DMD Fast Forward. Published on October 12, 2011 as doi:10.1124/dmd.111.042259
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DMD #42259

TITLE PAGE

Simultaneous absolute protein quantification of transporters, cytochrome P450s and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities

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RUNNING TITLE PAGE

a) Running title: Protein levels of CYPs, UGTs and transporters in human liver

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c) Number of text pages: 35

Number of tables: 6

Number of figures: 5

Number of references: 39

Number of words in *Abstract*: 207

Number of words in *Introduction*: 670

Number of words in *Discussion*: 1426

Non-standard abbreviations

ABC, ATP binding cassette; BCRP, breast cancer resistance protein; CNT, concentrative nucleoside transporter; CYP, cytochrome P450; LC-MS/MS, liquid

chromatography–tandem mass spectrometry; LOQ, lower limit of quantification; LRP-1, low density lipoprotein receptor-related protein 1; MRM, multiple reaction monitoring; MRP, multidrug resistance-associated protein; NTCP, sodium/taurocholating polypeptide; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OST, organic solute transporter; P450R, NADPH-cytochrome P450 reductase; PEPT, oligopeptide transporter; UGT, UDP-glucuronosyltransferases

Abstract

The purpose of the present study was to determine the absolute protein expression levels of multiple drug-metabolizing enzymes and transporters in 17 human liver biopsies, and to compare them with the mRNA expression levels and functional activities in order to evaluate the suitability of the three measures as parameters of hepatic metabolism. Absolute protein expression levels of 13 cytochrome P450 (CYP) enzymes, NADPH-cytochrome P450 reductase (P450R) and 6 UDP-glucuronosyltransferase (UGT) enzymes in microsomal fraction and 22 transporters in plasma membrane fraction were determined using liquid chromatography–tandem mass spectrometry. CYP2C9, CYP2E1, CYP3A4, CYP2A6, UGT1A6, UGT2B7, UGT2B15 and P450R were abundantly expressed (more than 50 pmol/mg protein) in human liver microsomes. The protein expression levels of CYP3A4, CYP2B6 and CYP2C8 were each highly correlated with the corresponding enzyme activity and mRNA expression levels, while for other CYPs, the protein expression levels were better correlated with the enzyme activities than the mRNA expression levels were. Among transporters, the protein expression level of organic anion transporting polypeptide 1B1 was relatively highly correlated with the mRNA expression level. However, other transporters showed almost no correlation. These findings indicate that protein expression levels determined by the present simultaneous quantification method are a useful parameter to assess differences of hepatic function between individuals.

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Introduction

Hepatic disposition and metabolism are key factors determining inter-individual differences in the pharmacokinetics of drugs. The uptake of most drugs from the circulating blood into the liver at the sinusoidal membrane of hepatocytes involves active transport. The drugs subsequently undergo biotransformation by intracellular enzymes such as cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT), and the parent drug or its metabolites are eventually excreted from the hepatocytes by canalicular and/or sinusoidal transporter proteins. Therefore, the activities and expression levels of drug-metabolizing enzymes and transporters involved in these processes have been a focus of interest for pharmacokinetic research for a long time, and have been analyzed in attempts to evaluate individual differences of hepatic disposition and metabolism (Shimada et al., 1994; Parkinson et al., 2004; Meier et al., 2006; Johansson and Ingelman-Sundberg, 2011).

Assessment of activities of CYP enzymes is performed in vitro and in vivo by the use of specific probe substrates (Tassaneeyakul et al., 1993; Goldstein et al., 1994; Powell et al., 1996; Schmider et al., 1997; Kenworthy et al., 1999; Faucette et al., 2000; Li et al., 2002; Frye, 2004). However, the availability of suitable specific substrates for UGTs (Court, 2005), and especially for drug transporters, is still very limited. Although mRNA expression levels have been thoroughly analyzed, this approach suffers from the disadvantage that mRNA expression levels do not necessarily reflect protein expression or functional activity of the corresponding enzymes or transporters. For example, Sumida et al. demonstrated that mRNA expression of CYP3A4 was well correlated

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with activity in human liver samples, whereas mRNA expression of CYP2E1 did not show such a correlation (Sumida et al., 1999). Several other reports have demonstrated poor correlations between mRNA expression and activity of CYP2B6, CYP2D6, CYP2E1 and CYP3A4 (Rodriguez-Antona et al., 2001; Sy et al., 2002; Hayashi et al., 2011).

Compared to mRNA, protein expression is considered to be a more suitable parameter of the functional activity of enzymes and transporters. Sy et al. demonstrated that protein expression of CYP3A4 correlated well with activity, but not with mRNA expression in human liver samples (Sy et al., 2002). Hayashi et al. also reported that protein expression of CYP2E1, but not mRNA expression, correlated with activity in samples of human intestinal tissue. Despite these advantages, the limited availability of specific antibodies, amongst other factors, has restricted quantitative analyses of drug-metabolizing enzymes and drug transporters at the protein level.

To overcome these problems, we have recently developed liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based protein quantification methods that do not require antibodies (Ohtsuki et al., 2011). In this method, the target protein concentration in a sample is determined after enzymatic digestion by quantifying one or more peptide fragments specific for the target molecule. Trypsin digestion is commonly applied for analyte peptide generation. Synthetic peptides of known concentrations are used as calibration standards and stable isotope-labeled peptides are added to the samples as internal standards. Currently, 37 different proteins can be quantified simultaneously with high reliability and robustness (Sakamoto et al., 2011). We

have previously reported the quantification of 34 transporter proteins in mouse tissues, 114 membrane proteins in human brain microvessels, 51 membrane proteins in monkey brain microvessels and 11 CYP enzymes in microsomes of human liver autopsies (Kamiie et al., 2008; Ito et al., 2011; Kawakami et al., 2011; Uchida et al., 2011). Therefore, this novel protein quantification method can provide valuable data on absolute protein expression levels of drug-metabolizing enzymes and transporters, and this should lead to a better understanding of hepatic disposition and metabolism of drugs.

The purpose of the present study was to determine the absolute protein expression levels of 20 membrane-associated drug-metabolizing enzymes, such as CYPs and UGTs, and 22 drug transporters, including those classified as key transporters by the International Transporter Consortium (Giacomini et al., 2010), in tissue samples of 17 human liver biopsies by using LC-MS/MS. Furthermore, mRNA expression levels and activities were measured and compared with protein expression levels to evaluate the suitability of protein expression level as a parameter for analyzing differences of hepatic metabolism among individuals.

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Materials and Methods

Materials. Substrates, metabolite standards, internal standards and other chemicals were obtained from the following sources: acetaminophen, amodiaquine, dehydronifedipine, dextromethorphan, dextrorphan, diclofenac, 4'-hydroxydiclofenac, 4'-hydroxymephenytoin, 1'-hydroxymidazolam, 6β-hydroxytestosterone, NADPH, nifedipine, phenacetin and testosterone were from Sigma-Aldrich (Deisenhofen, Germany). Bupropion was from LGC Promochem (Luckenwalde, Germany). Hydroxybupropion and [$^{2}H_3$]4'-hydroxymephenytoin were from Toronto Research Chemicals (North York, Ontario, Canada). [$^{2}H_8$]Hydroxybupropion, [$^{13}C_6$]4'-hydroxydiclofenac and [$^{2}H_6$]dehydronifedipine were synthesized by Syncom (Groningen, The Netherlands). [$^{2}H_4$]Acetaminophen, [$^{2}H_5$]desethylamodiaquine, [$^{2}H_3$]dextrorphan and [$^{2}H_8$]6β-hydroxytestosterone were from Cerilliant (Round Rock, TX, USA). Desethylamodiaquine was from BD Biosciences (Woburn, MA, USA). *S*-Mephenytoin was from Enzo Life Sciences (Plymouth Meeting, PA, USA). Midazolam (Dormicum®) was from Roche Pharma AG (Grenzach-Whylen, Germany). [^{14}C]Lauric acid was from ARC (St. Louis, MO, USA). Other reagents and solvents used were from standard suppliers and were of reagent or HPLC grade.

Preparation of microsomal and plasma membrane fractions. Frozen human liver tissue biopsy samples from 17 donors were purchased from XenoTech, LLC (Lenexa, KA, USA) and UKHTB (Leicester, UK). Donor demographics are shown

in Table 1. The research protocols for the present study were approved by the Ethics Committees of the Graduate School of Pharmaceutical Sciences, Tohoku University. The study has been carried out in accordance with the Declaration of Helsinki.

Frozen liver tissues were dissected and homogenized using a Potter-Elvehjem homogenizer in buffer A (0.1 M KCl-phosphate buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO)). Microsome preparation for CYP activity measurement was performed in the absence of protease inhibitors. For protein quantification, cells were additionally subjected to nitrogen cavitation (450 psi for 15 min at 4°C) in buffer A (Hunter and Commerford, 1961; Kamiie et al., 2008). The obtained homogenates were centrifuged at 10,800 × g for 20 min at 4°C and the supernatants were collected and ultracentrifuged at 100,000 × g for 60 min at 4°C. The microsomal pellet was suspended in buffer A and ultracentrifuged at 100,000 × g for 60 min at 4°C. The resulting pellet was suspended in buffer B (20 mM Tris-HCl-buffer containing 0.25 M saccharose and 5.4 mM EDTA), and part of the solution was stored at -80 °C as microsomal fraction (Kawakami et al., 2011). The remaining portion was layered on top of a 38% (w/v) sucrose solution and centrifuged at 100,000 x g for 30 min at 4°C. The turbid layer at the interface was recovered, suspended in buffer B, and centrifuged at 100,000 x g for 30 min at 4°C. The plasma membrane fraction was obtained from the resulting pellet, which was suspended in buffer B. Protein concentrations were measured by the Lowry method using the DC protein assay reagent (Bio-Rad, Hercules, CA) or using the bicinchoninic assay according to the manufacturer`s protocol (Pierce Biotechnology, Rockford,

IL, USA).

The plasma membrane fraction was suggested to contain canalicular membranes, since canalicular localized transporters (MDR1, BCRP, BSEP, MRP2 and MATE1) were quantified in plasma membrane fractions of human and/or mouse liver in our present and previous studies (Kamiie et al., 2008). Furthermore, our previous manuscript demonstrated insulin-dependent subcellular translocation of low density lipoprotein receptor-related protein 1 (LRP-1) to plasma membrane of rat hepatocytes (Tamaki et al., 2007). The amount of LRP-1 in rat liver plasma membrane fractions increased under the fed condition, while its amount in whole liver lysate was not affected. This suggests that plasma membrane was concentrated in the liver plasma membrane fraction, and intracellular membrane was less concentrated in the fraction.

LC-MS/MS-based protein quantification analysis. Protein quantitation of the target molecules was simultaneously performed by HPLC-MS/MS or nanoLC-MS/MS with multiplexed multiple reaction monitoring (multiplexed MRM) as described previously (Uchida et al., 2011). Protein expression levels were determined by quantifying specific target peptides produced by trypsin digestion. Absolute amounts of each target peptide were determined by using an internal standard peptide, which is a stable isotope-labeled peptide with the identical amino acid sequence to that of the corresponding target peptide. The target peptide for quantification was selected based on in-silico selection criteria as described previously (Kamiie et al., 2008). Quantification of human transporters and other membrane proteins was based on the MRM conditions

reported previously (Kamiie et al., 2008; Ohtsuki et al., 2011; Sakamoto et al., 2011; Shawahna et al., 2011; Uchida et al., 2011). The peptide sequence for NADPH-cytochrome P450 reductase (P450R) was FAVFGLGNK and L was labeled with stable isotope. The MRM transitions for P450R were set as m/z 476.6, 635.4, 734.4, 488.3 and 805.5 for unlabeled peptide and m/z 480.1, 642.4, 741.4, 495.3 and 812.5 for labeled peptide (Q1, Q3-1, Q3-2, Q3-3, Q3-4, respectively).

Protein samples were suspended in suspension buffer containing 7 M guanidium hydrochloride and 10 mM EDTA. Samples were reduced with dithiothreitol at room temperature for 60 min under a nitrogen atmosphere, and S-carbamoylmethylated by iodoacetamide at room temperature for 60 min. The alkylated proteins were precipitated with a mixture of methanol and chloroform. The precipitates were dissolved in 6 M urea, diluted with 100 mM Tris-HCl (pH 8.0) and digested with tosylphenylalanyl chloromethyl ketone-treated trypsin at an enzyme/substrate ratio of 1:100 at 37°C for 16 hours.

The tryptic digests were spiked with stable isotope-labeled internal standard peptides and acidified with formic acid for analysis with the nano LC system or HPLC system, which was connected to an electrospray ionization triple quadrupole mass spectrometer (API5000 or 4000 QTrap (for CYP2B6 and 2C19), AB Sciex, Foster City, CA, USA) operated in positive ionization mode. LC was performed with C18 capillary columns. Linear gradients of 1-45% acetonitrile in 0.1% formic acid were applied to elute the peptides at a flow rate of 100 nl/min for nano LC and 50 μl/min for conventional LC. The mass spectrometer was set up to run a multiplexed-MRM experiment for peptides. The

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ion counts in the chromatograms were determined by using the quantitation procedures in Analyst software (AB Sciex).

One specific peptide was selected for quantification of each target protein, and measured at 4 different MRM transitions. The amount of each peptide was determined as an average of 3 or 4 MRM transitions from one sample. In cases where signal peaks of less than 3 transitions were obtained, the amount of peptide in the sample was as defined as under the limit of quantification. The data of individual donors are presented as the mean ± SEM of the values determined by using the different MRM transitions (Tables S5 and S6). The data of 17 donors are presented as the mean ± SD of the mean values of individual donors.

The absolute expression amount of CYP3A4 was calculated from the quantitative data obtained for a peptide generated from both CYP3A4 and CYP3A43, and subtracting the value obtained for a peptide that is specific for CYP3A43.

Enzyme activity assays. Liver tissue biopsy samples were assayed for CYP activities by *in vitro* incubation of microsomal preparations. Assay details are provided in the supplemental data (Table S1). The substrate concentrations used were as high as possible, depending on the solubility in the assay buffer. The assay conditions, such as protein concentration, substrate concentration and incubation time, were validated to provide linear enzyme kinetics. In general, microsomes were diluted with buffer (100 mM Tris, pH 7.4), magnesium chloride (5 mM), substrate and NADPH (1 mM) at 4°C to a final incubation volume of 200

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µL, and warmed to 37°C in a 96-well temperature-controlled thermocycler (Biometra, Göttingen, Germany). The final solvent concentration (acetonitrile) was ≤1%. Incubations were terminated by heating to 80°C for 2 min, followed by cooling to 4°C. After centrifugation of the incubation mixture (20 min at 10,000 x g), 20 µL aliquots of the supernatants were diluted with 180-480 µL of quench solution (20% methanol or acetonitrile with 0.1% formic acid) containing the stable isotope-labeled metabolite as an internal standard. A 5 µL aliquot of diluted sample was directly injected into the LC-MS/MS system. Standard curve and quality control samples consisted of the same matrix as incubation samples. The following equipment was used for LC-MS/MS: HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), 1100 series binary pump and degasser (Agilent, Waldbronn, Germany), column switch thermostat Spark 880 (MayLab, Vienna, Austria), 4000 QTrap (AB Sciex) equipped with an orthogonal Turbo VTM ion source operated in negative or positive electrospray ionisation mode. Chromatographic separation of incubation samples was performed on YMC-Pack ProC18, 50 × 2.1 mm, 5 µm (YMC Europe, Dinslaken, Germany) and X-Terra MS C18, 50 × 2.1 mm, 5 µm (Waters, Eschborn, Germany) analytical columns using validated methods. Integration of chromatograms as well as determination of peak areas was performed by Analyst software version 1.4.2 (AB Sciex). Formation of 11- and 12-hydroxylauric acid was monitored using HPLC on a X-Terra RP C18, 150 × 2.1 mm, 5 µm (Waters, Eschborn, Germany) analytical column, with a radioactivity detector (PerkinElmer, Dreieich, Germany). A 20 µL aliquot of the incubation supernatant was directly injected into the HPLC system. Integration of the chromatograms as well as determination of the peak

areas was done by Chromeleon software (Dionex, Idstein, Germany). For additional calculations, data were transferred to Microsoft® Excel 2007.

TaqMan RT-PCR Gene Expression Analysis. Total RNA from liver tissue was isolated using the RNeasy (Qiagen, Hilden, Germany) protocol. Yield of extracted RNA was evaluated spectrophotometrically at 260 nm. Purity and integrity of RNA were assessed using a RNA 6000 LabChip with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). For first-strand cDNA synthesis, 200 ng of total RNA was reverse-transcribed in a final volume of 20 µL following the protocol for the High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). cDNA samples were diluted to 60 µL with water and stored at -20 °C. Volumes of 1 µL of diluted cDNA were used for quantitative real-time PCR. CYP and UGT analyses were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems), transporter analyses was performed on an iQ5 Real Time PCR System (BioRad, Hercules, CA). Assays were performed as technical triplicates. Inventoried assays as well as primer and probe sequences used in this study are provided as supplemental data (Tables S3 and S4).

Data Normalization and Analysis. Gene expression data were normalized to β-actin as an endogenous control in the same sample (duplexed RT-PCR), or on the same plate for inventoried assays. Relative expression differences were calculated using the comparative ΔΔCt method, taking account of the actual PCR efficiency for each target and run.

Results

Protein expression levels of drug-metabolizing enzymes in human liver microsomal fraction.

Protein expression levels of 13 CYP enzymes, P450R and 6 UGT enzymes were measured in human liver microsomal fractions from 17 donors (Table 1). As shown in Table 2, CYP enzymes could be quantified in liver microsomes from all 17 donors, except for CYP3A43 (n=14) and CYP3A7 (n=13). CYP2C9, CYP2E1, CYP3A4 and CYP2A6 showed the highest mean expression levels of more than 50 pmol/mg protein.

CYP3A4 exhibited the greatest inter-individual differences in protein expression level, with a 43.4-fold difference between the donors with the highest and lowest levels. CYP3A4 was expressed at high levels in donors No.16, 1, 10 and 17 at 270, 202, 142 and 94.9 pmol/mg protein, respectively, while other samples showed levels in the range from 6.22 to 42.0 pmol/mg protein. High variation in protein expression (more than 10-fold) was observed for CYP2A6, CYP1A2, CYP2C8 and CYP2C19. CYP2C19 showed high expression in donors No. 1, 9 and 3 at 22.2, 12.9 and 10.1 pmol/mg protein, respectively, while the levels in other samples ranged from 2.08 to 4.31 pmol/mg protein. CYP3A5 showed high expression in only a single sample (No. 7, 17.1 pmol/mg protein), while it showed lower expression in the narrow range of 2.48 to 3.61 pmol/mg protein in the other 16 samples.

Among the UGT enzymes that were analyzed, the highest mean expression level was detected for UGT1A6, which also showed the highest

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inter-individual variability of 6.2-fold. The other 5 UGT enzymes, 1A1, 1A3, 1A9, 2B7 and 2B15, were detected at more than 10 pmol/mg protein on average. UGT1A4 was below the limit of quantification (0.5 pmol/mg protein) in all samples.

Except for CYP3A7, no significant correlation was observed between protein expression levels of enzymes and the age of liver tissue donors (data not shown). As shown in Fig. 1, one group of donors (No. 1, 2, 8, 9, 10, 11, 14, 16 and 17) showed an age-dependent increase of CYP3A7 protein expression with a correlation coefficient of r^2=0.973 by least-squares regression analysis, but the rest of the donors showed low and age-independent CYP3A7 expression.

No significant difference in any enzyme level was observed between smoking and non-smoking groups. Significant gender difference ($p<0.05$) was detected only in the expression of CYP3A43: 5.04 ± 0.62 pmol/mg protein in the female group (n=9) and 3.04 ± 2.52 pmol/mg protein in the male group (n=9). However, the mean CYP3A43 concentration in the group of female tissue donors was only 1.7-fold greater than that in male group.

Correlation of protein expression to mRNA expression of drug-metabolizing enzymes.

Protein expression levels of 12 CYPs and 5 UGTs were compared with mRNA expression levels (Table 3). For CYP3A4, CYP2B6, CYP2C8 and UGT1A6, a high correlation was observed with r^2 values of more than 0.6 (in particular, r^2 for CYP3A4 was 0.944). Many CYP enzymes that showed a high-to-moderate correlation between protein and mRNA quantities, such as 3A4, 2C8, 2C19 and

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3A7, exhibited relatively similar 1/slope values from 26.2 to 57.1, suggesting similar protein-to-mRNA expression ratios among these enzymes. Fig. 2 summarizes the data of individual samples and suggests a relatively narrow expression range of proteins, but a wider expression range of mRNAs. For example, the mRNA level of CYP2B6 varied by over 3 orders of magnitude, whereas a range of only 3.7-fold was observed for protein concentration (Fig 2A). There was a higher correlation between protein and mRNA expressions of CYP2B6 in the range of higher protein expression, but a lower correlation in the range of low protein expression, <7 pmol/mg protein (Table 3 and Fig. 2A).

Correlation of protein and mRNA expression levels with enzyme activities of CYP enzymes.

Enzyme activities of 9 CYP enzymes were measured using human liver microsomes and specific probe substrates (see supplemental materials). The enzyme activities (for absolute activity data, see supplementary materials) are compared with protein and mRNA expression levels in Figs. 3, 4 and Table 4. Except for CYP2B6, there was a better correlation of enzyme activity to protein expression than to mRNA expression (Table 4). In the case of CYP2B6, mRNA expression correlated slightly better to enzyme activity than it did to protein expression. The activities of CYP3A4 as determined with three different probe substrates, viz. testosterone, midazolam and nifedipine, showed consistently high correlations to both protein and mRNA levels. However, in the low expression range, protein expression showed a better correlation to activity than mRNA expression (Fig. 4 bottom). Activity of CYP4A11, measured in terms of

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lauric acid 12-hydroxylation, was not correlated with either protein or mRNA level. Two distinct patterns of activity-to-protein correlation were observed. In the cases of CYP1A2, CYP2C8 and CYP3A4, the correlations are represented by lines with a y-axis intercept that is close to the origin, whereas a y-intercept clearly greater than zero was seen in the cases of CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1.

Protein expression levels of transporters in plasma membrane fraction of human liver tissue.

Protein expression levels of 30 transporters and 2 membrane marker proteins were assessed. As shown in Table 5, 22 transporters and 2 membrane marker proteins could be quantified, while the protein concentrations of 8 transporters, ABCA2, ABCG8, OATP1C1, OATP4C1, CNT3, PEPT1, OSTα and OSTβ, were below the limit of quantification (< 0.1-0.6 pmol/mg protein). The highest average expression levels, which exceeded 5 pmol/mg protein, were detected for OCT1, ABCA6 and NTCP. The largest inter-individual differences among the 17 samples were observed for MDR3 (8.4-fold) and OCT1 (5.8-fold).

Among the group of canalicular-localized transporters, the mean expression levels of all detected transporters except BCRP were similar at 1.07-1.88 pmol/mg protein. In contrast, BCRP expression was only one-third to half of those of the other canalicular transporters.

With regard to the group of sinusoidal membrane transporters, 4 MRPs and 3 OATPs were detected. MRP1 and MRP4 were quantifiable in only 15 and 3 samples out of 17, respectively. In the 3 samples for which MRP4 could be

quantified, a much lower expression of MRP4 was observed compared to the other MRP transporters. OATP1B3 could be quantified in all 17 samples, whereas OATP1B1 and 2B1 were detected in only 8 and 5 samples, respectively. The levels in quantifiable samples were 5.82 ± 3.20 pmol/mg protein (n=8) for OATP1B1, and 1.58 ± 0.93 pmol/mg protein (n=5) for OATP2B1, indicating higher overall expression and larger variability of OATP1B1.

Neither significant age dependence nor gender difference in expression was observed for the quantified transporters. MRP1, OATP1B3 and CNT1 expression levels were significantly elevated in smokers (n=4) versus non-smokers (n=6), namely 4.43 ± 1.24 pmol/mg protein in smokers and 1.81 ± 1.23 pmol/mg protein in non-smokers for MRP1 ($p<0.05$), 2.22 ± 0.25 pmol/mg protein and 1.69 ± 0.31 pmol/mg protein for OATP1B3 ($p<0.05$) and 0.905 ± 0.117 pmol/mg protein and 0.735 ± 0.111 pmol/mg protein for CNT1 ($p<0.05$).

Correlation of protein expression to mRNA expression of transporters.

Table 6 shows the correlation of protein expression levels of 12 transporters with their mRNA levels. Only OATP1B1 exhibited a substantial correlation between protein and mRNA with an r^2 value of 0.727. This was also reflected by the 1/slope value of 59.3, which is similar to that found for CYP3A4. As for the CYP enzymes, Fig. 5 shows a narrower range of protein concentrations compared to the larger range of mRNA expression levels. NTCP for example showed a 3 orders of magnitude variation in mRNA expression among donors, but only a 2.8-fold range of protein concentration (Fig. 5B ◊).

Discussion

In the present study, we used LC-MS/MS to measure the absolute protein expression levels of 20 enzymes, including 13 CYPs and 6 UGTs, in microsomal fraction, and 22 transporters in plasma membrane fraction from 17 human liver biopsy samples. CYP enzyme activities, assessed by the use of specific probe substrates, were well correlated with the CYP protein levels, except for CYP4A11 (Table 4). On the other hand, mRNA expression levels were less well correlated with enzymatic activities, except in the cases of CYP2B6 and CYP3A4, for which protein and mRNA expression levels were both highly correlated with functional activity. The present results clearly support the view that protein expression data are to be preferred over mRNA data for predicting the drug-metabolizing activity of CYPs in liver tissue.

CYP3A4 activity was correlated well with both protein and mRNA levels (Tables 3 and 4). These data suggest that mRNA expression of CYP3A4 can serve as a surrogate of enzymatic activity. However, in the lower range of expression, mRNA levels of CYP3A4 were less well correlated with activity (Fig. 4). Studies conducted at other labs have reported conflicting results on the correlation between mRNA expression and activity of CYP3A4 (Sumida et al., 1999; Rodriguez-Antona et al., 2001; Sy et al., 2002). These apparent contradictions may be explained by the expression-range-dependent correlation found here. Rodriguez-Antona et al found a poor correlation between protein and mRNA expressions (r^2=0.52) compared to the present result (r^2=0.944). However, the range of CYP3A4 protein expression levels in their case was below 80 pmol/mg protein, which corresponds to the lower range shown in Fig 4.

Protein expression levels are determined not only by the level of transcription, but also by post-transcriptional processes, such as translation, translocation, protein stabilization and degradation, etc.. Therefore, it is conceivable that the protein expression level of CYP3A4 in microsomes could be predominantly controlled by post-transcriptional processes in the low-expression range, whereas in the high-expression range, transcription becomes the major factor.

The protein and mRNA expression levels of CYP2B6 also showed high correlations with activity, though the activity-to-protein expression regression line gave a y-intercept of nearly 5 pmol/mg protein (Table 4 and Fig. 2). CYP2C9, 2D6 and 2E1 also showed highly correlated regression lines with a marked y-intercept. One of the possible explanations for this is the existence of non-active forms of the enzymes, such as apo-enzyme and/or inactivated enzyme. Hanna et al. reported that a band with lower molecular weight than the main band was consistently detected with anti-CYP2B6 antibody in all human liver microsome samples examined, and they suggested it is likely to be non-active CYP2B6 (Hanna et al., 2000).

Relatively poor correlations of activity to protein and mRNA expression levels were observed for CYP4A11 (Table 4). Several possible reasons can be considered. One is lack of specificity of lauric acid 12-hydroxylation for CYP4A11. Castle et al. reported that additional CYP forms (e.g. CYP3A4) may catalyze the hydroxylation of lauric acid at the 12-carbon atom (Castle et al., 1995). Another possibility is instability of CYP4A11 activity and/or protein during microsome preparation and storage, since pronounced effects of storage on lauric acid 12-hydroxylation activity have been observed (Pearce et al., 1996).

To our knowledge, the present study is the first to examine the protein-mRNA correlation of multiple drug transporters expressed in human liver (Table 6 and Fig. 5). Except for OATP1B1, protein and mRNA expression levels of the investigated transporters were not well correlated. This finding is in contrast to CYP enzymes, and may be explained by the prominent role of post-transcriptional processes and intracellular trafficking in controlling the protein levels of transporters in the plasma membrane (Marinelli et al., 2005; Gu and Manautou, 2010). Therefore, mRNA expression cannot be considered as a suitable surrogate for expression levels of transmembrane proteins in plasma membranes, and indeed, may be highly misleading. Since transmembrane proteins are partly localized to intracellular membranes, it remains possible that mRNA levels would correlate to their protein levels measured in whole tissue lysates instead of plasma membrane fractions.

Although the number of samples examined was limited, the present study uncovered several interesting correlations of protein expression levels to donor status. The protein expression of CYP3A7 was highly correlated to donors' age in one group (Fig. 1). CYP3A7 is a major CYP3A enzymes in human fetal and neonatal liver, though it has been reported that high mRNA expression of CYP3A7 was found in about 10% of human liver samples (Burk et al., 2002; Stevens et al., 2003). This result raised the possibility that CYP3A7 protein levels can be classified in high and low protein expression phenotypes, and the high expression phenotype may be manifested with increasing age. Further studies with larger numbers of donors would be necessary to evaluate age-dependent changes of CYP3A7 protein expression. The absolute

expression profile of transporters revealed significant differences in the expression of MRP1, OATP1B3 and CNT1 between smokers and non-smokers. The difference in the case of MRP1 was as high as 2.45-fold (Table 5). Since MRP1 is the most abundant MRP family member expressed at the sinusoidal membrane in human liver, the mechanism of its induction by smoking and its influence on hepatic metabolism would be worth studying in the future.

UGT1A4 and ABCG8 protein were reported to be expressed in human liver (Klett et al., 2004; Izukawa et al., 2009; Uchihashi et al., 2011). However, these molecules were not quantifiable in the present study (Tables 2 and 5). One possible explanation is that protein expression of these molecules was not high enough to be detectable by the present LC-MS/MS-based protein quantification, while antibody-based assays have higher sensitivity. This possibility is supported by the result that ABCG8 protein was quantified in mouse liver using the same peptide as used in the present study for human liver (Kamiie et al., 2008). In the case of UGT1A4, another possibility can be considered; the N-terminal 28 amino acids of UGT1A4 may serve as a signal peptide according to the UniProt database, and the peptide used for quantification is located in this region (from 4 to 11). Protein sequencing of purified rabbit liver UGT gave the N-terminal amino acid sequence of rabbit UGT2B16 corresponding to that just after the signal sequence (Li et al., 1997). Therefore, it is possible that the signal sequence in UGT is removed in human liver microsomes, so that UGT1A4 lacks the sequence used for quantification.

Differences in the quality of liver samples and in the sample preparation procedures may also affect quantitation results. The absolute amount profile of

DMD Fast Forward. Published on October 12, 2011 as DOI: 10.1124/dmd.111.042259
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CYP enzymes in Table 2 matches that of commercial pooled human liver microsomes previously quantified by LC-MS/MS (Kawakami et al., 2011), and this result suggests that the microsomal fractions used in this study were of comparable quality to the commercial products. Furthermore, expression of Na^+/K^+-ATPase is widely used for normalizing protein expression (Tables 2 and 5). The maximum differences in Na^+/K^+-ATPase were 2.1-fold in microsomal fraction and 3.3-fold in plasma membrane fraction. Therefore, the influence of sample condition and preparation procedures on the observed protein expression is likely to lie within the range of variation of Na^+/K^+-ATPase expression. However, the average expression level of Na^+/K^+-ATPase in plasma membrane was less than that in microsomal fraction, even though Na^+/K^+-ATPase was expected to be concentrated in plasma membrane. It has been reported that Na^+/K^+-ATPase is internalized from plasma membrane of rat cholangiocytes under conditions of ATP depletion (Doctor et al., 2000). It is also expected that Na^+/K^+-ATPase would be internalized into hepatocytes during the processes of collection and storage, resulting in an apparently lower expression level in plasma membrane fraction. Although the above report demonstrated that ATP depletion did not affect SGLT1 expression on the plasma membrane, an influence of sample condition on absolute amounts cannot be ruled out in the case of human tissue samples, and comparisons should be done among human samples prepared and examined under conditions as consistent as possible.

In conclusion, the present study showed that LC-MS/MS quantification of drug-metabolizing enzymes and transporters can provide information about expression levels, variability of expression and relationships of protein

expression to mRNA expression and functional activity. Quantitative data on enzyme and transporter expression are not only indispensable for advanced physiological pharmacokinetic modeling, but also may serve as a bridge between in vitro test systems and the intact organ. This study is only a first step in creating a broader database of enzyme and transporter expression levels. It needs to be expanded in several dimensions, e.g., by including additional molecular entities, increasing the number of biological samples, and examining internal and external influences on expression levels.

Acknowledgements

We thank Monika Schuster and Thomas Link for enzyme activity assessments, Ikue Kitahara for assistance with mRNA analysis and Takashi Igarashi for collaborative support.

Authorship contributions

Participated in research design: Ohtsuki, Schaefer, Schwellinger, Ishiguro, and Terasaki.

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Contributed new reagents or analytic tools: Kawakami, and Inoue.

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Wrote or contributed to the writing of the manuscript: Ohtsuki, Schaefer, Ebner, Ludwig-Schwellinger, and Terasaki.

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Footnotes

This study was supported in part by grants for Development of Creative Technology Seeds Supporting Program for Creating University Ventures from Japan Science and Technology Agency (JST); Strategy Promotion of Innovative Research and Development from JST; the Industrial Technology Research Grant Program from New Energy and the Industrial Technology Development Organization (NEDO) of Japan.

The method of quantifying membrane protein by using a mass spectrometer is patented: US Patent 7,901,942 B2 (08 March, 2011) and Japanese patent 4,670,060 (28 January, 2011).

DMD Fast Forward. Published on October 12, 2011 as DOI: 10.1124/dmd.111.042259
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Legends for Figures

Figure 1 Age-dependent increase of CYP3A7 protein expression in human liver microsomal fraction.

Age-dependent increase of CYP3A7 protein expression of 9 donors (closed circles, No. 1, 2, 8, 9, 10, 11, 14, 16 and 17). Open circles, the lower expression of 4 donors did not correlate with age (No. 5, 6, 7 and 15). Closed triangles, the data were under the limit of quantification (No. 3, 4, 12, and 13). Each data point represents the mean ± SEM of the values determined by using 2-4 different MRM transitions.

Figure 2 Comparison between protein and mRNA expression levels of CYPs and UGTs.

A. High-correlated CYP enzymes; B. Moderately correlated CYP enzymes; C. Poorly correlated CYP enzymes; D. UGT enzymes.

mRNA expression levels were quantified by quantitative RT-PCR as described in materials and methods, and expressed as relative amounts to β-actin. Each data point represents the mean ± SEM of the values determined by using 2-4 different MRM transitions for protein quantification and by 3 experiments for mRNA quantification.

Figure 3 Correlation of enzyme activity with protein or mRNA expression of CYP enzymes.

DMD Fast Forward. Published on October 12, 2011 as DOI: 10.1124/dmd.111.042259
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Closed circle and solid line, activity-protein expression correlation; open square and dot line, activity-mRNA expression correlation. Enzyme activities of CYP2E1 and CYP4A11 were determined in terms of activity for 11-hydroxylation and 12-hydroxylation of lauric acid, respectively. The correlation coefficients (r^2) are summarized in Table 4. Each data point represents the mean ± SEM of the values determined by using 3-4 different MRM transitions for protein quantification and by 3 experiments for activity assay and mRNA quantification.

Figure 4 Correlation of enzyme activity to protein and mRNA expression levels of CYP3A4.

Closed circle and solid line, activity-protein expression correlation; open square and dot line, activity-mRNA expression correlation. The plots of 3A4-1, 3A4-2 and 3A4-3 show the activities measured using testosterone, midazolam and nifedipine, respectively, as shown in Table 4. The bottom three plots are those in lower ranges corresponding to the upper plots. The correlation coefficients (r^2) are summarized in Table 4. Each data point represents the mean ± SEM of the values determined by using 3-4 different MRM transitions for protein quantification and by 3 experiments for activity assay and mRNA quantification.

Figure 5 Comparison between protein and mRNA expression levels of transporters.

A. Canalicular-localized and localization-unknown transporters; B. Sinusoidal-localized transporters.

mRNA expression levels were quantified by quantitative RT-PCR as described in

materials and methods, and expressed as relative amounts to β-actin.

Each data point represents mean ± SEM of the values determined by using 3-4 different MRM transitions for protein quantification and by 3 experiments for mRNA quantification.

Tables

Table 1. Donor demographics of liver tissue biopsies

No.	Sex	Age (yr)	Ethnicity	Cause of death	Smoker	Drug history
1	M	24	C	Trauma (head and chest) following road traffic accident	No	None
2	M	29	C	Anoxia	Unknown	Unknown
3	M	38	C	Subarachnoid hemorrhage	Yes	None
4	M	48	C	Stroke	Yes	None
5	M	49	C	Head trauma	Unknown	Unknown
6	M	54	C	Intracranial hemorrhage	No	None
7	M	56	C	Cerebral hypoxia post cardiac arrest	Yes	Thyroxin/ phospinax/ vitamin D/ calcium
8	M	74	C	Cerebrovascular accident	Unknown	Unknown
9	F	20	C	Head injury	No	None
10	F	27	C	Head trauma	Unknown	Unknown
11	F	43	C	Subarachnoid hemorrhage	No	None
12	F	47	C	Cerebral Infarct	No	Labetalol/ acyclovir/ cefotaxime
13	F	49	C	Subarachnoid hemorrhage	Yes	Occasional steroids /noradrenaline/ T3/ insulin
14	F	54	A	Brain stem bleed	No	Frusemide/ atenalol/ lisinopril
15	F	54	C	Cerebrovascular accident	Unknown	Unknown
16	F	63	C	Subarachnoid hemorrhage	Unknown	Anti-hypertensive
17	F	65	C	Cerebrovascular accident	Unknown	Unknown

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Table 2. Protein expression levels of drug-metabolizing enzymes in human liver microsomal fraction.

	No. of quantified donors	Protein amount (pmol/mg protein)				
		Mean	SD	MAX	min	Max/min
CYP2C9	17	76.3	26.6	115	40.2	2.9
CYP2E1	17	66.1	27.4	147	36.3	4.0
CYP3A4	17	60.4	75.0	270	6.22	43.4
CYP2A6	17	56.2	45.0	168	5.45	30.8
CYP2C8	17	30.0	22.6	83.5	5.66	14.8
CYP1A2	17	25.1	21.2	65.5	3.26	20.1
CYP4A11	17	21.8	8.7	46.5	9.29	5.0
CYP2D6	17	17.2	8.4	36.4	6.16	5.9
CYP2B6	17	6.72	3.19	14.9	4.05	3.7
CYP2C19	17	5.31	5.20	22.2	2.08	10.7
CYP3A43	14	4.10	2.01	6.42	<LOQ	-
CYP3A5	17	3.86	3.43	17.1	2.48	6.9
CYP3A7	13	2.40	3.09	9.39	<LOQ	-
P450 R	17	71.6	17.0	99.0	41.7	2.4
UGT1A6	17	114	54	277	45.0	6.2
UGT2B7	17	84.3	29.5	146	53.1	2.8
UGT2B15	17	61.8	21.5	103	24.2	4.3
UGT1A1	17	33.2	11.5	59.7	20.8	2.9
UGT1A9	17	25.9	6.3	38.0	15.5	2.5
UGT1A3	17	17.3	7.8	37.1	8.16	4.5
Na^+/K^+-ATPase	17	12.1	2.3	15.8	7.42	2.1

The expression of enzymes was determined in microsomal fraction of human liver tissues from 17 donors. Each value represents the mean ± SD (n=17). When the quantification value was under the limit of quantification, the value was taken as 0 pmol/mg protein for calculating the mean value of each protein. Amounts of CYP3A4 were determined by using the equation shown in Experimental Procedures. <LOQ, under the lower limit of quantification. UGT1A4 was under the limit of quantification in all samples.

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Table 3. Correlation between protein and mRNA expression levels of drug-metabolizing enzymes.

	1/Slope	r^2
High-correlation		
CYP3A4	45.5	0.944
CYP2B6	3.75	0.702
CYP2C8	26.2	0.649
Medium-correlation		
CYP2C19	45.7	0.586
CYP2D6	151	0.521
CYP3A7	57.1	0.487
CYP3A5	84.9	0.441
Low-correlation		
CYP1A2	58.0	0.163
CYP2C9	72.0	0.114
CYP2A6	-	<0.100
CYP2E1	-	<0.100
CYP4A11	-	<0.100
UGT1A6	550	0.622
UGT1A9	106	0.309
UGT2B7	58.5	0.282
UGT2B15	905	0.250
UGT1A1	-	<0.100

Least-squares regression analysis was performed for protein expression levels (x-values, pmol/mg protein) against mRNA expression levels (y-values, relative amount to β-actin). Data points are shown in Fig. 2. 1/Slope, reciprocal slope of regression line; r^2, correlation coefficient of regression line.

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Table 4. Correlation of enzyme activity to protein level or mRNA expression level of drug-metabolizing enzymes.

		r^2	
Enzyme	Probe substrate	Protein	mRNA
CYP1A2	Phenacetin	0.679	0.400
CYP2B6	Bupropion	0.849	0.904
CYP2C8	Amodiaquine	0.800	0.751
CYP2C9	Diclofenac	0.625	0.261
CYP2C19	S-Mephenytoin	0.952	0.473
CYP2D6	Dextromethorphan	0.778	0.499
CYP2E1	Lauric acid	0.778	0.499
CYP4A11	Lauric acid	0.261	<0.100
CYP3A4-1	Testosterone	0.969	0.963
CYP3A4-2	Midazolam	0.952	0.862
CYP3A4-3	Nifedipine	0.978	0.911

Least-squares regression analysis was done for protein or mRNA expression levels (x-values) against enzyme activity (y-values). Enzyme activity of CYP2E1 and CYP4A11 was evaluated in terms of 11-hydroxylation and 12-hydroxylation of lauric acid, respectively. Data points and regression lines are shown in Fig. 3. r^2, correlation coefficient of regression line.

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Table 5. Protein expression levels of transporters in human liver plasma membrane fraction.

	No. of quantified donors	Protein amount (pmol/mg protein)				
		Mean	SD	MAX	min	MAX/min
Canalicular localized						
MDR3	17	1.88	1.16	4.75	0.567	8.38
MDR1	17	1.50	0.44	2.35	0.964	2.44
BSEP	17	1.48	0.44	2.23	0.636	3.50
MRP2	16	1.46	0.65	2.51	<LOQ	
MATE1	16	1.07	0.35	1.65	<LOQ	
BCRP	14	0.419	0.219	0.698	<LOQ	
γ-gtp	17	3.95	1.19	6.09	1.79	3.41
Sinusoidal localized						
OCT1	17	7.35	3.26	14.6	2.53	5.78
NTCP	17	5.54	1.67	10.1	3.62	2.79
OATP1B1	8	2.74	3.67	12.3	<LOQ	
MRP1	15	2.53	1.62	5.86	<LOQ	
MRP6	17	2.24	0.69	3.59	1.11	3.25
OAT2	17	1.91	0.58	3.11	0.928	3.35
OATP1B3	17	1.70	0.45	2.51	0.967	2.59
MRP3	17	1.00	0.48	2.73	0.610	4.47
OATP2B1	5	0.463	0.872	3.18	<LOQ	
MRP4	3	0.0681	0.1659	0.605	<LOQ	
Na^+/K^+ ATPase	17	7.34	1.77	11.1	3.35	3.30
Localization unknown						
ABCA6	17	7.23	2.84	12.4	3.85	3.22
MCT1	17	1.63	0.60	3.38	0.811	4.17
ENT1	17	1.37	0.30	1.93	0.888	2.17
ABCA8	16	1.28	0.53	2.19	<LOQ	
CNT1	17	0.842	0.277	1.80	0.597	3.02
MRP5	3	0.361	0.807	2.18	<LOQ	

The expression of transporters was determined using the plasma membrane fraction of human liver tissue from n=17 donors. Data represents mean ± SD. In the case of <LOQ, a value of 0 pmol/mg protein was taken for calculation of the mean. <LOQ, under the lower limit of quantification.

DMD Fast Forward. Published on October 12, 2011 as DOI: 10.1124/dmd.111.042259
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Table 6. Correlation between protein and mRNA expression levels of transporters.

	1/Slope	r^2
Canalicular localized		
MRP2	25.9	0.223
MDR1	131.1	0.185
BSEP	-	<0.100
MATE1	-	<0.100
BCRP	-	<0.100
Sinusoidal localized		
OATP1B1	59.3	0.727
OATP2B1	-21.2	0.380
MRP1	-2,400	0.246
OCT1	-	<0.100
NTCP	-	<0.100
OATP1B3	-	<0.100
Localization unknown		
ABCA8	-	<0.100

Least-squares regression analysis was done for protein expression levels (x-values, pmol/mg protein) against mRNA expression levels (y-values, relative amount to β-actin); each data point is shown in Fig. 4. 1/Slope, the reciprocal number of slope of regression line; r^2, correlation coefficient of regression line.

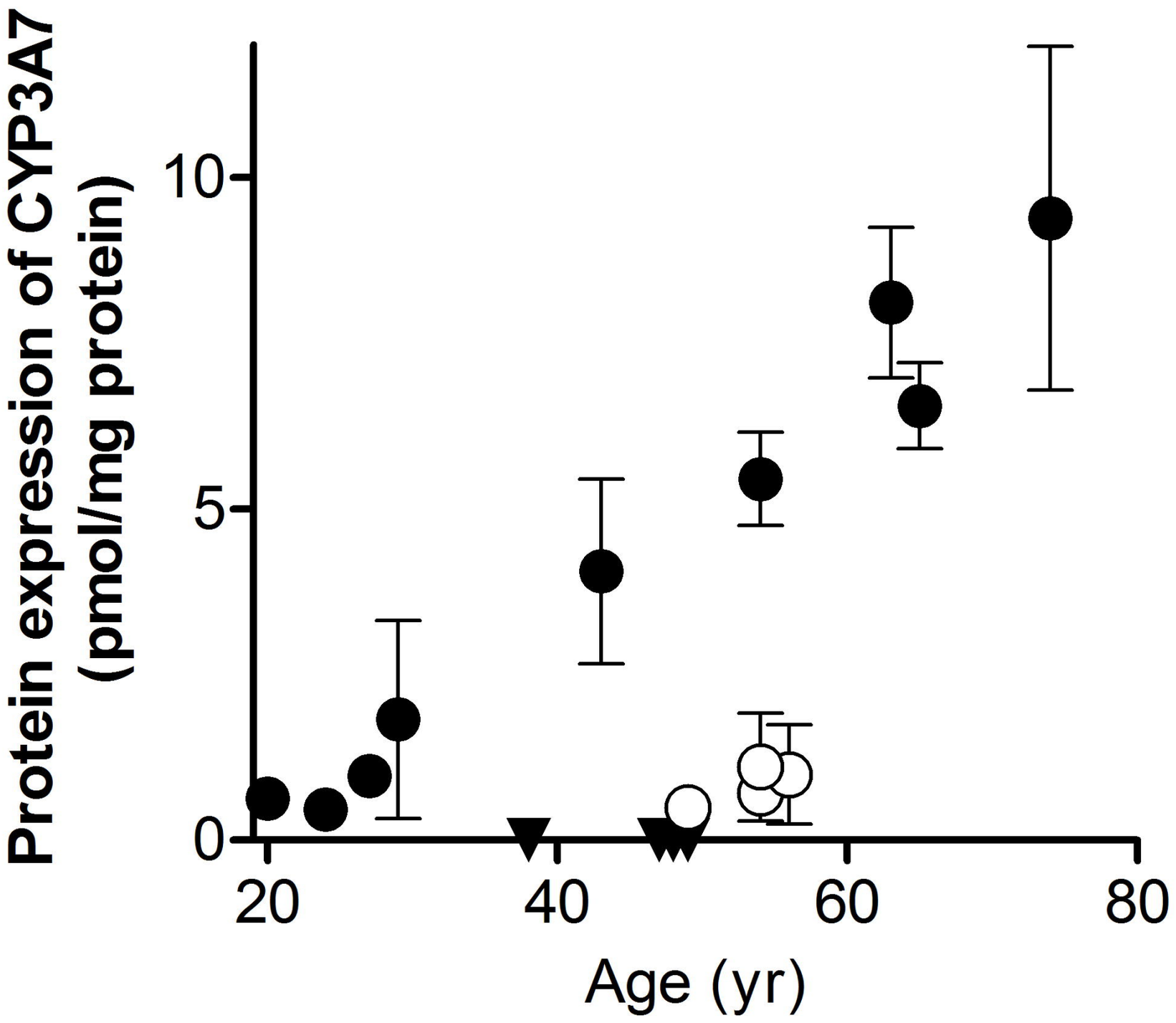
Protein expression of CYP3A7
(pmol/mg protein)
10
5
0
20
40
60
80
Age (yr)

Figure 1

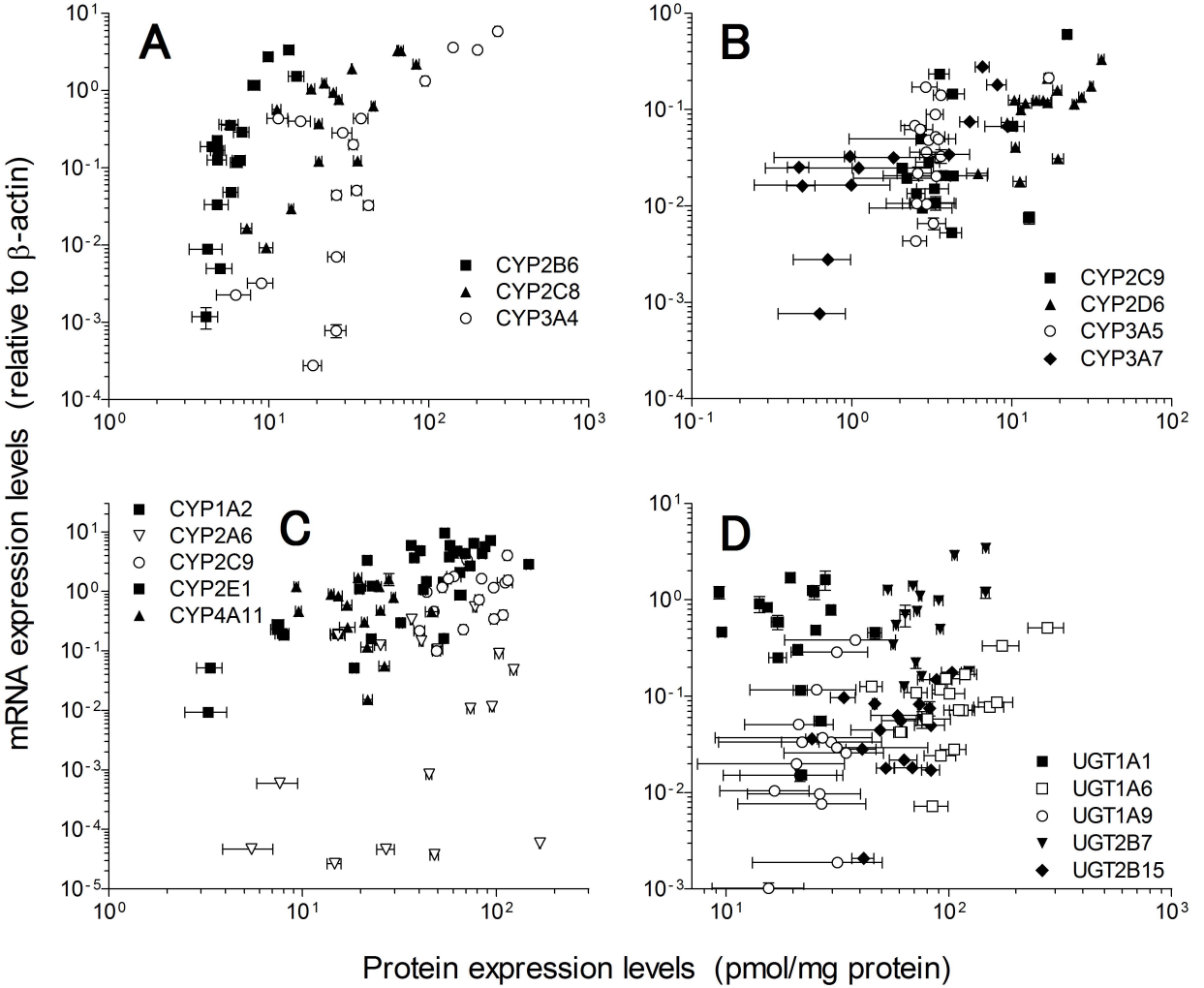
A
CYP2B6
CYP2C8
CYP3A4
B
CYP2C9
CYP2D6
CYP3A5
CYP3A7
C
CYP1A2
CYP2A6
CYP2C9
CYP2E1
CYP4A11
D
UGT1A1
UGT1A6
UGT1A9
UGT2B7
UGT2B15
mRNA expression levels (relative to β-actin)
Protein expression levels (pmol/mg protein)

Figure 2

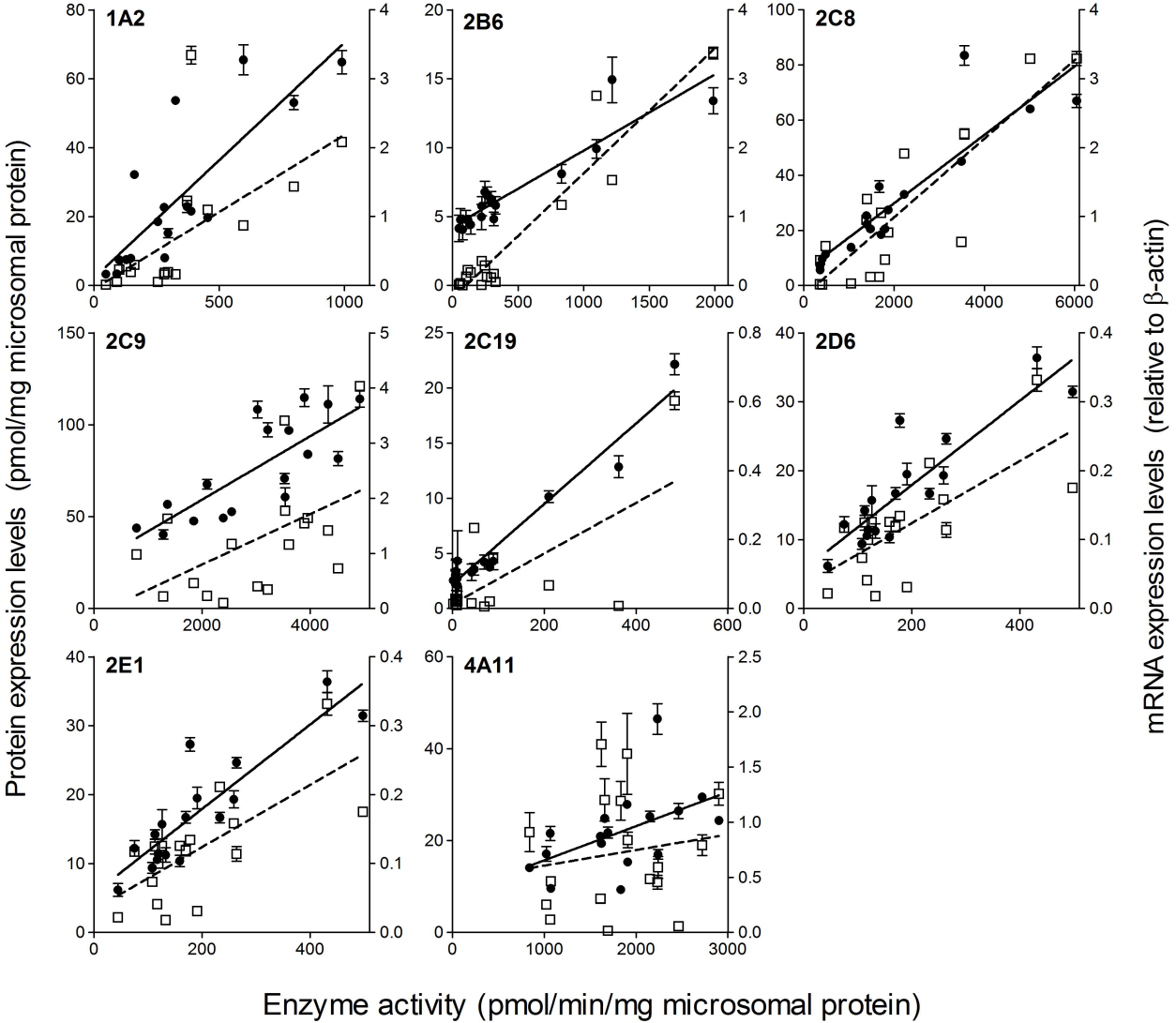
1A2
2B6
2C8
2C9
2C19
2D6
2E1
4A11
Protein expression levels (pmol/mg microsomal protein)
mRNA expression levels (relative to β-actin)
Enzyme activity (pmol/min/mg microsomal protein)

Figure 3

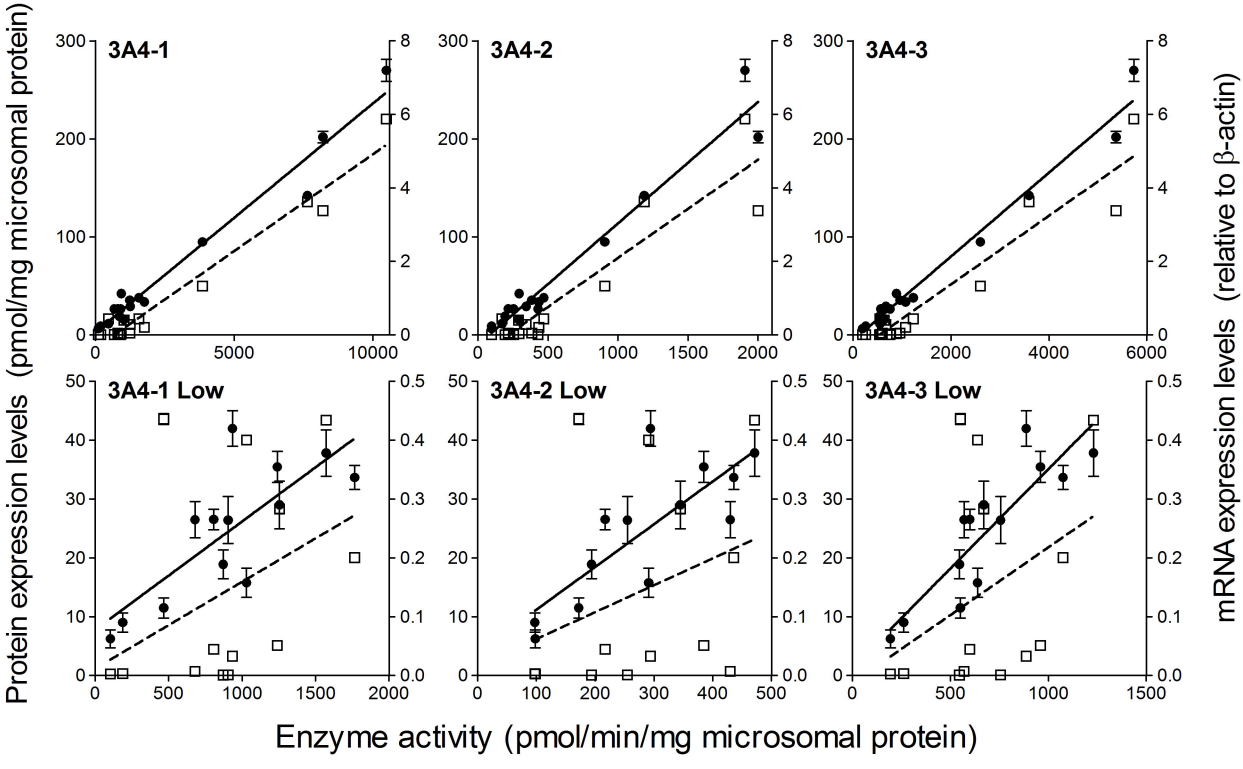
3A4-1
3A4-2
3A4-3
3A4-1 Low
3A4-2 Low
3A4-3 Low
Protein expression levels (pmol/mg microsomal protein)
mRNA expression levels (relative to β-actin)
Enzyme activity (pmol/min/mg microsomal protein)

Figure 4

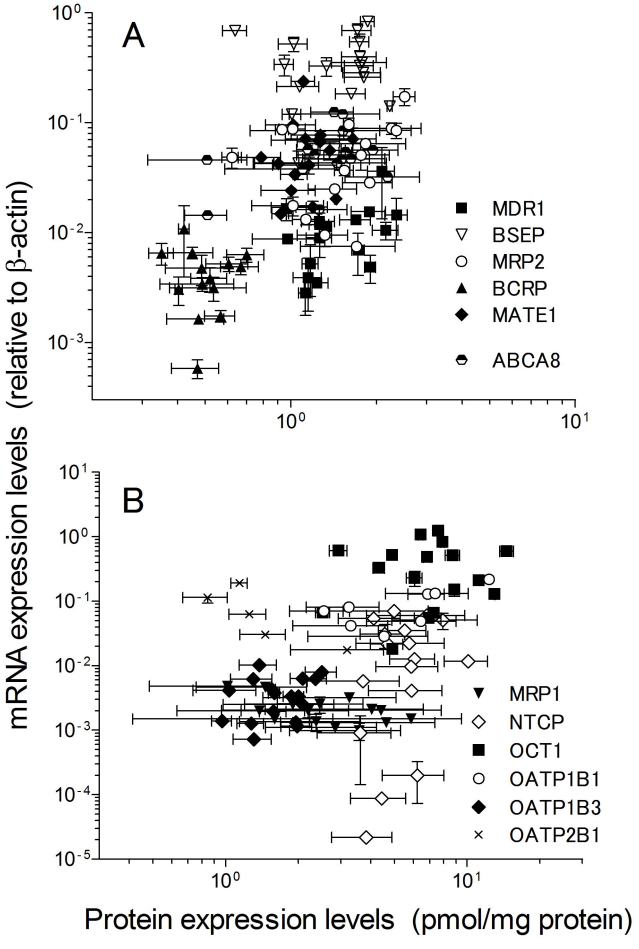
A
B
MDR1
BSEP
MRP2
BCRP
MATE1
ABCA8
MRP1
NTCP
OCT1
OATP1B1
OATP1B3
OATP2B1
mRNA expression levels (relative to β-actin)
Protein expression levels (pmol/mg protein)

Figure 5