Quantitative Transporter Proteomics by Liquid Chromatography with Tandem Mass Spectrometry: Addressing Methodologic Issues of Plasma Membrane Isolation and Expression-Activity Relationship

Short Communication

ABSTRACT
To predict transporter-mediated drug disposition using physiologically based pharmacokinetic models, one approach is to measure transport activity and relate it to protein expression levels in cell lines (overexpressing the transporter) and then scale these to in vitro to in vivo extrapolation (IVIVE). This approach makes two major assumptions. First, that the expression of the transporter is predominantly in the plasma membrane. Second, that there is a linear correlation between expression level and activity of the transporter protein. The present study was conducted to test these two assumptions. We evaluated two commercially available kits that claimed to separate plasma membrane from other cell membranes. The Qiagen Qproteome kit yielded very little protein in the fraction purported to be the plasma membrane. The Abcam Phase Separation kit enriched the plasma membrane but did not separate it from other intracellular membranes. For the Abcam method, the expression level of organic anion-transporting polypeptides (OATP) 1B1/2B1 and breast cancer resistance protein (BCRP) proteins in all subcellular fractions isolated from cells or human liver tissue tracked that of Na+-K+ ATPase. Assuming that Na+-K+ ATPase is predominantly located in the plasma membrane, these data suggest that the transporters measured are also primarily located in the plasma membrane. Using short hairpin RNA, we created clones of cell lines with varying degrees of OATP1B1 or BCRP expression level. In these clones, transport activity of OATP1B1 or BCRP was highly correlated with protein expression level ($R^2 > 0.9$). These data support the use of transporter expression level data and activity data from transporter overexpressing cell lines for IVIVE of transporter-mediated disposition of drugs.

Introduction
Physiologically based pharmacokinetic models are increasingly used in drug development to predict drug disposition in humans based on in vitro data. Such in vitro to in vivo extrapolation (IVIVE) has been most successful for drugs cleared predominantly by cytochrome P450 metabolism. This is because scaling factors relating the expression of the enzyme in vitro (in microsomes) to that in vivo are available. The increased focus on designing compounds that are metabolically stable has resulted in the development of drugs that are significantly cleared by transporters. However, until recently, the corresponding scaling factors for transporters were lacking primarily because the traditional method for measurement of transporter expression, namely, Western blotting, yields only relative expression data (because pure standards of membrane proteins are not available) that are semiquantitative at best. To overcome these deficiencies of Western blotting, proteomics, based on liquid chromatography with tandem mass spectrometry (LC-MS/MS) and surrogate unique peptide(s), is increasingly being used to quantify transporter protein expression (Prasad and Unadkat 2014). This method is both quantitative and independent of the availability of pure transport protein standards.

Irrespective of the method used, it should ideally measure transporter protein that is active and present in the plasma membrane. However, the current approaches used for transporter proteomics either do not isolate the plasma membrane from other intracellular membranes (Prasad et al., 2013, 2014; Qiu et al., 2013) or give poor yield (Suski et al., 2014). In addition, for IVIVE, the method assumes that expression and activity are directly proportional to each other. This makes it possible to extrapolate the activity and expression of the transporter measured in cell lines (overexpressing the transporter) to that in vivo where the expression of the transporter may be variable and likely lower. Therefore, our study determined whether 1) pure plasma membrane can be isolated from tissues and cells using commercially available kits and 2) the expression of transporter protein (in total membrane) is correlated with activity when the former is varied.

Materials and Methods
Short hairpin RNA (shRNA) or scrambled shRNA lentiviral particles (for ABCG2 and SLCO1B1), polybrene and puromycin dihydrochloride were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ProtoExtract native membrane

ABBREVIATIONS: AU, arbitrary fluorescence unit; BCRP, breast cancer resistance protein; CHO, Chinese hamster ovary cells; EG, estradiol 17β glucuronide; [3H]-EG, tritium-labeled estradiol 17β glucuronide; HEK293, human embryonic liver 293 cells; HL, human liver cells; IVIVE, in vitro to in vivo extrapolation; Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2':1,2](3,4,5)-indole-3-propanoic acid 1,1-di-methyl ester; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LY335984, (2R)-1-[[4-[[1a(R),10bS]-1,1-Dichloro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopenta[c][7]annulen-6-yl]-3-(quinolin-5-yl)propan-2-yl]-MDCKII, Madin-Darby canine kidney II cells; OATP, organic anion-transporting polypeptide; PBS, phosphate-buffered saline; shRNA, short hairpin RNA.
Transporter Expression-Activity Correlation

Protein extraction kits were procured from Calbiochem (Temecula, CA). The plasma membrane protein extraction kits, ab65400 and Qproteome, were procured from Abcam (Cambridge, MA) and Qiagen (Hilden, Germany), respectively. The protein quantification BCA kit, dithiothreitol, iodoacetamide, and MS grade trypsin were purchased from Pierce Biotechnology (Rockford, IL). Synthetic signature peptides for organic anion-transporting polypeptide (OATP) 1B1/2B1 and breast cancer resistance protein (BCRP) (Prasad et al., 2013, 2014) were obtained from New England Peptides (Boston, MA). The corresponding stable isotope-labeled peptides were obtained from Thermo Fisher Scientific (Rockford, IL). Tritium-labeled estradiol 17β glucuronide (13H-EG) (specific activity: 50 Ci/mmol, concentration: 1 mCi/ml) was purchased from American Radiolabeled Chemicals (St. Louis, MO). ScintiVerse BD Cocktail liquid scintillant was purchased from Fisher Scientific. Tritium-labeled estradiol 17β and 4′-tritiated estrone 3-sulfate sodium salt were obtained from Thermo Fisher Scientific (Rockford, IL). Tritium-labeled estradiol was reconstituted with Buffer PM of the kit with protease inhibitors to the pellet, and the beads were removed. We added 500 μl of Lysis Buffer PM of the kit with protease inhibitors to the pellet, and the beads were resuspended and incubated on ice for 5 minutes. The tube was placed on a magnetic separator for 1 minute, and the supernatant was collected (washing). We added 500 μl of Elution buffer PME to the pellet, mixed well, and incubated on ice for 5 minutes. The tube was placed on a magnetic separator for 1 minute, and the supernatant and beads were collected (Eluent and Beads, respectively). Samples from each of these steps were analyzed by LC-MS/MS for OATP2B1 and subcellular marker expression (see Fig. 1).

For the Abcam protocol, 3 × 10⁶ HEK293 cells expressing OATP2B1 or 1 g of human liver tissue (HL-105, HL-115, or HL-129) were used. The cells were washed with 1 ml of ice-cold PBS, and the pellet was resuspended in 1 ml of the homogenization buffer mix of the kit in an ice-cold Dounce homogenizer. Homogenization was performed on ice for 30-50 times. The liver tissue was homogenized in 3 times volume of the IX Homogenization Buffer Mix until it was completely lysed (40 times). The homogenate was transferred to a 1.5-ml tube on ice for 5 minutes. The tube was placed on a magnetic separator for 1 minute, and the supernatant was collected (washing). We added 500 μl of Elution buffer PME to the pellet, mixed well, and incubated on ice for 5 minutes. The tube was placed on a magnetic separator for 1 minute, and the supernatant and beads were collected (Eluent and Beads, respectively). Samples from each of these steps were analyzed by LC-MS/MS for OATP2B1 and subcellular marker expression (see Fig. 1).

Plasma Membrane Protein Extraction

Two different commercially available kits were used to isolate plasma membrane: Qproteome (Qiagen, Hilden, Germany) and ab65400 (Abcam, Cambridge, MA).

For the Qproteome protocol, 1 × 10⁷ HEK293 cells expressing OATP2B1 were centrifuged for 5 minutes at 450 g, and the cell pellet was washed with phosphate-buffered saline (PBS). The washed cell pellet was resuspended in 2 ml of Lysis Buffer PM without protease inhibitors and incubated for 15 minutes at 4°C. Complete cell disruption was achieved by multiple aspiration through a needle (26 or 21 gauge), and the lysate was centrifuged at 12,000g for 4°C for 20 minutes (Pellet 12k g; see Fig. 1A).

The supernatant was transferred to a new microcentrifuge tube, and 20 μL of the reconstituted Binding Ligand PBL was added. The equilibrated Strep-Tactin magnetic beads were added to the reaction mix and incubated with gentle agitation for 30 minutes on ice. The tube was kept on a magnetic separator for 1 minute, and the supernatant was removed (Bead supernatant). We added 500 μl of Lysis Buffer PM of the kit with protease inhibitors to the pellet, and the beads were resuspended and incubated on ice for 5 minutes. The tube was placed on a magnetic separator for 1 minute, and the supernatant was collected (washing). We added 500 μl of Elution buffer PM to the pellet, mixed well, and incubated on ice for 5 minutes. The tube was placed on a magnetic separator for 1 minute, and the supernatant and beads were collected (Eluent and Beads, respectively). Samples from each of these steps were analyzed by LC-MS/MS for OATP2B1 and subcellular marker expression (see Fig. 1).

Transporter Expression-Activity Correlation

Protein expression in sequential steps used in isolation of plasma membrane with the (A) Qproteome (Qiagen) or (B) Abcam kit. OATP2B1 expression tracks expression of Na⁺-K⁺ ATPase, a plasma membrane marker, but not that of other markers, namely, GS28 (Golgi), calreticulin (ER), Tim23 (mitochondria), GAPDH (cytosol), or histone 3 (nucleus). In the Qproteome and Abcam kits, the eluent and fraction 4 are supposed to respectively contain only the plasma membrane.
the pellet was recovered as the plasma membrane fraction (fraction 4). The lower phase was also collected (fraction 5). Each of these listed fractions was analyzed by LC-MS/MS for OATP2B1 and subcellular marker expression (see Fig. 1B).

Generation of Cells Expressing Varying Levels of OATP1B1 or BCRP Using shRNA

For these experiments, OATP1B1-expressing CHO cells were selected over OATP1B1-expressing MDCKII cells because of the higher expression of OATP1B1 in these cells. OATP1B1 (CHO)—or BCRP (MDCKII)—expressing cells were transduced with the shRNA or control (scrambled) lentiviral particles using the manufacturer’s protocol with some optimization. Briefly, before viral infection, cells were plated in a 12-well plate for 24 hours with 1 ml of Dulbecco’s modified Eagle’s medium containing fetal bovine serum and penicillin/streptomycin. On day 2, the medium was removed and replaced with Dulbecco’s modified Eagle’s medium containing Polybrene (sc-134220) (final concentration, 5 μg/ml for MDCKII cells and 10 μg/ml for CHO cells). Then varying amounts (12.5–125 μl) each μl has about 5000 lentiviral particles carrying shRNA) of lentiviral particles were added to the cells. On day 3, the medium was removed and replaced by 1 ml of complete medium (without Polybrene), and the cells were grown for the next 24 hours. The cells were split on day 4 and incubated for 48 hours in complete medium. Stable clones expressing shRNA (or scrambled shRNA) were selected by adding an optimized concentration of puromycin dihydrochloride: 5 μg/ml for OATP1B1 (CHO cells) or 10 μg/ml for BCRP (MDCKII cells).

Transporter Activity Assay

OATP1B1. [3H]-estradiol glucuronide ([3H]-EG) was used as a substrate to measure OATP1B1 transport activity. CHO cells grown in T75-flasks until 80–90% confluent were harvested using trypsin, seeded into 12-well poly-D-lysine-coated plates (density of approximately 75,000 cells per well), and allowed to grow for the next 24 hours. Then the cells were lysed with 160 ml of this lysate solution for total protein estimation using the BCA method, and 90 μl to analyze total radioactivity by Tri-Carb Liquid Scintillation Counters (PerkinElmer). Transporter and Na+/K+ ATPase expression was measured by LC-MS/MS as described herein.

Next, the cells were lysed with 160 μl of 0.2% (w/v) aqueous sodium dodecyl sulfate solution. We used 30 μl of this lysate solution for total protein estimation using the BCA method, and 90 μl to analyze total radioactivity by Tri-Carb Liquid Scintillation Counters (PerkinElmer). Transporter and Na+/K+ ATPase expression was measured by LC-MS/MS as described herein.

To confirm that the decrease in OATP1B1 transport activity in the knock-down cells was due to a change in Vmax and not in VM, these parameters were estimated in control (transduced with scrambled shRNA) and maximal knock-down cells. A simplified two-point approach was used to determine the Vmax and VM. Preliminary data indicated that [3H]-EG uptake (Vmax) was achieved in the presence of 120 μM EG. Therefore, the velocity of [3H]-EG uptake by the cells was determined in the presence of 120 μM EG (Vmax) or 20 nM EG (Vmax/Km). The reported values are mean ± S.D. of triplicates.

BCRP. The cellular accumulation of Hoechst 33342 dye was used to indirectly measure BCRP efflux activity as described elsewhere (Kim et al., 2002). Hoechst 33342 emits blue fluorescence when bound to double-strand DNA. MDCKII cells grown in T75-flasks until 80–90% confluent were harvested using trypsin, seeded into black 96-well plates (density of approximately 40,000 cells per well), and allowed to adhere overnight. Then the culture medium was removed, and the cell monolayer was washed twice with Krebs-Hepes buffer and resuspended in 100 μl buffer: LY335984 (32R)-(1-4-[1(αR,10bS)-1,1-Dichloro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopenta[c]phenanthrene]-6-(2-methylpropyl)-1,4-dioxopyrazino[1',2',1':6,1]pyrido[3,4-b]jindole-3-propanoic acid 1,1-dimethylethyl ester) (1 μM) were used to selectively inhibit MDR1 (endogenous) and BCRP activity, respectively. After 30 minutes of preincubation with inhibitors at 37°C, the cells were incubated with varying concentrations of Hoechst 33342 solution (0.5 μM, 1 μM, 2.5 μM, 5 μM, 10 μM, and 30 μM). The fluorescence (excitation at 355 nm and an emission at 460 nm) in each well was immediately measured at 4-minute intervals (at 37°C) up to 40 minutes. After correcting for background fluorescence, the rate of cellular uptake of Hoechst 33342 was assessed by taking the linear slope of the difference in fluorescence between the fluorescence signal in the presence of both the LY335984 and Hoechst 33342 solution (0.5 μM, 1 μM, 2.5 μM, 5 μM, 10 μM, and 30 μM). The fluorescence (excitation at 355 nm and an emission at 460 nm) in each well was immediately measured at 4-minute intervals (at 37°C) up to 40 minutes.

For expression-activity correlation, various BCRP knockdown clones were incubated with 2.5 μM Hoechst 33342 for 40 minutes, and activity was assessed as the fluorescence Au (arbitrary fluorescence unit) difference between passive

Fig. 2. Transporter protein expression (normalized to the expression of Na+/K+ ATPase) for sequential steps used in the isolation of plasma membrane using the Abcam kit. The y-axis represents the ratio (%) of peak areas of transporter versus membrane marker (Na+/K+ ATPase) in each fraction after each is normalized to the homogenate. Cells expressing BCRP or OATP1B1/2B1 as well as human liver tissues (HL 1–3) were used. Experiments 1 and 2 represent two independent experiments. Transporter protein expression tracked that of Na+/K+ ATPase. Fraction 4 is supposed to contain only the plasma membrane. BCRP expression was not detectable in fraction 3 or 5.
LC-MS/MS Quantification of Transporters and Membrane Markers

The protein expression of BCRP, OATP1B1/2B1, and subcellular markers (e.g., Na⁺-K⁺ ATPase) in cell lines or liver tissues was quantified using LC-MS/MS. The expression of these transporters was highly correlated in various clones of cells expressing OATP1B1 (A) or BCRP (B) created by gene knockdown. OATP1B1 and BCRP transport activity were measured by [3H]-EG uptake and intracellular accumulation of Hoechst 3342 dye, respectively. Transport activity was expressed as a percentage of that observed in control cells transduced with scrambled shRNA.

**Results**

**Plasma Membrane Isolation.** The expressions of OATP2B1 and signature proteins representing various intracellular organelles (e.g., Na⁺-K⁺ ATPase, GS28 for Golgi, calreticulin for endoplasmic reticulum, TIM23 for mitochondria, GAPDH for cytosol, and histone 3 for nucleus) in all subcellular fractions showed that neither method could separate plasma membrane from other intracellular membranes. The Qproteome method yielded very little protein and no enrichment (Ko14 + LY335984 inhibitor) and BCRP efflux (LY335984 inhibitor). Most of the reports on transporter quantification (including from our laboratory) are based on measurements of total transporter protein (plasma membrane plus intracellular) and not just that present in the plasma membrane. Indeed, the expression of OATP1B1, OATP2B1, and BCRP tracked that of Na⁺-K⁺ ATPase in overexpressed cells and the three individual human livers (Fig. 2).

**Expression-Activity Correlation in Cells Expressing OATP1B1 and BCRP.** OATP1B1 and BCRP transporter activity was highly correlated with the expression of these transporters in knockdown cells (Fig. 3). As expected, the decrease in transporter activity in the knockdown cells was explained by a decrease in transporter Vₘₐₓ and not a change in Kₘ (Table 1). The Kₘ values were comparable to those reported by others (Yamazaki et al., 2005; Gui et al., 2008).

**Discussion**

Most of the reports on transporter quantification (including from our laboratory) are based on measurements of total transporter protein (plasma membrane plus intracellular) and not just that present in the plasma membrane. The latter is more relevant because it is the transporter expressed in the plasma membrane that is responsible for compound cellular efflux or uptake and therefore needed in extrapolating the in vitro transporter activity to cell lines to that in vivo. Therefore, we investigated methods that could be used to routinely separate plasma membranes from other intracellular membranes. Though sucrose gradients and biotinylation have been used for this purpose, both methods have limitations, as the former requires a large quantity of cells or tissue and the latter is best applied to intact cells but not tissues (Elia, 2012).

Therefore, we evaluated two kits that claimed to separate plasma membrane (in tissues and cells) from intracellular membranes: Qiagen Qproteome and Abcam ab65400. Neither method was able to separate the plasma membrane from intracellular membranes. The Qproteome kit gave poor yield of protein purported to be the plasma membrane. In contrast, when we used the Abcam kit, the expression of OATP1B1, OATP2B1, and BCRP tracked that of Na⁺-K⁺ ATPase in overexpressed cell lines as well as in HL tissue. As Na⁺-K⁺ ATPase has been shown to be predominantly localized in the plasma membrane (Padilla-Benavides et al., 2010), our data suggest that the expression of OATP1B1/2B1 and BCRP is also predominantly in the plasma membrane.

**TABLE 1**

Gene knockdown of OATP1B1 or BCRP significantly reduces the Vₘₐₓ of the transporter without affecting its Kₘ

<table>
<thead>
<tr>
<th>Overexpressed Transporter Cell Lines</th>
<th>Kinetic Parameters</th>
<th>Control Cells</th>
<th>Gene Knockdown Cells</th>
<th>P value^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1 (CHO cells)</td>
<td>Vₘₐₓ (fmol/min/mg protein)</td>
<td>4583 ± 364</td>
<td>539 ± 192</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>Kₘ (μM)</td>
<td>4.06 ± 1.03</td>
<td>4.48 ± 1.44</td>
<td>.71</td>
</tr>
<tr>
<td>BCRP (MDCKII cells)</td>
<td>Vₘₐₓ (AU/min/40,000 cells)</td>
<td>31.9 ± 3.31</td>
<td>24.8 ± 1.95</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>Kₘ (μM)</td>
<td>3.45 ± 1.10</td>
<td>3.76 ± 0.89</td>
<td>.72</td>
</tr>
</tbody>
</table>

^aAU, arbitrary fluorescence unit.
^bCells transduced with control (scrambled shRNA).
^cUnpaired t test.
Immunolocalization data of OATP and BCRP in cell lines support this conclusion (Kopplov et al., 2005; Xia et al., 2005).

Because the two kits failed to isolate pure plasma membrane and we are not aware of any other routine method that can do so, an alternative and relevant question would be, is the plasma membrane expression of the transporter as a percentage of total cellular expression in overexpressed cell lines comparable to that in liver tissue? If it is, we can use with confidence the activity and expression of the transporter measured in cell lines for IVIVE. Assuming that Na⁺-K⁺ ATPase is predominately located in the plasma membrane, our data suggest that this assumption is valid. Therefore, we used Na⁺-K⁺ ATPase as a normalizing factor in analyzing the expression-activity correlation described herein.

Another assumption made when conducting IVIVE of transporter activity and expression in cell lines is that transporter expression and activity are linearly correlated. Using knockdown with lentiviral shRNA, we showed that transporter expression was highly correlated with activity ($r^2>0.9$) for cells expressing OATP1B1 and BCRP. Moreover, this was due to a reduction in $V_{\text{max}}$ of the transporter and not due to a change in $K_m$.

Collectively, our data support the use of cell lines overexpressing transporters for IVIVE of transporter-based disposition of drugs. Whether such IVIVE will be accurate needs to be verified.

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References


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Participated in research design: Kumar, Prasad, Patilea, Evers, Gupta, Salphati, Hop, Unadkat.

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Quantitative transporter proteomics by LC-MS/MS: addressing methodological issues of plasma membrane isolation and expression-activity relationship

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Drug Metabolism and Disposition

Supplement Table 1: MRM parameters used for quantification of various subcellular marker proteins.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Surrogate Peptide</th>
<th>Precursor Ion (m/z)</th>
<th>Product Ions (m/z)</th>
<th>Fragmentor voltage (V)</th>
<th>Collision Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺-K⁺ ATPase</td>
<td>AAVPDAGVGK</td>
<td>414.2</td>
<td>685.4, 586.3</td>
<td>125</td>
<td>13</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>EQFLDGDWTSR</td>
<td>705.8</td>
<td>893.4, 778.4</td>
<td>150</td>
<td>16</td>
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<tr>
<td>GS28</td>
<td>QLENELDLK</td>
<td>551.3</td>
<td>375.2, 242.2</td>
<td>135</td>
<td>14</td>
</tr>
<tr>
<td>TIM23</td>
<td>YLVQDTEFILPTGANK</td>
<td>962.5</td>
<td>700.4, 587.3</td>
<td>160</td>
<td>20</td>
</tr>
<tr>
<td>GAPDH</td>
<td>LVINGNPITIFQER</td>
<td>807.5</td>
<td>1003.6, 579.3</td>
<td>160</td>
<td>18</td>
</tr>
<tr>
<td>Histone H3</td>
<td>STELLIR</td>
<td>416.3</td>
<td>643.4, 514.4</td>
<td>130</td>
<td>13</td>
</tr>
</tbody>
</table>

LC conditions were the same as that used for analysis of OATPs and BCRP