Review Article

Theme: Targeted Proteomics Quantification for Membrane Proteins

Guest Editors: Marilyn E. Morris and Yurong Lai

Mass Spectrometry-Based Targeted Proteomics as a Tool to Elucidate the Expression and Function of Intestinal Drug Transporters

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Abstract. Intestinal transporter proteins affect the oral bioavailability of many drugs in a significant manner. In order to estimate or predict their impact on oral drug absorption, data on their intestinal expression levels are needed. So far, predominantly mRNA expression data are available which are not necessarily correlated with the respective protein content. All available protein data were assessed by immunoblotting techniques such as Western blotting which both possess a number of limitations for reliable protein quantification. In contrast to this, mass spectrometry-based targeted proteomics may represent a promising alternative method to provide comprehensive protein expression data. In this review, we will summarize so far available intestinal mRNA and protein expression data for relevant human multidrug transporters. Moreover, recently observed mass spectrometry-based targeted proteomic data will be presented and discussed with respect to potential functional consequences. Associated to this, we will provide a short tutorial how to set up these methods and emphasize critical aspects in method development. Finally, potential limitations and pitfalls of this emerging technique will be discussed. From our perspective, LC-MS/MS-based targeted proteomics represents a valuable new method to comprehensively analyse the intestinal expression of transporter proteins. The resulting expression data are expected to improve our understanding about the intestinal processing of drugs.

KEY WORDS: drug transporters; intestinal; mass spectrometry; quantification; targeted proteomics.

INTRODUCTION

The oral drug absorption is a very complex process which is influenced by many poorly predictable factors such as gastrointestinal motility, intestinal water content, drug liberation from the administered dosage form, drug dissolution properties, passive diffusion, intestinal phase I and II metabolism as well as uptake and efflux transport (1,2). Therefore, it is not surprising that the oral administration of drugs, although being the most favourable administration route, is associated with several problems such as (1) low and highly variable (inter- and intra-subject) bioavailability for many drugs; (2) substantial impact of the administered dosage form on drug absorption for many compounds; and (3) poorly predictable drug–drug or drug–food interactions (1,3,4). As a consequence, it is still challenging to predict the oral absorption of drugs or to estimate intestinal DDIs despite the availability of highly sophisticated physiologically based pharmacokinetic modeling and prediction tools (5,6).

During the last decade, it was clearly pointed out that the oral bioavailability of many drugs is significantly influenced by several intestinal transporter proteins (7). With reference to this, ABC (ATP binding cassette) transporters such as ABCB1 (P-glycoprotein), ABCC2 (MRP2) and ABCG2 (BCRP) act as efflux transporters thereby limiting the intestinal absorption of many compounds by pumping them back to gut lumen (Fig. 1). Thus, co-administration of inducers or inhibitors and substrates of these proteins were shown to cause clinically relevant drug–drug interactions (DDIs, Table I) (3,8–17). Moreover, several uptake carriers from the SLC transporter family have been reported to mediate the intestinal uptake of many endogenous compounds (e.g. bile acids by ASBT, sugars by GLUTs) and drugs (e.g. β -lactam antibiotics and ACE inhibitors by PEPT1) (7,18). However, their intestinal expression and contribution to intestinal drug absorption are less well documented and understood and in part controversially discussed (e.g. intestinal expression of OATPs, see section “[Available Intestinal Expression Data](#)”) (19). The same is true for drug transporters that are expressed at the basolateral membrane of the enterocytes which could act as functional uptake (ABCC1, ABCC3, OSTalpha) or efflux carriers (e.g. OCT1).

The intestinal processing of drugs is even more complicated by that fact that the expression of different metabolizing enzymes and transporter proteins is most likely not homogeneous along the human intestine as indicated by several mRNA expression studies (see section “[Available Intestinal Expression](#)”).

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Fig. 1. Schematic overview of clinically relevant intestinal uptake (blue) and efflux (red) transporters as well as cytochrome P450 enzymes (CYP), UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT) which were shown to affect the oral absorption of many drugs (according to Giacomini *et al.* 2010 (7), Paine *et al.* 2006 (20), Riches *et al.* 2009 (21) and Harbourt *et al.* 2012 (22))

Data”). Therefore, the extent of intestinal drug absorption may be influenced by the intestinal site of drug release and, thus, be determined by the administered dosage form (e.g. immediate vs. sustained release) or co-administered drugs that affect gastrointestinal motility (e.g. anticholinergic drugs) or transporter function (inhibition/induction). This assumption is supported by some experimental clinical studies that demonstrated site-dependent intestinal absorption of drugs that are known to be transporter substrates (23–26). For example, the intestinal absorption of the ABCB1 substrate talinolol was shown to be significantly higher from the upper small intestine than from

lower parts, which is in well agreement to the observation that the intestinal ABCB1 expression increases from duodenum to ileum (25–27). In parallel to their impact on intestinal drug absorption, different expression pattern of transporters along the gut may also affect their probability to contribute to intestinal DDIs (3,28). Accordingly, one would expect predominately interactions involving transporters that are highly expressed in the small intestine (e.g. ABCB1), i.e. the site of predominant drug absorption, but not for those that are most abundant in more distal parts of the small intestine or even in colon.

Table I. Examples for Clinically Relevant Drug–Drug Interactions Caused by Induction or Inhibition of Intestinal Transporter Proteins				
Substrate (victim)	Inhibitor/inducer	PK change	Reason	Reference
Digoxin	Rifampicin (600 mg, 10 days)	F↓ 30%	Induction of ABCB1	Greiner <i>et al.</i> 1999 (17)
Digoxin	Clarithromycin (250 mg, b.i.d., 3 days)	AUC↑ 64%	Inhibition of ABCB1	Rengelshausen <i>et al.</i> 2003 (13)
Ezetimibe	Rifampicin (600 mg, 8 days)	AUC ^a ↓ 63%	Induction of ABCB1, ABCC2	Oswald <i>et al.</i> 2006 (11)
Ezetimibe	Rifampicin (600 mg, SD)	AUC ^a ↑ 92%	Inhibition of ABCB1, ABCC2, OATP1B1	Oswald <i>et al.</i> 2006 (12)
Talinolol	Rifampicin (600 mg, 8 days)	F↓ 35%	Induction of ABCB1	Westphal <i>et al.</i> 2000 (16)
Talinolol	Erythromycin (500 mg, SD)	AUC↑ 52%	Inhibition of ABCB1	Schwarz <i>et al.</i> 2000 (14)
Talinolol	Grapefruit juice (SD)	AUC↓ 56%	Inhibition of intestinal OATPs	Schwarz <i>et al.</i> 2005 (15)
Fexofenadine	Rifampicin (600 mg, 8 days)	CL/F↑ 87–164%	Induction of ABCB1	Hamman <i>et al.</i> 2001 (9)
Fexofenadine	Rifampicin (600 mg, SD)	CL/F↓ 73–76%	Inhibition of ABCB1, ABCC2, OATP1B1/3	Kusuhara <i>et al.</i> 2013 (10)
Fexofenadine	Grapefruit juice (SD)	AUC↓ 63%	Inhibition of intestinal OATPs	Dresser <i>et al.</i> 2002 (8)

^a Sum of ezetimibe and ezetimibe glucuronide

However, only very limited data on intestinal transporter protein expression are available to draw reliable conclusions on their relevance for drug absorption or DDIs in general. On the other side, these data are of great interest for industry and academia to estimate or even to predict the relevance of the aforementioned transporters in oral drug absorption (5,6,28,29).

The majority of so far available information is based on mRNA expression data which must not necessarily be correlated with the respective protein expression (30–40). The few available protein data were generated by former methods of choice for protein quantification, namely immunohistochemistry and Western blotting (11,16,27,41,42). However, these methods possess a number of substantial limitations which make them appear inappropriately for reliable protein quantification (see section “Protein Quantification by Immunoblotting”). This is especially true when one considers the emerging role of transporter proteins in drug development and regulatory affairs and the need for robust and reliable protein expression data from *in vitro* or *in vivo* models to conclude on their pharmacokinetic relevance (43).

A novel and promising approach for the determination of transporter proteins is liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based targeted proteomics (44,45). Here, amino acid sequence-dependent mass transitions of protein specific peptides generated by tryptic digestion are monitored for quantification. Because the tryptic digestion of the respective protein is supposed to be complete, the molarity of the measured peptide acts as a surrogate for the respective protein expression. Therefore, these methods are expected to assess the absolute protein expression levels by using synthetic peptide standards and isotope labelled internal standards. These methods have been successfully used to quantify several ABC (e.g. ABCB1, ABCC2 and ABCG2) and SLC transporters (e.g. OATP1B1, OATP2B1, OATP1B3 and NTCP) in human tissue (liver, brain and kidney), human cells (hepatocytes, platelets) and transfected cell lines (46–64). However, to our knowledge, this emerging technique has so far not been applied to quantify these transporter proteins in human intestinal tissue.

In this review, we will summarize so far available mRNA and protein expression data for clinically relevant multidrug transporters in the human intestine. Moreover, we will present own mass spectrometry-based protein quantification data and discuss advantages and limitations of this novel method as well as potential functional implications of the observed transporter protein expression in the human intestine.

AVAILABLE INTESTINAL EXPRESSION DATA

The expression of transporter proteins in the human intestine has been investigated by several studies (27,30–39,41,42). Tables II and III summarize so far available intestinal expression data, which represent predominately mRNA expression data (Table II). This is comprehensible from the technical point of view as intestinal samples are mostly taken by biopsies which provide not sufficient amount of sample for tissue consuming protein quantification procedures such as immunoblotting but is still adequate for a multiplex gene expression analysis (30,32,33,36,40).

Although the data are heterogeneous, the majority of these records indicate considerable differences in the expression levels of multidrug transporter genes along the human intestine (Table II). With reference to this, the expression of ABCB1, ABCC2, ABCG2, ASBT and PEPT1 was shown to be highest in proximal parts of the intestine (30–33,36,39,40), while the abundance of ABCC3, OCT1, OCT3 and MCT1 was highest in the colon (30,31,36,39,40). The expression levels of OATP2B1 were moderate and homogenous along the entire human intestine (30,32,33,36,40). The relative rank order of gene expression within and between the different gut segments varied markedly between the studies. Potential reasons for these discrepancies may be due to the fact that available human data were predominately derived from patients suffering from different gastrointestinal diseases including gastrointestinal cancer or inflammatory diseases that may affect gene expression as clearly shown for ulcerative colitis and Crohn's disease (65,66).

Another potential confounder may be merging of expression data, i.e. expression data from different individuals and intestinal segments were matched to each other to conclude on the transporter expression along the human gut. However, considering the high variability in the expression of intestinal transporters that have been reported, this approach remains very questionable (11,67).

Finally, intestinal mRNA expression data must not necessarily be correlated with the respective protein abundance or function (37,56,68). In this regard, in previous clinical studies, we were not able to find significant correlations between the mRNA and protein expression for ABCB1 and ABCC2 in human duodenum using quantitative immunohistochemistry for protein quantification ((11,69–71); Fig. 2).

Moreover, a low expression of target genes which is compensated by using a high number of PCR cycles may generate expression signals that can easily cause misleading observations such as the reported intestinal expression of the liver-specific uptake transporters OATP1B1 and OATP1B3 (72). The same is true for the reported mRNA expression of intestinal OATP1A2 which could also not be confirmed by other studies (32,33,35,36).

Taken together, protein data are needed and appear to be a more reliable surrogate for intestinal transporter function than available gene expression data. To our knowledge, there were so far only three studies published which quantified the protein content of human multidrug transporters (ABCB1, ABCC2 and ABCG2) along the intestine (Table III) (27,41,42). All of these studies used Western blotting for protein quantification (limitations see next section). Mouly *et al.* verified on protein level the reported increase in ABCB1 expression from duodenum to ileum (27). This is in contrast to findings from Berggren who observed a substantial drop in intestinal expression from proximal to distal small intestine for ABCB1 and ABCC2 (41). A recent study by Tucker *et al.* investigated the expression of ABCB1, ABCC2 and ABCG2 solely in duodenal tissue and observed the following rank order of transporter protein expression: ABCG2 ≥ ABCB1 > ABCC2 (42). This study reported for the first time absolute protein expression values which are a prerequisite for prediction of transporter function (5,6). However, the duodenum is not supposed to be the predominate site for intestinal drug absorption. In this regard, many of the aforementioned studies generated their expression

Table II. Overview of so Far Available mRNA Data Expression for Transporter Proteins in the Human Intestine (+++, High Expression; ++, Moderate Expression; +, Low Expression; n.d., Not Detectable; -, No Data Available)

Protein Alias	ABCB1 P-gp	ABCC1 MRP1	ABCC2 MRP2	ABCC3 MRP3	ABCG2 BCRP	ASBT IBAT	OATP1A2 OATP-A	OATP2B1 OATP-B	OCT1	OCT3 EMT	PEPT1
Gene	<i>ABCB1</i>	<i>ABCC1</i>	<i>ABCC2</i>	<i>ABCC3</i>	<i>ABCG2</i>	<i>SLC10A2</i>	<i>SLCO1A2</i>	<i>SLC22B1</i>	<i>SLC22A1</i>	<i>SLC22A3</i>	<i>SLC15A1</i>
Duodenum	+/++	+	+++	++	++	+	n.d.	++	+	-	++
Jejunum	++	+	+++	++	++	++	n.d.	++	+	+	+++
Ileum	+++	+/++	++	++	+/+++	+++	n.d.	++	+	-	+++
Colon	+/++	+/++	+	+++	+/++	+	n.d.	+/++	+/++	+/n.d.	+

According to: Terada *et al.* 2005 (40), Gutmann *et al.* 2005 (31), Hilgendorf *et al.* 2007 (32), Seithel *et al.* 2006 (36), Englund *et al.* 2006 (30), Berggren *et al.* 2007 (41), Zimmermann *et al.* 2005 (39), Meier *et al.* 2007 (33)

data from intestinal parts that are accessible by endoscopic procedures (duodenum, terminal ileum, and colon segments), thereby omitting the intestinal section that is expected to be most important for drug absorption, i.e. the jejunum (30,31,33,34,38,39).

There are also some intestinal protein expression data from rat and dog available (73,74). However, it remains uncertain how predictive these transporter expression data are for the situation in human (75). In conclusion, there are substantial gaps in knowledge which counteract a comprehensive understanding or a reliable prediction about the impact of intestinal drug transporters on oral drug absorption due to the lack of absolute protein expression data.

PROTEIN QUANTIFICATION BY IMMUNOBLOTTING

The previous methods of choice for specific quantification of membrane transporters and proteins in general were immunological methods such as Western blotting or quantitative immunohistochemistry. The analytical principle of both methods is binding of specific antibodies to the protein of interest which enables afterwards detection of an optical densitometric, fluorescence or radioactive signal caused by a secondary antibody.

Although these methods are supposed to be protein specific, a major limitation is the uncertain specificity of the used antibody, i.e. cross-reactivity with other proteins or even a lack of functionality (76,77). This is not surprising when one keeps in mind that an epitope which is recognized by specific antibodies has normally the length of five to ten amino acids and its distinct binding motif is mostly unknown. Other limitations which could be associated with antibodies are their high prize or their lack of commercial availability.

Both methods are well-accepted bioanalytical techniques for protein detection. However, they are also frequently used for protein quantification (especially Western blot). In this regard, and in addition to the aforementioned limitations, one has to consider further aspects which could interfere with reliable protein quantification. Firstly, there are mostly no data on the analytical quality for immunoblotting assays available, i.e. specificity, limit of detection, accuracy and precision. Associated to this, we recently observed a high variability and analytical error (i.e. poor reproducibility; Fig. 3) for Western blot-based protein quantification of human serum albumin (HSA) and hepatic OATP transporters overexpressed in HEK293 cells (78).

Secondly, the linear analytical range, i.e. a linear correlation between protein amount and analytical signal, is mostly unknown when quantifying proteins by Western blot using chemiluminescent signals for detection (76,79). Associated to this, we could demonstrate a highly variable but linear correlation for the water-soluble protein HSA but rather a quadratic correlation for hepatic OATP transporters (78). Considering that this correlation is mostly unknown and differs between the different proteins and used antibodies, the blind assumption of a linear correlation, e.g. doubling the optical density means twofold higher protein expression, may cause misleading results.

Thirdly, Western blotting has only a very limited sample throughput and is characterized by a poor reproducibility (i.e. highly variable within- and between accuracy and precision, Fig. 3). Consequently, without a minimal method validation,

Table III. Overview of so Far Available Protein Expression Data for Drug Transporter Proteins in the Human Intestine (+++, High Expression; ++, Moderate Expression; +, Low Expression; n.d., Not Detectable; –, No Data Available)

Reference	Mouly <i>et al.</i> 2003 (27)	Berggren <i>et al.</i> 2007 (41)		Tucker <i>et al.</i> 2012 (42)		
Quantitative assay	Western blot (N=5)	Western blot (N=15 ^a)		Western blot (N=14)		
Segment/protein	ABCB1	ABCB1	ABCC2	ABCB1 (pmol/mg)	ABCC2 (pmol/mg)	ABCG2 (pmol/mg)
Duodenum	+	–	–	205±153	49±52	227±184
Jejunum	++	++	+++	–	–	–
Ileum	+++	++	++	–	–	–
Colon	–	++	++	–	–	–

^aSamples for each segment were from different donors (jejunum=3, Ileum=4, colon=7)

all quantitative data generated by immunoblotting remain questionable.

PROTEIN QUANTIFICATION BY MASS SPECTROMETRY-BASED TARGETED PROTEOMICS

Mass spectrometry-based targeted proteomics may be a promising approach to overcome most of the aforementioned limitations (44,45). This novel methodology enables the simultaneous and label-free absolute quantification of several proteins of interest. The first methods have been published in the early 1990s of the last century and were applied to quantify human endogenous compounds such as beta-endorphin and apolipoprotein A-I (80,81). Nowadays, this method is a frequently used standard technique which is used to quantify a broad variety of target proteins including serum proteins, hormones and other biomarkers (82–84).

In 2008, mass spectrometry-based targeted proteomics were introduced to the field of membrane transporters by Tetsuya Terasaki and Yurong Lai (49,51). Both groups and others contributed in the meanwhile several manuscripts that quantified different ABC- and SLC transporters as well as metabolizing enzymes in different human tissues (liver, brain and kidney), human cells (hepatocytes, platelets), transfected cell lines and animals (46–64).

The basic principle of the method is to measure proteospecific peptides generated by a tryptic digest as surrogates for the respective protein. Figure 4 demonstrates the general workflow of the method with ABCB1 as an example.

Method Development

The first and most critical step is the selection of an appropriate peptide candidate for protein quantification. A very easy way to do so is to perform an *in silico* trypsin digestion for the protein of interest by using well-established proteomic web tools such as UniProtKB/Swiss-Prot (<http://web.expasy.org>) or ProteinProspector (<http://prospector.ucsf.edu>). However, these prediction algorithms will simply identify all possible tryptic peptides (cleavage after arginine and lysine) but will ignore important biological facts which have to be considered in the following selection process. Here, the following aspects should be taken into account: (1) the mass of the selected peptide must be within the mass range of the used mass spectrometer (but at least seven amino acids to assure protein specificity); (2) the peptide should not contain posttranslational modifications (only few experimentally proven modifications, prediction via online tools such as NetPhos, NetNGlyc, NetOGlyc); (3) the peptide should not contain amino acid exchanges due to genetic polymorphisms (if so, allele frequency should be below 1%); (4) the peptide should not be located within a transmembrane region of the protein to exclude inefficient trypsin digestion; (5) the amino acid sequence should not contain repeated sequences of arginine and lysine due to the risk of missed cleavage by trypsin; (6) the peptide should not contain cysteine, methionine and tryptophan due to stability issues and (7) the protein (and species) specificity must be verified by BLAST search analysis.

Finally, the selection procedure will result in a list of potential peptide candidates eligible for protein quantification.

Fig. 2. Correlation between duodenal mRNA and protein expression of ABCB1 (*left*) and ABCC2 (*right*) in 12 healthy volunteers (Oswald *et al.* 2006) (11). Similar findings have been observed by Haenisch *et al.* 2008 (71) and Giessmann *et al.* 2004 (69,70)

Fig. 3. Analytical data for the Western blot quantification of OATPs in overexpressing HEK293 cells (*left*) and human serum albumin (*right*). Graphs represent correlations between protein content and analytical signal (optical density) which were derived in each case from six calibration curves prepared and measured on six different days. Tables below summarize within-day accuracy and precision derived from in each case six quality control sample sets measured on 1 day

Fig. 4. Graphical summary of the workflow needed to set up a reliable LC-MS/MS for transporter protein quantification

However, in the case of large proteins such as ABCB1 (1,280 amino acids), there is still a substantial number of peptides remaining; in the case of ABCB1 17 peptides. To assure that we will pick the right peptide candidate, we always perform in parallel wet-lab trypsin digestions for the protein of interest using transporter over-expressing cell lines or commercially available membrane preparations. Afterwards, we perform a so-called shot-gun proteomics experiment to identify the experimentally observable peptides (85). Only the peptides which were theoretically predicted and experimentally proven are considered as reliable candidates for quantification. The final step is to identify the peptide specific mass transitions, the so-called multiple reaction monitoring (MRM) mass to charge (m/z) ratios. For this purpose, we order crude peptides for all final candidates and perform a mass spectrometric optimization comparable to that for small molecules. For each peptide, we use the three MRMs of the highest signal intensity for detection.

There are also free web-based or vendor-specific prediction tools available to forecast appropriate MRMs such as Skyline, MIDAS workflow, ProteinPilot, MRMPilot, PinPoint, PeptideAtlas or PeptideArt (86). However, most of these algorithms are based on empirical data from different training sets of proteins, which bear the risk that superior peptides or mass transitions may be overseen.

After collection of all relevant data for mass spectrometric detection, the chromatographic behaviour has to be investigated. Due to the complexity of the digested biological matrix, the chromatography is exclusively performed with gradient elution (mostly going from 1–5% to 30–50% acetonitrile over 20–60 min) using reverse phase C18 columns. In contrast to peptide identification via shotgun experiments, MRM quantification methods mostly use a more robust high flow rate chromatography (200–500 $\mu\text{l}/\text{min}$), the efficiency of which can be markedly increased by using UHPLC systems. Considering a run time of approximately 20–30 min, the daily sample throughput of these methods is moderate (30–60 samples).

Although MRM methods allow multiplex quantification of several proteins in one analytical run, the number of simultaneously measured peptides is not unlimited but restricted by the following aspects: (1) a practical analytical run time (length of gradient); (2) the number of peptides monitored per protein; (3) the number of monitored mass transitions per peptide (e.g. six for each transporter peptide and its internal standard peptide when using three MRMs per peptide); (4) a reliable dwell time (time needed for each MRM experiment) of at least 30 ms; and (5) a feasible cycle time (cycle time = number of proteins $\times$ number of peptides/protein $\times 2$ (factor for internal standard peptides) $\times$ number of MRMs/peptide $\times$ dwell time/MRM). Considering typical LC-MS experiments with peak widths of about 10–20 s, the cycle time should be adjusted to allow at least eight data points per peak for a reliable quantification (i.e. cycle times of 1–2 s are acceptable). Figure 5 demonstrates the influence of dwell time and cycle time on the LC-MS/MS analysis of a single peptide (three transitions). One can easily see that the shorter the dwell time, the more data points are generated per peak. However, associated to this the shape, symmetry and height of the peak become more and more worse. Although some authors recommend a dwell time below 10 ms (50,60–63), we can only recommend 30 ms as a lower limit for reliable quantification (Fig. 5). Using this dwell time, the number of simultaneously quantified proteins is limited to six

assuming the quantification of one peptide/protein, the use of three MRMs/peptide and typical peak widths of 10 s. To increase this number, the principle of scheduled MRM was introduced; i.e. the mass spectrometry method is divided into different time segments in which different mass transitions will be monitored in dependence on the known retention time of the respective peptide peak. These methods were shown to quantify dozens of peptides in one analytical run (87).

Finally, the peptide(s) which result in the highest intensity and the best chromatographic properties (i.e. no interference with signals from the biological matrix, co-elution of all mass transitions from one peptide) will be ordered in high analytical quality as light and stable isotope labelled version (internal standard) for further method validation.

It is beyond the scope of this article to give a comprehensive tutorial to set up these methods, but we can highly recommend excellent reviews for this (44,45,88). There are also several free online prediction tools and online data repositories for MRM-based quantification of proteins available which also contain data for transporter proteins such as PeptideAtlas, SRMAtlas or the Global Proteome Machine Database. In addition to the aforementioned predicted data and own experimental data, these information may be helpful to set up sound quantitative methods for targeted transporter proteomics.

Method Validation and Application of Targeted Proteomics to Human Intestinal Samples

Before the analytical assay can be applied to biological samples, the method has to be comprehensively validated to assure reliable measurements. Although this step is obligatory and well accepted for the quantification of small molecules, validation data for quantitative proteomic assays are rarely found in the literature (51,60).

As targeted proteomics assays are multistep methods, all steps need to be optimized or standardized and if possible validated, i.e. sample preparation, membrane protein extraction, protein digestion and LC-MS/MS analysis. Very recently, we described a comprehensive method validation procedure for the quantification of ten clinically relevant transporter proteins in human intestinal tissue (89).

A minimal method validation should contain at least the following parameters as suggested by bioanalytical method validation guidelines (FDA and EMA): (1) selectivity (no interference with signals from the matrix or other peptides); (2) linearity (definition of LLOQ); (3) within-day and between-day accuracy and precision; (4) peptide stability (e.g. freeze–thaw stability, post-preparative stability, stability during digestion, stability under storage conditions) and (5) digestion efficiency (time course of protein digestion). In order to assure reproducible sample preparation, membrane protein extraction and protein digestion, we apply standardized protocols and use if possible commercially available kits such as the ProteoExtract® Native Membrane Protein Extraction kit (89).

One challenging aspect during the process of method validation is that there is mostly no biological blank matrix available. On the other side, it is important to perform the method validation in a matrix comparable to the biological samples of interest because it is expected that the sample preparation and peptide quantification are influenced by the matrix itself (e.g. digestion efficiency, ion suppression in the

Fig. 5. Relationship between the applied dwell time and cycle time of mass spectrometric detection of three mass transitions from one peptide and resulting chromatographic peaks. Table below demonstrates resulting dwell time for unscheduled MRM methods in dependence on the number of monitored proteins at a cycle time of 1.2 s which allows a sufficient peak quantification for a peak width of 10 s (*asterisk* sum for unlabeled and stable isotope labeled peptides)

mass spectrometer). Strategies to overcome this issue are preparation of an artificial matrix mimicking the biological samples (e.g. a digested human protein of identical concentration) or to apply the method of standard addition to a pooled matrix of biological tissue. Finally (if possible), the accuracy of the method should be compared with a different quantitative method (e.g. Western blotting) (49).

We applied our recently developed and validated method to quantify the expression of clinically relevant intestinal uptake and efflux transporters (ABCB1, ABCC2, ABCC3, ABCG2, ASBT, OATP1A2, OATP2B1, OCT1, OCT3 and PEPT1) along the entire human intestine from eight body donors (90).

Applying this method to intestinal samples from our recently established intestinal tissue bank, we assessed the expression pattern given in Fig. 6. These data show that the expression levels of ABCB1, ABCC2, ABCG2 and OATP2B1 are comparable (0.2–0.6 pmol/mg), whereas that of PEPT1 was found to be 7–20-fold higher in human jejunum, i.e. the predominate site of drug absorption. Interestingly, in ileum the expression of ABCB1, ABCC2 and PEPT1 was considerably

higher. Although these conclusions are in this case based on protein expression data from only four samples from jejunum and ileum, similar findings have been observed in our more comprehensive expression study using in each case ten samples from the entire intestinal tract from eight donors (90). A similar increase in ABCB1 protein expression from jejunum to ileum was also reported by Mouly *et al.* (27).

The transporter protein expression was in accordance with the respective mRNA expression data in jejunum but not in ileum which may be due to different tissue-specific regulation. This again demonstrates the limitation of mRNA expression data alone. In line with this assumption, we were only able to identify substantial correlations between mRNA and protein for ABCB1 and PEPT1 in our recent expression study (90).

A very recent study by Tucker *et al.* published absolute protein expression data from the human duodenum as observed by Western blotting (42). These expression data (Table III) were markedly different to our data from human jejunum; i.e. ABCB1 (377 vs. 205 fmol/mg), ABCC2 (581 vs. 49 fmol/mg) and ABCG2 (422 vs. 227 fmol/mg). However, we recently observed

substantial differences in the expression pattern of several transporter proteins along the small intestine. Thus, these differences may be partly explained by site-dependent differences in intestinal transporter expression. Moreover, as this study also used the crude membrane fraction for analysis, differences in membrane preparation and/or protein extraction procedures may have contributed to these differences (see also “[Limitations and Future Perspectives](#)”). Finally, our data are based on the analysis of four donors only, whereas the study of Tucker *et al.* included tissues from 14 patients which has thereby a markedly higher explanatory power. On the other side, no validation data of the respective Western blot assays which would allow an estimate of the analytical quality have been provided (see further limitations under “[Protein Quantification by Immunoblotting](#)”).

In addition to the expression analysis of human intestinal samples, we also characterized the transporter protein expression in Caco-2 cells which is one of the most frequently used *in vitro* models to characterize the intestinal drug absorption (91) (Fig. 7). Here, we found that the expression of ABCB1 and PEPT1 is very comparable to that in human jejunum. Thus, for these transporters Caco-2 cells may appear as an appropriate *in vitro* model to conclude on drug absorption in human. However, the expression of OATP2B1 was fourfold higher, while ABCC2 was found to be ~80% lower than in human jejunum. Surprisingly, the expression of ABCG2 was even below the limit of quantification. These differences in transporter expression may have a substantial impact on interpretation of Caco-2 monolayer studies. Considering this finding and the well-documented variability in the transporter expression in Caco-2 cells in dependence on different laboratory conditions (92), it appears to be mandatory to characterize the transporter protein expression in Caco-2 cells for each transport experiment to avoid misinterpretation of the generated transport data. This information may help to standardize or normalize the respective experimental data comparable to recognize passive diffusion and paracellular transport by monitoring the transfer of propranolol and mannitol.

POTENTIAL IMPLICATIONS OF INTESTINAL EXPRESSION DATA

From our perspective, the potential value of reliable intestinal transporter protein expression data are (1) to know which transporter proteins are expressed in the intestine; (2) the impact of transporter proteins for the intestinal absorption of different drugs could be estimated or predicted; (3) the contribution of different transporters to intestinal drug–drug interactions caused by transporter induction or inhibition

Fig. 7. Protein expression of clinically relevant multidrug transporters in human jejunum ($N=4$) and in Caco-2 cells ($N=4$). Mean $\pm$ SD are given (asterisk indicates values below limit of quantification)

could be elucidated and; (4) site-dependent differences in the intestinal could be explained.

In the following paragraph, we will discuss briefly the potential impact of intestinal expression and function of ABCB1 (P-gp), PEPT1 and OATPs.

The expression data presented in Fig. 6 and from our recent expression analysis study observed in the human jejunum substantial amounts of ABCB1 the expression of which was even higher in the ileum (27,90). This expression pattern fits to site-dependent intestinal absorption phenomena of ABCB1 substrates such as talinolol that have been described. For this beta 1-selective adrenergic blocker, the intestinal absorption after direct administration into the intestine via a catheter was shown to be decreased the more distal the drug was administered most likely caused by a higher ABCB1-mediated efflux in the lower small intestine (25). The same conclusion can be derived from a previous clinical study in which we compared the absorption of talinolol after administration of different dosage forms, namely a capsule and an enteric-coated tablet (26). Again, the drug release in more distal intestinal segments by the enteric-coated tablet caused a substantial loss in oral bio-availability (AUC, $1.33 \mu\text{g} \times \text{h/ml}$ vs. $2.64 \mu\text{g} \times \text{h/ml}$, $p < 0.05$), while the absorption of paracetamol which was present in the same dosage form was not affected (AUC, $3.72 \mu\text{g} \times \text{h/ml}$ vs. $3.96 \mu\text{g} \times \text{h/ml}$, NS). The differences in oral absorption of

Fig. 6. Intestinal mRNA (left) and protein (right) expression of clinically relevant uptake (OATP2B1, PEPT1) and efflux (ABCB1, ABCC2, ABCG2) transporters in human jejunum and ileum from four donors

digoxin in dependence on the administered dosage form may also be due to a higher ABCB1 expression in more distal parts of the small intestine. While digoxin solution is nearly completely absorbed, the bioavailability of elixirs and immediate release tablets which are absorbed in deeper intestinal segments is incomplete and accounts for 60–80% and 70–85% (93).

Considering the nearly parallel expression pattern of ABCB1 and PXR along the intestine (own unpublished mRNA data), it is not surprising that co-administration of prototypic enzyme/transporter inducers such as rifampicin or St. John's wort results in substantial DDIs (3,8–17). The same is true for inhibition of ABCB1 for example by macrolide antibiotics (Table I).

With respect to SLC transporters, PEPT1 showed the highest expression of all investigated transporters and was almost exclusively expressed in the small intestine with highest abundance in ileum, whereas the OATP2B1 expression was markedly lower but homogeneous in jejunum and ileum. Interestingly, we could not detect neither mRNA nor protein expression for OATP1A2 in our previous expression analysis (90). This is an interesting finding because OATP1A2 was reported to be expressed in the human intestine and is frequently speculated to be a potential intestinal uptake carrier for several drugs such as talinolol, fexofenadine and aliskiren (8,15,19,72). Moreover, several interaction studies with grapefruit juice were explained by inhibition of OATP1A2 as the grapefruit juice flavonoid naringin was shown to be a potent OATP1A2 inhibitor *in vitro* (94). However, in view of our expression data, we assume that rather OATP2B1 than OATP1A2 may be the responsible transporter behind these interactions because this protein is expressed along the entire intestine, accepts OATP1A2 substrates (e.g. talinolol) and can also be inhibited by fruit juices (95).

The high intestinal expression of the di- and tripeptide transporter PEPT1 suggests a substantial role in the intestinal uptake of peptide-like compounds. Indeed, β -lactam antibiotics such as amoxicillin or cefadroxil, ACE inhibitors (captopril, benazepril, enalapril, fosinopril) and antiviral drugs valacyclovir and oseltamivir have been described as PEPT1 substrates (29). Considering the predominate expression of PEPT1 in the upper small intestine, one could speculate that PEPT1 substrates may be exclusively absorbed in these intestinal segments. This assumption is supported by few clinical studies, which demonstrated only a substantial drug absorption after intestinal infusion of the PEPT1 substrates amoxicillin and benazepril to the small intestine but not to colon (23,24). However, systematic data on drug affinity or DDIs for PEPT1 are not available which may also be due to its narrow substrate spectrum or its transport characteristics (low affinity/high capacity).

In conclusion, intestinal transporter protein data could provide deeper insights into the intestinal drug processing. Using sophisticated *in silico* tools such as SimCyp or GastroPlus, these data may allow prediction of intestinal drug absorption, DDI and site-dependent intestinal absorption phenomena (5,6,28). Finally, these *in vivo* predictions could be refined by using Ussing chamber perfusion data from human intestinal tissue which allows biorelevant insights into the intestinal transporter function (96).

LIMITATIONS AND FUTURE PERSPECTIVES

Mass spectrometry-based targeted proteomics is a promising novel tool to determine simultaneously the expression of several transporters in a reliable manner by using validated assays. Consequently, this method was frequently used in recent applications to study the expression, regulation and function of transporters in *in vitro* models, animals and human tissues (46–64). These expression data may improve our current understanding of transporter-mediated drug possessing, e.g. at the intestinal absorption barrier. In association with prediction algorithms, these data may enable predictions of transporter function *in vivo*.

Despite the convincing quality of mass spectrometry-based protein quantification methods and first promising expression data, one has to keep in mind also some limitations of targeted proteomics which apply to all so far available methods and applications.

Firstly, all methods published so far (including our data) are based on the quantification of a single proteospecific peptide only. Assuming different trypsin digestion efficiency at different regions of the proteins and potential posttranslational modifications that may occur, the appearance of tryptic peptides may differ. Thus, it appears necessary to verify the protein expression at least by using two to three different peptides. The lack of experimentally proven posttranslational modifications of transporter proteins (mostly prediction tools such as NetPhos are used) also illustrates the need of verification by independent peptide candidates.

Secondly, the digestion efficiency between the different transporter proteins being monitored in one assay may differ. However, due to the lack of purified transporter proteins this issue is hard to address. It is also worth to notice that the digestion efficiency can also differ somewhat for one protein in different samples, which can be normalized by using SILAC-labelled internal standard proteins as described recently (46).

Thirdly, the observed protein expression may not necessarily be associated with the protein function due to intracellular localisation of the transporter. This aspect also directs the attention to the membrane fraction used for tryptic digestion. Although only the portion of transporter proteins localized in the outer membrane (plasma membrane) can contribute to transporter function, it is very challenging to isolate the plasma membrane fraction alone in a reliable and reproducible manner. Moreover, considering only the protein that is expressed in the plasma membrane may neglect any protein regulation or protein traffic to the cell surface which was shown to take place within few minutes (97,98). Consequently, we and others decided to consider the whole membrane fraction.

Finally, the protein expression data should be normalized to a cell or tissue-specific protein comparable to normalization of mRNA expression data by housekeeping genes. These protein markers that should be measured in parallel to the transporters of interest normalize for differences in membrane protein isolation when using a plasma membrane marker such as Na/K-ATPase or differences in the fraction of a specific cell type in a tissue sample (e.g. villin-1 for enterocytes). However, as an essential prerequisite, these reference proteins must be stably expressed in the tissue of interest.

All of the aforementioned limitations are from our point of view no knockout criteria for available protein expression

data generated by targeted proteomics but needs to be addressed by further research projects to assure that this thrilling technique generates reliable protein expression data.

Because it is today well established that the intestine is equipped with a complex network of phase I and II enzymes and transporter proteins which work in concert to limit the intestinal absorption of xenobiotics, the so far available intestinal expression data for transporters need to be extended by expression data for relevant metabolizing enzymes which has so far only been done by mRNA expression analysis (99–101).

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