Short Communication

Retrospective Analysis of P-Glycoprotein–Mediated Drug-Drug Interactions at the Blood-Brain Barrier in Humans

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ABSTRACT

To date, the in vitro–in vivo correlation (IVIVC) of P-glycoprotein (P-gp)–mediated drug-drug interaction (DDI) at the blood-brain barrier is limited. The cutoff value for significantly affect the brain penetration of digoxin was [I_{unbound}/K_i] of 1, where I_{unbound} is the unbound plasma concentration of P-gp inhibitors. On the basis of the IVIVC in rats, we speculated that clinically used P-gp inhibitors do not cause DDI at the human BBB, because none of the compounds studied was [I_{unbound}/K_i]>1 at therapeutic doses. Recently, positron emission tomography studies with P-gp substrates, such as [11C]verapamil, [11C]-N-desmethyl loperamide, and [11C]loperamide, together with potent P-gp inhibitors, have indicated that increases in the influx rate constant for brain entry were observed in humans. Therefore, we aimed to retrospectively analyze the results of P-gp-mediated DDIs with in vitro P-gp inhibition assays to confirm the appropriate cutoff value. In vitro P-gp inhibition assays using verapamil, N-desmethyl loperamide, and loperamide as P-gp probe substrates were performed in human multidrug resistance protein 1-expressing LLC-PK1 cells. The influx ratios decreased in the presence of P-gp inhibitors, and the K_i of tarquidar was 10 nmol/L, regardless of probe substrates. Taking the in vitro K_i and unbound plasma concentrations in clinical DDI studies together, the criterion [I_{unbound}/K_i] of 1 was an appropriate cutoff limit to observe significant P-gp–mediated DDI at the BBB in humans. On the other hand, no significant DDI was observed in cases in which [I_{unbound}/K_i] was less than 0.1. This criterion was comparable to the previous IVIVC result in rats.

INTRODUCTION

P-glycoprotein [P-gp; multidrug resistance protein 1 (MDR1)] is a member of the ATP-binding cassette transporters that is encoded by the ABCB1 gene in humans. P-gp is ubiquitously expressed in normal tissues, including the small intestine, liver, kidney, and blood-brain barrier, that are involved in drug absorption, elimination, and disposition (Tsuji et al., 1992; Tsuji, 2002; Chan et al., 2004). A wide range of structurally diverse compounds, including anti-cancer drugs, HIV protease inhibitors, cardioactive drugs, and immunosuppressant agents, can be actively transported by P-gp (Eyal et al., 2009). Recently, the clinical relevance of P-gp–mediated drug-drug interactions (DDIs) has been summarized by the draft guidance published by the US Food and Drug Administration (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf). Accordingly, it is important to assess whether drug candidates have potential risks of P-gp–mediated DDI in clinical studies.

In the brain capillaries, P-gp is localized at the luminal membrane of the endothelial cells and pumps drugs and xenobiotics out from the brain into the blood. In vivo studies using mdr1a knockout mice indicated that P-gp regulates the brain penetration of several drugs, such as dexamethasone, digoxin, and cyclosporin A (Schnikel et al., 1995). However, current clinical data indicate that there are no consistent examples in which P-gp inhibition is clinically relevant at the human brain-blood barrier (BBB). In addition, there is very limited information available regarding quantitative prediction of the magnitude of P-gp–mediated DDI. We have recently reported the quantitative investigation of the impact of P-gp inhibition on digoxin transport across the rat BBB. The in vitro–in vivo correlation (IVIVC) in rats indicated that the cutoff value to significantly affect the brain penetration of digoxin was [I_{unbound}/K_i] of 1, where I_{unbound} is the unbound plasma concentration of P-gp inhibitors. Verapamil acts only as a substrate, whereas N-desmethyl loperamide is one of the most promising P-gp probe substrates using positron emission tomography imaging, because further demethylated metabolites have limited entry into the brain. Kreisl et al. reported that the influx rate constant for the brain entry of [11C]N-desmethyl

ABBREVIATIONS: BBB, blood-brain barrier; DDI, drug-drug interaction; I_{unbound}, unbound plasma concentration; IVIVC, in vitro–in vivo correlation; MCFR, modified corrected flux ratio; MDR1, multidrug resistance protein 1; MS, mass spectrometry; P-gp, P-glycoprotein.
loperamide after intravenous administration of tariquidar (6 mg/kg) increased by 3.7-fold relative to the baseline (Kreisl et al., 2010). Loperamide has no opioid adverse effects on the central nervous system, because P-gp avidly restricts its entry into brain. Passchier et al. reported that the influx rate constant for brain entry of \( ^{14} \text{C} \) loperamide was increased by 1.3-fold in the presence of quinidine (Passchier et al., 2008). In addition, concurrent administration of quinidine at a dose of 600 mg/man resulted in opioid-induced respiratory depression that lasted for a couple of hours (Sadeque et al., 2000).

To date, in vitro P-gp inhibition studies in human MDR1 have not been reported for these probe substrates. Moreover, the quantitative investigation for the impact of P-gp inhibition on brain penetration in humans has not been fully examined. Therefore, the aims of the present study are to retrospectively evaluate the magnitude of P-gp-mediated DDIs at the human BBB with use of in vitro P-gp inhibition data and to confirm the appropriate cutoff value.

### Materials and Methods

**Materials.** Cyclosporin A, N-desmethyl loperamide, and saquinavir were purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Loperamide was from MP Biomedicals, LLC (Tokyo, Japan). Lucifer yellow was from Sigma-Aldrich (St. Louis, MO). Clarithromycin, verapamil, and quinidine were from Wako Pure Chemicals Industries (Osaka, Japan). Tariquidar was from Sigma-Aldrich (St. Louis, MO). Clarithromycin, verapamil, and quinidine were purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada).

**Cell Culture.** The LLC-PK1 cells expressing human MDR1 were cultured as previously described with slight modification (Takeuchi et al., 2006). In brief, cells were collected by adding 0.05% trypsin/EDTA solution, were suspended in the cell culture media (Dulbecco’s modified Eagle’s medium with 20% fetal bovine serum, 500 mmol/l glucose, 150 mmol/l sodium) and this condition was maintained for 0.2 minute, followed by a linear increase of B to 95% over 0.2 minute, and held for 1 minute. The initial concentration was then reinstated and held for 1.2 minutes for re-equilibration. Detailed MS conditions are shown in Table 1.

The permeability coefficient of probe substrates \( (P_{\text{app}}) \) was calculated according to Eq. 1, and the efflux ratio was calculated using Eq. 2:

\[
P_{\text{app}} = \frac{\text{Amount}}{\text{Area} \times C_0 \times \text{Time}}
\]

\[
\text{Efflux ratio} = \frac{P_{\text{app, BioA}}}{P_{\text{app, BioB}}}
\]

where “amount” is the amount of transported substrates per well of the cell monolayer, “area” is the surface area of the cell monolayer (0.143 cm\(^2\) in a 96-well plate), and this condition was maintained for 0.2 minute, followed by a linear increase of B to 95% over 0.2 minute, and held for 1 minute. The initial concentration was then reinstated and held for 1.2 minutes for re-equilibration. Detailed MS conditions are shown in Table 1.

**P-gp Inhibition Assay for \( ^{3} \text{H} \) Verapamil, N-Desmethyl Loperamide, and Loperamide Transport.** The P-gp inhibition assay for \( ^{3} \text{H} \) verapamil, N-desmethyl loperamide, and loperamide transport across LLC-PK1 cells expressing human MDR1 was performed according to the previously described method (Sugimoto et al., 2011b). Samples from the receiver side (25 mmol/l of tariquidar) were almost 1 (unpublished data). Therefore, the above Eq. 3 could be simplified to the following Eq. 4.

\[
\text{MCFR} - 1 \times \text{[initial concentration of probe substrates]}
\]

\[
\text{ER} - 1 \times \text{[initial concentration of probe substrates]}
\]

**Determination of the Plasma Protein Binding.** The unbound fraction in human plasma for tariquidar (final concentration: 1 mmol/l) was determined using the 96-well equilibrium dialysis apparatus (HTDialysis LLC, Gales Ferry, CT) according to the previously described method (Sugimoto et al., 2011b).

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ionization mode</th>
<th>Detection mode</th>
<th>( m/z )</th>
<th>Source temp. (°C)</th>
<th>Mass spectrometric parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CE (eV)</td>
</tr>
<tr>
<td>N-desmethyl loperamide</td>
<td>Electrospray ionization</td>
<td>Multiple reaction monitoring</td>
<td>463.3/252.4</td>
<td>550</td>
<td>33</td>
</tr>
<tr>
<td>Loperamide</td>
<td>(Positive ion mode)</td>
<td>MRM</td>
<td>477.2/265.8</td>
<td>49</td>
<td>39</td>
</tr>
<tr>
<td>Tariquidar</td>
<td></td>
<td></td>
<td>647.4/335.3</td>
<td>71</td>
<td>25</td>
</tr>
<tr>
<td>Alprenolol (I.S.)</td>
<td></td>
<td></td>
<td>250.3/116.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CE, collision energy; CXP, collision cell exit potential; DP, declustering potential; EP, entrance potential; I.S., internal standard.
Co. Ltd. The total run time, flow rate, and column temperature were 2.6 minutes, 0.5 ml/min, and 50°C, respectively. The mobile phases A and B consisted of 10 mmol/l ammonium formate and acetonitrile. The initial concentration of mobile phase B was 10%, and the condition was maintained for 0.2 minute, followed by linear increase of B to 95% in the next 0.2 minute, held for 1 minute. The condition was then returned initial concentration and held for 1.2 minutes for re-equilibration. Detailed MS conditions are shown in Table 1.

**Results and Discussion**

To investigate the inhibitory potency of P-gp inhibitors, which were used in the clinical DDI studies, on \[^3\text{H}\]verapamil, \textit{N}-desmethyl loperamide, and loperamide transport, a transcellular transport study in human MDR1-expressing cells was performed. In vitro \(K_i\) values were ascertained from the intersection of the Dixon plot described in Fig. 1.

![Dixon plots for the inhibitory effect of tariquidar (A; 0.003, 0.01, 0.03 \text{μmol/L}), cyclosporine A (B; 0.1, 0.3, 1 \text{μmol/L}), and clarithromycin (C; 20, 60, 200 \text{μmol/L}) on \[^3\text{H}\]verapamil efflux ratio; tariquidar (D; 0.001, 0.003, 0.01 \text{μmol/L}) on \textit{N}-desmethyl loperamide efflux ratio; and tariquidar (E; 0.001, 0.003, 0.01 \text{μmol/L}), quinidine (F; 0.1, 0.3, 1 \text{μmol/L}), ketoconazole (G; 0.3, 1, 3 \text{μmol/L}), and saquinavir (H; 20, 60, 200 \text{μmol/L}) on loperamide efflux ratio in human MDR1-expressing cells. The transport study was conducted at 37°C for 2 hours. Each result represents the mean value (n = 4).](image)

**Table 2**  
\(K_i\) values for verapamil, \textit{N}-desmethyl loperamide, and loperamide transport via human MDR1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>(K_i) \text{μmol/L}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>Tariquidar</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin A</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Clarithromycin</td>
<td>434</td>
</tr>
<tr>
<td>\textit{N}-desmethyl loperamide</td>
<td>Tariquidar</td>
<td>0.010</td>
</tr>
<tr>
<td>Loperamide</td>
<td>Tariquidar</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>Quinidine</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>Saquinavir</td>
<td>148</td>
</tr>
</tbody>
</table>

Mean (n = 4)
<table>
<thead>
<tr>
<th>Dose and route of administration (substrates)</th>
<th>Inhibitor</th>
<th>Dose and route of administration (inhibitors)</th>
<th>Central nerve system end point</th>
<th>Inhibitor peak plasma concentration $\mu$mol/l</th>
<th>Unbound fraction of inhibitor in human plasma $[I_{\text{unbound}}]/K_i$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe substrate: verapamil</td>
<td>Tariquidar</td>
<td>8 mg/kg i.v. infusion for 30 min</td>
<td>3.3-fold increase of the influx rate constant</td>
<td>2.03</td>
<td>0.0063 ± 0.0021</td>
<td>1.28</td>
</tr>
<tr>
<td>379 ± 9 MBq of (R)-[11C]verapamil i.v. administration</td>
<td>Tariquidar</td>
<td>2 mg/kg i.v. infusion for 30 min</td>
<td>1.49-fold increase of the influx rate constant</td>
<td>0.76 ± 0.26</td>
<td>0.0063 ± 0.0021</td>
<td>0.48</td>
</tr>
<tr>
<td>384 ± 13 MBq of (R)-[11C]verapamil i.v. administration</td>
<td>Cyclosporin A</td>
<td>2.5 mg/kg/h i.v. infusion for 1 hour</td>
<td>1.88-fold increase of the $AUC_{\text{brain}}/AUC_{\text{blood}}$ ratio</td>
<td>2.80 ± 0.40</td>
<td>0.070 ± 0.020</td>
<td>0.44</td>
</tr>
<tr>
<td>7.4 MBq/kg of [11C]verapamil i.v. administration</td>
<td>Cyclosporin A</td>
<td>2.5 mg/kg/h i.v. infusion for 1 hour</td>
<td>1.8-fold increase of the influx rate constant</td>
<td>2.80 ± 0.40</td>
<td>0.070 ± 0.020</td>
<td>0.44</td>
</tr>
<tr>
<td>745 ± 16 MBq of (R)-[11C]verapamil i.v. administration</td>
<td>Clarithromycin</td>
<td>400 mg/day oral administration (b.i.d. for 2 days)</td>
<td>None</td>
<td>2.00</td>
<td>0.44 ± 0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Probe substrate: N-desmethyl loperamide</td>
<td>Tariquidar</td>
<td>6 mg/kg i.v. administration</td>
<td>3.7-fold increase of the influx rate constant</td>
<td>1.86</td>
<td>0.0063 ± 0.0021</td>
<td>1.17</td>
</tr>
<tr>
<td>Probe substrate: loperamide</td>
<td>Quindine</td>
<td>600 mg oral administration</td>
<td>1.33-fold increase of the influx rate constant</td>
<td>6.00</td>
<td>0.16 ± 0.03</td>
<td>1.17</td>
</tr>
<tr>
<td>343 ± 31 MBq of [11C]N-desmethyl loperamide i.v. administration</td>
<td>Quindine</td>
<td>600 mg oral administration</td>
<td>Respiratory depression</td>
<td>6.00</td>
<td>0.16 ± 0.03</td>
<td>1.17</td>
</tr>
<tr>
<td>16 mg loperamide Single oral administration</td>
<td>Tariquidar</td>
<td>150 mg/500 ml i.v. infusion for 30 min</td>
<td>None</td>
<td>0.64</td>
<td>0.0063 ± 0.0021</td>
<td>0.41</td>
</tr>
<tr>
<td>16 mg loperamide Single oral administration</td>
<td>Ketoconazole</td>
<td>400 mg oral administration</td>
<td>None</td>
<td>9.17</td>
<td>0.0071 ± 0.0017</td>
<td>0.07</td>
</tr>
<tr>
<td>16 mg loperamide Single oral administration</td>
<td>Saquinavir</td>
<td>600 mg oral administration</td>
<td>None</td>
<td>1.41</td>
<td>0.014 ± 0.004</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
and summarized in Table 2. Tariquidar showed the most potent P-gp inhibitory effect ($K_i$, 0.01 μmol/l) among the P-gp inhibitors tested, followed by cyclosporin A ($K_i$, 0.45 μmol/l) and quinidine ($K_i$, 0.82 μmol/l). Recent reports suggest that P-gp may contain multiple drug binding sites rather than a single site of broad substrate specificity by using radioligand binding techniques and the X-ray structure of P-gp (Martin et al., 2000; Aller et al., 2009). However, the substrate dependency of P-gp inhibition has not been fully understood, and further investigation should be required to clarify the issue by using a number of substrates that bind to different binding sites of P-gp. Thus, it is important to design the in vitro P-gp inhibition assay with use of the combination of substrates and inhibitors that are used in the clinical trials.

The relationship between the end point of the central nerve system in P-gp-mediated clinical DDI studies and unbound plasma concentrations of P-gp inhibitors ([I\text{unbound}]/K) divided by in vitro $K_i$ values was retrospectively investigated to set the appropriate criterion to observe significant DDI in humans (Table 3). The plasma concentrations of the concomitant drugs in humans were obtained or assumed on the basis of the previous report (Janisz-Dow et al., 1997; Stewart et al., 2000; Kharasch et al., 2004; Brunton et al., 2005). The [I\text{unbound}/K] values of tariquidar after intravenous administration were 1.28 and 1.17, where 3.3 and 3.7-fold increases of influx rate constant for brain entry of [11C]verapamil and [11C]N-desmethyl loperamide were observed, respectively. The [I\text{unbound}/K] values of quinidine (600 mg/man) after oral administration was 1.17, where the respiratory depressant effects of loperamide were observed. Of note, the dose of quinidine in this study was relatively higher than the conventional therapeutic dosage (Brunton et al., 2005). Accordingly, the relationship of IVIVC indicated that significant DDI at the BBB occurred in the presence of the P-gp inhibitors with [I\text{unbound}/K] > 1 in humans. In the presence of the P-gp inhibitors with 0.1 < [I\text{unbound}/K] ≤ 1, P-gp-mediated DDIs may or may not be observed. The [I\text{unbound}/K] values of tariquidar after i.v. infusion for 30 minutes at a dose of 2 mg/kg was 0.48, where slight but notable increase of influx rate constant for brain entry was observed. The [I\text{unbound}/K] of cyclosporin A after i.v. infusion for 1 hour at a dose of 2.5 mg/kg/h was 0.44, where the AUC ,brain/AUC, blood of verapamil increased by 88% (Sasongko et al., 2005). However, in the clinical study, the precise quantitation of P-gp function in the presence of a P-gp inhibitor by measuring total [11C]radioactivity may be impaired, because the metabolites of [11C]verapamil also have the properties of P-gp substrates (Lubberink et al., 2007). In addition, the peak plasma concentration achieved in the clinical study (2.80 μmol/l) was more than twice as much as the reported one at the therapeutic concentration (1.11 μmol/l) (Brunton et al., 2005). The [I\text{unbound}/K] values of tariquidar after i.v. infusion for 30 minutes at a dose of 6 mg/kg was 0.87, where the moderate DDI compared with higher dosage was observed (data not shown). However, loperamide’s opioid brain effect in humans was not observed after i.v. administration of 150 mg tariquidar, even though the [I\text{unbound}/K] was 0.41 (Kurnik et al., 2008). The unbound plasma concentrations of tariquidar in this study may be inadequate to significantly increase the brain penetration of loperamide to induce opioid-induced toxicity. Further investigations may be required to investigate the changes of the influx rate constant of loperamide in the presence of tariquidar. In the presence of the P-gp inhibitors with [I\text{unbound}/K] < 0.1, no clinically relevant DDI was reported. Therefore, the criterion [I\text{unbound}/K] of 1 was a clear cutoff to observe significant DDI at the BBB in humans. On the other hand, no significant DDI was observed in cases where [I\text{unbound}/K] was less than 0.1. This finding was consistent with our previous report using rats as an experimental model for P-gp-mediated DDIs (Sugimoto et al., 2011a).

In addition, at the conventional dose of clinically used P-gp inhibitors, P-gp-mediated DDI at the BBB in humans may be limited. The further clinical data accumulation will be necessary to define the precise cutoff to avert the P-gp-mediated DDIs.

Although the cutoff value for P-gp-mediated DDI at the BBB was found to be identical in rats and humans, the magnitude of DDI in humans is relatively lower than that in rodents (Lee et al., 2010). One of the possible reasons is the difference in the inhibition pattern between human MDR1 and rat Mdr1a. The relationship between plasma tariquidar concentration and the influx rate constant of [11C]verapamil indicated that Hill coefficients in humans were higher than that in rats, suggesting that there was a different cooperative binding site to human MDR1 and rat Mdr1a (Bauer et al., 2012). The accumulation of in vitro P-gp inhibition data will be necessary for a further understanding of the inhibition pattern between human MDR1 and rat Mdr1a. Another possible reason for the species difference is the influence of cerebral blood flow. The cerebral blood flow rate in rats receiving isoflurane anesthesia (1.11 ± 0.43 ml/g/min) was approximately 3-fold higher than that in humans (0.348 ± 0.023 ml/g/min) (Wei et al., 1993; Kreisel et al., 2010). When the function of P-gp is completely inhibited, the influx rate constant from blood to brain would approach to the cerebral blood flow. In fact, the influx rate constant of [11C]verapamil increased from 0.16 ml/g/min (baseline) to 1.16 ml/g/min in rats after bolus administration of 30 mg/kg tariquidar (Bauer et al., 2012). Therefore, the species difference of cerebral blood flow may at least partially be attributed to the different influence of P-gp inhibition on influx rate constant from blood to brain. The other possible factor for the species difference is the expression level of P-gp at the BBB between rats and humans or their functional activities. Although the P-gp expression levels at the BBB in rodents is 2.3-fold higher than that in humans (Uchida et al., 2011), Syvānen et al. suggested that the intrinsic P-gp activity at the BBB in humans may be less than that in rats (Syvānen et al., 2009). Further studies regarding species difference of intrinsic P-gp function between humans and rats will be required to address these issues.

In conclusion, this retrospective study indicated that significant DDI at the BBB in humans may occur in the presence of P-gp inhibitor with [I\text{unbound}/K] > 1. On the contrary, no significant DDI would be observed in cases where [I\text{unbound}/K] was less than 0.1. This criterion was comparable to the previous IVIVC results in rats. This investigation will facilitate the understandings of the appropriate cutoff to avert the risk of P-gp-mediated DDIs in human BBB.

**References**


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