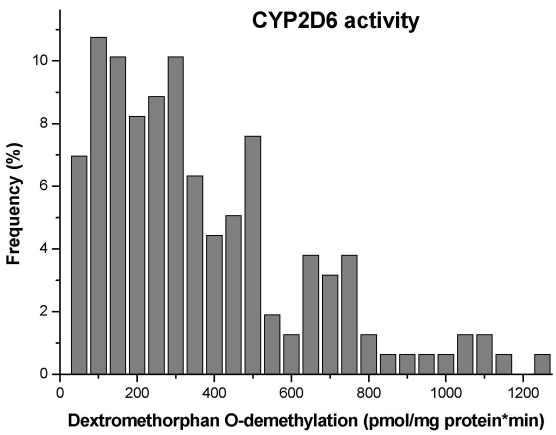
Figure 1

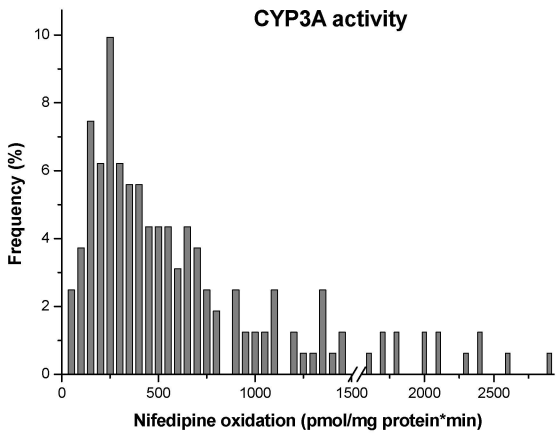

CYP1A2 activity
Frequency (%)
Phenacetin O-dealkylation (pmol/mg protein*min)

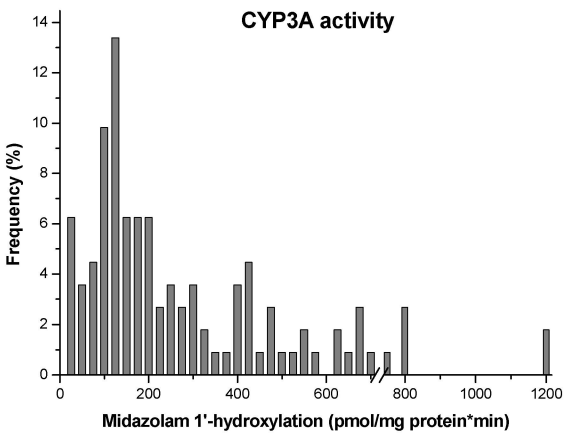

CYP2B6 activity
Frequency (%)
Mephenytoin N-demethylation (pmol/mg protein*min)


CYP2C9 activity
Frequency (%)
Tolbutamide 4-hydroxylation (pmol/mg protein*min)


CYP2C19 activity
Frequency (%)
Mephenytoin 4'-hydroxylation (pmol/mg protein*min)


CYP2D6 activity
Frequency (%)
Dextromethorphan O-demethylation (pmol/mg protein*min)


CYP3A activity
Frequency (%)
Nifedipine oxidation (pmol/mg protein*min)


CYP3A activity
Frequency (%)
Midazolam 1'-hydroxylation (pmol/mg protein*min)

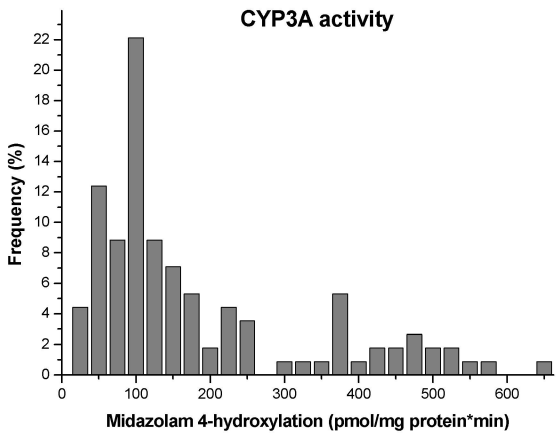
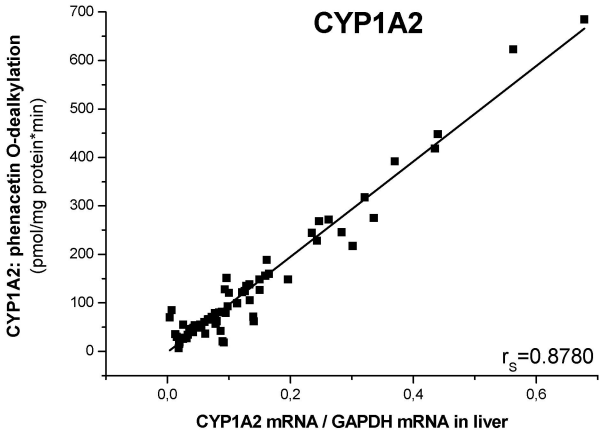
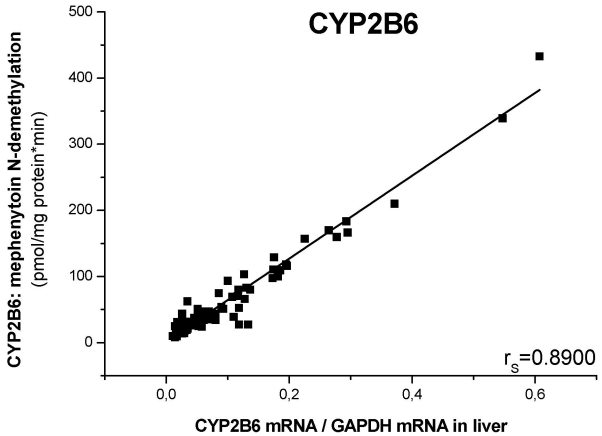
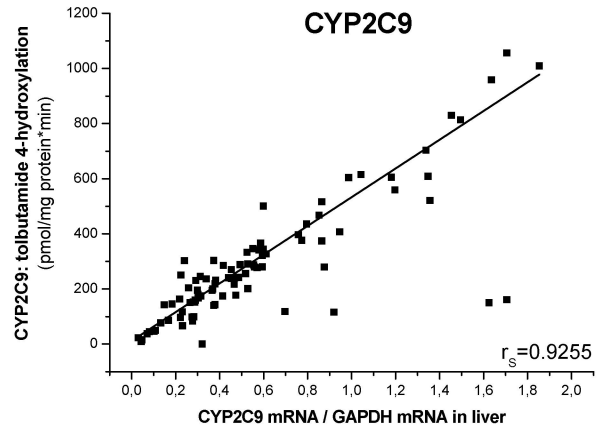
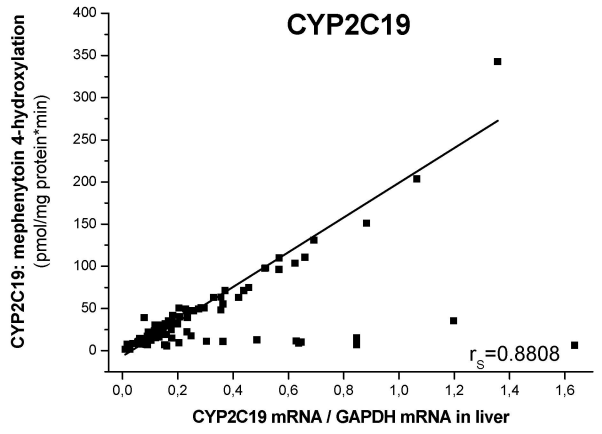

CYP3A activity
Frequency (%)
Midazolam 4-hydroxylation (pmol/mg protein*min)

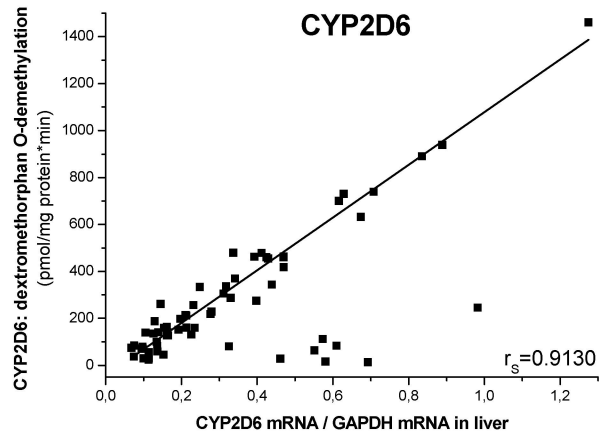
Figure 2

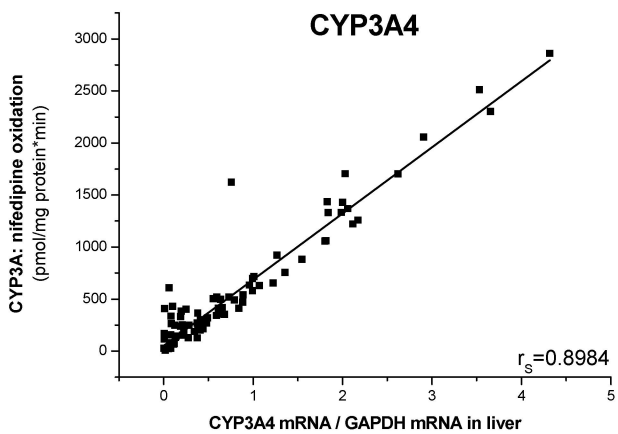

CYP1A2
CYP1A2: phenacetin O-dealkylation
(pmol/mg protein*min)
CYP1A2 mRNA / GAPDH mRNA in liver
r_S=0.8780

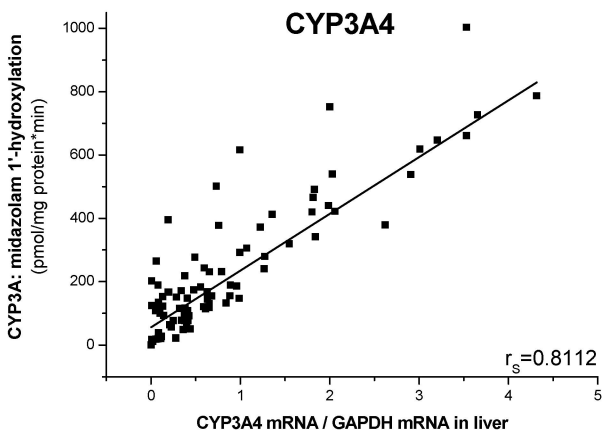

CYP2B6
CYP2B6: mephenytoin N-demethylation
(pmol/mg protein*min)
CYP2B6 mRNA / GAPDH mRNA in liver
r_S=0.8900


CYP2C9
CYP2C9: tolbutamide 4-hydroxylation
(pmol/mg protein*min)
CYP2C9 mRNA / GAPDH mRNA in liver
r_S=0.9255


CYP2C19
CYP2C19: mephenytoin 4-hydroxylation
(pmol/mg protein*min)
CYP2C19 mRNA / GAPDH mRNA in liver
r_S=0.8808


CYP2D6
CYP2D6: dextromethorphan O-demethylation
(pmol/mg protein*min)
CYP2D6 mRNA / GAPDH mRNA in liver
r_S=0.9130


CYP3A4
CYP3A: nifedipine oxidation
(pmol/mg protein*min)
CYP3A4 mRNA / GAPDH mRNA in liver
r_S=0.8984


CYP3A4
CYP3A: midazolam 1'-hydroxylation
(pmol/mg protein*min)
CYP3A4 mRNA / GAPDH mRNA in liver
r_S=0.8112

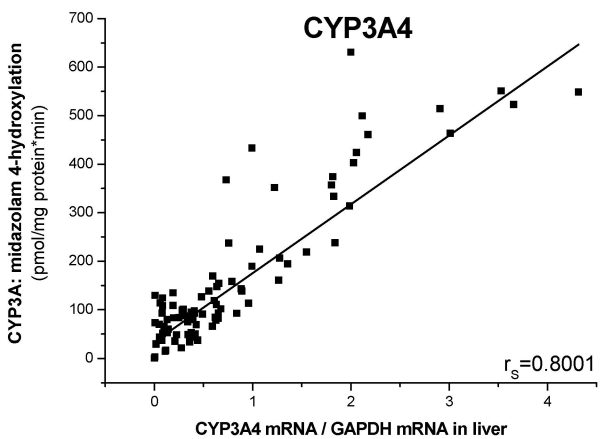
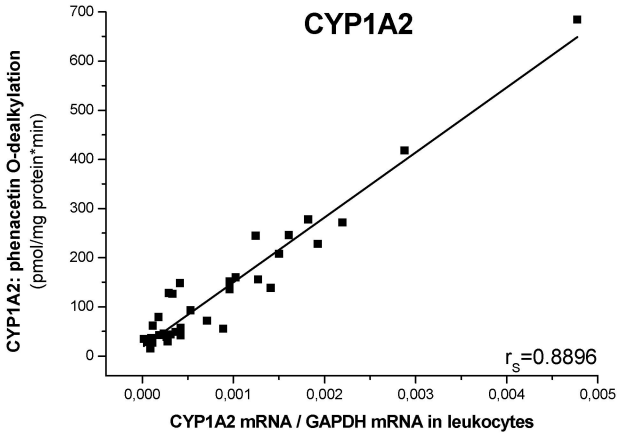
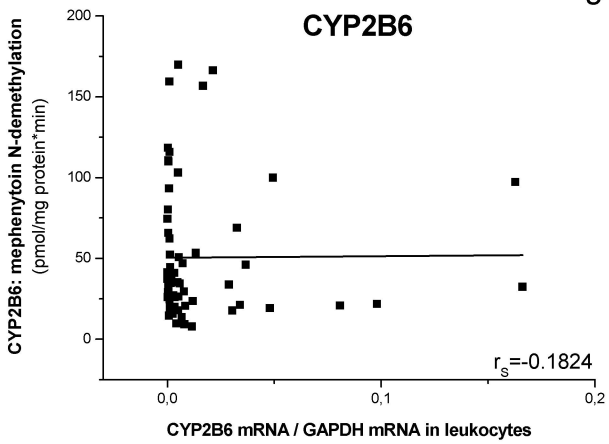
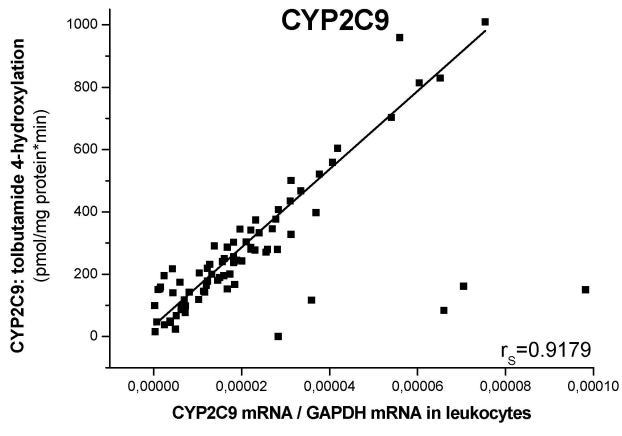
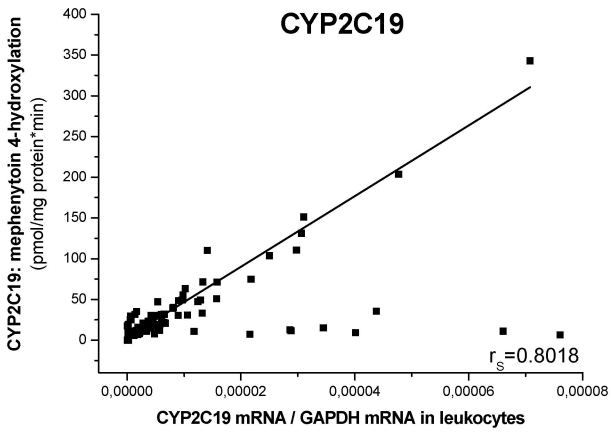

CYP3A4
CYP3A: midazolam 4-hydroxylation
(pmol/mg protein*min)
CYP3A4 mRNA / GAPDH mRNA in liver
r_S=0.8001

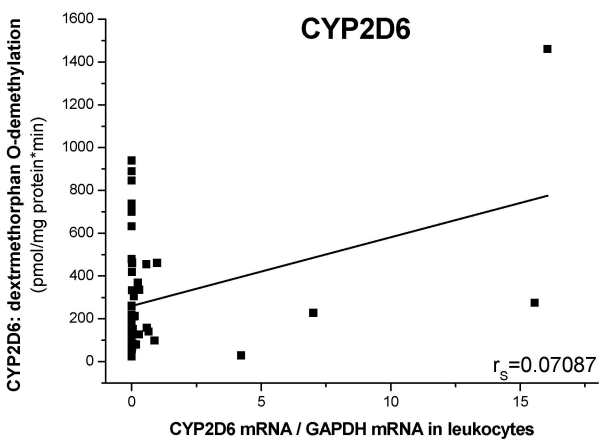
Figure 3

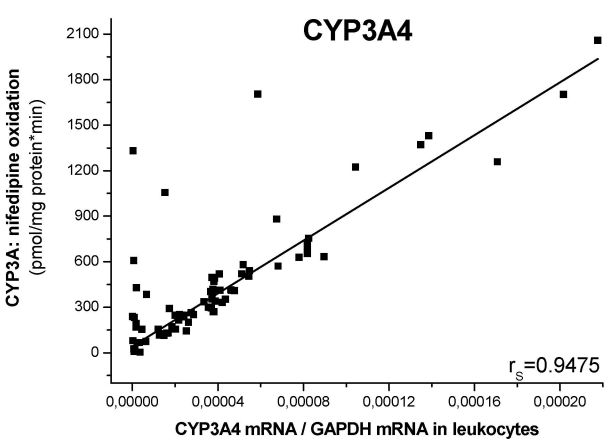

CYP1A2
CYP1A2: phenacetin O-dealkylation
(pmol/mg protein*min)
CYP1A2 mRNA / GAPDH mRNA in leukocytes
r_S=0.8896

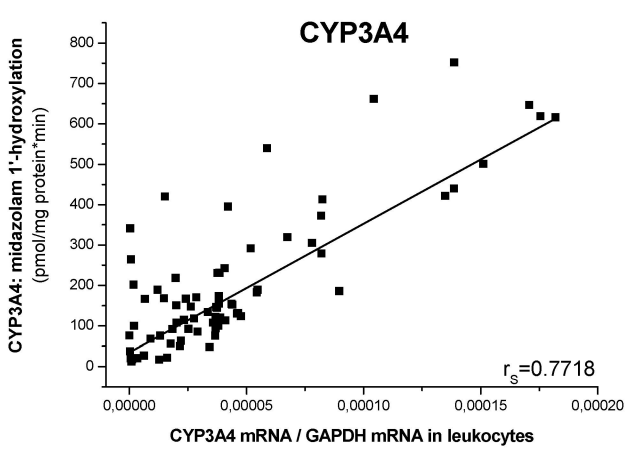

CYP2B6
CYP2B6: mephenytoin N-demethylation
(pmol/mg protein*min)
CYP2B6 mRNA / GAPDH mRNA in leukocytes
r_S=-0.1824


CYP2C9
CYP2C9: tolbutamide 4-hydroxylation
(pmol/mg protein*min)
CYP2C9 mRNA / GAPDH mRNA in leukocytes
r_S=0.9179


CYP2C19
CYP2C19: mephenytoin 4-hydroxylation
(pmol/mg protein*min)
CYP2C19 mRNA / GAPDH mRNA in leukocytes
r_S=0.8018


CYP2D6
CYP2D6: dextrmethorphan O-demethylation
(pmol/mg protein*min)
CYP2D6 mRNA / GAPDH mRNA in leukocytes
r_S=0.07087


CYP3A4
CYP3A: nifedipine oxidation
(pmol/mg protein*min)
CYP3A4 mRNA / GAPDH mRNA in leukocytes
r_S=0.9475


CYP3A4
CYP3A4: midazolam 1'-hydroxylation
(pmol/mg protein*min)
CYP3A4 mRNA / GAPDH mRNA in leukocytes
r_S=0.7718

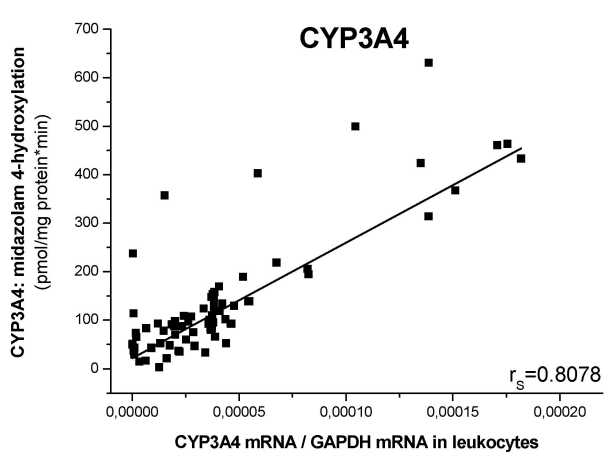

CYP3A4
CYP3A4: midazolam 4-hydroxylation
(pmol/mg protein*min)
CYP3A4 mRNA / GAPDH mRNA in leukocytes
r_S=0.8078

Figure 4