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Direct Quantification of Cytochromes P450 and Drug Transporters – A Rapid, Targeted Mass Spectrometry-Based Immunoassay Panel for Tissues and Cell Culture Lysates

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Abbreviations: AhR, aryl-hydrocarbon receptor; AR, constitutive androstane receptor; CYP, cytochrome P450; LLOQ - lower limit of quantification; nLC - nano-Liquid Chromatography**;** CMF, crude membrane/nuclei fraction; POR, cytochrome P450 oxidoreductase; PXR, pregnane X receptor; tSIM, targeted Selected Ion Monitoring; TXP, Triple X Proteomics

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Abstract

The quantification of drug metabolizing enzymes and transporters has recently been revolutionized on the basis of targeted proteomic approaches. Isotope-labeled peptides are used as standards for the quantification of the corresponding proteins in enzymatically fragmented samples. However, hurdles in these approaches are low throughput and tedious sample pre-fractionation steps prior to mass spectrometry read-out. We have developed an assay platform using sensitive and selective immunoprecipitation coupled with mass spectrometric read-out allowing the quantification of proteins directly from whole cell lysates using less than 20,000 cells per analysis. Peptide group-specific antibodies (Triple X Proteomics -antibodies) enable the enrichment of proteotypic peptides sharing a common terminus. These antibodies were employed to establish a MS-based immunoassay panel for the quantification of 14 cytochrome P450 enzymes and 9 transporters. We analyzed the cytochrome P450 enzyme and transporter levels in genotyped liver tissue homogenates, microsomes and in samples from a time course induction experiment in human hepatocytes addressing different induction pathways. Since for the analysis of P450 enzymes and transporters only a minute amount of sample is required and no prefractionation is necessary, the assay platform bears the potential to bridge cell culture model experiments and results from whole organ tissue studies.

Introduction

Many xenobiotic compounds can induce their own as well as the elimination of other substances by increasing the abundance of the proteins that catalyze their metabolism and transport (Zanger and Schwab, 2013). Phase I and II enzymes as well as transporter proteins are relevant for drug-drug interactions and objectives for a precise understanding of the drug metabolism system. Of particular importance for drug metabolism in humans are the cytochrome P450 (CYP) isoforms 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4/5, as well as the drug-relevant transporters ABCB1, ABCG2, OATP1B1, OATP1B3, SLC22A2, SLC22A6, and SLC22A8, among others. Current protocols and experimental design of such induction experiments have been described by Chu and others (Chu et al., 2009). These protocols and also the FDA recommend using mRNA as a surrogate for the protein induction, since protein methods like western blots rely on antibodies, which often cannot discriminate between members of the same subfamily (FDA, 2012).

Over the last years mass spectrometry has become a widely used method for the indirect quantification of proteins, by measuring proteotypic peptides in enzymatically fragmented samples as protein surrogates. The quantification of the peptide and indirectly of the protein can be achieved by adding isotope-labeled peptide standards in a defined amount to the digested sample (Gerber et al., 2003; Nature methods, 2013). By referencing the signals derived from the endogenous peptide and the peptide standard, quantification can be achieved. In several studies, this method was applied to analyze drug metabolizing enzymes and transporters from membrane fractions of tissue and cell culture (Oswald et al., 2006; Ohtsuki et al., 2012; Schaefer et al., 2012). The introduction of an immunoprecipitation step using peptide-specific antibodies prior mass spectrometric read-out resulted in better sensitivity and higher through-put (Anderson et al., 2004; Jiang et al., 2007). More recently, we introduced peptide-group specific antibodies (Triple X Proteomics (TXP) antibodies) to this workflow. These antibodies bind short C-terminal sequences of proteotypic tryptic peptides, allowing to enrich peptides with high sequence similarities using just a single antibody for several analytes (Weiss et al., 2015; Groll et al., 2016; Marx-Stoelting et al., 2017). This method was used to

analyze CYP and transporter proteins directly from cell culture or tissue samples without any pre-fractionation steps, like membrane enrichment (Kawakami et al., 2011; Ohtsuki et al., 2012; Schaefer et al., 2012) or SDS PAGE (Langenfeld et al., 2009; Miliotis et al., 2011).

Here, we extended this concept for the analysis of 14 CYP enzymes, 9 transporters, and the cytochrome P450 oxidoreductase (POR) by the use of 13 TXP-antibodies. We demonstrate the suitability of the method for analyses of tissue samples. Moreover, comparison of whole tissue and microsome samples suggests that membrane enrichment as a pre-purification step can be omitted for the analysis of such proteins. The method is semi-automated and highly parallelized allowing a throughput of up to 100 runs per day and mass spectrometer. Most importantly, the method is highly sensitive allowing the analysis to be performed from less than 10 µg protein extract. Since primary hepatocytes are the most precious part of induction experiments, this sensitive method enables the design of experiments in 96-well format, thereby saving costs and material.

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Material and Methods

Total protein. Protein concentrations were determined using a BCA assay kit (Thermo Scientific, Waltham, USA).

Peptide Standards. Isotopically-labeled standard peptides (Intavis, Tübingen, Germany) were quantified by amino acid analysis on a HPLC system (Agilent 1100 Series HPLC Value System, Agilent Technologies, Santa Clara, USA).

Epitope-motif analysis of antibodies - protein digestion. 150 µg total protein generated by lysis of HepG2 cells were diluted in triethanolamine buffer (50 mM, pH 8.5) and 0.5% octyl-β-D-glucopyranoside, reduced by TCEP (5 mM) and denatured by heating up to 99°C for 5 min. Afterwards proteins were alkylated by adding iodoacetamide (10 mM) and incubated for 30 min at RT. Trypsin was added in a ratio of 1:40 (Trypsin:Protein). After 16 h incubation at 37°C the digestion procedure was stopped by heating the samples up to 99°C for 5 min and adding PMSF to a final concentration of 1 mM.

Epitope-motif analysis of antibodies - LC-MS procedure. Specificities of TXP-antibodies were tested by performing immunoprecipitation in triplicates from tryptically digested cell lysate (HepG2). For this step 20 µg digested lysate was incubated with 5 µg antibody. 25 µL protein G-coated magnetic microspheres were used to precipitate peptide antibody complexes. Elution was performed in 20 µL 1% formic acid. 10 µL eluate were separated on an UltiMate 3000 RSLCnano LC system (Thermo Scientific, Waltham, USA). For desalting a PepMap100 µ-precolumn (0.3 mm I.D. x 5 mm, Thermo Scientific, Waltham, USA) and for separation an Acclaim Rapid Separation LC (RSLC) Column (75 µm I.D. x 150 mm, Thermo Scientific, Waltham, USA) was used. The samples were loaded with a buffer containing 98% ddH2O, 2% ACN, and 0.05% TFA onto the column. A linear gradient starting with 4% B and ending after 20 min at 55% B was applied. Mobile phase A was composed of 99.9% ddH2O and 0.1% FA, mobile phase B of 80% acetonitrile with 0.1% formic acid. Sample measurement using a Full-

MS method on a Q Exactive™ Plus (Thermo Scientific, Waltham, USA) and data analysis were performed as described previously (Weiss et al., 2015).

Dynamic range of MS-based immunoassays. Dilution series were prepared in triplicates in analyte-free buffer containing proteolytically fragmented fish gelatin (50 mM Tris-HCl, 150 mM NaCl, and pH 7.4 - Blocking Reagent, Roche Diagnostics, Mannheim, Germany). 100 nM isotopically-labeled peptides (Intavis, Tübingen, Germany) were serially diluted eight times in a ratio of 1:3, while the non-labeled peptides (Intavis, Tübingen, Germany) were kept constant at a concentration of 10 nM. For immunoprecipitation, total peptide amounts ranging from 1000 to 0.15 fmol were used. Immunoprecipitation procedures were performed using 1 µg antibody. After separation by nano-LC (UltiMate 3000 RSLCnano, Thermo Scientific, Waltham, USA), peptide ratios of endogenous und isotopic-labeled peptides were determined using targeted Selected Ion Monitoring (tSIM - QExactive Plus™, Thermo Scientific, Waltham, USA) as described earlier (Gallien et al., 2012).

Immunoprecipitation. Enzymatically fragmented proteins were mixed with TXP-antibodies and stable isotopic standards. After an incubation period of 1 h, peptide-antibody-complexes were precipitated using protein G-coated magnetic microspheres (Dynabeads® Protein G, Life Technologies, Carlsbad, USA) in a magnetic particle processor (KingFisher™, Thermo Scientific, Waltham, USA). Peptide-antibody-microsphere-complexes were washed twice in PBS + 0.3% Chaps and three times in ammonium bicarbonate + 0.3% Chaps. Elution was performed in 20 µL 1% formic acid.

Peptide quantification using nano-liquid chromatography **(nLC) -tSIM.** CYP- and transporter peptides were measured with the same instrumental set-up, including columns and buffer composition, but different LC- methods. 5 µL eluate were separated using step gradients and subsequent washing procedures (UltiMate 3000 RSLCnano, Thermo Scientific, Waltham, USA). CYP peptides were separated with a gradient starting at 10% phase B and ending after 8 min with 35% phase B using a flow rate of 300 nL/min. The column oven temperature was held at 40°C. Including wash procedure the total run time was 18 min. Transporter peptides were separated with a flow rate of 1000 nL/min at 55°C using

a gradient spanning 10% to 25% phase B over 2.75 min. The total run time was 10 min. Peptides were quantified using targeted Selected Ion Monitoring (tSIM - QExactive Plus™, Thermo Scientific, Waltham, USA).

Data analysis. Raw data were processed with Pinpoint 1.4 (Thermo Scientific, Waltham, USA). Peak areas of isotopically-labeled peptides representing known peptide amounts and endogenous signals were set in relation to one another on parent ion level. Influences of non-labeled standard peptide impurities on the correctness of quantification were avoided by defining the LLOQ as either 1% of the spiked standard peptide amount or the lower limit of the working range (SD <20% , recovery:80-120%) whichever is the higher value. Data from different tissue fractions were compared by calculation of Pearson correlation coefficients.

Recombinant proteins - protein digestion. 250 µg of single recombinant protein in triethanolamine buffer (50 mM, pH 8.5) and 0.5% octyl-β-D-glucopyranoside were reduced with TCEP (5 mM) and denatured at 99°C for 5 min. After alkylation for 30 min at RT with iodoacetamide (10 mM), trypsin was added in a ratio of 1:40 (Trypsin:Protein). After 16 h, trypsin was added again in a ratio of 1:40. The digestion procedure was stopped after 40 h by a heating step and the addition of PMSF (1 mM).

Liver tissue samples. Liver tissue samples for fraction comparison and genotype/phenotype analysis (set A, n=10) were provided by the Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology. Set A contained samples from five female and five male patients aged between 47 and 75 years which were diagnosed with primary liver cancer or liver metastasis. For assay reproducibility and tissue analysis, liver tissue (set B, n=15) was provided from Uppsala University. Set B contained liver biopsies from twelve males and three females aged between 42 and 79 years. The diagnoses were, clear cell carcinoma, hepatocellular carcinoma, colorectal cancer or renal cell carcinoma. The biopsies do not contain tumor tissue, but healthy liver tissue. The studies were approved by the local ethics committees and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient

Microsomal versus whole tissue preparation - microsomal and nuclei/membrane fractionation. Microsomes, cytosols and crude membrane/nuclei fraction were taken from one preparation process each as described earlier by Hitzl and Lang (Lang et al., 2001; Hitzl et al., 2003). Homogenates of corresponding liver samples were prepared as described below.

Liver tissue analysis - tissue preparation. Liver tissues were homogenized using a ball mill (Micro – dismembrator S, Sartorius, Göttingen). Lysis buffer was added with a 10-fold volume (µL) of the weighed liver piece value (mg), homogenates were directly processed or snap frozen in liquid nitrogen and stored at -80°C until preparation. Samples were incubated for 1 h at 8°C under continuous rotation.

Liver tissue analysis - protein digestion. 50 µg protein were digested in triethanolamine buffer (50 mM, pH 8.5) and 0.5% octyl-β-D-glucopyranoside. For reduction TCEP (5 mM), for alkylation iodoacetamide (10 mM) was used (see *Analysis of antibody specificity - protein digestion*). For enzymatic fragmentation, trypsin was added in a ratio of 1:10 (Trypsin:Protein). After 16 h trypsin was spiked in again in a ratio of 1:10. After 40 h the digestion procedure was stopped by heating and adding PMSF (1 mM).

Expression induction study - cultivation of cryopreserved hepatocytes. Induction-qualified plateable human hepatocytes (HU8148 - 55 years old woman - Life Technologies, Carlsbad, USA) were cultivated as monolayer in collagenized 96-well plates at a density of 50,000 cells per well. Medium containing 'Primary Hepatocyte Thawing and Plating Supplements', L-Glutamin, and William's E Medium was used for thawing and adhesion. Medium was changed to maintenance medium (Primary Hepatocyte Maintenance Supplements, L-Glutamin, William's E Medium) after 24 h. After an adhesion phase of 48 h, cells had been treated with rifampicin (10 µM), phenobarbital (3 mM), and omeprazole (100 µM) for 72 h, 48 h, and 24 h respectively. The total cultivation time was 72 h for all cells. Control treatments were performed using maintenance medium containing 0.1% DMSO. Cells were seeded and harvested at the same time, but treatment was started at different time points. Standard inducers were applied

to the cell culture media 72h, 48h, 24h and 0h prior to harvesting. To study the basal enzyme level during cultivation, controls were collected after the adhesion phase (T0) and the total cultivation time using maintenance medium without DMSO (T72).

Expression induction study - lysis and protein digestion. For lysis, cells were washed once with ice-cold PBS and afterwards lysed using 50 µL lysis buffer. After incubation at 8°C for 1 h, cells were transferred to a 96-well-PCR-plate (Thermo Scientific, Waltham, USA). 11 µg protein were digested in triethanolamine buffer (50 mM, pH 8.5) and 0.7% octyl-β-D-glucopyranoside. Proteins were reduced using TCEP (5 mM) and alkylated by iodoacetamide (10 mM) (see *Analysis of antibody specificity - protein digestion*). Trypsin was added in a ratio of 1:3 (Trypsin:Protein). After 16 h trypsin was added again taking an equal amount. After 40 h the digestion procedure was stopped by heating the samples and adding PMSF (1 mM). Since protein amounts were lower than 15 µg per well after cultivation only the quantification of CYP enzymes and the transporter ABCB1 could be performed.

Results

Generation of antibodies. For the identification of suitable antigens, we aligned the C-termini of all peptides generated from 14 CYP enzymes, 9 transporters, and the POR after a tryptic *in-silico* digestion. In total 13 C-terminal epitopes were required to address peptides representing the respective human CYP enzymes and transporters. The C-terminal sequence FSGR for instance was shared by five proteotypic CYP peptides derived from 2B6, 2C8, 2C18, 2E1, and 2F1. Four members of the CYP family 3 and one transporter shared the terminus LPNK (3A4/3A43, 3A5, 3A7, and ABCB1) (Weiss et al., 2015). The sequence LQEEIDAVLPNK can be found both in CYP3A4 and CYP3A43. However, for CYP3A43 no or only very low expression levels were observed in previous liver quantification studies (Kawakami et al., 2011; Schaefer et al., 2012). The termini YIPK (1A1, 1A2), LAER (2C9, 2C19), GSLR (ABCC1, ABCC2), DLFR (SLC22A7, SLC22A8) address two targets each and the termini SVLK (CYP2D6), FIPK (CYP3A43), GGEK (ABCB11), GDLK (SLC10A1), QDEK (SLC22A9), PSSK (SLCO1B1), and FVEK (POR) only one. Immunization and purification of the TXP-antibodies were performed as described previously (Hoeppe et al., 2010).

Analysis of antibody specificity. We performed a detailed epitope analysis of the generated TXP-antibodies by LC-MS/MS measurements of immunoprecipitates from digested HepG2 lysates (Planatscher et al., 2010; Planatscher et al., 2014). Eluated peptides were separated by nLC and detected using a high resolution tandem mass spectrometer (LC-MS/MS, QExactive Plus™). All peptide identifications obtained by MASCOT- and SEQUEST-data analysis were investigated for enrichment analogies. Epitope motifs of the antibodies were determined considering statistical significance of the enrichment procedure (p-value = 0.05). All single-epitopes were weighted for the number of identified peptides sharing the same single-epitope and combined to a motif logo. For example, the immunoprecipitation procedure using anti-LAER resulted in a combined motif logo consisting of 14 single-motifs due to the polyclonal properties of the antibody. Overall the logo is based on 159 significantly enriched peptides (Figure 1). 45 peptides comprise the sequence “LAER” at the C-terminus. So, 22% of the theoretical number of all proteome wide “LAER”-peptide fragments (201)

were observed in the HepG2 digest. Peptide sequences sharing the motifs “LAER” (45 of 201), “LAEK” (17 of 209) and “LAQR” (16 of 147) are numerically the most frequently identified single-epitopes of the epitope motif. In comparison, the antibody anti-FVEK provided 25 peptide identifications comprising the C-terminus “FVEK”. The antibody was capable of enriching 40% of all theoretically existing “FVEK”-peptides - 64 peptides in total, assuming all known proteins are expressed and present in detectable peptide amounts (all epitope motifs are provided in Supplementary Figure S1).

Dynamic range of MS-based immunoassays. We determined the lower limit of quantification (LLOQ) according to the FDA’s recommendation for bioanalytical method validation. Mean values of accuracy (variation) and precision (recovery) were within 15% at the LLOQ and did not exceed 20% at the LLOD. For peptides derived from CYP1A1, CYP2C18, CYP3A4/CYP3A43, CYP3A5, and CYP3A7 LLOQs of 460 amol were achieved, reflecting a peptide concentration of 46 pM. The proteotypic peptides of CYP2C9, CYP2C19, ABCB1, and SLC22A9 could be quantified down to 1.4 fmol (140 pM). The assays for the peptides of CYP1A2, CYP2B6, CYP2C8, CYP2E1, CYP2F1, SLC10A1, SLC22A7, and SLCO1B1 revealed LLOQs of 4.1 fmol (410 pM), the peptides of ABCC1, ABCC2, and SLC22A8 were quantifiable at 12.3 fmol (1.2 nM). For ABCB11 and POR LLOQs of 3.7 nM were observed. The peptide derived from CYP2D6 could not be quantified below 111.1 fmol (11.1 nM) within a deviation of <20% (Figure 2, Supplementary Figure 2 and 3).

Digestion kinetics. To ensure a complete proteolyzation of lysates, which is needed for absolute quantification, we analyzed the digestion kinetics of the chosen peptide set by treating a liver lysate with trypsin for varying time periods. Peptides derived from CYP enzymes and transporters were quantified after a digestion period of 2, 6, 16, 18, 24, 42, and 66 h (Figure 3). After 16 h most CYP enzymes and transporters showed a high relative digestion rate. After 24 h several target peptides showed diminished values. By comparison, the CYP2E1-peptide showed a decelerated release. For this peptide, the highest signal was achieved after an incubation period of 66 h. In contrast to that, considerable signal decrease of the CYP1A2-peptide was already observed after 6 h digestion which could be due to surface adsorption.

Recombinant proteins. The recovery of the assays was tested by the analysis of commercially available 12 recombinant CYP proteins and POR usually used for enzyme-substrate assays (Cypex, Bactosomes). Using our MS-based immunoassays we quantified the targets in the respective single-expression samples and compared the results to the data provided by the manufacturer by means of UV-spectral analysis of the heme-iron group. The results, presented in Figure 4, are generally very similar to the manufacturer's spectral data. However, for CYP1A1 and CYP3A7 we observed higher amounts of protein compared to the specified amounts.

Microsomal versus whole tissue preparation. Microsomal preparations are commonly used as starting material for mass spectrometry-based quantification of CYPs. We analyzed the amount of the different CYPs in liver tissue, microsomal and other cell fraction preparations of ten liver tissue samples (set A) to investigate if a direct analysis of the whole tissue extract is feasible. The quantitative CYP enzyme data obtained for whole tissue homogenates and microsomes correlated very well with correlation coefficients between 0.86 and 1.00 (Supplementary Table 1). The enrichment factor in the microsomal preparation was 3.4 on average with a variance of 51% across all CYP analytes. If the results for CYP3A7 are not considered, the average enrichment factor was 2.9 with a much lower variance of 12% (Supplementary Table 2). As shown in Figure 5 all analytes could be detected in the tissue lysates as well in the microsomal preparations, suggesting that the microsomal enrichment step is not necessary for the quantification of CYP enzymes. Transporters were quantified from tissue extract, microsomes and a crude membrane/nuclei-enriched fraction (CMF) prepared during the microsomal preparation (Lang et al., 2001; Hitzl et al., 2003). The data correlated with values between 0.51 and 0.98. For transporters, enrichment factors between 1.2 and 3.9 were observed.

Assay reproducibility. To determine the reproducibility of the TXP quantification workflow, three liver tissue samples from set B were analyzed for CYP enzymes and transporters in triplicates on three different days. Inter-day assay variances are displayed in Figure 6 and listed in Supplementary Table 3. For CYP enzymes the analysis revealed variances ranging from 0.7% to 8.8% for intra-day measurements and from 1.9% to 11.0% for inter-day. For transporters, intra-day variances ranged from

0.3% to 13.0% and inter-day variances from 1.5% to 37.9%. If results for ABCC2 were excluded from the analysis CVs improved to 1.5% to 20.1%

Liver tissue analysis. A set of 15 patient-derived liver samples (set B) were investigated for CYP and transporter levels (Figure 7). CYP2C9 was determined as the highest expressed target analyte averaging 15 fmol/µg, and CYPs 2C8, 1A2, 2D6, 3A4, and 2E1 showed mean expression levels between 6.5 and 3.6 fmol/µg, exceeding that of POR (3.4 fmol/µg). The remaining CYPs 2C19, 2B6, 3A5, 3A7, 2C18, and 1A1 were expressed at lower concentrations than POR (0.9 fmol/µg and less). Thus, taking the enrichment factor for microsomal proteins into account, our quantified analyte levels corresponded approximately with expression levels determined by Ohtsuki et al. (Ohtsuki et al., 2012). Transporters were determined in a concentration range of 0.1 to 0.9 fmol/µg. Comparison of transporter expression levels in the liver tissue confirmed that SLC22A9 and SLC22A1 had the highest expression (Ohtsuki et al., 2012; Schaefer et al., 2012).

Genotype versus protein expression of CYP2C19, 2D6, and 3A5. Ten liver samples were selected according to available genotype data for the polymorphic enzymes CYP2C19, CYP2D6, and CYP3A5. The protein expression levels were generally in line with the corresponding CYP genotype. For instance, no or very low enzyme expression was observed for carriers of the homozygous poor metabolizer genotypes *CYP2C19*2/*2* and *CYP2D6*4/*4*, both of which lead to absence of liver-expressed protein due to erroneous splicing (Zanger and Schwab, 2013). In contrast, in a carrier of the CYP2D6 duplication (*1x2/*2) protein levels were increased (Figure 8). Similar results had been observed by Langenfeld and colleagues in a previous MS analysis of CYP2D6 (Langenfeld et al., 2009). Conversely, an approximately ten-fold increased CYP3A5 protein level was observed in three liver donors with the genotype **1/*3* compared to the more frequent poor metabolizer genotype (**3/*3*). The gene product of the *CYP3A5*3* allele is a non-functional shortened protein, also due to erroneous splicing missing the target peptide sequence LPNK, whereas the reference **1* allele encodes functional protein (Lamba et al., 2012).

Expression induction study. The applicability of the MS-based immunoassay method in protein induction experiments was tested with the typical standard inducers rifampicin, phenobarbital and omeprazole in 96-well cell culture experiments (Chu et al., 2009). When comparing enzyme expression levels after adhesion phase (T0) and 72h cultivation, we observed cultivation effects on protein levels due to dedifferentiation effects. For CYP1A2, 2C18, 3A4 and ABCB1, 1.2 to 3.2-fold increased amounts were observed whereas CYP2B6, 2C8, 2C9, 2D6, 2E1, 3A5, and POR showed 0.2 to 0.7-fold decreased concentration levels after 72 h cultivation (Figure 9 and Supplementary Figure 4). The expression levels of CYP2A13, 2C19, 2F1, and 3A7 were lower than the LLOQ of the established assays (data not shown). Since dedifferentiation effects with influence on the expression of the CYP system are often observed after cultivating hepatocytes over longer time spans (Baker et al., 2001; Hengstler et al., 2009), these effects were taken into account by doing a time course experiment. Cells were seeded and harvested at the same time, but treatment was started at different time points. Standard inducers were applied to the cell culture media 72h, 48h, 24h and 0h prior to harvesting.

Treatment of hepatocytes with the known inducers rifampicin, phenobarbital and omeprazole led as expected to differential increases of CYP proteins. These compounds are known to affect the CYP expression mainly via the receptor pathways of constitutive androstane receptor (CAR), pregnane X receptor (PXR), and aryl-hydrocarbon receptor (AhR). Omeprazole treatment induced CYP1A2 by a factor of 11. CYP2B6 was only slightly induced by a factor of 2.4, although the CYP2B6 level was increased by all three compounds. The treatment with phenobarbital showed a 5.2-fold CYP2B6 induction after 48 h. Rifampicin induced CYP2B6 by a factor of 3.2, omeprazole by a factor of 1.9. The expression of CYP3A4/3A43 and CYP2C8 is inducible via PXR, and here the maximal induction was observed under rifampicin treatment after 48 h. Rifampicin induced a 4.6-fold expression of CYP2C8. Based on a basal concentration of 2.6 fmol/µg, CYP3A4 was induced 19-fold to 50.5 fmol/µg.

Discussion

We developed and validated 24 MS-based immunoassays, which allow the quantification of CYP enzymes and transporters without the need for pre-fractionation procedures such as microsomal preparation by ultra-centrifugation. Even low expressed CYPs, e.g. CYP1A1, could be directly analyzed in tissue and cell cultures. The assays are suitable for conducting protein induction studies to complement mRNA- and activity data. In our procedure the application of multi-specific TXP-antibodies directed against small C-terminal peptide sequences, facilitates the simultaneous enrichment of several analytes (Weiss et al., 2014; Weiss et al., 2015). The confirmed recognition of short peptide sequences supports the suitability of the TXP strategy for addressing homologous protein families and underlines its generic applicability. Finally, multiplexing of several TXP-antibodies enables the specific, fast, and extensive quantification of a multitude of peptides.

The generation of the 13 group-specific antibodies was performed as published previously (Hoeppe et al., 2010). The in-depths analysis of the antibodies´ epitopes revealed epitope motifs similar to the antigen used for antibody generation. In 6 out of 12 cases the epitope contained one variable position, in three we observed two and in one case three variable positions. In just one case - the PSSK antigen - we observed cross reactivity based on four varying amino acids. However, the PSSK sequence was still overrepresented in the motif (> 60%). All generated antibodies could be used to set up quantitative assays for the targeted CYP-enzymes and transporters. The most valuable antibody in terms of multi-usability was the anti-FSGR antibody. The antibody could be applied for assay development of five enzymes – CYP2B6, 2C8, 2C18, 2E1, and 2F1. However, the initial selection of suitable proteotypic peptide sequences also plays a major role for the development of MS-based immunoassays. The use of concatamers – artificial proteins comprising sequentially arranged peptides from proteins of interest – can potentially improve the presented assay accuracy since endogenous and reference target peptides are simultaneously released by the enzymatic digestion (Beynon et al., 2005; Achour et al., 2014). However, it has to be ensured that the release of both peptide variants – from the endogenous and the artificial protein (concatamer) - is comparable. As for all MS-based peptide-centric

quantification methods, peptide properties like hydrophobicity, length, ionization potential and/or a potential delayed release during protein digestion influence the peptide availability and therefore the sensitivity of the following analysis steps. As observed during assay development, sensitivities and linear range of the developed tests differed due to peptide properties. For example, assay performance using the peptides GTTLITNLSSVLK (CYP2D6) and DIEINGVFIPK (CYP3A43) exhibited a linear range of just one order of magnitude, while most of the developed assays showed linear qualities of at least 3 dimensions. Therefore, the selection of peptides has a big impact on assays´ sensitivity and linearity. However, the observed values for all proteins of interest were within the dynamic ranges of the developed assays.

The reference analysis of commercially available recombinant proteins regarding the question of the assay's accuracy revealed differences between the information given by the manufacturer and our analysis. In most cases the MS-based quantification resulted in higher CYP content than stated by the manufacturer. Whereas mostly the measured values were 1.2 to 2-fold higher, the largest discrepancy was observed for CYP1A1. Here, a 10-fold higher content was measured. The discrepancies could be due to the different analytical approaches. On one hand, the enzymes have been quantified by the manufacturer using UV-spectral analysis of the heme group, determining the functional portion (holo-protein) of the expressed protein. The herein described method uses a mass spectrometry read-out measuring the total expressed protein including the apo-protein (no heme). Hence, this could be an explanation for the deviation between the two results. This should be also taken into account if peptide-based quantifications of CYPs are compared with enzyme activities.

Subcellular fractionation is often required to reduce complexity and to enrich targets prior to protein quantification. Unfortunately, subcellular fractionation generally results in incomplete protein yields (Wegler et al., 2017). In this work, we provide a solution to this problem. We demonstrated that the direct quantification of CYP enzymes and POR from tissue extract highly correlates with data generated by analysis of the corresponding microsomal preparation. So, our test system represents a possibility to avoid the extensive and time-consuming procedure of preparing microsomal fractions that are

susceptible to errors due to many processing steps. However, the observed average enrichment factor for CYPs in the microsomal preparation was about 3-fold. Taking this into account, our results were in line with previous studies from others using microsomal preparation prior to nLC-MS read-out (Kawakami et al., 2011; Ohtsuki et al., 2012; Schaefer et al., 2012). We observed high amounts of CYP proteins in the crude membrane/ nuclei fraction, suggesting that a certain amount of the CYP containing endoplasmatic reticulum membranes are lost within the first steps during microsomal preparation. However, this preparation procedure is an established and optimized method to get pure microsomal preparations. On the other hand, the amount of transporters in these membrane/nuclei enriched fractions was higher compared to other fractions.

We confirmed the reproducibility of our assays by determination of intra- and inter-assay performance. Three different liver tissues which comprised a wide concentration range of the analyzed proteins were used for this analysis. In regard to the CYP enzyme and transporter levels we could demonstrate that liver tissues could be studied in a very robust and sensitive way. Hence, we have validated the assay panel “fit for purpose”. It can be used to address academic questions. In an industrial/ pharmaceutical setting the assay requires more extensive validation including quality controls, external calibration curves, determination of sample and reagent stability etc.

Furthermore, we were able to clearly differentiate between different functional and clinically relevant genotypes of CYP2C19, 2D6, and 3A5 at the protein level. The different metabolizer phenotypes (poor, intermediate, extensive and ultrarapid) were also reflected by the concentration of the respective CYP enzymes. Since we analyzed only a very low number of selected samples with respect to genotype, these findings have to be confirmed in a larger cohort of samples.

Finally, the assay system was also proven for the analysis of induction studies performed in 96-well plate cultures using cryopreserved hepatocytes. Actually, results confirmed trans-activation processes after administering the PXR-inducer rifampicin and the CAR-inducer phenobarbital. The observed effects resulted from hetero-dimerization of the nuclear receptors with retinoid X receptor and the

consequential activation of the CYP2B-, CYP2C-, and CYP3A-isoforms (Xie et al., 2000; Gerbal-Chaloin et al., 2001; Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Luo et al., 2002; Chen et al., 2004; Ferguson et al., 2005).

In summary, we have developed a multiplex assay system capable of detecting relevant drug metabolizing CYPs, POR and transporters in tissue and primary cell cultures. The enzymes and transporters can be measured directly from crude cell lysates, since the rapid immunoprecipitation replaces the microsomal preparation step. As primary human hepatocytes have to be isolated upon surgery and thus are very precious and rarely available, the new assay platform supports their efficient use in the 96-well plate format. Therefore, CYP-protein analysis could be established in future workflows of drug-drug interaction studies and complement mRNA and enzyme activity data.

Authorship Contributions

Participated in research design: Weiß, Hammer, Poetz, Klein, Zanger, Artursson and Wegler

Conducted experiments: Weiß, Hammer, Klein, and Norén

Performed data analysis: Weiß, Hammer and Planatscher

Wrote or contributed to the writing of the manuscript: Weiß, Pötz, Klein, Zanger, Artursson, Wegler, Hammer and Joos

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Footnotes

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Figure legends.

Figure 1: Frequency of C-termini after immunoprecipitation from proteolyzed HepG2-lysate (technical replicates, n=3) using the TXP-antibodies anti-LAER (A) and anti-FVEK (B). Peptides were analyzed by high resolution tandem-mass spectrometry and identified combining the MASCOT- und SEQUEST-algorithms. Enrichment analogies were identified considering statistical significance (p-value = 0.05). Single-epitopes were weighted for the number of identified peptides and combined in a motif logo. Size of the letter represents percentage of identified peptides carrying the respective amino acid at this position.

Figure 2: Accuracy and recovery of selected MS-based immunoassays. Isotope-labeled peptides were serially diluted in analyte-free buffer containing proteolytically fragmented fish gelatin, while sequence-identical non-labeled peptides were kept constant (technical replicates, n=3). For immunoprecipitation, absolute peptide amounts ranging from 1000 to 0.15 fmol were provided. By using the signal of the non-labeled peptide, the isotope-labeled peptide amount was back-calculated and plotted as recovery. Linearity of approaches is demonstrated over at least three orders of magnitude. Mean and SD are depicted.

Figure 3: Digestion kinetics. CYP enzymes and transporters were quantified considering different digestion periods. Maximum quantified peptide concentrations were set to 100% and declared as normalized amount. The optimal digestion duration for the analysis of all analytes in one experiment is indicated in grey. Representative experiment is shown.

Figure 4: Quantification of recombinant Cytochrome P450 proteins. CYP enzymes expressed in bacterial membranes and standardized using a spectral determination method, were quantified using MS-based immunoassays. The ratio between TXP quantification and spectral quantification was determined. (technical replicates, n=3, mean and standard deviation are shown).

Figure 5: A) Quantification of CYP enzymes and transporters from liver tissue T (set A) and three different fractions (crude membrane/nuclei fraction CMF, microsomes M, and cytosol C), from one preparation from tissue material of donor 4 (technical replicates: T, CMF and C n=3; microsomal preparation was measured as single replicate due to limited amount of sample. Mean and standard deviation are shown). B) Enrichment of analytes by CMF and microsomal preparation. Ratio between either CMF or microsomal preparation (M) and direct quantification strategy from tissue (T) was determined. Results are depicted as box blot (Box: 25,75 percentile, whiskers: < 1.5 IQR, all values are depicted as dots, minimal and maximal values are indicated as x)

Figure 6: Reproducibility and variation of MS-based immunoassays (technical replicates, n=9). Three tissue samples (from set B) had been selected. CYP enzymes and transporters were quantified in triplicates on three different days (*: <LOQ).

Figure 7: Tissue studies. Quantification of CYP enzymes and transporters in human liver tissues of 15 liver samples (set B) Results are depicted as box blot (Box: 25,75 percentile, whiskers: 5-95 percentile, all values are depicted as dots, minimal and maximal values are indicated as x, technical replicates: n=3).

Figure 8: Quantification of CYP2C19, 2D6, and 3A5 with reference to the donor's genotype. Ten Liver tissues selected according to previously determined relevant genotypes were analyzed with the direct quantification strategy from tissue (set A). Mean and SD are given (technical replicates: n=3)

Figure 9: Induction studies. Induction qualified human cryopreserved hepatocytes were cultivated in 96-well plates. Cells were seeded at the same time and treated with rifampicin (10 µM), phenobarbital (3 mM), and omeprazole for 72h, 48h, 24h and 0h prior to harvesting. The CYP enzymes 1A2, 2B6, 2C8, 2C9, and 3A4 were quantified using MS-based immunoassays (biological replicates, n=3) (A-E). The protein expression after cultivating cells for 72 h using maintenance medium without DMSO, is used as initial point of the visualization (T72, treatment duration: 0 h). Dedifferentiation effects on the

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expression of the CYP system were observed by comparing basal enzyme levels after adhesion phase (T0), with expression rates after the whole cultivation time (T72) (F-J).

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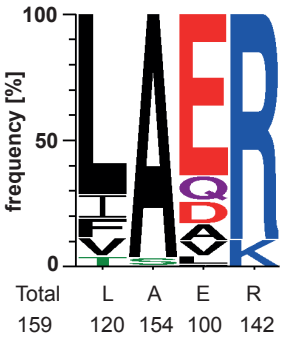
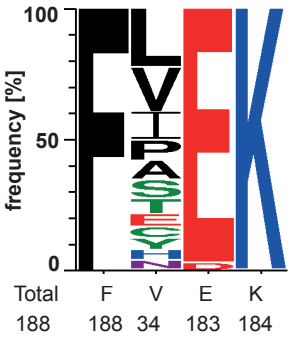
Fig. 1

A



C-terminus	observed peptides	theoretical peptides
LAER	45	201
LAEK	17	209
LAQR	16	147
VAER	11	107
LAAR	11	277
FAER	10	51
LAVR	10	162
IADR	6	43
IAER	6	50
TAER	6	84
LADR	6	116
LALR	6	314
LSER	5	168
LAIR	4	81
Total	159	2010

B



C-terminus	observed peptides	theoretical peptides
FLEK	44	157
FVEK	25	64
FIEK	19	51
FPEK	14	51
FSEK	14	80
FAEK	13	59
FTEK	11	59
FEEK	9	78
FCEK	7	31
FYEK	7	32
FHEK	6	29
FNEK	6	33
FVDK	5	33
FFEK	4	22
FVER	4	47
Total	188	826

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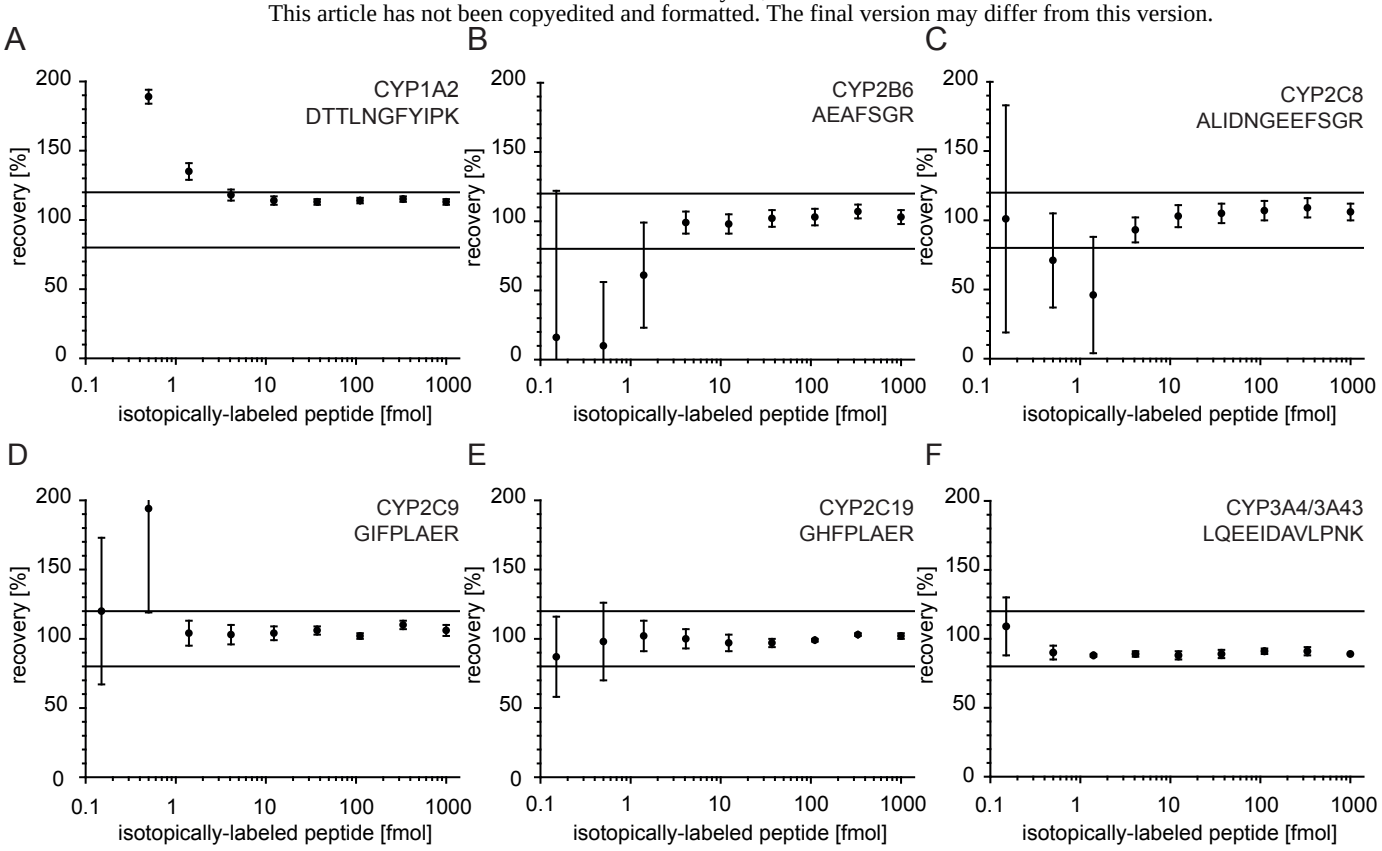

Fig. 2

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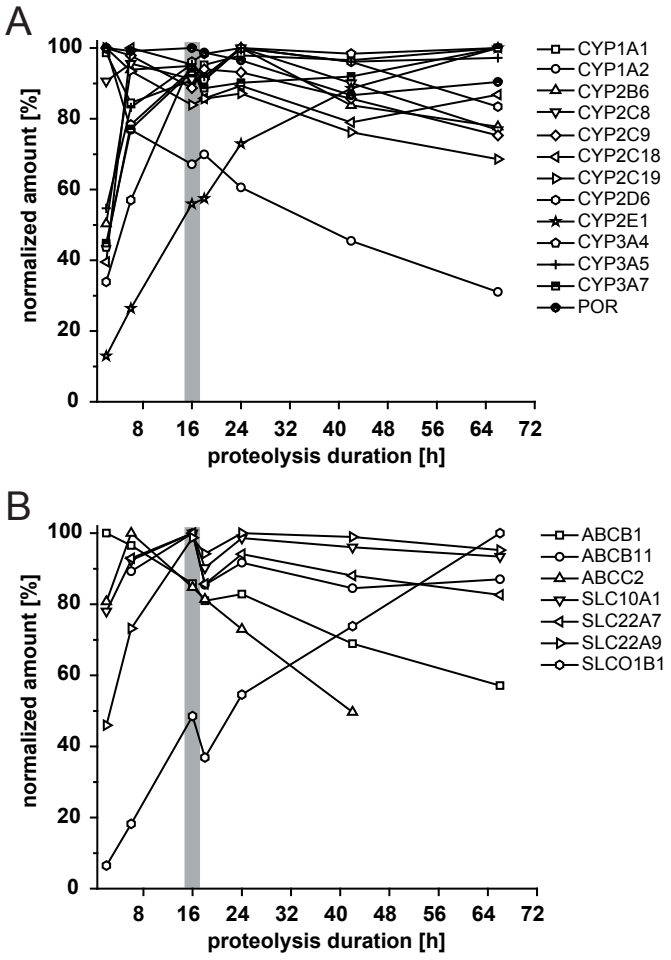

Fig. 3

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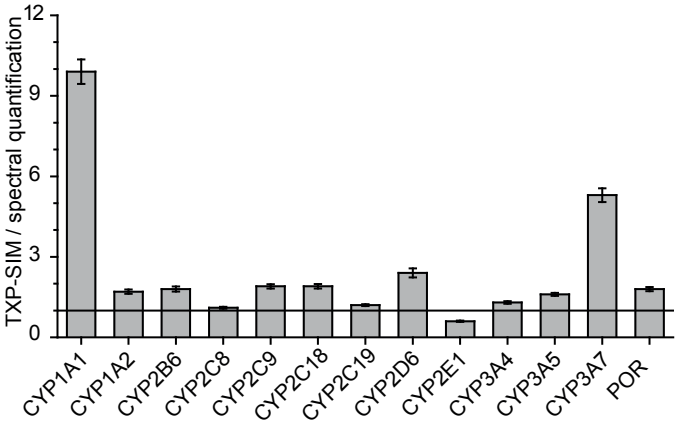
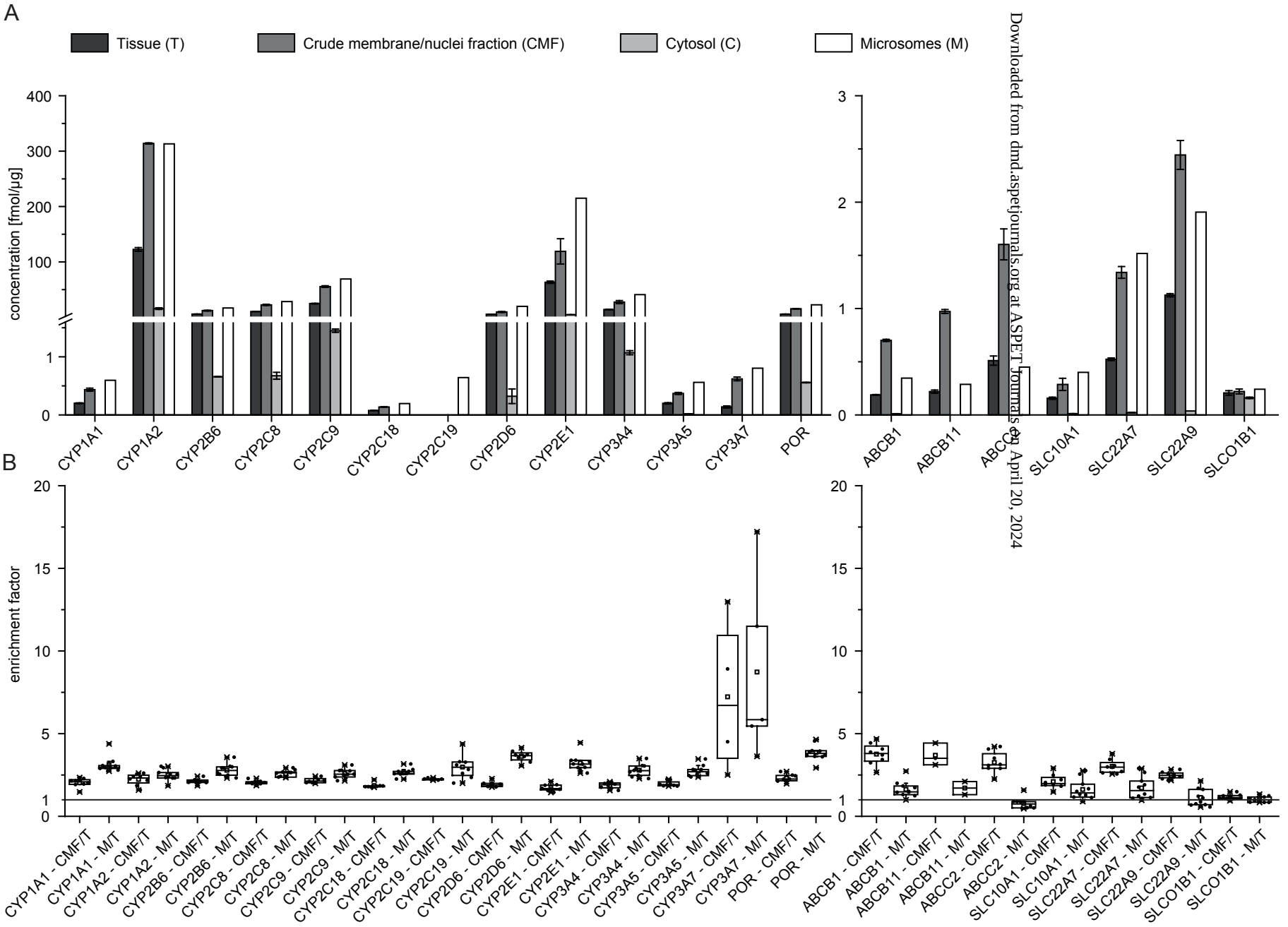


Fig. 4

Fig. 5



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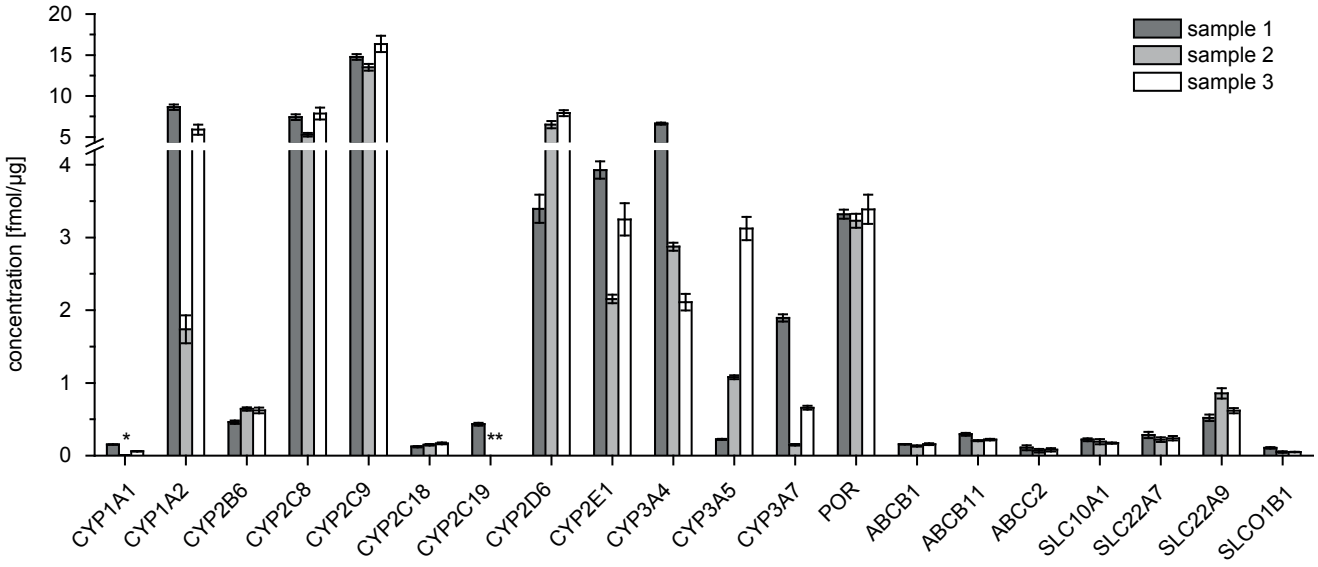

Fig. 6

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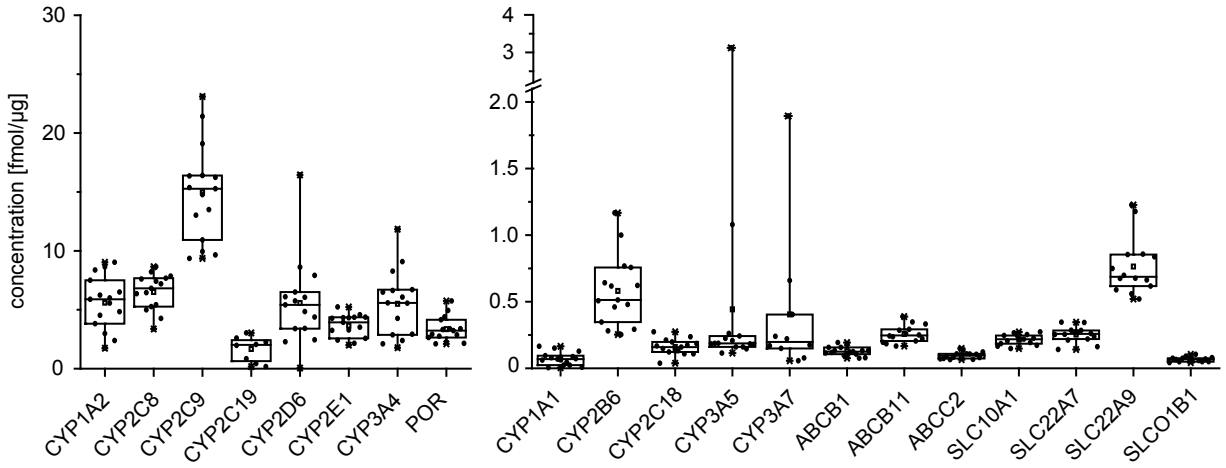

Fig. 7

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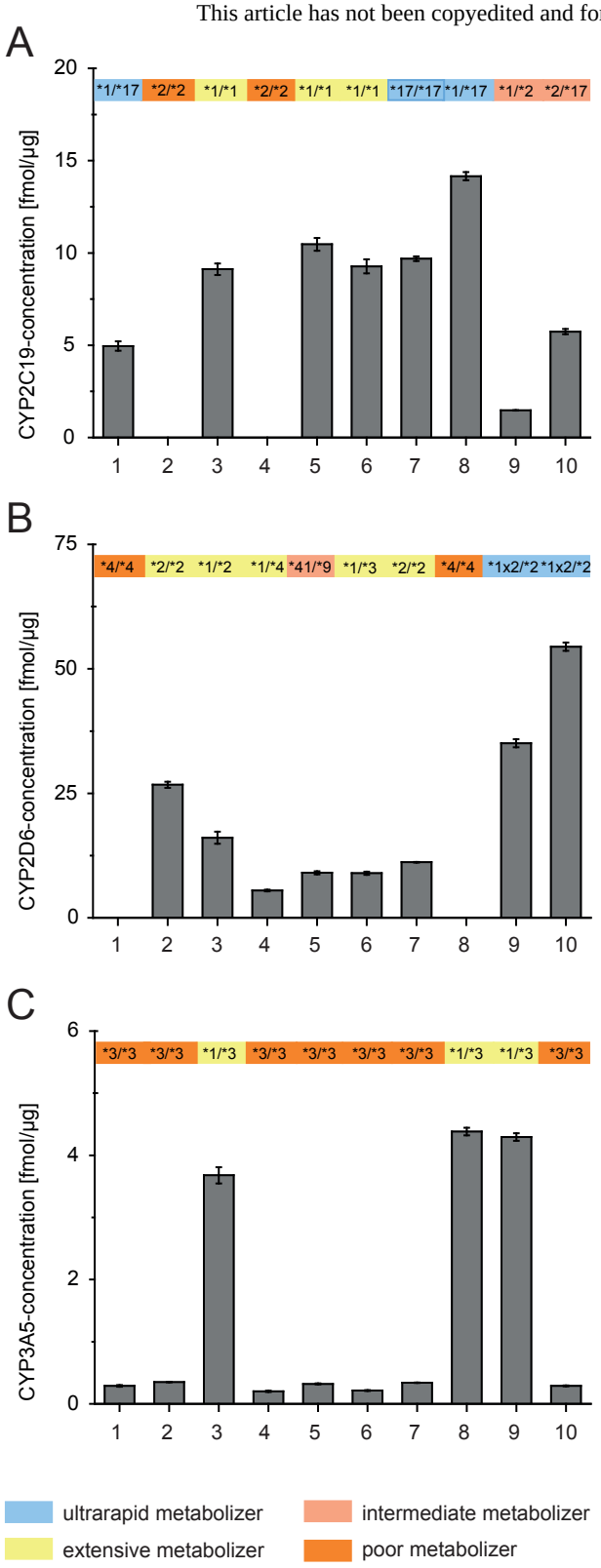

Fig. 8

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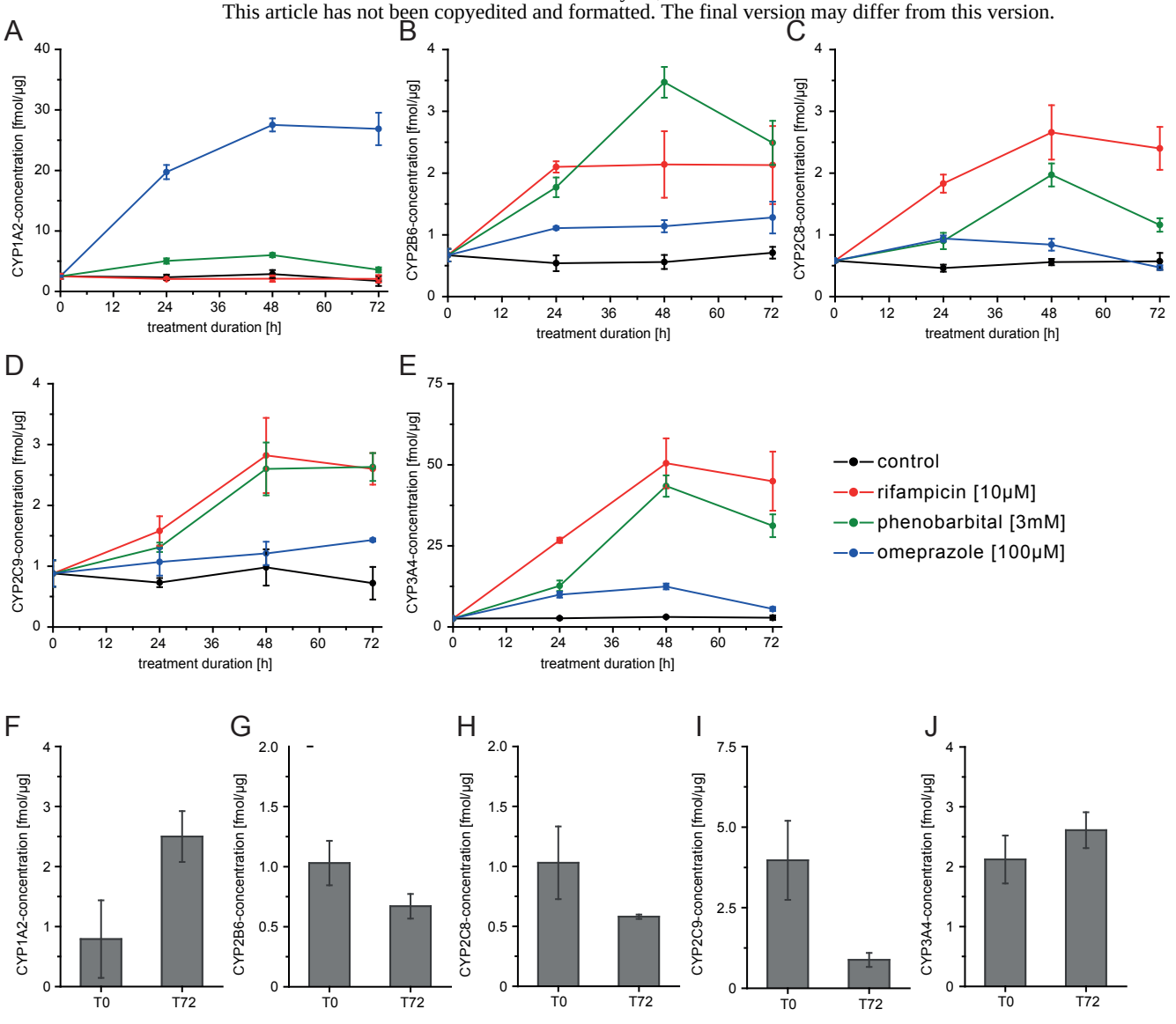

Fig. 9