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Comparison of Proteomic Quantification Approaches for Hepatic Drug Transporters: Multiplexed Global Quantitation Correlates with Targeted Proteomic Quantitation[S]

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ABSTRACT

Targeted protein quantification using liquid chromatography–tandem mass spectrometry with stable isotope-labeled standards is recognized as the gold standard of practice for protein quantification. Such assays, however, can only cover a limited number of proteins, and developing targeted methods for larger numbers of proteins requires substantial investment. Alternatively, large-scale global proteomic experiments along with computational methods such as the "total protein approach" (TPA) have the potential to provide extensive protein quantification. In this study, we compared the TPA-based quantitation of seven major hepatic uptake transporters in four human liver tissue samples using global proteomic data obtained from two multiplexed tandem mass tag experiments (performed in two independent laboratories) to the quantitative data from targeted proteomic assays. The TPA-based quantitation of these hepatic transporters [sodium-taurocholate cotransporting polypeptide (NTCP/SLC10A1), organic anion transporter 2 (OAT2/SLC22A7), OAT7/SLC22A9, organic anion-transporting polypeptide 1B1 (OATP1B1/SLCO1B1), OATP1B3/SLCO1B3, OATP2B1/SLCO2B1, and organic cation transporter (OCT1/SLC22A1)] showed good-to-excellent correlations (Pearson r = 0.74–1.00) to the targeted data. In addition, the values were similar to those measured by targeted proteomics with 71% and 86% of the data sets falling within 3-fold of the targeted data. A comparison of the TPA-based quantifications of enzyme abundances to available literature data showed that the majority of the enzyme quantifications fell within the reference data intervals. In conclusion, these results demonstrate the capability of multiplexed global proteomic experiments to detect differences in protein expression between samples and provide reasonable estimations of protein expression levels.

Introduction

Quantification of transport proteins is an important component to facilitate in vitro–in vivo extrapolation (IVIVE), and thus the mechanistic modeling of drug disposition (Prasad and Unadkat, 2014; Vildhede et al., 2016). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is generally considered the method of choice for the identification and quantification of proteins (Han et al., 2008). Typically, the proteins are enzymatically digested into peptides, which are then separated and analyzed by LC-MS/MS (Walther and Mann, 2010). Peptide concentrations are thus measured as surrogates of protein abundance.

There are essentially two main strategies for mass spectrometry-based proteomic analysis. In targeted proteomics, a predefined set of peptide ions, uniquely identifying proteins of interest, and one or more of their fragment ions are selected and monitored for accurate quantification of each peptide using an external calibration curve. Because of the reproducibility and high selectivity and sensitivity of the method, targeted proteomics is considered the gold standard for protein quantification (Peterson et al., 2012). It does, however, suffer from limited throughput, as there are practical restrictions to the number of peptides that can be targeted in a single run with reliable quantification (Lange et al., 2008).

In contrast to targeted proteomics, global or shotgun proteomics aims to identify as many proteins as possible in a sample. This is achieved by operating the mass spectrometer in data-dependent acquisition mode (Zhang et al., 2013). Precursor ions are detected in a survey scan followed by fragmentation of a subset, typically selected by highest signal abundances. From the fragment spectra, the identities of the peptides can be determined by sequence database searching, and the peptides are then assigned to proteins. To convert the spectral intensities to protein concentrations, a computational method termed the "total protein approach" (TPA) was recently proposed and validated (Wiśniewski et al., 2012). The method does not require standards and is applicable to any large-scale proteomic data set. It thus offers an efficient way to quantify all identified proteins in a sample. This approach is, however, expected to be less reproducible than targeted proteomic quantifications owing to the stochastic sampling of precursor ions for fragmentation, thus affecting run-to-run reproducibility (Tabb et al., 2010).

To reduce the impact of run-to-run variability when comparing protein abundances between different samples, multiplexing at the sample level is possible. For example, tandem mass tags (TMT) can be used to introduce isotope-based differences to the samples via chemical

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ABBREVIATIONS: CYP, cytochrome P450; LC, liquid chromatography; MS/MS (MS2), tandem mass spectrometry; MS/MS/MS (MS3); triple-stage mass spectrometry; NTCP, sodium taurocholate cotransporting polypeptide; OAT2, organic anion transporter 2; OAT7, organic anion transporter 7; OATP1B1, organic anion-transporting polypeptide 1B1; OATP1B3, organic anion-transporting polypeptide 1B3; OATP2B1, organic anion-transporting polypeptide 2B1; OCT1, organic cation transporter 1; TMT, tandem mass tag; TPA, total protein approach; UGT, UDP-glucuronosyltransferase.

labeling (Thompson et al., 2003). This approach was initially reported to suffer from interference, resulting in ratio distortions, a problem that was solved by an additional fragmentation step, i.e., triple-stage mass spectrometry (MS3) (Ting et al., 2011).

In this study, we aimed to evaluate the quantitative ability of the total protein approach using MS3 spectral data obtained from two independently performed TMT experiments. To assess quantification accuracy, calculated protein levels were compared with the abundances determined by targeted proteomics for a set of hepatic uptake transporters that are of relevance in drug disposition.

Materials and Methods

Human Liver Tissue. Normal human liver tissue samples from four different donors were obtained from the Pfizer Tissue Bank (Groton, CT). Collection of these samples was conducted in a manner compliant with Pfizer policies, including ethical approval.

Targeted Proteomic Quantification of Hepatic Uptake Transporters. Surrogate peptide levels for sodium taurocholate cotransporting polypeptide (NTCP), organic anion transporter 2 (OAT2), OAT7, organic anion transporting polypeptide 1B1 (OATP1B1), OATP1B3, OATP2B1, and organic cation transporter 1 (OCT1) were measured by modification of previously published quantitative targeted proteomic methods (Kimoto et al., 2012; Qiu et al., 2013), and with guidance on the choice of proteotypic surrogate peptides from other publications (Sakamoto et al., 2011; Groer et al., 2013). Details of the targeted proteomic methodology are provided in Supplemental Methods. In brief, membrane proteins were extracted from the human liver samples and digested with trypsin (Supplemental Fig. 1). The resulting mixture of peptides was spiked with known quantities of stable isotope-labeled peptides as internal standards. Peptides were then separated and analyzed by LC-MS/MS in multiple-reaction monitoring mode. Chromatographic peak areas of target fragment ions in relation to their respective stable isotope-labeled internal standard were used to calculate peptide concentrations (as surrogate of protein levels) with an external calibration curve prepared in digested human serum albumin matrix.

Multiplexed Global Proteomic Analysis of Liver Tissue Samples and Quantification of Protein Abundances. Liver tissue samples from the same donors included in the targeted proteomic quantification were analyzed by multiplexed global proteomics in two independent laboratories: MS Bioworks (Ann Arbor, MI) and IQ Proteomics (Cambridge, MA) using their in-house protocols. For the global proteomic experiments, proteins (both soluble and membrane-bound) were extracted by homogenization and lysis of the liver tissue samples (Supplemental Fig. 1). Proteins were digested with trypsin (laboratory 1) or Lys-C and trypsin in tandem (laboratory 2), and the resulting peptide samples were labeled with isobaric TMT reagents in technical duplicates. After labeling, samples were mixed and peptides were subjected to high pH reverse-phase fractionation, followed by LC-MS/MS/MS analysis on Orbitrap Fusion Lumos instruments (Thermo Fisher Scientific, Waltham, MA). MS3 spectral intensities of the reporter ions, formed upon fragmentation, were used to calculate protein abundances under the assumption that the MS signal for a given protein in relation to the summed MS signals for all identified proteins reflects its partial abundance in the sample (Wiśniewski, 2017). A more detailed description of the TMT experiments is provided in Supplementary Methods.

Statistical Analysis. Correlations of protein abundance measurements across laboratories were assessed by Pearson's correlation coefficients (r). Protein abundance values calculated from the TMT data were compared with those determined in the targeted proteomic experiment by Dunnett's multiple comparison tests and by calculating fold errors.

Results and Discussion

To evaluate the quantitative merits of the multiplexed global proteomics approach, and to justify its utility in quantitative translation needed for system pharmacokinetics, we compared transporter protein levels in human liver samples acquired from TMT data to the abundances determined by validated targeted proteomic methods. For this comparison, TMT data obtained from two independent laboratories with different sample processing protocols were used.

Surrogate peptide abundance levels of the major hepatic uptake transporters involved in drug disposition, namely NTCP, OAT2, OAT7, OATP1B1, OATP1B3, OATP2B1, and OCT1, were measured in four human liver tissue samples. All these transporters were detected in the liver tissue samples analyzed with measured abundances within the ranges observed in previous studies (Kimoto et al., 2012; Qiu et al., 2013; Peng et al., 2015; Wang et al., 2015). For the four samples included in this study, NTCP showed ~6-fold intersubject variability, whereas the variability of the other transporters was ~2- to 3-fold (Table 1).

Human liver tissue samples from the same donors included in the targeted proteomic quantification were analyzed in two separate TMT experiments, performed in two independent laboratories. The samples were analyzed in technical duplicates. Very high correlations (Pearson $r > 0.99$) in MS3 intensities for the replicate measurements were observed, indicating a good precision of the TMT method. More than 5000 proteins were identified in each TMT experiment, demonstrating a sufficient depth of analysis for the TPA-based quantification of protein abundances (Wiśniewski, 2017). Among the identified proteins were the seven uptake transporters of interest (Table 1). For laboratory 1, however, NTCP was not detected in MS3 mode and, thus, MS2 data were used instead. The lack of NTCP detection in MS3 mode was likely a result of the shorter MS acquisition time for this TMT experiment compared with the other one (24-hour compared with 36-hour).

TABLE 1

Comparison of the average protein abundance and range (interindividual variability) in protein abundance for four human liver tissue samples measured with two different proteomic methodologies, targeted proteomics, and multiplexed global proteomics using tandem mass tag (TMT) reagents

LC-MS/MS/MS run time was 24 hours for laboratory 1 and 48 hours for laboratory 2.

Transporter	Average Protein Abundance ± S.D. (pmol/mg membrane protein)			Range in Protein Abundance (max/min)		
	Targeted Data	TMT Data		Targeted Data	TMT Data	
		Laboratory 1	Laboratory 2		Laboratory 1	Laboratory 2
NTCP	1.28 ± 0.67	1.09 ± 0.39[a]	4.81 ± 1.52*	6.03	2.60[a]	2.32
OAT2	1.15 ± 0.45	0.74 ± 0.32	2.74 ± 0.94*	2.50	2.71	2.30
OAT7	5.42 ± 1.77	1.32 ± 0.55*	4.16 ± 1.38*	1.97	2.53	1.97
OATP1B1	8.08 ± 2.24	2.80 ± 0.85*	4.28 ± 1.47*	2.07	2.24	2.52
OATP1B3	4.39 ± 1.88	1.24 ± 0.24*	2.11 ± 0.98*	2.60	1.58	2.69
OATP2B1	1.86 ± 0.73	1.72 ± 0.50	2.29 ± 0.79	2.23	1.96	2.35
OCT1	2.92 ± 0.80	5.69 ± 2.31*	3.37 ± 1.00	1.79	2.32	1.86

*Statistically different from the targeted proteomic data (adjusted $P < 0.05$).
[a]NTCP values represent MS2 data for laboratory 1 because of lack of detection of NTCP in MS3 mode.

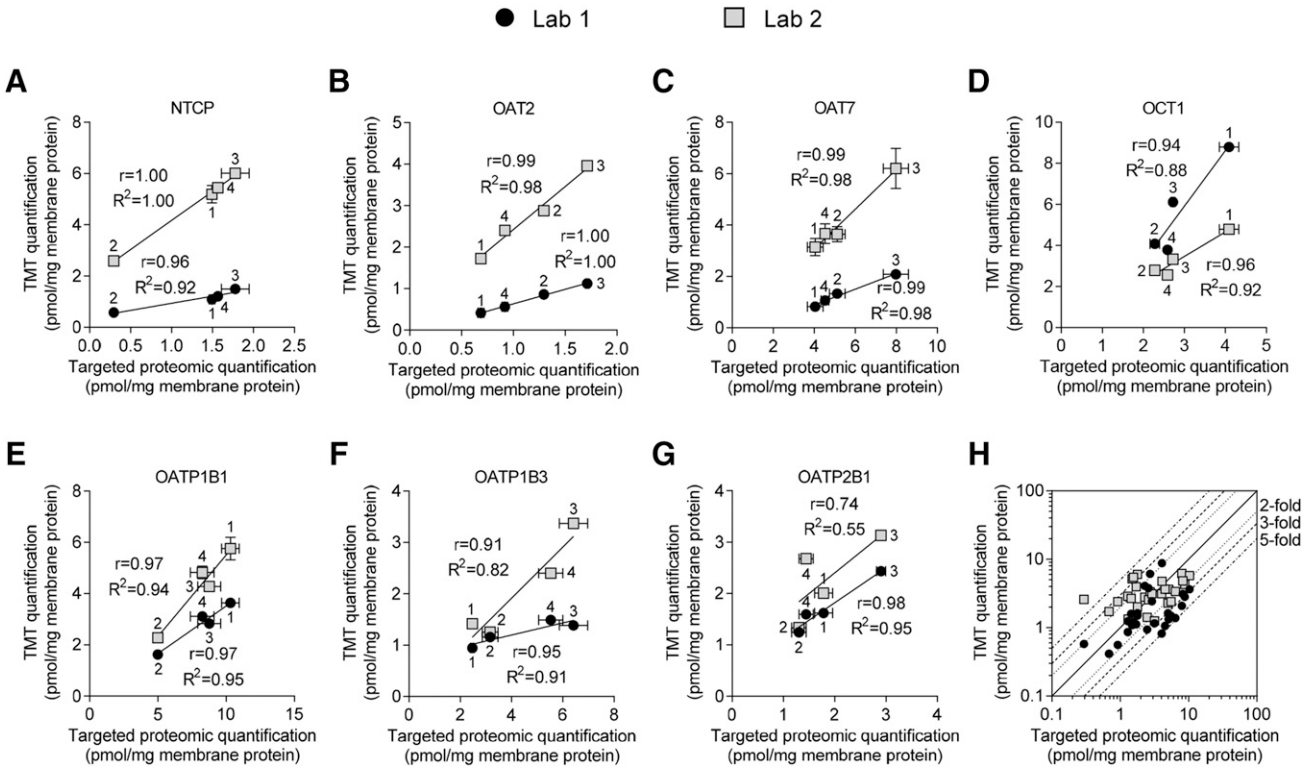

Fig. 1. Comparison of protein quantifications by multiplexed global proteomics (*y*-axis) and targeted proteomics (*x*-axis). Cross-laboratory correlation analysis of protein abundance measurements for NTCP (A), OAT2 (B), OAT7 (C), OCT1 (D), OATP1B1 (E), OATP1B3 (F), and OATP2B1 (G). Data are presented as arithmetic means with standard deviations (n = 3 for the targeted proteomic data, n = 2 for the TMT data). r = Pearson correlation coefficient. Quantitative agreement of protein abundances calculated from the multiplexed global proteomic data to those measured by targeted proteomics (H). The solid line represents the line of unity, dotted lines represent 2-fold deviations, dashed lines represent 3-fold deviations, and the dot-dash lines represent 5-fold deviations. LC-MS/MS/MS run time was 24 hours for laboratory 1 and 48 hours for laboratory 2. NTCP was not detected in MS3 mode for laboratory 1, and hence, MS2 data were used instead.

Moreover, because of the stochastic element of precursor ion fragmentation in data-dependent acquisition mode, proteins of interest are not always identified in complex samples (Tabb et al., 2010). This is especially true for less abundant proteins, such as NTCP, when the selection criterion for fragmentation is chosen on the basis of peptides with the highest signal abundances. To increase the probability of identifying the proteins of interest, enrichment through subcellular fractionation is possible, although isolated fractions are reported to be associated with lower yields than expected (Wiśniewski et al., 2016; Wegler et al., 2017). Another option is to perform data-independent acquisition on the proteome-scale using a recently introduced technique known as SWATH-MS (Gillet et al., 2012).

The calculated protein abundances from the TMT experiments were compared with the targeted protein quantifications (Fig. 1). For this comparison, a membrane-to-total protein ratio of 0.233 (in-house

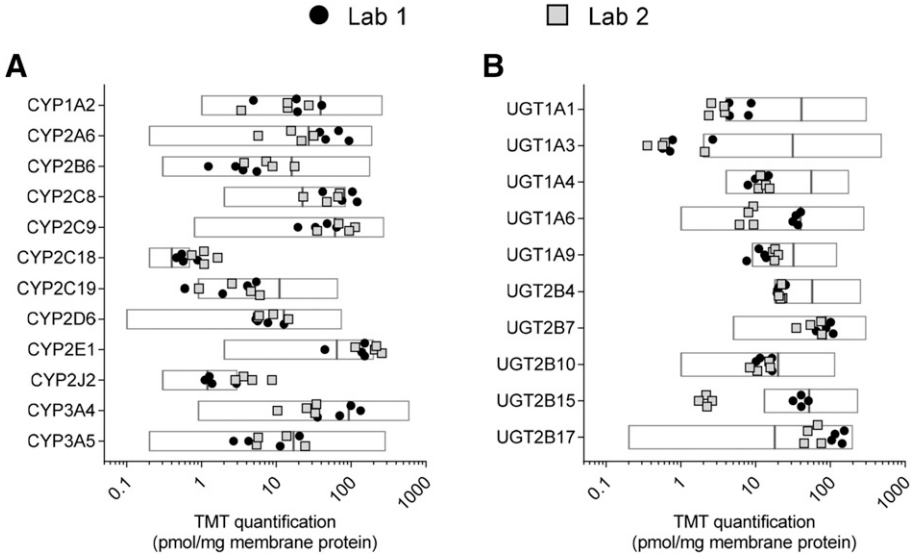

Fig. 2. Comparison of cytochrome P450 (A) and UGT (B) enzyme abundances, quantified by multiplexed global proteomics, to the range (white bars) and weighed mean (solid gray line) from two recent meta-analyses (Achour et al., 2014a,b).

data, $n = 42$) was used to convert the measured abundances to the same unit. The TMT data showed good-to-excellent correlations (Pearson r = 0.74–1.00) to the targeted proteomic data (Fig. 1, A–G). We attribute this cross-laboratory agreement to the confident identification of peptides in the TMT experiments and to the thorough analytical validation of the targeted proteomic assays to avoid false positive identifications caused by matrix interference (Balogh et al., 2012; Qiu et al., 2013; Carr et al., 2014). The good correlations observed in our study are in stark contrast to the predominantly poor correlations of transporter and enzyme protein levels across laboratories reported elsewhere (Achour et al., 2017; Wegler et al., 2017). In such cases, correlations with activity measurements are necessary to determine which proteomic methodology results in expression data that align with functional activity. Unfortunately, it is not possible to measure transport activities in frozen liver tissue samples.

In addition to the excellent correlations of measured protein concentrations across methods observed in our study, the transporter abundances calculated from the TMT data resulted in values comparable to those measured by targeted proteomics with absolute fold errors within 3-fold for 71% and 86% of the data set for the two TMT experiments, respectively (Fig. 1H). Furthermore, the range (interindividual variability) in protein expression was very similar across methods (Table 1), suggesting that the multiplexed global proteomic approach can be used to reliably detect differences in protein levels across samples.

The better quantitative agreement for laboratory 2 suggests that longer run-times are required for more reliable quantifications. This observation is in agreement with the finding that higher quantification accuracy is obtained when proteins are identified by a larger number of peptides (Wiśniewski and Rakus, 2014). Differences in the sample processing protocols, including the use of sequential enzymatic digestion with Lys-C and trypsin (laboratory 2) compared with digestion with trypsin alone (laboratory 1), can also add to the quantitative differences observed, when the former is expected to reduce missed cleavages and enhance protein sequence coverage (Guo et al., 2014).

To further assess the accuracy of the TPA-based quantifications, we compared the abundances of cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) drug-metabolizing enzymes with literature data (Fig. 2). Most of the enzyme quantifications fell within the ranges reported in literature. In addition, there was a good quantitative agreement between the two different TMT experiments with an average absolute fold error of 2.1 for the CYP enzymes and 3.5 for the UGT enzymes, respectively.

In summary, our results suggest that multiplexed global proteomic experiments with sufficient proteome coverage can provide reasonable estimates of protein concentrations, as demonstrated with a set of major hepatic uptake transporters, as well as drug-metabolizing enzymes. More importantly, such experiments accurately capture differences in protein levels across samples and may thus be used to compare in vitro and in vivo expression for IVIVE exercises. The global proteomic approach also supports the simultaneous quantification of thousands of other proteins, thus providing a much larger dataset than that obtained by targeted proteomics. Such data can, for example, be useful when studying the age-, gender-, ethnicity-, disease-, and drug-dependent expression of transporters and other drug disposition-related proteins in various tissues or when assessing the appropriateness of an in vitro model compared with the in vivo counterpart. Our study is the first to present evidence suggesting that MS3 intensities from TMT experiments can be applied to TPA-based protein quantifications. Additional validation employing the multiplexed global proteomic approach presented herein can further strengthen its application in quantitative proteomics.

Pharmacokinetics, Dynamics and Metabolism (A.V., E.K., A.D.R., M.V.S.V.) and Discovery Sciences (C.N., F.B.), Medicine Design, Pfizer Worldwide R&D, Groton, Connecticut; IQ Proteomics, Cambridge, Massachusetts (B.K.E., R.C.K.); and MS Bioworks, Ann Arbor, Michigan (R.J.)

Anna Vildhede
Chuong Nguyen
Brian K. Erickson
Ryan C. Kunz
Richard Jones
Emi Kimoto
Francis Bourbonais
A. David Rodrigues
Manthena V.S. Varma

Authorship Contributions

Participated in research design: Vildhede, Nguyen, Erickson, Kunz, Jones, Kimoto, Bourbonais, Rodrigues, Varma.

Conducted experiments: Vildhede, Kunz, Jones.

Performed data analysis: Vildhede, Nguyen.

Wrote or contributed to the writing of the manuscript: Vildhede, Nguyen, Erickson, Kunz, Jones, Kimoto, Bourbonais, Rodrigues, Varma.

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Address correspondence to: Dr. Manthena V. Varma, Pharmacokinetics, Dynamics, and Metabolism, MS 8220-2451, Pfizer Global Research and Development, Pfizer Inc., Groton, CT 06340. E-mail: manthena.v.varma@pfizer.com

SUPPLEMENTAL MATERIAL

Comparison of proteomic quantification approaches for hepatic drug transporters: multiplexed global quantitation correlates with targeted proteomic quantitation

Anna Vildhede, Chuong Nguyen, Brian K. Erickson, Ryan C. Kunz, Richard Jones, Emi Kimoto, Francis Bourbonais, A. David Rodrigues, Manthena V. Varma

DRUG METABOLISM AND DISPOSITION

SUPPLEMENTAL METHODS

Targeted proteomic quantification of hepatic uptake transporters

Liver tissue samples were homogenized in a FastPrep 24 bead mill homogenizer (MP Biomedicals, Santa Ana, CA). Membrane proteins were isolated from the samples using the ProteoExtract™ Native Membrane Protein Extraction Kit (EMD Millipore, Billerica, MA) per manufacturer instructions. Protein concentrations of the extracted membrane fractions were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). The extracted membrane proteins were reduced, denatured, alkylated and digested as described elsewhere with minor modifications (Balogh et al., 2012, Kimoto et al., 2012).

Aliquots containing 200µg (or less) of membrane protein were prepared in triplicate in 40 mM ammonium bicarbonate with 1.36 w/v% sodium deoxycholate. The proteins were reduced with 6 mM dithiothreitol (DTT) for 5 minutes at 95°C followed by alkylation with 15 mM iodoacetamide for 20 minutes, protected from light. Aliquots containing 200µg of human serum albumin were processed under the same conditions for subsequent use as matrix in the construction of an external calibration curve. Proteins were digested with trypsin at 37°C for 24 hours using a trypsin to protein ratio of 1:20. The reaction was quenched by acidification with 0.2% formic acid (FA). Stable isotope labelled (SIL) peptides were added to each sample as internal standards and unlabelled peptides were spiked into the human serum albumin matrix samples to serve as standards for the calibration curve. The samples were centrifuged at 16,000g, 4°C, to pellet the acid-precipitated sodium deoxycholate, and the supernatant was concentrated by evaporation under nitrogen flow prior to reconstitution in 0.1% formic acid for LC-MS/MS analysis.

Targeted LC-MS/MS quantification of NTCP, OAT2, OAT7, OATP1B1, OATP1B3, OATP2B1, and OCT1 peptide surrogates was conducted on a SCIEX 6500 triple quadrupole mass spectrometer operating in ESI mode coupled to a Shimadzu LC-30AD unit. The mobile phases were 0.1% FA in water (A) and 0.1% FA in 90:10 acetonitrile:water (B). A 10 µl sample was injected onto a Phenomenex Kinetex C18 analytical column (2.6 µm, 100 Å, 100 x 3mm) and analytes were separated at a flow rate of 0.5 ml/min using a gradient program starting at 5.5% B for 5 minutes, followed by a linear gradient of 5.5 to 33.3% mobile phase B over 40 minutes, a hold at 33.3% B for 5 min, a washing step using 100% mobile phase B for 5 minutes, and finally re-equilibration for 4 minutes. The doubly charged parent to singly charged product

ion transitions for the analyte peptides and their respective SIL peptides were monitored using optimal declustering potentials, collision energies, and collision cell exit potentials for each peptide (Supplemental Table 1) with the following mass spectrometer instrument settings; 5 kV ion spray voltage, 10 V entrance potential, 10 V collision cell exit potential, and 500°C source temperature. Three transitions were monitored for each peptide, of which one transition was used for quantification and the other two were used for peptide verification. Data were processed using Analyst 1.6.2 (MDS SCIEX, Ontario, Canada).

Multiplexed global proteomic analysis of liver tissue samples

Lab 1. Liver samples were lysed in a Next Advance Bullet Blender (Averill Park, NY) using 1 mm silica beads. Samples were heated to 100°C for 15 min and clarified by centrifugation. Each sample was subjected to trichloroacetic acid precipitation and resultant pellets were resuspended in 8 M urea, 50 mM Tris HCl, pH 8.0 with the addition of Complete Protease Inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were quantified by Qubit fluorometry (Life Technologies, Carlsbad, CA). Duplicate aliquots of 100µg protein of each sample were reduced with 12 mM DTT for 1 hour and alkylated with 15 mM iodoacetamide for another hour at room temperature. Proteins were digested with trypsin overnight using an enzyme to substrate ratio of 1:20. The samples were acidified in FA and subjected to solid phase extraction (SPE) on an Empore SD C18 plate (3M, Maplewood, MN). Samples were lyophilized and reconstituted in 140 mM HEPES, pH 8.0, 30% acetonitrile for Tandem Mass Tag (TMT) labelling. Peptide labelling was carried out according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). After labelling, samples were pooled and subjected to SPE. Labelled peptides were subjected to high pH reverse phase fractionation on a Waters XBridge C18 column (2.1x150 mm, 3.5 µm) at a flow rate of 0.3 ml/min. Fractions were collected every 30 second from 1-49 min and thereafter pooled to create 12 combined fractions, frozen, and lyophilized, followed by reconstitution in 0.1% trifluoroacetic acid (TFA) for LC-MS/MS analysis.

Peptides were analysed by nano LC-MS/MS and LC-MS/MS/MS using an Easy-nLC 1200 UPLC system interfaced to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were loaded onto a trapping column (75 µm x 2 cm) and eluted over an analytical column (75 µm x 75 cm) packed with 2 µm C18 PepMap resin. Each

sample was separated over a 2h gradient at a flow rate of 300 nl/min. The data were acquired two ways: data-dependent MS2 mode and synchronized precursor selection (SPS) MS3 mode. In both cases MS1 scans were acquired in the Orbitrap at a resolution of 120,000. For the MS2 method, precursors were isolated with the quadrupole mass filter set to a width of 0.7 m/z and fragmented using higher energy collisional dissociation (HCD) at 40% normalized collision energy (NCE). Product ions were acquired in the Orbitrap at 50,000 resolution. For the MS3 method, MS2 scans were acquired in the ion trap using collision induced dissociation (CID) at 35% NCE. Five product ions were isolated using synchronized precursor selection and fragmented further using HCD at 65% NCE. MS3 scans were acquired in the Orbitrap at a resolution of 50,000 from m/z 100 to 200. For both methods the mass spectrometer was operated with a 3 second cycle time.

Lab 2. Liver samples were homogenized in 8 M urea, 1% SDS, 50 mM Tris HCl, pH 8.5 with the addition of Complete Protease and PhosSTOP phosphatase Inhibitor cocktails (Roche, Basel, Switzerland). Protein concentrations in the lysates were quantified using the Pierce micro BCA assay (ThermoFisher Scientific, Waltham, MA). Aliquots of 200 µg of protein were reduced with 5 mM DTT and alkylated with 15 mM iodoacetamide, followed by protein precipitation with methanol/chloroform/water (4:2:3 ratio). The protein pellets were resuspended in 8 M urea, 100 mM EPPS, pH 8.0 and the solutions were diluted to 4M urea with 100 mM EPPS, pH 8.0. LysC (Wako Chemicals USA, Richmond, VA) was added at 20 ng/µl (1:50 protease to protein ratio) and the proteins were digested at 25°C for 12 h. Following the LysC digestion, the urea concentration was diluted to 1 M with 100 mM EPPS, pH 8.0 and trypsin (Promega, Madison, WI) was added at 10 ng/µl (1:25 protease to protein ratio). Trypsin digestion was carried out at 37°C for 8 h and the reaction was quenched with TFA. Peptide samples were desalted on Waters tC18 SepPak cartridges (Waters Corporation, Milford, MA) and dried by centrifugal evaporation. Dried peptides were resuspended in 200 mM EPPS, pH 8.0 and peptide concentrations were quantified using the Pierce peptide fluorescence assay (ThermoFisher Scientific, Waltham, MA). The samples were split into duplicates and labelled with TMT 10-plex reagents at a 8:1 ratio (TMT reagent:peptide), incubating at 25°C for 2h. TMT reactions were quenched with 0.5% hydroxylamine and samples were acidified with TFA and combined. Labelled peptides were subjected to orthogonal basic-pH reverse phase fractionation on a 4.6x250 mm column packed

with 3 µm ZORBAX Extend C18 material (Agilent, Santa Clara, CA), utilizing a 45 min linear gradient from 8% buffer A (5% acetonitrile in 10 mM ammonium bicarbonate, pH 8) to 35% buffer B (acetonitrile in 10 mM ammonium bicarbonate, pH 8) at a flow rate of 0.8 ml/min. Ninety six fractions were consolidated into 24 samples, acidified with FA and vacuum dried. The samples were resuspended in 5% FA, desalted on StageTips packed with Empore C18 material (3M, Maplewood, MN), and vacuum dried. Peptides were reconstituted in 5% formic acid, 5% acetonitrile for LC-MS/MS/MS analysis.

Twelve of the 24 fractions were analysed by nano LC-MS/MS/MS on an Orbitrap Fusion Lumos mass spectrometer coupled to an Easy-nLC 1200 UPLC pump. Peptides were separated on an in-house packed 75 µm inner diameter column containing 0.5 cm of Magic C4 resin (5 µm, 100 Å, Michrom Bioresources) followed by 25 cm of GP-C18 resin (1,8 µm, 120 Å, Sepax Technologies). A gradient of 5 to 30% acetonitrile with 0.125% FA was applied over 180 min at a flow rate of 400 nl/min. The mass spectrometer was operated in data-dependent mode. MS1 scans were acquired at a resolution of 120,000. The ten most intense ions were selected for MS/MS. Previously interrogated precursor ions were excluded using a dynamic window of 75 seconds ± 10 ppm. The MS2 precursors were isolated with a quadrupole mass filter set to a width of 0.5 m/z. MS2 scans were acquired in the ion trap using collision induced dissociation at 35% NCE. Ten product ions were isolated using synchronized precursor selection and fragmented using higher energy collisional dissociation of 55% NCE.

Quantification of protein concentrations using the total protein approach

Multiplexed global proteomic data were processed in MaxQuant v. 1.6.0.13 (www.maxquant.org) using protein and peptide false discovery ratios of 0.01 (calculated based on forward/decoy database searching). Protein abundances were calculated from reporter ion intensities using the total protein approach (Wisniewski 2017). This computational method is based on the assumption that the summed MS signal for a given protein in relation to the summed MS signals from all proteins reflects its partial abundance in the sample. By combining this assumption with the molecular weight of the protein, its concentration was calculated according to Equation 1.

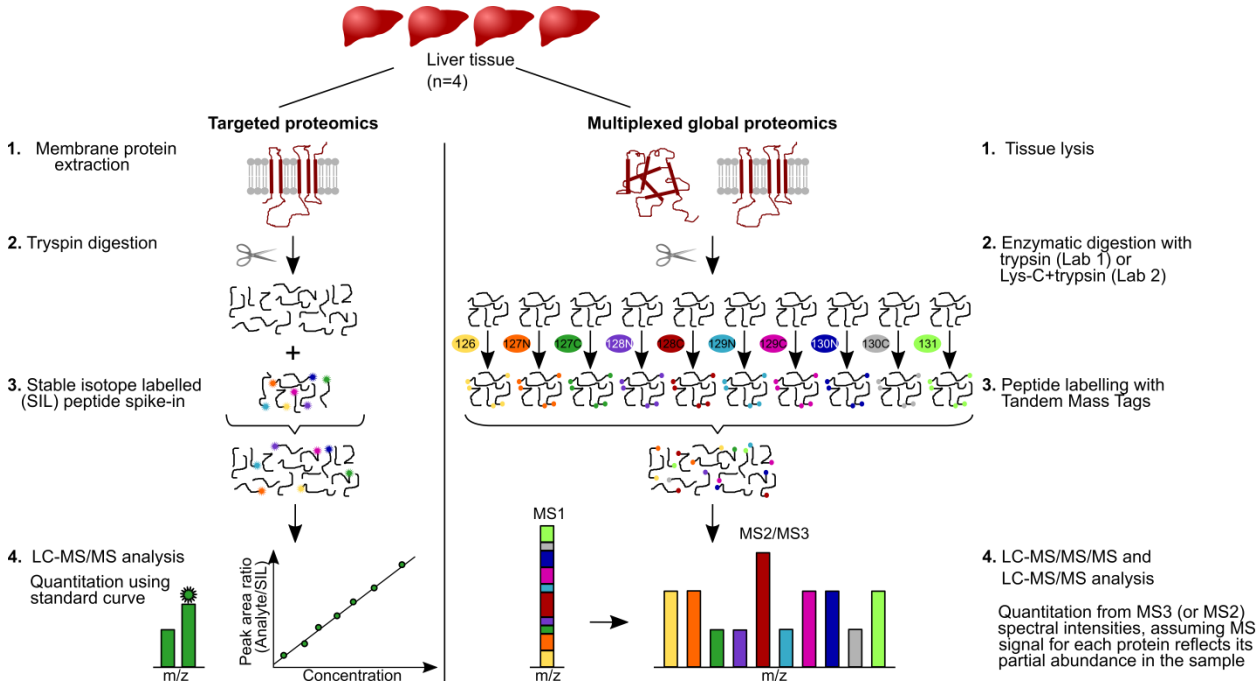
$$Protein\ concentration\ (mol/g) = \frac{MS\ signal_{protein\ i}}{Total\ MS\ signal \times M_{w\ (protein\ i)}} \qquad \text{Equation 1}$$

Supplemental Table 1. Optimized parameters for multiple reaction monitoring (MRM) of surrogate peptides selected for targeted proteomic analysis of hepatic uptake transporters.

Transport protein	Peptide sequence[a]	MRM transitions		DP (V)	CE (V)	CXP (V)
		Precursor ion (m/z)	Product ion (m/z)			
NTCP	GIYDGDLK	440.9	710.4	50	20	15
		440.9	547.3	50	20	15
		440.9	432.2	50	25	5
	GIYDGDL***K***	444.9	718.3	50	20	15
		444.9	555.3	50	20	15
		444.9	440.2	50	25	5
OAT2	NVALLALPR	484.1	753.5	70	30	10
		484.1	569.4	70	30	10
		484.1	456.3	70	30	10
	NVALLALP***R***	489.1	763.5	70	30	10
		489.1	579.4	70	30	10
		489.1	466.3	70	30	10
OAT7	DTLTLEILK	523.6	716.5	100	25	10
		523.6	829.5	100	25	10
		523.6	615.4	100	25	10
	DTLTLEIL***K***	527.6	724.5	100	25	10
		527.6	837.5	100	25	10
		527.6	623.4	100	25	10
OATP1B1	NVTGFFQSFK	587.8	961.5	80	25	16
		587.8	860.4	80	25	16
		587.8	656.3	80	25	16
	NVTGFFQSF***K***	591.8	969.5	80	25	16
		591.8	868.5	80	25	16
		591.8	664.3	80	25	16
OATP1B3	NVTGFFQSLK	570.9	927.4	80	23	16
		570.9	826.4	80	23	16
		570.9	622.4	80	23	16
	NVTGFFQS***L***K	574.3	934.6	80	23	16
		574.3	833.6	80	23	16
		574.3	629.6	80	23	16
OATP2B1	SSPAVEQQLLVSGPGK	798.8	711.9	80	35	16
		798.8	1155.6	80	35	16
		798.8	445.2	80	35	16
	SSPAVEQQLLVSGPG***K***	802.9	715.9	80	35	16
		802.9	1163.6	80	35	16
		802.9	453.2	80	35	16
OCT1	ENTIYLK	441.0	423.6	50	17	10
		441.0	260.2	50	17	10
		441.0	536.6	50	17	10
	ENTIYL***K***	445.1	431.6	50	17	10
		445.1	268.2	50	17	10
		445.1	544.5	50	17	10

DP, declustering potential; CE, collision energy; CXP, collision cell exit potential

[a] Stable isotope labelled amino acid is shown in bold and italic.



Supplemental Figure 1. Illustration of the workflow applied for the targeted proteomic and multiplexed global proteomic experiments. Proteins were extracted from the tissue samples, and subsequently reduced, alkylated, and enzymatically digested into peptides. For the targeted proteomic quantification, stable isotope labelled (SIL) peptides were added as internal standards post-digestion and peptide concentrations were determined by LC-MS/MS using an external calibration curve. For the multiplexed global proteomic quantification, peptides were labelled with tandem mass tag (TMT) reagents in technical duplicates and then the samples were pooled and fractionated, followed by LC-MS/MS/MS and LC-MS/MS analysis. Spectral intensities for the TMT reporter ions, formed upon fragmentation of the tags, were used to calculate protein abundances using the total protein approach (Wisniewski 2017).

SUPPLEMENTAL REFERENCES

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