Detection of Statin Cytotoxicity Is Increased in Cells Expressing the OATP1B1 Transporter

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Cytotoxicity of a compound is determined by the intracellular concentration mediated both by passive permeability and active uptake through drug transporters. However, the major liver uptake transporters were either absent or expressed at significantly lower levels in human liver cell lines than in human liver. When comparing cytotoxicity of five statins, the organic anion transporting polypeptide 1B1 expressing HEK cells showed a significantly higher sensitivity than the wild-type HEK cells. The IC₅₀ shifts ranged from 9- to >100-fold, and the potency shifts collapsed in the presence of rifampicin, the inhibitor for OATPs. The extent of the shifts correlated with the permeability of the statins with high permeable compounds having smaller shifts and low permeable compounds having larger shifts. The changes in statin potency in transporter-transfected cells reflect the active uptake of statins into the cells, and the increased intracellular drug concentration lead to increased toxicity. The data suggested that uptake transporters have a significant impact on the outcomes of a cell-based assay and should be considered during the early stages of compound toxicity screening in drug discovery. For compounds with low permeability that are likely to undergo transporter-mediated uptake, it is important to test them in transporter-competent cell models.

Key Words: cytotoxicity; uptake transporter; OATP1B1; statin; permeability.

Drug development is a complicated, costly, and time-consuming process with high risk of failures. Two decades ago, poor pharmacokinetics and lack of efficacy were the top two reasons of attrition with each of them contributing 39 and 30% to drug failures, respectively (van de Waterbeemd and Gifford, 2003). With the successful implement of early screening of absorption, distribution, metabolism, and excretion properties, the failure rate due to poor pharmacokinetic properties has dropped to less than 10%. Analysis of recent phase II and phase III clinical trial failures indicated that safety has surpassed poor pharmacokinetics and become the second major reason for late-stage drug failures after efficacy (Arrowsmith, 2011a, b). These data highlight the importance in addressing toxicity issues early in drug discovery. In recent years, much effort has been put forward to develop in vitro tools for early safety prediction while reducing animal usage. One of the widely used in vitro screening assays for toxicity is the cell death assay (Kepp et al., 2011). Though systems such as zebrafish, yeast, and primary cells have been used in cell death–oriented screens for toxicity (Kepp et al., 2011), most cell death assays are developed in immortalized cell lines for good reproducibility and low cost. Regardless of the underlying mechanisms and detection methods, in vitro cytotoxicity assays are valuable tools for predicting in vivo toxicity. Analyses of Pfizer proprietary compounds have shown that in vitro cytotoxicity screens in THLE and HepG2 cells correlated well with in vivo toxicity findings in preclinical exploratory toxicity studies (Greene and Song, 2011; Greene et al., 2010).

In recent years, drug transporters have been shown to be increasingly important in drug discovery and development (Mizuno et al., 2003; Zolk and Fromm, 2011). They are found in various tissues, such as liver, kidney, small intestines, and brain, etc. Transporters account for 1.7% of the human genome (Venter et al., 2001) and can be classified into many families. The most important drug transporter families are the solute carrier proteins (SLC) and the ATP-binding cassettes (ABC) families. The SLC and ABC transporters function as gatekeepers to facilitate the uptake and efflux of endogenous substances as well as a wide variety of therapeutic agents into and out of cells. The uptake transporters are especially important for molecules that do not readily cross cell membranes due to their size or hydrophilicity, such as sugars, bile acids and their conjugates, and many drugs. It has been widely acknowledged that the activities of drug transporters have great impact on drug efficacy and toxicity given their important roles in drug distribution (Giacomini et al., 2010; Huang and Woodcock, 2010; Mizuno et al., 2003; Monks et al., 2007).

Transporter expression profiles vary from tissue to tissue (Nishimura and Naito, 2005), and these transporters are key determinants of the drug concentration in the tissue that are directly related to the toxic effects of the drugs. It is important
to recognize that free tissue concentration may be different from systemic exposure of free drug when transporters are involved in the distribution process. For example, a previous study on thiopurine-induced hematopoietic toxicity showed that Mrp4 knockout mice experienced a gene dosage-dependent toxicity caused by accumulation of toxic 6-thioguanine nucleotides (6-TGNs) in their myelopoietic cells. However, there was no difference in 6-TGNs plasma concentration between the knockout and wild-type mice (Krishnamurthy et al., 2008). This example suggests that considering systemic exposure alone may underestimate the true risk associated with asymmetric drug distribution between plasma and tissue compartments.

In drug discovery, compounds are routinely screened in cell-based in vitro assays to assess dose-dependent efficacy or toxicity. The concentration of the dosing solution is assumed to be the same as intracellular free drug concentration, leading to the pharmacological effect or toxicity. This is likely to be incorrect for transporter substrates because concentration gradients will be developed across the membrane by transporters at the steady state. For uptake transporter substrates, the intracellular free drug concentration is likely to be higher than the media at the steady state. Cell-based screening assays using cell lines that do not express uptake transporters are likely to be less sensitive than those in which transporters are expressed. To understand the transporter profiles of commonly used cell lines for toxicity studies, the gene expression level of major transporters was measured in various in vitro liver cells. The impact of transporters on cellular responses to drug treatment was investigated using statins as an example. Statins are known substrates for polypeptide 1B1 (OATP1B1), and different statins show distinct membrane permeability (Hamelin and Turgeon, 1998; Hirano et al., 2004; Hsiang et al., 1999; Nezasa et al., 2003). The role of uptake transporters in mediating drug-induced cytotoxicity was further validated by OATP1B1 inhibitor, rifampicin, and intracellular concentration measurement.

**MATERIALS AND METHODS**

**Chemicals.** Cerivastatin, fluvastatin, pravastatin, rifampicin, and doxourubicin were purchased from Sigma-Aldrich (St Louis, MO). Pitavastatin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rosuvastatin was obtained from Pfizer Global Material Management (Groton, CT). Microcystin-LR was purchased from Enzo (Plymouth Meeting, PA).

**Cell culture.** T-Rex HEK293 cells were purchased from Invitrogen (Carlsbad, CA). OATP1B1 overexpression cell lines were developed at Pfizer Sandwich (UK) in T-Rex HEK293 background. All cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (4.5 g/l glucose) supplemented with 10% FBS, 2mM Glutamine, and 5 µg of Blasticidin (Invitrogen), and cultured at 37°C, 5% CO2, and 95% relative humidity. Cells under 15 passages were used for screening, and the OATP1B1 expression levels were monitored for every screen. HepG2 cells were purchased from ATCC and maintained in DMEM (4.5 g/l glucose) supplemented with 10% FBS and 2mM Glutamine. THLE-2 cells were purchased from ATCC (CRCL 2706) and maintained in complete growth media composed of BEGM media (BEGM Bullet Kit) (Lonza, MD), 10% FBS, heEFG (5 µg/ml unfiltered), and phosphoethanolamine (70 µg/ml).

**Cytotoxicity assay and data analysis.** T-Rex 293 and OATP1B1 cells were plated in Poly-D-Lysine coated 96-well plates (BD Falcon) at a density of 10,000 cells/well in the volume of 100 µl. Twenty-four hours after plating, the cell growing media in the plates were removed and replaced with fresh media supplemented with test compounds at various concentrations. A 10-point 1:3 serial dilution was performed in DMSO for each compound with a maximum concentration of 300 µM. The final concentration of DMSO in each well was 1%. Cell proliferation assay WST-1 (Roche, IN) was performed 48 h after exposure to test compounds following the manufacturer’s protocol. IC50 values of each compound in HepG2, HEK-WT, and HEK-OATP1B1 cells were calculated individually by Prism (GraphPad Software Inc., San Diego, CA) using a sigmoidal dose-response model. Cells treated with DMSO only were defined as 100%. For inhibition assay, rifampicin (80 µM) was coincubated with compounds for 48 h before cytotoxicity was measured by WST-1 assay.

**OATP1B1 uptake assay.** Uptake buffer at pH 7.4 was prepared using Hanks’ Balanced Salt Solution supplemented with 20mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid. Wild-type HEK293 and HEK-OATP1B1 were seeded at a density of 112,500 cells per well in 48-well poly-D-Lysine–coated plates and cultured for 48 h. Cell plates were rinsed thrice with 0.2 ml of prewarmed uptake buffer, leaving the final rinse volume on the cells for 15 min to allow the cells to adjust to the buffer. After removal of the final rinse, uptake was started by the addition of 0.2 ml uptake buffer containing probe. The plates were then incubated at 37°C with shaking at 150 rpm. The experiment was stopped at the desired interval by removing the uptake buffer followed by three washes with 0.2 ml ice-cold uptake buffer per well.

**Sample preparation.** Samples from OATP uptake incubations were retrieved by lysing the cells with 0.2 ml per well of internal standard (IS) solution in 100% methanol. Cell lysates were transferred to 96-well polystyrene deep-well plates and evaporated under warm nitrogen gas. Samples were reconstituted and centrifuged prior to injection. Total cell protein per well was determined from representative wells with the bicinchoninic acid protein assay (Pierce) using methods as recommended by the supplier.
**Quantification of statins by liquid chromatography-tandem mass spectrometry.** Samples were injected onto an AB SCIEX Triple Quad API5500 (AB Sciex, Framingham, MA) liquid chromatography-tandem mass spectrometry (LC/MS/MS) system. All samples were analyzed for both probe and IS. The mobile phase consisted of (A) 95% 2mM ammonium acetate, 5% 50/50 acetonitrile/methanol and (B) 90% 50/50 acetonitrile/methanol, 10% 2mM ammonium acetate. The flow rate was set to 1.5 ml/min using an Optimize Technologies SP Small Molecule Trap column. The m/z transitions were 460.4 → 356.2 (Cerivastatin), 412.2 → 223.9 (Fluvastatin), 422.2 → 290.1 (Pitavastatin), 423.0 → 100.7 (Pravastatin), 482.2 → 258.2 (Rosuvastatin), and 685.2 → 149.4 (IS). Intracellular concentrations of probe (in nmol) were calculated with Analyst v1.5.1 software using linear regression. The amount of probe uptake was calculated as nmol/mg protein.

**RESULTS**

**Gene Expression Levels of Major Liver Transporters in Cell Lines and Tissues**

Gene expression levels of 15 major liver transporters (Giacomini et al., 2010) were measured in the human liver cell lines HepG2, Huh7, and THLE and in human tissue samples (liver, kidney, and smooth muscle). Among the 15 transporters, NTCP, OCT1, OAT2, OATP1B1, OATP1B3, OATP2B1, and OAT7 are uptake transporters located in the basolateral membrane of the hepatocytes. These uptake transporters facilitate the uptake of endogenous substances and drugs from the systemic circulation into the hepatocytes. Also in the basolateral membranes are the efflux transporters MRP3, MRP4, and MRP6. There are five major efflux transporters located in the apical (canalicular) membrane of the hepatocytes (BCRP, P-gp, BSEP, MRP2, and MATE1), and they are important for biliary excretion of various substances (Giacomini et al., 2010). As expected, most of these transporters were expressed at similar or much higher levels in the liver compared with the kidney and muscle tissue with the exception of MRP4 (Table 1). When comparing the transporter expression levels between liver cell lines and liver tissue, it was observed that all the uptake transporters located in the basolateral membrane of the hepatocytes were either not expressed or expressed at significantly lower levels in liver cell lines than in the human liver sample. On the other hand, the majority of the liver efflux transporters were detected in at least one liver cell line. Overall, the mRNA levels in the cell lines were lower than those of liver tissue. The gene expression levels of efflux transporters also varied from cell line to cell line. THLE cells only expressed one efflux transporter, MRP3, at an abundant level. In contrast, Huh7 and HepG2 cells expressed MRP6, P-gp, MRP2, MATE1, and BCRP at levels similar to liver tissue.

We then examined the expression levels of the same liver transporters in human cryopreserved hepatocytes over 3 days in sandwich culture. Overall, the expression levels of transporters in sandwich-cultured hepatocytes on day 0 are comparable to the expression levels in the liver. However, there was noticeable day-to-day variation in both uptake and efflux transporters. The mRNA levels of uptake transporters on the basolateral side of the hepatocytes showed a dramatic drop on day 1 (Fig. 1). Although there was a slight increase during the rest of the culture period, the levels of the uptake transporters were much lower at the end of the 3-day culture compared to day 0. The mRNA levels of OAT2, OATP1B1, and OATP1B3 on day 3 were only 21, 29, and 11% of the initial values, respectively. The other uptake transporters were approximately 50% of what was observed on day 0. In contrast to the uptake transporters, efflux transporters showed an overall increase in gene expression levels on day 1 and beyond, especially for MRP4 and MRP2. On day 3, the mRNA levels of MRP2, MRP3, and MRP4 were two- to four-fold higher than that of human liver (data not shown).

**Cytotoxicity of Statins in HepG2 and HEK293**

The cytotoxicity of five statins cerivastatin, fluvastatin, pitavastatin, rosvastatin, and pravastatin was measured after 48-h exposure in a cell viability WST-1 assay in HepG2 and HEK293 cells. The calculated IC50 values of all statins were comparable between HepG2 and HEK293 cells (Fig. 2). Among the statins, cerivastatin and pitavastatin showed the most significant cytotoxicity (IC50 < 25μM), followed by fluvastatin (50μM < IC50 < 100μM). Pravastatin and rosvastatin showed no effects on cell viability at the highest concentration tested, 300μM, in either HepG2 or HEK293 cells.

**Uptake of Statins by HEK293 Cells Transfected with OATP1B1**

Although statins are similar in molecular weight and acid pKa, they differ significantly in membrane permeability due to different lipophilicity. Compounds can enter the cells by transcellular passive diffusion and through transporters. Statins are known substrates for OATPs (Hirano et al., 2004; Hsiang et al., 2004).

<table>
<thead>
<tr>
<th>Relative to</th>
<th>THLE</th>
<th>Huh7</th>
<th>HepG2</th>
<th>Human liver</th>
<th>Human kidney</th>
<th>Human muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTCP</td>
<td>N.D.</td>
<td>0.02</td>
<td>0.01</td>
<td>11.44</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>OCT1</td>
<td>0.09</td>
<td>0.10</td>
<td>0.18</td>
<td>272.78</td>
<td>0.21</td>
<td>0.08</td>
</tr>
<tr>
<td>OAT2</td>
<td>N.D.</td>
<td>0.04</td>
<td>0.77</td>
<td>37.97</td>
<td>27.17</td>
<td>N.D.</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>N.D.</td>
<td>0.12</td>
<td>0.01</td>
<td>25.41</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>0.09</td>
<td>1.76</td>
<td>0.02</td>
<td>52.08</td>
<td>0.40</td>
<td>N.D.</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>0.03</td>
<td>12.93</td>
<td>6.52</td>
<td>102.90</td>
<td>22.72</td>
<td>4.83</td>
</tr>
<tr>
<td>OAT7</td>
<td>N.D.</td>
<td>10.38</td>
<td>1.67</td>
<td>7.84</td>
<td>0.04</td>
<td>N.D.</td>
</tr>
<tr>
<td>MRP3</td>
<td>40.07</td>
<td>7.54</td>
<td>15.56</td>
<td>74.30</td>
<td>27.43</td>
<td>0.26</td>
</tr>
<tr>
<td>MRP4</td>
<td>3.21</td>
<td>7.52</td>
<td>3.28</td>
<td>0.94</td>
<td>18.01</td>
<td>3.00</td>
</tr>
<tr>
<td>MRP6</td>
<td>0.68</td>
<td>10.81</td>
<td>19.37</td>
<td>36.24</td>
<td>25.53</td>
<td>0.38</td>
</tr>
<tr>
<td>P-gp</td>
<td>0.96</td>
<td>15.06</td>
<td>16.92</td>
<td>14.41</td>
<td>17.60</td>
<td>0.11</td>
</tr>
<tr>
<td>BSEP</td>
<td>0.02</td>
<td>0.17</td>
<td>N.D.</td>
<td>35.41</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>MRP2</td>
<td>0.09</td>
<td>20.30</td>
<td>45.00</td>
<td>74.63</td>
<td>16.81</td>
<td>0.22</td>
</tr>
<tr>
<td>BCRP</td>
<td>0.15</td>
<td>6.66</td>
<td>3.60</td>
<td>3.72</td>
<td>1.73</td>
<td>0.14</td>
</tr>
<tr>
<td>MATE1</td>
<td>3.20</td>
<td>7.34</td>
<td>22.23</td>
<td>41.11</td>
<td>76.73</td>
<td>11.54</td>
</tr>
</tbody>
</table>

Note: All values are expressed as mean of two independent experiments, triplicates each. N.D.: not detected.
Therefore, the intracellular concentration of the statins is measured in HEK-OATP1B1 cells and HEK-WT cells (no detectable OATP1B1 mRNA). The mRNA level of OATP1B1 in HEK293-OATP1B1 was approximately eight times higher than human liver, and the overexpression level was stable over at least 15 passages. No significant differences in expression levels of other transporters were observed between wild-type and OATP1B1-transfected HEK293 cells by real-time PCR (data not shown).

In the substrate uptake rate assay, each statin (1µM) was incubated in HEK-WT or HEK-OATP1B1 cells, and intracellular drug concentration was measured at various intervals using LC/MS/MS. The uptake amount in HEK-WT represents the baseline drug level due to passive permeability as well as active transport.
endogenous transporters and nonspecific binding to the cell membrane. The uptake difference between HEK-OATP1B1 and HEK-WT indicates the net drug uptake mediated by OATP1B1. Both cerivastatin and fluvastatin have higher uptake than pitavastatin, pravastatin, and rosuvastatin (Fig. 3) in HEK-WT cells, suggesting higher passive permeability of these two statins. The OATP1B1 uptake ratio at each time point was calculated by comparing the total amount between HEK-OATP1B1 and HEK-WT. The OATP1B1 uptake ratios for cerivastatin and fluvastatin were 2- and 2.7-fold at 2 min, respectively. In contrast, the uptake of pitavastatin, pravastatin, and rosuvastatin in HEK-WT cells was negligible compared with the uptake in HEK-OATP1B1. At 2 min, the uptake ratios for pitavastatin, pravastatin, and rosuvastatin were 11, 19, and 32, respectively.

Effects of OATP1B1 Expression on the Sensitivity of the Cytotoxicity Assay for Statins

To test whether the expression of OATP1B1 transporters can affect the outcomes of the cell viability assay, statins and two positive controls (doxorubicin and microcystin-LR) were tested in both HEK-WT and HEK-OATP1B1–transfected cells. Doxorubicin is a cytotoxic compound that readily crosses the cell membrane by passive diffusion (Decorti et al., 1998). Microcystin-LR is a known OATP1B1 substrate and exerts its hepatotoxicity via OATP1B1-mediated uptake (Evers and Chu,
Forty-eight hours after drug exposure, cell viability was measured in both cell lines, and IC_{50} values were calculated for each compound. As expected, the permeable compound doxorubicin showed no differences in its dose-response curve between the two cell lines. In contrast, the known OATP1B1 substrate microcystin-LR showed potent cytotoxicity only in the HEK-OATP1B1–transfected cells but not in the wild-type cells, suggesting that OATP1B1-mediated uptake is required for its cytotoxicity (Fig. 4A). The dose-response curves of all statins showed a significant left shift toward lower IC_{50} when comparing OATP1B1-transfected cells with the wild-type cells (Fig. 4B). In HEK-OATP1B1 cells, all statins showed cytotoxicity with IC_{50} values ranging from 0.03 to 11µM (Table 2). The increased sensitivity of the OATP1B1-transfected cells is likely due to the increased intracellular free drug concentrations mediated by OATP1B1 uptake. It is worth noting that cerivastatin and fluvastatin exhibited a less pronounced IC_{50} shift compared with pravastatin and rosuvastatin, which did not show any effects in the cell viability assay in either HepG2 or wild-type HEK293 cells. Pitavastatin also showed a significant shift in IC_{50} even though its cytotoxicity was prominent in wild-type HEK cells. The cytotoxicity rank ordering of the five statins in the OATP1B1-transfected cells was pitavastatin > cerivastatin > fluvastatin = rosuvastatin > pravastatin, which was different from that in the wild-type HEK cells: cerivastatin > pitavastatin > fluvastatin > rosuvastatin = pravastatin. To further confirm that the IC_{50} shift was indeed due to the increased drug exposure mediated by OATP1B1, an inhibition assay was performed using the known OATP1B1 inhibitor, rifampicin. A dose-response experiment was carried out to determine that 80µM rifampicin showed almost full inhibition of OATP1B1 in the absence of cytotoxicity (data not shown). Thus, cell viability assays for statins were repeated in the presence of rifampicin at 80µM (Fig. 3B). As expected, rifampicin coinoculation did not change the dose-response curves for any of the statins in the wild-type cells. In the OATP1B1-transfected cells, rifampicin showed no effect on the cytotoxic response of doxorubicin. However, in the presence of rifampicin, the dose-response curves for microcystin-LR and all the statins shifted toward those of the wild-type cells. The effects of rifampicin on the shift of IC_{50} values generally correlated with the IC_{50} shift mediated by OATP1B1, i.e., rifampicin had less effect on the more permeable statins than the low permeable ones. Rifampicin only caused a 2.5- to 5-fold changes in IC_{50} values for cerivastatin and fluvastatin, but the shift in IC_{50} values for pitavastatin, rosuvastatin, and pravastatin was between 25- to 50-fold, demonstrating the importance of OATP1B1 uptake in compound cytotoxicity.

**DISCUSSION**

Immortalized cell lines are widely used for the assessment of biological effects of compounds. It is well known that many cancer cell lines overexpress efflux transporters (e.g., MDR1, BCRP, MRP1) that lead to drug resistance in cytotoxicity assays (Cole and Deeley, 1998; Doyle et al., 1998; Gottesman et al., 2002; Riordan et al., 1985). Compared to efflux transporters, less is known about the expression levels of uptake transporters in cell lines and their impact on compound performance in cell-based assays. In our study, we compared the gene expression levels of 15 important liver transporters (Giacomini et al., 2010) in liver tissue, human hepatocytes, and three human liver cell lines. Interestingly, our data showed that all the uptake transporters located on the basolateral side of the hepatocytes were significantly downregulated or completely suppressed compared with the liver tissue. In contrast, the majority of the efflux transporters were expressed in at least one cell line at comparable levels to that of the human liver. THLE, a transformed human liver epithelial cell line, showed much lower levels of efflux transporters than the cancer-derived Huh7 or HepG2 cell lines. These findings are in agreement with previous studies showing that liver cell lines differ from liver tissue in their transporter expression profiles (Hilgendorf et al., 2007; Libra et al., 2006). For example, Hilgendorf et al. profiled gene expression levels of 36 drug transporters in human intestine, liver, kidney, and their corresponding cell lines, Caco-2, HepG2, and Caki-1, respectively. The only correlation between tissue and cell line was observed in human jejunum and Caco-2 cell line. Most uptake transporters expressed in the liver and kidney were suppressed in their respective cell lines (Hilgendorf et al., 2007).

It is worth noting that human hepatocytes showed time-dependent changes in expression levels for both uptake transporters and efflux transporters in 3-day sandwich cultures. The change of expression levels occurred within 24h in culture. For uptake transporters, after a dramatic drop at day 1, the mRNA levels returned to a partial level of day 0. For instance, the mRNA levels of OAT2, OATP1B1, and OATP1B3 at the end of the 3-day culture were less than 30% of the day 0 levels, whereas most efflux transporters experienced an increase in expression levels except BSEP. Our results are consistent with a recent study in which the OATP1B1/1B3 expression levels and activities were evaluated in human primary hepatocytes over time in culture (Ulvestad et al., 2011). This study showed that OATP1B1/1B3 expression levels and activities decreased significantly as early as 2h in culture, and the rate of decrease varied from donor to donor. Taken together, the expression levels of uptake transporters in liver cell lines and primary hepatocytes were lower than that of the liver tissue. This decrease may result from loss of differentiation due to either pathological changes or adaption to the *in vitro* culture conditions (Obaidat et al., 2012; Vander Borght et al., 2005).

Loss or change of uptake transporter activities on the basolateral side of the cells affects the uptake of substrate compounds. One important and well-studied liver uptake transporter is OATP1B1. OATP1B1 mediates sodium-independent uptake of various structurally independent, mainly amphipathic organic compounds, including bile salts, hormones and their conjugates,
FIG. 4. Cytotoxicity of statins in HEK293 wild-type (circle) and HEK293-OATP1B1–transfected cells (square) in the presence and absence of the OATP1B1 inhibitor rifampicin. (A) Cytotoxicity of doxorubicin and microcystin-LR was used as positive controls for passive diffusion and OATP1B1-mediated cytotoxicity, respectively. (B) Cytotoxicity of five statin compounds. Cytotoxicity of all compounds was measured by WST-1 assay after 48 h of compound incubation. X-axis, concentration in µM; y-axis, % of cell viability normalized to DMSO control. Dose-response curves of each compound in the presence (dashed line) and absence (solid line) of rifampicin (80 µM) were plotted using Prism Sigmoidal curve fit. Each data point represents the mean ± SEM of three independent measurements performed on different days.
Cytotoxicity of Statin Compounds in HEK293 and HEK293-
OATP1B1 Cells in the Presence and Absence of OATP1B1
Inhibitor Rifampicin (80μM)

<table>
<thead>
<tr>
<th>Compound name</th>
<th>HEK293, no rifampicin</th>
<th>HEK293- OATP1B1, no rifampicin</th>
<th>HEK293 + OATP1B1 + rifampicin</th>
<th>HEK293- OATP1B1 + rifampicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerivastatin</td>
<td>2.23±0.10</td>
<td>0.25±0.02</td>
<td>1.52±0.18</td>
<td>0.61±0.14</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>75.99±9.48</td>
<td>2.03±0.20</td>
<td>56.15±14.10</td>
<td>10.59±2.57</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>12.67±0.72</td>
<td>0.03±0.00</td>
<td>4.47±1.25</td>
<td>1.41±0.27</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>300.00±0.00</td>
<td>11.30±0.27</td>
<td>300.00±0.00</td>
<td>300.00±0.00</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>269.50±30.50</td>
<td>2.04±0.29</td>
<td>249.00±51.00</td>
<td>71.78±13.69</td>
</tr>
</tbody>
</table>

Note. Data are expressed as mean ± SEM (n = 3–4).

Toivola et al., 1998). We next evaluated the roles of permeability and transporter-mediated uptake in determining the cytotoxicity responses of statins in the cell viability assay after 48h of drug exposure. The data showed that all five statins had lower IC₅₀ values in OATP1B1-expressing cells compared with wild-type cells, but the magnitude of change varied dramatically. Statins with higher permeability, such as cerivastatin and fluvastatin, showed the least changes in IC₅₀ values when comparing the wild-type and the OATP1B1-expressing cells. However, the more hydrophilic pitavastatin, pravastatin, and rosuvastatin showed more prominent responses in OATP1B1-expressing cells than the wild-type cells. In fact, cytotoxicity of pravastatin and rosuvastatin was not detected in the wild-type HEK293 cells. Moreover, the IC₅₀ shift of statins between HEK-WT and HEK-OATP1B1 qualitatively correlated with the statin uptake in the same cell types. Pitavastatin, pravastatin, and rosuvastatin only showed negligible uptake in the wild-type cells. Therefore, the cytotoxicity of these three statins in HEK-OATP1B1 cells is mostly impacted by OATP1B1-mediated uptake. For cerivastatin and fluvastatin, their relatively high lipophilicity results in higher membrane permeability that leads to cytotoxicity in the absence of OATP1B1. Interestingly, the cytotoxicity of permeable statins is further enhanced by the expression of OATP1B1 in the cells. To further prove that the IC₅₀ shift was indeed due to the expression of OATP1B1, we coincubated the statins with the known OATP1B1 inhibitor, rifampicin, at a noncytotoxic dose. The data showed that in the presence of the OATP1B1 inhibitor, the dose-response curves shifted back toward that of the wild-type cells for all the statins and our positive control, microcystin-LR.

Microcystins are cyclic peptide produced by cyanobacteria. They are potent inhibitors for phosphatases type 1 (PP1) and 2A (PP2A) and cause severe hepatotoxicity in animals and humans (Gupta et al., 2003; Jochimsen et al., 1998; Tachi et al., 2007; Toivola et al., 1994). Different forms of microcystins exhibit distinct toxicity in vivo, and a recent study showed that the differential toxicities among microcystin congeners were due to the differential cellular uptake by OATP1B1/1B3 rather than differences in phosphatase inhibition potency (Fischer et al., 2010). In fact, OATP1B1 expression is required for microcystin to manifest its hepatotoxicity based on studies in OATP-transfected cell lines and Oatp1b2 knockout mice (Evers and Chu, 2008; Lu et al., 2008; Zaher et al., 2008). However, it is less clear how the loss of OATP1B1/1B3 expression in the cell lines may affect the outcomes of a cytotoxicity assay for therapeutic agents. Unlike an uptake assay, toxicity assays often require compound incubation longer than 24h when distribution equilibrium between intracellular compartment and media has been established. This is especially important for compounds with low passive permeability because transporter-mediated uptake is likely to be the dominant pathway for these drugs to enter the cells. Therefore, we investigated the cytotoxicity responses of five statins in cell lines in the presence and absence of the OATP1B1 transporter. All statins have similar molecular weight and pKa but different lipophilicity (Hamelin and Turgeon, 1998). We next evaluated the roles of permeability and transporter-mediated uptake in determining the cytotoxicity responses of statins in the cell viability assay after 48h of drug exposure. The data showed that all five statins had lower IC₅₀ values in OATP1B1-expressing cells compared with wild-type cells, but the magnitude of change varied dramatically. Statins with higher permeability, such as cerivastatin and fluvastatin, showed the least changes in IC₅₀ values when comparing the wild-type and the OATP1B1-expressing cells. However, the more hydrophilic pitavastatin, pravastatin, and rosuvastatin showed more prominent responses in OATP1B1-expressing cells than the wild-type cells. In fact, cytotoxicity of pravastatin and rosuvastatin was not detected in the wild-type HEK293 cells. Moreover, the IC₅₀ shift of statins between HEK-WT and HEK-OATP1B1 qualitatively correlated with the statin uptake in the same cell types. Pitavastatin, pravastatin, and rosuvastatin only showed negligible uptake in the wild-type cells. Therefore, the cytotoxicity of these three statins in HEK-OATP1B1 cells is mostly impacted by OATP1B1-mediated uptake. For cerivastatin and fluvastatin, their relatively high lipophilicity results in higher membrane permeability that leads to cytotoxicity in the absence of OATP1B1. Interestingly, the cytotoxicity of permeable statins is further enhanced by the expression of OATP1B1 in the cells. To further prove that the IC₅₀ shift was indeed due to the expression of OATP1B1, we coincubated the statins with the known OATP1B1 inhibitor, rifampicin, at a noncytotoxic dose. The data showed that in the presence of the OATP1B1 inhibitor, the dose-response curves shifted back toward that of the wild-type cells for all the statins and our positive control, microcystin-LR.

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A recent approach to minimize off-target effects associated with systemic exposure is to use liver-specific uptake transporters for liver targeting (Oballa et al., 2011; Pfefferkorn et al., 2012). This design strategy leads to a shift of chemical space toward high polarity and hydrophilic and low passive permeability. It is generally acknowledged that both passive diffusion and carrier-mediated drug transport coexist in cells and tissues. The relative contribution of each route to the total uptake of compounds into cells depends on the cell type and physicochemical properties of compounds (Sugano et al., 2010). Current in vitro cell-based assays still rely on permeability for cytotoxicity detection due to the absence or decrease of uptake transporter activities in the cell models. As a result, they may not sufficiently address the change of the chemical space and could underestimate the cytotoxicity for the low permeable, transporter-mediated compounds. We and others (Cihlar et al., 1999; Ho et al., 2000; Wong et al., 2011) have shown that using engineered cell lines that express uptake transporters can have significant impact on toxicity.
on the assay outcomes and may overcome the limitation of current assays due to the lack of uptake transporters. For instance, the drastically enhanced cytotoxicity of the antiviral drugs adefovir and cidofovir in cells expressing the kidney-enriched Organic Anion Transporter 1 (OAT1) correlated well with the nephrotoxicity associated with these drugs, and the OAT1-expressing cells were useful to screen similar compounds with nephrotoxicity liability (Cihlar et al., 1999; Wong et al., 2011). Given the physicochemical properties of OATP1B1 substrates, OATP1B1-expressing cells will be a more appropriate model for studying the cellular responses caused by acidic or, to a lesser degree, neutral compounds with low permeability. In summary, our studies have demonstrated that the transporter expression level can have significant impact on the functional endpoints of a cell-based assay. Therefore, the drug uptake routes (passive diffusion vs. transporter-mediated uptake) should be carefully considered when selecting or developing cell-based assays to assess compound-induced effects.

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