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Membrane Protein Quantification by Peptide-Based Mass Spectrometry Approaches: Studies on the Organic Anion-Transporting Polypeptide Family

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Abstract

The reliable quantification of drug transport proteins is a key element that is necessary to establish relative scaling factors for human pharmacokinetic predictions and to account for pharmacokinetic variations across populations. Herein, we report a detailed evaluation of targeted mass spectrometry-based quantification methods from the perspective of sample preparation for three major organic anion-transporting polypeptide (OATP) membrane proteins. We also demonstrate the first implementation of stable isotope labeling by amino acids in cell culture (SILAC) into the OATP quantification scheme, which hinges on label incorporation at the protein level to allow for a substantially accelerated sample preparation process with precise results. These methods will enable comparable quantifications to provide individual scaling factors as well as address potential inter-individual variability in OATP proteins that are central to transporter-mediated hepatic drug clearance. The evaluation procedures can also serve as a model for other proteolytically resistant transporters that have not been adequately detected by higher-throughput quantifications. In addition, results derived from various sample preparation conditions explicitly illustrate how the conditions have a differential impact on the quantification of different proteins. Consequently, the ratio between different proteins can be an artifact of the conditions rather than a reflection of the endogenous ratio for typical peptide-based quantifications where a stoichiometrical relationship between the proteolytic peptide and the protein is not established. To the extent that ratios determined under different sets of conditions can underlie different conclusions, our results highlight important limitations that have been underappreciated in recent drug transporter quantifications that extend peptide-based mass spectrometry tools beyond relative quantifications in order to directly compare the abundance of multiple drug transporters.

Keywords: Targeted proteomics; Absolute protein quantification; OATP; SILAC

Abbreviations: ABC: Ammonium Bicarbonate; AQUA: Absolute Quantification; CV: Coefficient of Variation; DOC: Deoxycholate; DTT: Dithiothreitol; Gdn: Guanidine; IS: Internal Standard; MRM: Multiple Reaction Monitoring; OATP: Organic Anion-Transporting Polypeptide; PMAX: ProteaseMAX™ Surfactant; S.E.M: Standard Error of the Mean; SIL: Stable Isotope Label; SILAC: Stable Isotope Labeling by Amino Acids in Cell Culture.

Introduction

The liver is a principal excretory organ that contributes to the elimination of endogenous substances, drugs, and metabolites through the processes of hepatic uptake, metabolism, and biliary excretion. Three members of the organic anion transporting polypeptide (OATP) superfamily (OATP1B1, OATP1B3, and OATP2B1) have been shown to play an important role in hepatic uptake by performing the rate limiting process in hepatic elimination [1]. Consequently, inhibition of each OATP can result in clinical drug-drug interactions that impact systemic exposure and potentially cause adverse effects [2-4]. Given that *in vitro* model- and species-dependent variations in transporter expression constitute a significant hurdle for *in vitro* - *in vivo* extrapolations [5], the quantification of individual proteins becomes critical to establish scaling factors for pharmacokinetic predictions implemented during drug discovery as well as to account for pharmacokinetic variations across different populations. These scaling factors are particularly important in biliary secretion predictions that are highly dependent upon *in vitro* and preclinical data due to lack of access to clinical bile samples. Furthermore, since three OATP isoforms are co-localized on the sinusoidal membrane of hepatocytes and exhibit overlapping substrate specificity, the contribution of each isoform to the hepatic uptake of a mutual substrate is of significant interest to understand key determinants of drug disposition and predict drug-drug interactions. The general lack of selective compounds combined with the potential caveats of multiple binding underscores the need to pursue additional means of estimating contributions to total transport by using relative expression factors in a manner similar to relative activity factors [6].

The application of liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomics has facilitated the identification and quantification of proteins with relevance to drug disposition as well as other aspects of drug discovery and development [7,8]. Generally, targeted proteins are digested into peptides and analyzed by LC-MS/MS as a surrogate measurement of protein levels. Ideally, purified target proteins of known concentration, which structurally mimic endogenous material throughout the entire workflow would be utilized to control levels of protein loss (or enrichment), extraction, denaturation and digestion, as exemplified by protein standard absolute quantification (PSAQ) approaches [9-12]. When such a standard is not available, which is often the case for

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integral membrane transporter proteins, it is important to appreciate the effect of each portion of the experimental design since primary and secondary structural differences among proteins can amount to varying levels of proteolysis. The accuracy of peptide-related approaches at the protein level therefore varies from method to method, and arguably more important, from protein to protein, thereby hindering reliable comparisons between the levels of different proteins. This concept (as well as other complexities/limitations) has been recognized within the fundamental proteomic literature [9,12-17], particularly in studies which exemplify the difficulty in identifying and quantifying membrane proteins [18-22]. Nevertheless, these methods can provide relative quantification values beyond those determined in traditional immunochemical and mRNA methods, which can be limited by the specificity and availability of antibodies and the discrepancy between mRNA and protein levels due to underlying post-translational mechanisms [23,24].

A variety of bottom-up techniques that apply the isotope dilution concept have been described for MS-based protein quantification [25-28]. Among the common method descriptors is the absolute quantification (AQUA) method in which synthesized stable isotope-labeled (SIL) peptide standards are added to digested samples [29]. Although the quantification of integral membrane proteins involves unique challenges predominantly derived from their hydrophobic, proteolytically resistant nature and low expression levels, targeted SIL peptide-based methods have been increasingly incorporated to evaluate drug transporters. For example, studies by Li et al. [30-33] and Zhang et al. [34] have utilized SIL peptides to examine individual ATP-binding cassette transporters in routinely used models of transport and determine their relative expression across tissues and across species that express highly homologous forms of each protein. In addition to these characterizations, a series of recent publications encompassing an extensive number of drug transporters, enzymes, and receptor proteins have focused on incorporating multiplexed selected reaction monitoring into higher-throughput quantifications [35-43]. Although many of the aforementioned studies, including previous reports from our laboratory, are often associated with the phrase "absolute quantification", strictly speaking this phrase refers to the absolute quantification of a surrogate *peptide* to analyze a *protein*, in which the values reflect the relative amounts of a specific protein in different samples. An intriguing paper by Kamiie et al. [35], which proposed the construction of a quantitative atlas encompassing multiple membrane proteins, was followed by related high-throughput studies that targeted hundreds of membrane proteins, many of which remain to be adequately detected. These studies utilize peptide quantification values not only to provide relative ratios for individual proteins, but also to rank or directly compare the abundance of different transporter proteins in human brain microvessels [41,43]. Based on preceding studies (immunoblots, additional peptides, and lack of >20 kDa bands detected by SDS-PAGE after trypsin digestion) [35], Uchida et al. [43] concluded that sample preparation procedures should not affect the quantification of the multidrug resistance protein 1 and breast cancer resistance protein transporters, however, insufficient solubilization and digestion may be a confounding factor for other proteins. In total, this particular application is confounded by the fact that peptide-based strategies are subject to differential error across proteins due to the protein-dependent nature of the sample preparation and digestion process.

Because peptide levels are ultimately used as protein surrogates, even inter-experimental comparisons for a single protein can benefit from more precise methodologies with reduced errors derived from the variability in native membrane protein extraction, denaturation and digestion. The stable isotope labeling by amino acids in cell culture (SILAC) approach is one such method which offers a metabolic-labeling strategy that permits label incorporation during culture [44]. Therefore, rather than introducing an internal standard (IS) later in the workflow, both heavy and light proteins can be combined and the peptides produced can be simultaneously analyzed by LC-MS/MS. In this scenario, the heavy isotope-labeled protein generated in culture eventually serves as the coeluting IS distinguishable by mass spectrometry.

Herein, we present the first detailed evaluation of mass spectrometry-based quantifications targeting three major OATP membrane proteins from the perspective of sample preparation and digestion. We focus on the crucial effects that differences in target protein denaturation/digestion have on MS-based quantifications, and how these effects can contribute to a high level of uncertainty in "absolute protein quantifications". While relative fold-variations for a single protein can be determined with high accuracy, a similar level of confidence in absolute protein concentrations is beyond the MS-based methods that have been employed without protein standards – an important concept to re-emphasize in light of the increasing interest among the drug transporter community in large-scale quantification studies that are directly comparing multiple proteins using peptide-based methods. An optimized preparation process developed to enhance OATP detection in complex samples was utilized to obtain expression levels as well as to compare the hepatic OATP levels with those previously reported in the literature. Furthermore, integration of the SILAC concept resulted in an ideal alternative strategy that allows for the precise analysis of samples, which would ultimately ensure reliable comparisons can be made between experiments.

Materials and Methods

Materials

The ProteoExtract® Native Membrane Protein Extraction Kit (M-PEK) was purchased from Calbiochem (San Diego, CA). Dulbecco's Modified Eagle's Medium-based SILAC Protein Quantitation Kit and the BCA Protein Assay Kit were purchased from Pierce Biotechnology (Rockford, IL). All research grade peptide standards were synthesized and purified by New England Peptide (Gardner, MA). Sequencing Grade Modified Trypsin and ProteaseMAX™ Surfactant (PMAX) were purchased from Promega (Madison, WI). Cell dissociation buffer was purchased from Invitrogen (Carlsbad, CA). ammonium bicarbonate (ABC), dithiothreitol (DTT), iodoacetamide, guanidine (Gdn) and deoxycholate (DOC) were purchased from Sigma (St. Louis, MO). The Protein Lobind tubes and plates were purchased from Eppendorf (North America sales).

Liver tissue and cell culture

Frozen human liver tissues were obtained from the Pfizer Tissue Bank (Groton, CT). Human OATP1B1 and OATP1B3 expressed in HEK293 cells were obtained from Prof. Yuichi Sugiyama (University of Tokyo, Tokyo, Japan) and wild-type (WT) and human OATP2B1 expressed in HEK293 cells were obtained from Prof. Dietrich Keppler (DKFZ, Heidelberg, Germany). HEK293 cell lines were cultured in SILAC media containing 10% dialyzed fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 400 µg/ml geneticin (hOATP1B3 and hOATP2B1), 3 µg/ml blasticidin (hOATP1B1), with either natural L-lysine and L-arginine or $^{13}C_6$ L-lysine and $^{13}C_6$, $^{15}N_4$ L-arginine

according to the manufacturer's recommendations. Samples were initially processed as described below to evaluate the degree of label incorporation and ensure sufficient doublings for incorporation in these cell lines. Once label incorporation was verified, natural and SILAC cell populations grown on T175-culture flasks were harvested using cell dissociation buffer. Subsets of unlabeled pellets were combined with SILAC-derived pellets prior to processing for comparison with pellets processed by the traditional method using external SIL peptides.

Membrane protein extraction

The membrane protein fraction was extracted using a M-PEK procedure based on the manufacturer's suggestions. Briefly, crushed liver tissues and transfected cell pellets were lysed using extraction buffer I containing a protease inhibitor cocktail. Following a 10 min incubation at 4°C, the samples were centrifuged at 16,000 x g for 15 min. The supernatant, which contains soluble proteins, was removed and the pellet was resuspended in extraction buffer II containing a protease inhibitor cocktail. Following a 60 min incubation at 4°C, the samples were centrifuged at 16,000 x g for 15 min and the supernatant containing the membrane fraction was removed for further analysis. Total protein concentrations in the membrane fractions were calculated using the BCA assay with bovine serum albumin as a standard.

Denaturation and digestion

Aliquots containing 80 µg of membrane protein were prepared in 25 mM ABC or 25 mM ABC containing 7 M Gdn and heat-denatured in the presence of 6 mM DTT for 5 min at 95°C. Additional aliquots prepared in 25 mM ABC with 1.25 or 10% w/v DOC or 0.2% w/v PMAX were incubated at room temperature for 10 min followed by reduction with 6 mM DTT for 20 min at 56°C. The selection of temperature was based on manufacturer's recommendations for PMAX and a previous study that indicated heat did not have a significant impact with DOC [16]. A subset of samples containing only the SILAC-derived protein (adjusted to 80 µg with human serum albumin) or 80 µg of human serum albumin alone were also processed under each condition for subsequent use in the quantification of external peptide standards. Extracted liver samples (200 µg each) were processed using 10% w/v DOC only. All samples were alkylated in the dark with 15 mM iodoacetamide at room temperature for 20 min. Trypsin was added to the samples at a 20:1 protein:trypsin ratio and samples were digested at 37°C. The DOC, PMAX, and Gdn concentrations were reduced to 1% w/v, 0.05% w/v, and 1 M, respectively, during the digestion. The ABC, DOC, and Gdn derived samples were collected between 3 and 44 hrs. PMAX derived samples were collected at the final time point only and incubated at 95°C for 5 min to effectively degrade the PMAX. The digestion was stopped by acidification with an equivalent volume of 0.2% formic acid alone (for SILAC incorporation evaluation samples only), 0.2% formic acid containing a peptide IS cocktail or 0.2% formic acid containing IS and a cocktail of unlabeled peptide standards (0.1-400 nM each) to construct each calibration curve. The concentration of SIL-peptide was selected based on preliminary estimates of endogenous OATP proteins in effort to stay within 10-fold of measured values. It should be noted that the OATP1B1 levels were underestimated with respect to the enhanced detection obtained with the additional denaturant during evaluations and the IS levels were adjusted accordingly in subsequent experiments (the SIL-peptide IS was within the linear range of detection in all cases). Samples were centrifuged at 14,000 x g for 5 min to effectively pellet the degraded PMAX and the acid-precipitated DOC and subsequently concentrated in a SpeedVac (Thermo Fisher Scientific) with resuspension in 80 µL prior to LC-MS/MS analysis.

Selection of surrogate OATP peptides

In silico predictive and experimental tools described in the literature [34,35] were used to select tryptic peptides for quantitative analysis. Among the unique peptides that were produced in an *in silico* digestion (http://prospector.ucsf.edu/prospector/mshome.htm), those not known to contain post-translational modifications, transmembrane regions, or sequences encompassing nucleotide polymorphisms were selected as candidate peptides. To ensure the best peptides were selected, each target peptide was verified in digested samples and analyzed by a high resolution instrument (AB Sciex TripleTOF 5600, Toronto, Canada). The compatible peptides with the best overall apparent detection sensitivity were selected as the quantification probes and the corresponding synthetic peptides were used for the analytical optimization of NVTGFFQSFK, IYNSVFFGR, and SSPAVEQQLLVSGPGK as surrogates of OATP1B1, 1B3, and 2B1, respectively (Table S-1). Additional surrogate peptides for OATP1B3 (NVTGFFQSLK) and OATP2B1 (VLAVTDSPAR) were chosen to examine the peptide-dependence of quantification values. The corresponding SIL peptides were synthesized and utilized as the IS with the exception of the SILAC experiments, which incorporated the heavy isotope-labeled proteins as the IS. Since enhanced levels of the alternate OATP1B3 peptide (NVTGFFQSLK) were observed, this peptide was used for the final quantifications of transfected cell lines and liver tissue. Both the analyte and labeled peptides in the complex mixture were simultaneously analyzed by LC-MS/MS using scheduled multiple reaction monitoring (MRM) where either the external SIL peptide or the SILAC-derived peptide served as the co-eluting IS peptide.

LC-MS/MS analysis

Analyses were conducted on an API-4000 triple quadrupole mass spectrometer with an atmospheric pressure electrospray ionization source (MDS SCIEX, Concord, Ontario, Canada). A 10 µL sample was injected onto a Kinetex C18 column (2.6 µm, 100 Å, 100 x 3.0 mm, Phenomenex, Torrance, CA) and eluted by a mobile phase with initial conditions of 5% solvent B for 5 min, followed by a linear gradient of 5% solvent B to 30% solvent B over 20 min (solvent A: 100% H2O with 0.1% formic acid; solvent B: 100% acetonitrile with 0.1% formic acid) at a flow rate of 500 µL/min. Since matrix complexity is one of the significant obstacles in quantification of endogenous material in which a true blank is not available, multichannel MRM analyses were conducted to monitor three transitions per peptide (Table S-2). The scheduled MRM acquisition methods were constructed with three tuned transitions and the optimal declustering potentials, collision energies, and collision cell exit potentials determined for each peptide with a 4.5 kV spray voltage, 10 eV entrance potential, and 450°C source temperature. The analyte and IS were quantified using Analyst 1.4.2 (MDS SCIEX, Ontario, Canada). The minimum signal to noise ratio considered for quantification was 10:1. Final quantifications are representative of the mean of three transitions (Table S-2) measured for each peptide for samples processed in duplicate (transfected cell lines) or triplicate (liver tissue samples), with the exception of the fourth liver tissue, which is representative of the mean of three transitions measured for each peptide for a single sample.

Statistical analysis

Method comparison data for samples processed from the same starting material was analyzed using a repeated measures analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. Differences were considered significant at the level of $p \leq 0.05$.

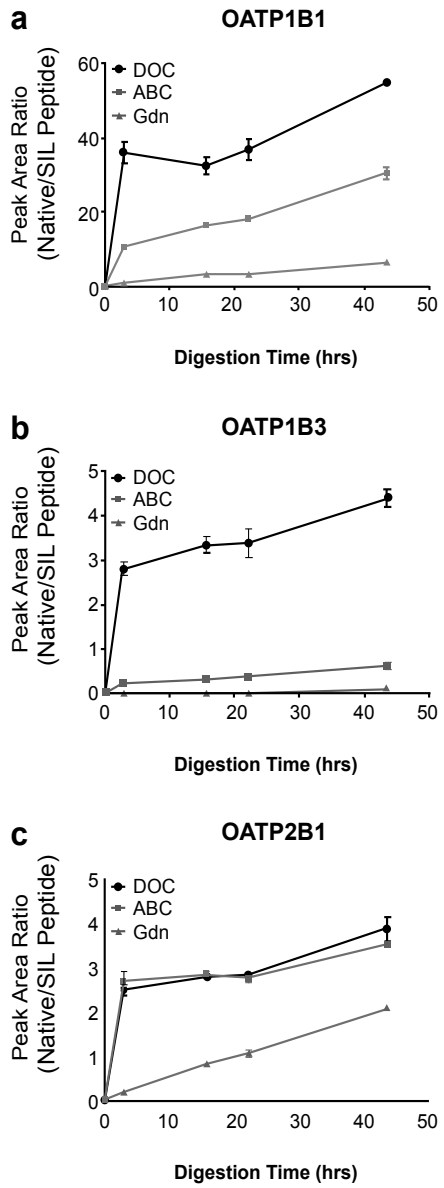

Figure 1: Time course evaluation of sample preparation methods. Plots illustrating the time- and denaturant-dependent production of target peptides from membrane fractions for (a) OATP1B1, surrogate-1, (b) OATP1B3, surrogate-1, and (c) OATP2B1, surrogate-1. All samples were processed from the same initial pellet for each transfected cell line to rule out variability in starting material. Membrane fractions were initially denatured in 25 mM ABC alone or 25 mM ABC containing 7 M Gdn, 0.2% PMAX, or 10% DOC prior to alkylation, dilution, and digestion. Data represent the mean ± standard error of the mean (S.E.M.) for three transitions measured for each peptide by LC-MS/MS at each time point.

Results

Quantification of OATP proteins under various sample preparation conditions

The time-course and denaturing conditions that initially consisted of heat denaturation in ABC buffer (designated as control), 7 M Gdn, or 10% DOC, were examined with OATP1B1, OATP1B3, and OATP2B1. The denaturant concentrations were reduced during the digestion as described in the methods section. In order to eliminate errors derived from sample identity, all digests were prepared from the same initial cell pellet for each transfected cell line. The results presented in Figure 1 and Figure 2 demonstrate that the variation in target peptide production is dependent not only on the preparation method, but on the specific OATP protein targeted. As determined from the final time point in our experiments (which also included an assessment of 0.2% PMAX), Gdn yielded quantification levels lower than controls while DOC provided the most efficient denaturation for OATP1B1 and OATP1B3 (Figure 2). Both DOC and PMAX were comparable with control for OATP2B1, whereas the levels obtained in the presence of Gdn were significantly lower, although still increasing beyond 40 hours (Figure 1c). Alternate surrogate peptides for OATP1B3 and OATP2B1 that met the *in silico* selection criteria were also evaluated and found to range from 1.7- to 4.1-fold of their corresponding partner levels under the conditions tested (Tables S-2 and S-3). Since enhanced levels of the alternate OATP1B3 peptide were observed, this peptide was used for the final quantifications reported below. As expected, the presence of the other two OATP proteins was not detected in each transfected cell line (data not shown).

In addition to the impact on levels determined for a single protein, a differential impact across proteins was evident as the magnitude of variation between preparation conditions was dependent upon the targeted protein. Collectively, the largest discrepancy was observed between DOC and Gdn, with differences of 8.6-, 33.5-, and 1.8-fold for OATP1B1, OATP1B3, and OATP2B1, respectively (Table 1). Based on the results observed with the transfected cell lines, the optimal preparation method with DOC was used to examine OATP proteins in human liver tissue (Table 2). As determined by the surrogate peptides, the levels of hepatic OATP ranged from 3.1 ± 0.1 to 14.9 ± 0.4, 2.4 ± 0.1 to 11.8 ± 0.2, and 1.2 ± 0.1 to 4.6 ± 0.2 fmol/μg membrane protein for OATP1B1, OATP1B3, and OATP2B1, respectively.

Incorporation of the SILAC strategy for the quantification of OATP proteins

The SILAC-labeled OATP proteins were obtained by exchanging lysine and arginine with their heavy isotope-labeled counterparts in culture. Initial LC-MS/MS evaluations demonstrated sufficient label incorporation (98%) after six doublings (Figure S-1). The corresponding batch of labeled OATP1B1 protein was applied to evaluate the utility and feasibility of the SILAC approach for our OATP quantifications. All samples were denatured in the presence of 1.25% DOC for this evaluation since this percentage does not require a large dilution prior to digestion but still provides a substantial increase in signal. A comparison of the results obtained from three digestions (2.5, 5, and 16.5 hrs), processed with and without the SILAC material is shown in Figure 3. Due to incomplete digestion at the earlier time points, the SIL-peptide results ranged from 21.4 ± 0.5 to 66.5 ± 1.0 fmol/μg membrane protein, whereas the OATP1B1 SILAC peptide simultaneously generated during digestion normalized for these inter-

sample differences and provided comparable quantification values between 62.9 ± 4.8 and 64.9 ± 5.8 fmol/μg membrane protein.

Discussion

In order for peptide-based protein quantifications to be accurate at the protein level, they must be reproducible as well as efficient. While the former of these has been the subject of recent transporter studies conducted from an LC-MS/MS perspective, the later remains to be fully appreciated. Any conclusions regarding the rank abundance or ratio of different proteins rely on the assumption that the solubilization and digestion efficiency do not have a differential impact on the quantification of different proteins. Studies that have examined the effect of multiple digestion schemes for plasma proteins [9,15,16] indicate this is not a valid assumption, particularly when faced with the intrinsic limitation of the typical enzymatic digestions employed, which involve a balancing act between facilitating protease access through denaturation without using conditions that impede proteolytic activity or interfere with subsequent MS analysis. Despite these findings, in addition to relative applications, current studies of significant interest within the drug transporter community also utilize peptide quantification values to directly compare the abundance of different transporter proteins [37,41,43]. For example, peptide values are directly equated with protein values to conclude BCRP is 1.6-fold more expressed than MDR1 and MRP4 is 10 times less abundant than MDR1 [41]. Peptide values were also directly equated with protein levels to conclude OATP1B1 has a higher expression level than that of the 1B3 and 2B1 isoforms [37]. Alternate sample processing methods were not examined in the aforementioned studies but the results presented here explicitly illustrate how the preparation conditions can have a differential impact on the quantification of different proteins and thus the ratio between different proteins can be an artifact of the preparation conditions (Table 1: Apparent Ratio) rather than a reflection of the endogenous ratio between proteins for typical peptide-based quantifications that are conducted in the absence of protein standards.

In light of the protein-dependent nature of the preparation process and the low OATP abundance encountered in tissues, we examined the effect of preparation conditions in order to further optimize reliable OATP detection as well as estimate the lower limit of error that would be associated with peptide quantifications extrapolated to the transporter protein level under these conditions. Based on a literature survey of widely used denaturants, we selected DOC, PMAX, and Gdn to compare with the controls heat-denatured in ABC buffer alone. DOC and PMAX were also chosen due to their particular compatibility with MS-based quantifications [16,21,45]. As detailed above, the variations

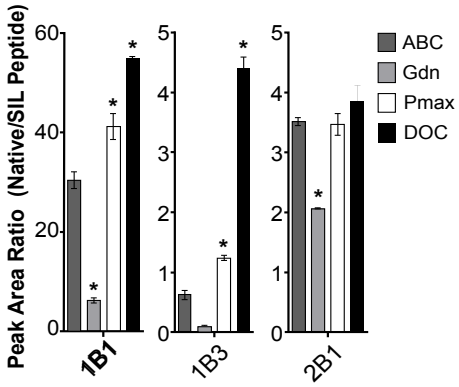

Figure 2: Comparison of sample preparation methods across proteins. Bar graphs emphasizing the denaturant- and protein-dependent response encountered in peptide-based quantifications. Data represent the mean ± S.E.M. for three transitions measured for each peptide by LC-MS/MS for 44 hr digestions. All samples were processed from the same initial pellet for each transfected cell line to rule out variability in starting material. Membrane fractions were initially denatured in 25 mM ABC alone or 25 mM ABC containing 7 M Gdn, 0.2% PMAX, or 10% DOC prior to alkylation, dilution, and digestion. Repeated measures ANOVA followed by Dunnett's Multiple Comparison Test were performed to compare each preparation method with control (designated as heat with ABC buffer alone). *Differences were considered significant at the level of $p \leq 0.05$.

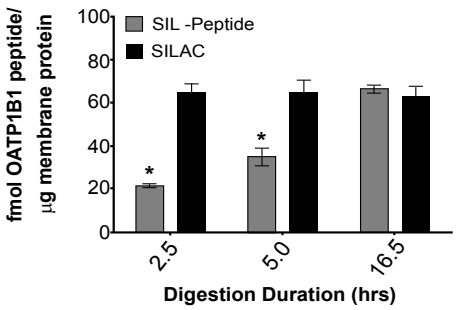

Figure 3: Comparison of SIL-peptide and SILAC quantification approaches. A OATP1B1-transfected cell membrane fraction extracted with and without the OATP1B1 SILAC material was initially denatured in the presence of 1.25% DOC. Digested samples were collected at (1) 2.5 hr, (2) 5 hr, and (3) 16.5 hr time points. Data was processed using the SIL-peptide IS or the SILAC-derived IS that is simultaneously generated during the tryptic digestion and thereby normalizes for inter-sample and inter-experimental error in SILAC samples. Data represent the mean ± S.E.M. for three transitions measured for each peptide by LC-MS/MS at each time point. Repeated measures ANOVA followed by Dunnett's Multiple Comparison Test were performed to compare all quantification values with the value determined for the SILAC approach at 16.5 hrs. *Differences were considered significant at the level of $p \leq 0.05$.

	fmol / μg membrane protein (%CV)[b]			Apparent Ratio[c]
Conditions[a]	OATP1B1	OATP1B3	OATP2B1	1B1 : 1B3 : 2B1
ABC	210.1 ± 4.9 (5.7)	33.0 ± 1.3 (7.7)	39.5 ± 0.9 (5.4)	5.3 : 0.8 : 1
ABC + Gdn	42.9 ± 1.8 (10.2)	4.1 ± 0.1 (5.1)	23.5 ± 0.4 (4.2)	1.8 : 0.2 : 1
ABC + PMAX	299.8 ± 14.9 (12.2)	66.5 ± 3.9 (11.7)	36.7 ± 0.8 (5.5)	8.2 : 1.8 : 1
ABC + DOC	367.2 ± 8.1 (5.4)	137.2 ± 4.7 (6.8)	43.3 ± 1.3 (7.2)	8.4 : 3.2 : 1

[a]Membrane fractions were initially denatured in 25 mM ABC alone or 25 mM ABC containing 7 M Gdn, 0.2% PMAX, or 10% DOC prior to alkylation, dilution, and digestion.

[b]Data represent the mean ± S.E.M. and coefficient of variation (CV%) for three transitions measured for each peptide by LC-MS/MS for 44 hr digestions processed in duplicate. Samples were processed from the same initial pellet for each transfected cell line to rule out variability in starting material.

[c]The apparent ratio between cell lines is provided to demonstrate how preparation conditions have a differential impact on the quantification of different proteins and thus alter the apparent ratio that is observed between different proteins.

Table 1: Quantification of OATP1B1 (surrogate-1), OATP1B3 (surrogate-2), and OATP2B1 (surrogate-1) peptides in transfected cell line membrane fractions under different sample preparation conditions.

in quantification values were found to be dependent upon the sample preparation conditions as well as the specific OATP analyzed. Although the trend with DOC was favorable for all three proteins, the level of enhancement over the minimum values observed with Gdn varied from 1.8- to 33.5-fold increases across the proteins.

Quantification values for a single protein have also been observed to be surrogate peptide-dependent [9,46]. Recently, the quantification of a second surrogate peptide under a single condition was proposed to serve as one confirmation that preparation procedures do not significantly affect quantification [35,43]. In light of this we quantified alternate surrogate peptides for OATP1B3 and OATP2B1 under multiple conditions to further assess the utility of this approach (Table S-1). Collectively, the values determined for both pairs of peptides (IYNSVFFGR / NVTGFFQSLK and SSPAVEQQLLVSGPGK / VLAVTDSPAR) fluctuated between 1.7- and 4.1-fold without a clear trend with regard to denaturant (Table S-3). Although the comparison of multiple peptides is of interest during peptide selection, this approach was not effective to predict the magnitude of the impact encountered with different sample preparation conditions. As an alternative, previous attempts have examined digestion efficiency through the use of extended surrogate peptides that contain a small number of amino acids from the surrounding sequence. However, when possible, PSAQ methods offer the optimal IS as the denaturation and accessibility of a short peptide under protease-compatible conditions is unlikely to reflect that of a large integral membrane protein.

While these results will presumably differ for additional proteins and methods, they indicate DOC would be a valuable addition to OATP quantification protocols. DOC is an ionic detergent reported to be compatible with both trypsin activity and downstream MS analysis after precipitation at low pH. Owing to its bile acid structure, DOC exhibits different properties from typical linear-chain ionic detergents; however, it still offers a beneficial level of solubilization and denaturation for some proteins [21,47]. Consequently, it can provide a MS-compatible alternative to sodium dodecyl sulfate, which can enhance digestion efficiencies that may aid not only in peptide coverage, but also in improved detection of certain proteolytically resistant proteins [16,45]. Despite this improvement, it is still important to note that methods can appear to reach completion at their own steady state even though digestion may not be complete with respect to the theoretical maximum (Figure 4). This complexity, which has also been described for several plasma proteins [16], undoubtedly hinders an assessment of protein level accuracy in the absence of full-length standards of known concentration as discussed above. Nonetheless, targeted method optimization can improve accuracy as well as detection by increasing the signal and closing the gap between the observed and the theoretical maximum of peptide production. Pursuit of alternate preparation approaches or quality membrane protein standards, perhaps by utilizing nanolipoprotein particles [48,49], may allow us to address any remaining gaps in quantification levels wherein the accuracy will only be limited by the initial assessment of standard material. Future work in this area is also needed to discriminate between active and inactive protein forms, as total membrane protein fractions may include components beyond functional transporter on the cell surface.

Recent high-throughput quantification studies by Sakamoto et al. [39] and Ohtsuki et al. [37] provide an opportunity to examine the different reports of liver OATP levels. Although there are additional factors such as inter-individual variation in expression levels and sample integrity that can contribute to differences in quantification

	fmol / µg membrane protein (%CV)[a,b]		
Donor (Gender, Age, Ethnicity)	**OATP1B1**	**OATP1B3**	**OATP2B1**
Male, 66, Unknown	14.9 ± 0.4 (7.8)	6.6 ± 0.2 (7.9)	3.6 ± 0.2 (13.7)
Male, 45, Asian	13.7 ± 0.4 (9.0)	11.8 ± 0.2 (4.9)	4.6 ± 0.2 (12.1)
Male, 50, Asian	3.1 ± 0.1 (12.0)	2.8 ± 0.1 (12.0)	1.2 ± 0.1 (12.0)
Female, 65, Unknown[c]	10.6 ± 0.3 (4.6)	2.4 ± 0.1 (3.8)	2.0 ± 0.1 (5.3)

[a]Membrane fractions were initially denatured in 25 mM ABC containing 10% DOC prior to alkylation, dilution, and digestion.

[b]Data represent the mean ± S.E.M. and coefficient of variation (CV%) for three transitions measured for each peptide by LC-MS/MS for 44 hr digestions processed in triplicate.

[c]Final quantification values for the fourth liver tissue are representative of the mean of three transitions measured for each peptide for a single sample.

Table 2: Quantification of OATP1B1 (surrogate-1), OATP1B3 (surrogate-2), and OATP2B1 (surrogate-1) peptides in human liver membrane fractions.

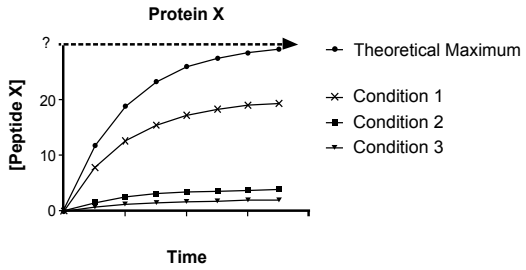

Figure 4: Digestion profile evaluation. The protein quantification results obtained with various sample processing methods may appear to reach completion although the digestion may not be complete with respect to the theoretical maximum for a specific protein. The optimization of native protein processing for a targeted protein can significantly improve digestion efficiency and thereby improve detection by closing the gap between the theoretical maximum and the observed peptide production under a given set of conditions.

values, the OATP1B1 values obtained from liver samples (reported to be processed and analyzed using the same method in these two studies) varied over 40-fold between these reports. Inspection of the individual values provided for 17 donors by Ohtsuki et al. [37] revealed a stark discrepancy among the donors as OATP1B1 was below the limit of quantification for 9 of the donors and as high as 12.3 pmol/mg in a single donor, the latter of which is comparable to the levels obtained with DOC (Table 2). Detailed method evaluations from the perspective of sample preparation and digestion were not reported in the aforementioned studies so it still remains to be determined what portion of the variability can be attributed to true inter-individual variability. The origin of our samples were a combination of Asian and unknown donors whereas those characterized by Ohtsuki et al. [37] were predominately Caucasian. Additional quality liver samples are clearly required to properly address genetic variation in different sample populations but the emerging variation underscores the need to implement an evaluated sample preparation and digestion method. The inclusion of an inter-study protein IS could also better ensure reliable comparisons are made as further discussed below.

Several IS approaches have been applied in MS-based protein quantifications, including chemical derivatization, synthetic SIL peptides, and metabolic incorporation of heavy-labeled amino acids [50]. SIL-based methods are a powerful tool for relative studies and may still represent the most feasible approach to evaluate the *in vitro* systems routinely used to characterize transport with potential drug candidates, particularly when relative scaling factors for individual proteins are sufficient to extrapolate data from existing well-characterized *in vitro* models. The adaptation of several quantifications to a single high-throughput format is not a trivial task given that both the primary and secondary structural differences among proteins can amount to varying levels of proteolysis with any given method. SIL-peptide methods are among the most common techniques previously used to evaluate the expression of drug transporters. However, due to different biochemical properties, SIL-peptides can only be added either during or post digestion to serve as the IS for the remaining portion of the experiment. One of the most important advantages recognized for SILAC is the ability to normalize for variability derived from any portion of the workflow [10,44,51-54]. This advantage renders SILAC particularly useful in approaches that hinge on label incorporation at the protein level to decouple results from errors encountered prior to digestion. In this study, we report what is to our knowledge the first incorporation of the SILAC strategy in the quantification of OATP proteins (Figure 3). Utilizing the OATP1B1 transfected cell line as a model, our comparison of a typical SIL-peptide method with the SILAC method demonstrated that the SILAC approach is feasible for our purposes. The capacity to normalize data using the SILAC material will allow for substantially accelerated preparations and more precise measurements to ensure reliable comparisons can be made between methods/studies.

The reliable determination of OATP proteins in *in vitro* models and the liver offers an important advancement with respect to pharmacokinetic predictions as well as inter-individual variability characterizations. The distributions will be particularly useful to understand for proteins such as the OATPs, which are not only important in hepatic clearance and drug-drug interactions, but are central to tissue targeting strategies that may be exploited in drug development [55]. The methods detailed here will enable comparable quantifications to better provide scaling factors and address the potential inter-individual variability for OATP membrane proteins. Furthermore, given the early introduction of the isotope label during the SILAC approach, alternative OATP sample enrichment techniques may now be utilized without further impact on variability. Similar in concept to the stable isotope standards and capture by anti-peptide antibodies (SISCAPA) method that is implemented at the peptide level to capture tryptic peptides and prevent the loss of low abundance peptides [56], the SILAC material is compatible with inclusion of immunoaffinity enrichment at the protein level. These components will be advantageous in future tissue quantifications where OATP or other transport proteins may constitute a small percentage of proteins in a biological sample. In total, these evaluation procedures can serve as a useful model for several proteolytically resistant transporters that have not been adequately detected by higher-throughput quantification attempts and importantly, they raise caution with respect to interpreting recent studies that report conclusions regarding the comparison of multiple transporter proteins, which are based on surrogate peptide analyses.

Supplementary Material

Additional tables (Tables S-1, S-2, and S-3) and Figure S-1, which includes label incorporation, MRM transitions, and dual peptide evaluations as noted in the text.

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References

1. Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, et al. (2010) Membrane transporters in drug development. Nat Rev Drug Discov 9: 215-236.
2. Lau YY, Huang Y, Frassetto L, Benet LZ (2007) Effect of OATP1B transporter inhibition on the pharmacokinetics of atorvastatin in healthy volunteers. Clin Pharmacol Ther 81: 194-204.
3. Poirier A, Funk C, Lave T, Noe J (2007) New strategies to address drug-drug interactions involving OATPs. Curr Opin Drug Discov Devel 10: 74-83.
4. Smith NF, Figg WD, Sparreboom A (2005) Role of the liver-specific transporters OATP1B1 and OATP1B3 in governing drug elimination. Expert Opin Drug Metab Toxicol 1: 429-445.
5. Lai Y (2009) Identification of interspecies difference in hepatobiliary transporters to improve extrapolation of human biliary secretion. Expert Opin Drug Metab Toxicol 5: 1175-1187.
6. Hirano M, Maeda K, Shitara Y, Sugiyama Y (2004) Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the hepatic uptake of pitavastatin in humans. J Pharmacol Exp Ther 311: 139-146.
7. Chen G, Mirza UA, Pramanik BN (2009) Macromolecules in Drug Discovery: Mass Spectrometry of Recombinant Proteins and Proteomics, in Advances in Chromatography. Taylor & Francis CRC Press.
8. Gross ML, Chen G, Pramanik BN (2011) Protein and Peptide Mass Spectrometry in Drug Discovery. John Wiley & Sons.
9. Brun V, Dupuis A, Adrait A, Marcellin M, Thomas D, et al. (2007) Isotope-labeled protein standards: toward absolute quantitative proteomics. Mol Cell Proteomics 6: 2139-2149.
10. Hanke S, Besir H, Oesterhelt D, Mann M (2008) Absolute SILAC for accurate quantitation of proteins in complex mixtures down to the attomole level. J Proteome Res 7: 1118-1130.
11. Ishihama Y, Sato T, Tabata T, Miyamoto N, Sagane K, et al. (2005) Quantitative mouse brain proteomics using culture-derived isotope tags as internal standards. Nat Biotechnol 23: 617-621.
12. Lebert D, Dupuis A, Garin J, Bruley C, Brun V (2011) Production and use of stable isotope-labeled proteins for absolute quantitative proteomics. Methods Mol Biol 753: 93-115.
13. Arsene CG, Ohlendorf R, Burkitt W, Pritchard C, Henrion A, et al. (2008) Protein quantification by isotope dilution mass spectrometry of proteolytic fragments: cleavage rate and accuracy. Anal Chem 80: 4154-4160.
14. Brun V, Masselon C, Garin J, Dupuis A (2009) Isotope dilution strategies for absolute quantitative proteomics. J Proteomics 72: 740-749.
15. Klammer AA, MacCoss MJ (2006) Effects of modified digestion schemes on the identification of proteins from complex mixtures. J Proteome Res 5: 695-700.
16. Proc JL, Kuzyk MA, Hardie DB, Yang J, Smith DS, et al. (2010) A quantitative study of the effects of chaotropic agents, surfactants, and solvents on the digestion efficiency of human plasma proteins by trypsin. J Proteome Res 9: 5422-5437.
17. Zhang G, Fenyo D, Neubert TA (2009) Evaluation of the variation in sample preparation for comparative proteomics using stable isotope labeling by amino acids in cell culture. J Proteome Res 8: 1285-1292.
18. Agarwal N, Lippmann ES, Shusta EV (2010) Identification and expression profiling of blood-brain barrier membrane proteins. J Neurochem 112: 625-635.
19. Grant KJ, Wu CC (2007) Advances in neuromembrane proteomics: efforts towards a comprehensive analysis of membrane proteins in the brain. Brief Funct Genomic Proteomic 6: 59-69.
20. Rabilloud T (2009) Membrane proteins and proteomics: love is possible, but so difficult. Electrophoresis 30: S174-S180.
21. Speers AE, Wu CC (2007) Proteomics of integral membrane proteins--theory and application. Chem Rev 107: 3687-3714.

22. Wu CC, Yates JR, 3rd (2003) The application of mass spectrometry to membrane proteomics. Nat Biotechnol 21: 262-267.

23. Haynes PA, Gygi SP, Figeys D, Aebersold R (1998) Proteome analysis: biological assay or data archive? Electrophoresis 19: 1862-1871.

24. Michaud GA, Salcius M, Zhou F, Bangham R, Bonin J, et al. (2003) Analyzing antibody specificity with whole proteome microarrays. Nat Biotechnol 21: 1509-1512.

25. Barr JR, Maggio VL, Patterson DG, Jr, Cooper GR, Henderson LO, et al. (1996) Isotope dilution--mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I. Clin Chem 42: 1676-1682.

26. Chen G, Pramanik BN (2008) LC-MS for protein characterization: current capabilities and future trends. Expert Rev Proteomics 5: 435-444.

27. Chen G, Pramanik BN (2009) Application of LC/MS to proteomics studies: current status and future prospects. Drug Discov Today 14: 465-471.

28. Zhang G, Annan RS, Carr SA, Neubert TA (2010) Overview of peptide and protein analysis by mass spectrometry. Curr Protoc Protein Sci Chapter 16: Unit16.1.

29. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci U S A 100: 6940-6945.

30. Li N, Bi YA, Duignan DB, Lai Y (2009) Quantitative expression profile of hepatobiliary transporters in sandwich cultured rat and human hepatocytes. Mol Pharm 6: 1180-1189.

31. Li N, Nemirovskiy OV, Zhang Y, Yuan H, Mo J, et al. (2008) Absolute quantification of multidrug resistance-associated protein 2 (MRP2/ABCC2) using liquid chromatography tandem mass spectrometry. Anal Biochem 380: 211-222.

32. Li N, Palandra J, Nemirovskiy OV, Lai Y (2009) LC-MS/MS mediated absolute quantification and comparison of bile salt export pump and breast cancer resistance protein in livers and hepatocytes across species. Anal Chem 81: 2251-2259.

33. Li N, Zhang Y, Hua F, Lai Y (2009) Absolute difference of hepatobiliary transporter multidrug resistance-associated protein (MRP2/Mrp2) in liver tissues and isolated hepatocytes from rat, dog, monkey, and human. Drug Metab Dispos 37: 66-73.

34. Zhang Y, Li N, Brown PW, Ozer JS, Lai Y (2011) Liquid chromatography/tandem mass spectrometry based targeted proteomics quantification of P-glycoprotein in various biological samples. Rapid Commun Mass Spectrom 25: 1715-1724.

35. Kamiie J, Ohtsuki S, Iwase R, Ohmine K, Katsukura Y, et al. (2008) Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. Pharm Res 25: 1469-1483.

36. Kawakami H, Ohtsuki S, Kamiie J, Suzuki T, Abe T, et al. (2011) Simultaneous absolute quantification of 11 cytochrome P450 isoforms in human liver microsomes by liquid chromatography tandem mass spectrometry with in silico target peptide selection. J Pharm Sci 100: 341-352.

37. Ohtsuki S, Schaefer O, Kawakami H, Inoue T, Liehner S, et al. (2012) simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. Drug Metab Dispos 40: 83-92.

38. Ohtsuki S, Uchida Y, Kubo Y, Terasaki T (2011) Quantitative targeted absolute proteomics-based ADME research as a new path to drug discovery and development: methodology, advantages, strategy, and prospects. J Pharm Sci 100: 3547-3559.

39. Sakamoto A, Matsumaru T, Ishiguro N, Schaefer O, Ohtsuki S, et al. (2011) Reliability and robustness of simultaneous absolute quantification of drug transporters, cytochrome P450 enzymes, and Udp-glucuronosyltransferases in human liver tissue by multiplexed MRM/selected reaction monitoring mode tandem mass spectrometry with nano-liquid chromatography. J Pharm Sci 100: 4037-4043.

40. Schaefer O, Ohtsuki S, Kawakami H, Inoue T, Liehner S, et al. (2012) Absolute Quantification and Differential Expression of Drug Transporters, Cytochrome P450 Enzymes, and UDP-Glucuronosyltransferases in Cultured Primary Human Hepatocytes. Drug Metab Dispos 40: 93-103.

41. Shawahna R, Uchida Y, Decleves X, Ohtsuki S, Yousif S, et al. (2011) Transcriptomic and quantitative proteomic analysis of transporters and drug metabolizing enzymes in freshly isolated human brain microvessels. Mol Pharm 8: 1332-1341.

42. Uchida Y, Ohtsuki S, Kamiie J, Terasaki T (2011) Blood-brain barrier (BBB) pharmacoproteomics: reconstruction of in vivo brain distribution of 11 P-glycoprotein substrates based on the bbb transporter protein concentration, in vitro intrinsic transport activity and unbound fraction in plasma and brain in mice. J Pharmacol Exp Ther 339: 579-588.

43. Uchida Y, Ohtsuki S, Katsukura Y, Ikeda C, Suzuki T, et al. (2011) Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. J Neurochem 117: 333-345.

44. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, et al. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1: 376-386.

45. Lin Y, Zhou J, Bi D, Chen P, Wang X, et al. (2008) Sodium-deoxycholate-assisted tryptic digestion and identification of proteolytically resistant proteins. Anal Biochem 377: 259-266.

46. Kuhn E, Wu J, Karl J, Liao H, Zolg W, et al. (2004) Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and 13C-labeled peptide standards. Proteomics 4: 1175-1186.

47. Helenius A, McCaslin DR, Fries E, Tanford C (1979) Properties of detergents. Methods Enzymol 56: 734-749.

48. Cappuccio JA, Hinz AK, Kuhn EA, Fletcher JE, Arroyo ES, et al. (2009) Cell-free expression for nanolipoprotein particles: building a high-throughput membrane protein solubility platform. Methods Mol Biol 498: 273-296.

49. Katzen F, Peterson TC, Kudlicki W (2009) Membrane protein expression: no cells required. Trends Biotechnol 27: 455-460.

50. Elliott MH, Smith DS, Parker CE, Borchers C (2009) Current trends in quantitative proteomics. J Mass Spectrom 44: 1637-1660.

51. Geiger T, Wisniewski JR, Cox J, Zanivan S, Kruger M, et al. (2011) Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. Nat Protoc 6: 147-157.

52. Harsha HC, Molina H, Pandey A (2008) Quantitative proteomics using stable isotope labeling with amino acids in cell culture. Nat Protoc 3: 505-516.

53. Ong SE, Mann M (2007) A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). Nat Protoc 1: 2650-2660.

54. Ong SE, Mann M (2007) Stable isotope labeling by amino acids in cell culture for quantitative proteomics. Methods Mol Biol 359: 37-52.

55. Oballa RM, Belair L, Black WC, Bleasby K, Chan CC, et al. (2011) Development of a liver-targeted stearoyl-CoA desaturase (SCD) inhibitor (MK-8245) to establish a therapeutic window for the treatment of diabetes and dyslipidemia. J Med Chem 54: 5082-5096.

56. Anderson NL, Jackson A, Smith D, Hardie D, Borchers C, et al. (2009) SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device. Mol Cell Proteomics 8: 995-1005.

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