Species Comparison of In Vivo P-Glycoprotein-Mediated Brain Efflux Using mdr1a-Deficient Rats and Mice

Christoffer Bundgaard, Christian Jes Nyberg Jensen, and Mats Garmer

Discovery DMPK, H. Lundbeck A/S, Valby, Denmark

ABSTRACT:
The experiments described herein compared the extent of in vivo P-glycoprotein (P-gp)-mediated brain efflux between rats and mice for a set of known central nervous system compounds. With use of newly introduced genetically modified mdr1a-deficient rats and their gene-competent counterparts, the brain to plasma distribution was assessed and compared with the distribution pattern in mdr1a-deficient and wild-type mice. Four compounds (aripiprazole, cilopram, risperidone, and venlafaxine) were administered using a continuous subcutaneous osmotic minipump infusion paradigm. Steady-state brain and plasma concentrations of the compounds, including selected metabolites (N-desmethyl-venlafaxine and O-desmethylenalafaxine) and N-desmethyl-venlafaxine) were measured in mdr1a-deficient rats and mice and their wild-type counterparts along with their free fractions to determine total and unbound brain to plasma distribution between genotypes within and between species. The results revealed qualitative as well as quantitative similarities between P-gp functionality in vivo at the blood-brain barrier level in rats and mice. All compounds tested were shown to have a significantly higher brain to plasma distribution in both mdr1a-deficient rats and mice compared with that in their wild-type counterparts. Moreover, the relative enhancement in extent of brain penetration between mdr1a-deficient and wild-type rats could be directly correlated to the enhancement ratios obtained in mice. From the unbound brain to unbound plasma distributions, the impact of P-gp on the overall brain penetration capabilities showed minor differences between rats and mice for the compounds tested. In conclusion, a comparable functional role of P-gp between rats and mice with respect to brain efflux mediated by this transporter is suggested.

Introduction
The physiology of the blood-brain barrier (BBB) with its endothelial cells lining the brain capillaries connected by tight junctions limits the extent of brain penetration for many xenobiotics. In addition to the physical barrier, numerous transport proteins are present at the BBB, acting as efflux pumps to actively exclude a wide variety of chemically diverse compounds from the central nervous system (CNS). One of the most studied members of this group of transporters is P-glycoprotein (P-gp, ABCB1), a mammalian plasma membrane phospholipid encoded by the multidrug resistance (MDR) 1 gene (mdr1 in rodents). P-gp belongs to the ATP-binding cassette (ABC) transporter superfamily and is located in the brain in the luminal membrane of the endothelial cells but is also expressed in other organs (Thiebaut et al., 1987). For compounds designed to exert their effect in the CNS, drug-P-gp interactions could influence the potential efficacy by reducing the brain concentration because of this efflux mechanism. Thus, P-gp has been shown in mice to have a profound influence on brain concentrations of many types of drug substrates including antidepressants and antipsychotics (Ejsing et al., 2007; Linnet and Ejsing, 2008). Therefore, screening for P-gp substrate recognition and assessment of the potential impact hereof are important parts of the profile evaluation of new chemical entities, particularly those targeted to the CNS (Wager et al., 2011).

During lead optimization programs and drug candidate characterization, in vivo pharmacology and pharmacokinetic studies are often performed in rats and mice. In many such studies it is important to understand the potential differences in the functional activity of P-gp between these species with respect to drug distribution to the brain to make proper comparisons and extrapolations (Kim et al., 2008; Syvänen et al., 2009). In both rats and mice, P-gp is the most highly expressed ABC protein in brain endothelial capillary cells (Warren et al., 2009). Rats and mice share two genes encoding for P-gp, mdr1a and mdr1b, which are similarly expressed between the two species, and in both of these species, mdr1a is the predominant form in the brain (Croop et al., 1989; Kwan et al., 2003). In addition, there is a high degree of homology in the amino acid content of P-gp between rats and mice of approximately 93% (Hubbard et al., 2007). Studies investigating differences in P-gp functionality between rats and mice have primarily been conducted in vitro, applying efflux ratios derived from bidirectional transport studies in cell systems transfected with mdr1 from each species. These studies suggest some correlation between rat and mouse P-gp-mediated efflux and also differences, depending on the substrate (Booth-Genthe et al., 2006; Katoh et al., 2006; Takeuchi et al., 2006). However, comparison of efflux ratios among species from in vitro transport studies is circumvented with indecisive
biological challenges such as total protein expression and density of the functional transporter in each cell line.

With respect to in vivo studies investigating quantitative species differences in P-gp function between rats and mice, a very limited amount of data has been published. Cutler et al. (2006) used infusion of the P-gp inhibitor GF-129198 (elacridar) and reported that similar total plasma concentrations of the inhibitor were required in rats and mice to achieve the same relative increase in the brain/plasma distribution ratio of an undisclosed P-gp substrate. However, species differences in plasma protein binding of the inhibitor or substrate are confounding factors that may influence the total plasma concentrations required to inhibit P-gp. Thus, such chemically induced knockout studies are not readily comparable between species.

With the advent of mdr1a/-deficient mice either from genetic knockout modifications or mutant origin (Schinkel et al., 1996), these animals has become widely applied in drug discovery to assess the role of P-gp in vivo on drug disposition including BBB transport studies. Use of these transporter-deficient animal models has suffered because they are only available in mouse species, making quantitative interspecies comparisons difficult. Recently, however, mdr1a/-knockout rats became commercially available through the application of zinc finger nuclease technology, enabling targeted gene knockout (Geurts et al., 2009; Chu et al., 2011). The purpose of this study was therefore to compare the P-gp functionality between mdr1a/-deficient rats and mice and their gene-competent counterparts (wild types) with respect to in vivo brain distribution. A range of known CNS compounds and their selected metabolites previously recognized to be P-gp substrates were evaluated in these animal models using a similar experimental design across species.

Materials and Methods

Animals. Homozygous mdr1a/-knockout and wild-type rats (SD-AbcB1a<sup>tm1sage</sup> /H9262; 350–500 g b.wt.) were obtained from SAGE Labs, Sigma Life Science (St. Louis, MO). mdr1a/-deficient mice and CF-1 (-non-Swiss) outbred background mice (Crl:CF1-Abcb1a<sup>mds</sup> mutants; 25–45 g b.wt.) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All animals arrived at the animal facility at least 5 days before being used in experiments. During acclimatization, animals were housed in pairs under controlled conditions of temperature (21 ± 2°C) and relative humidity (55 ± 5%) and a 12-h light/dark cycle (lights on at 6:00 AM). Food and tap water were freely available in the cage. Approval of all animal procedures was granted by The Danish National Committee for Ethics in Animal Experimentation.

Drugs and Dosing. The test compounds administered included citalopram, venlafaxine, risperidone, and aripiprazole, which all are extensively used CNS Drugs and Dosing. The test compounds administered included citalopram, venlafaxine, risperidone, and aripiprazole, which all are extensively used CNS compounds. In addition, these compounds and their selected metabolites previously recognized to be P-gp substrates were evaluated in these animal models using a similar experimental design across species.

Results

After 3 days of continuous treatment using minipumps, the four test compounds and three selected formed metabolites were measured in plasma and brains of wild-type and mdr1a/-deficient rats and mice. The observed total plasma and brain concentrations and corresponding K<sub>p</sub> values are summarized in Table 1 for rats and Table 2 for mice. For all compounds except 9-OH-risperidone, there were no statistically significant differences between the systemic plasma exposure in mdr1a/-deficient animals and their wild-type counterparts. For 9-OH-risperidone, plasma concentrations were significantly higher in mdr1a/-deficient mice compared with those in wild-type mice (p < 0.05). This trend was also observed for 9-OH-risperidone in mdr1a/-deficient rats versus wild-type rats although not to a statistically significant degree. From the derived K<sub>p</sub> values, all the compounds tested tended to display higher brain distribution in mice compared with that in rats when the species within in each genotype were measured under the same conditions.
Thus, from the as being substrates for P-gp at the BBB level in both rats and mice. 9-OH-risperidone and aripiprazole had the lowest wild-type controls (set as 100%) for each species. All the compounds evaluated, citalopram exhibited the highest Kp value in both species.

All the compounds in the data set that were tested were identified as being substrates for P-gp at the BBB level in both rats and mice. Thus, from the Kp values, all the compounds tested were shown to have significantly higher brain distribution in both mdr1a-deficient rats and mice compared with those in their wild-type counterparts. To quantitatively compare the impact of P-gp on brain distribution capabilities of the compounds between rats and mice, Kp values were normalized to account for differences in absolute total plasma and brain exposure. Figure 1 shows the normalized Kp values for the test compounds in mdr1a-deficient animals relative to those for their wild-type controls (set as 100%) for each species. All the compounds exhibited significant enhancement in their normalized Kp value in mdr1a-deficient animals. In both rats and mice, the most pronounced effect of P-gp-mediated brain efflux was observed for risperidone (835 ± 197% enhancement in mdr1a-deficient rats and 737 ± 221% in mice) and its metabolite 9-OH-risperidone (1100 ± 274% enhancement in mdr1a-deficient rats and 1200 ± 155% in mice). The weakest effect of P-gp on the relative brain distribution was seen with venlafaxine (192 ± 18% enhancement in mdr1a-deficient rats and 176 ± 20% in mice) and citalopram (330 ± 27% enhancement in mdr1a-deficient rats and 201 ± 8% in mice). As for the most pronounced P-gp substrates, the weaker relative effects of P-gp on brain distribution of venlafaxine and citalopram were also shown to be present in both species. When the impact of P-gp on brain distribution was ranked across all compounds tested, considerable overlap between rats in mice was observed. In rats this was (low to high) venlafaxine, O-desmethyl-venlafaxine, citalopram, aripiprazole, N-desmethyl-venlafaxine, risperidone, 9-OH-risperidone, and 9-OH-risperidone.

The corresponding ranking for mice was venlafaxine, citalopram, aripiprazole, O-desmethyl-venlafaxine, N-desmethyl-venlafaxine, risperidone, and 9-OH-risperidone.

The overall relationship between the enhancements in the normalized Kp values in mdr1a-deficient rats and mice is visualized in Fig. 2. Regression analysis revealed a linear correlation between the two species with a slope close to unity (y = 1.08x – 102; r² = 0.95, p = 0.0002).

To assess the impact of P-gp deficiency on the brain to plasma distribution of the compounds independent of binding to plasma proteins and brain tissue, the plasma and brain free fractions determined in vitro were combined with measured total brain and plasma concentrations at steady state to calculate Kp,uu for each compound in wild-type and mdr1a-deficient animals (Table 3). In wild-type rats, all compounds had Kp,uu values well below unity, consistent with active efflux dominating the BBB distribution of these compounds. The Kp,uu values in mdr1a-deficient rats were increased to approximately unity for the majority of the compounds. In mice, all the compounds except for citalopram exhibited Kp,uu values below unity. Citalopram was shown to have a Kp,uu value of 2.4 in wild-type mice, which was increased to 4.8 in mdr1a-deficient mice, whereas the other compounds were increased to values closer to unity compared with the respective values in wild-type animals.

### Discussion

The experiments described herein compared the prevalence and magnitude of in vivo P-gp-mediated brain efflux between rats and mice for a set of known CNS compounds. To compare the extent of brain distribution between rats and mice and the impact of P-gp, all experiments were performed at steady state. This design allowed for calculations of Kp and Kp,uu between brain and plasma with species-dependent pharmacokinetics taken into account.

### Table 1

**Total plasma and brain concentrations and derived Kp values of administered test compounds and formed metabolites in wild-type and mdr1a-deficient rats after a 3-day continuous minipump treatment**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wild-Type Rats</th>
<th>mdr1a-Deficient Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Brain</td>
</tr>
<tr>
<td>Citalopram</td>
<td>27 ± 2.4</td>
<td>265 ± 16</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>50 ± 3.7</td>
<td>158 ± 4.6</td>
</tr>
<tr>
<td>O-Desmethyl-venlafaxine</td>
<td>4.5 ± 1.4</td>
<td>4.4 ± 0.89</td>
</tr>
<tr>
<td>N-Desmethyl-venlafaxine</td>
<td>6.8 ± 1.5</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td>Risperidone</td>
<td>194 ± 34</td>
<td>41 ± 0.88</td>
</tr>
<tr>
<td>9-OH-risperidone</td>
<td>153 ± 24</td>
<td>22 ± 1.6</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>18 ± 5.3</td>
<td>3.0 ± 0.60</td>
</tr>
</tbody>
</table>

### Table 2

**Total plasma and brain concentrations and derived Kp values of administered test compounds and formed metabolites in wild-type and mdr1a-deficient mice after a 3-day continuous minipump treatment**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wild-Type mice</th>
<th>mdr1a-Deficient Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Brain</td>
</tr>
<tr>
<td>Citalopram</td>
<td>8.8 ± 0.40</td>
<td>191 ± 19</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>85 ± 9.8</td>
<td>403 ± 63</td>
</tr>
<tr>
<td>O-Desmethyl-venlafaxine</td>
<td>5.7 ± 0.35</td>
<td>10 ± 0.87</td>
</tr>
<tr>
<td>N-Desmethyl-venlafaxine</td>
<td>17 ± 2.8</td>
<td>25 ± 5.1</td>
</tr>
<tr>
<td>Risperidone</td>
<td>43 ± 10</td>
<td>21 ± 2.3</td>
</tr>
<tr>
<td>9-OH-risperidone</td>
<td>45 ± 8.4</td>
<td>13 ± 1.7</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>51 ± 8.7</td>
<td>25 ± 4.8</td>
</tr>
</tbody>
</table>
From the $K_p$ values obtained in mdr1a-deficient rats and mice and their wild-type counterparts, the experiments revealed qualitative as well as quantitative species similarities in P-gp functionality at the BBB level. Thus, all compounds evaluated were shown to be substrates in both species. Moreover, as shown in Fig. 2, the extent of the increase in the normalized $K_p$ values between mdr1a-deficient animals and their wild-type counterparts could be directly correlated between rats and mice. The indication of a comparable quantitative role of in vivo P-gp functionality at the BBB between rats and mice observed in this study correlates with expression levels and protein homology of mdr1 between these species (Kim et al., 2008). In addition, in vitro studies in mdr1a-transfected cells indicate a comparable functional role of P-gp between these species although some substrate dependence has been reported (Booth-Genthe et al., 2006; Katoh et al., 2006; Takeuchi et al., 2006).

The compounds tested have previously been reported to be substrates for P-gp in vivo at the BBB in mice as shown by increased brain to plasma distribution in P-gp knockout versus that in wild-type mice. Citalopram has been shown to have a 2- to 3-fold higher $K_p$ value in P-gp knockout mice (Uhr and Grauer, 2003; Doran et al., 2005; Uhr et al., 2008), in line with the present data in mice (201% increase). In mdr1a-deficient rats, citalopram showed an enhancement ratio of 330%, which could indicate that this compound has a slightly higher propensity for P-gp-mediated brain efflux in rats compared with that in mice. Assessment of bidirectional transport properties across the BBB including the impact of P-gp can be based on the ratio between unbound concentrations in brain and plasma at equilibrium conditions, expressed as $K_{puu}$. If $K_{puu}$ is close to unity, the BBB transport is predominantly mediated by passive diffusion or the impact of influx and efflux transport is equal. If $K_{puu}$ is below unity, efflux transport may explain this result, whereas $K_{puu}$ above unity is an indication that active uptake processes are involved (Ham-
In both mice and rats, it has been shown that knockout of P-gp in mice can result in a 99% increase in 
K\textsubscript{f, uu} values in the present study. First, it has been shown that knockout of P-gp in mice can result in 
alterations in the mRNA expression levels of other transporter proteins (Cisternino et al., 2004). Thus, if the compound under investigation 
has substrate affinity for other efflux or uptake transporters besides P-gp, this could potentially affect the brain to plasma distribution 
observed in mdr1a-deficient species, but the aforementioned variance in N-desmethyl-venlafaxine exposure 
and the very high free fraction of O-desmethyl-venlafaxine in mice suggest that these values should be interpreted with caution.

Development of the genetically mdr1a-deficient rat model provides a simple tool for evaluating the functional consequence of P-gp efflux 
at the BBB by the measurement of drug concentrations in brain and plasma at steady state. However, some drug- and/or species-dependent 
limitations in this model and methodology probably exist that may affect interpretation of the obtained 
K\textsubscript{p, uu} estimates. First, it has been shown that knockout of P-gp in mice can result in 
alterations in the mRNA expression levels of other transporter proteins (Cisternino et al., 2004). Thus, if the compound under investigation 
has substrate affinity for other efflux or uptake transporters besides P-gp, this could potentially affect the brain to plasma distribution 
obtained in mdr1a-deficient models. A recent characterization of the mdr1a-deficient rat model suggests that only minor compensatory 
changes in expression of other transporter-related genes occur at the BBB level in male rats lacking P-gp (Chu et al., 2011). Second, 
continuous drug administration may increase P-gp functionality at the BBB as a result of mdr1 induction. This has been suggested for 
venlafaxine in vitro in human cell systems (Ehret et al., 2007; Bachmeier et al., 2011) and in vivo in rats after 3 weeks of continuous 
treatment (de Klerk et al., 2010). Thus, if species differences exist in terms of liability and extent of mdr1 induction after subchronic 
treatment, this could theoretically hamper a quantitative comparison of P-gp-mediated efflux between rats and mice. Third, intrabrain drug 
distribution governed by processes other than nonspecific binding may potentially affect the estimates of K\textsubscript{p, uu} in the present study. 
Hence, determining brain free fractions using the homogenate methodology only measures nonspecific binding and therefore does not 
count for other intrabrain distributional aspects such as sequestration into lysosomes that might affect cellular retention for basic 
compounds. In addition, the homogenization process may alter the binding properties by unmasking binding sites that are not accessible 
to a drug in intact brain tissue. However, recent studies suggest that a

<table>
<thead>
<tr>
<th>Compound</th>
<th>Free Fraction, Rats</th>
<th>K\textsubscript{p, uu}, Rats</th>
<th>Free Fraction, Mice</th>
<th>K\textsubscript{p, uu}, Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>f\textsubscript{u, plasma}</td>
<td>f\textsubscript{u, brain}</td>
<td>WT</td>
<td>P-gp Def</td>
</tr>
<tr>
<td>Citalopram</td>
<td>50</td>
<td>3.1</td>
<td>0.61</td>
<td>2.1</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>64</td>
<td>9.2</td>
<td>0.45</td>
<td>0.89</td>
</tr>
<tr>
<td>O-Desmethyl-venlafaxine</td>
<td>89</td>
<td>35</td>
<td>0.38</td>
<td>1.2</td>
</tr>
<tr>
<td>N-Desmethyl-venlafaxine</td>
<td>32</td>
<td>8.0</td>
<td>0.34</td>
<td>2.0</td>
</tr>
<tr>
<td>Risperidone</td>
<td>11</td>
<td>9.0</td>
<td>0.17</td>
<td>1.4</td>
</tr>
<tr>
<td>9-OH-risperidone</td>
<td>20</td>
<td>15</td>
<td>0.11</td>
<td>1.1</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>0.10</td>
<td>0.10</td>
<td>0.19</td>
<td>0.64</td>
</tr>
</tbody>
</table>

WT, wild type; P-gp Def, mdr1a-deficient. *f\textsubscript{u, plasma} values of 99% was used to estimate K\textsubscript{p, uu} for venlafaxine and O-desmethyl-venlafaxine.

mardun-Udenaes et al., 1997, 2008). Of interest, citalopram displayed a K\textsubscript{p, uu} value in wild-type mice of 2.4, which was increased to 4.8 in mdr1a-deficient mice, indicating that, despite clear P-gp-mediated efflux, some involvement of uptake transporters is also suggested to contribute to the overall BBB distribution of citalopram. This pattern was also observed for citalopram in rats although to a lesser extent. A putative involvement of active uptake is supported by in vitro trans-
port studies across bovine brain endothelial cells, where citalopram 
could be considered as a means to a more accurate determination of potential species differences in the P-gp-mediated effects of this compound. K\textsubscript{p, uu} estimates of venlafaxine and its metabolites in mdr1a-deficient and wild-type animals indicated that passive diffusion was the dominant factor for BBB distribution without the presence of P-gp across the parent-metabolite system. K\textsubscript{p, uu} values of N-desmethyl-venlafaxine in rats and O-desmethyl-venlafaxine in mice increased to values greater than 2 in the mdr1a-deficient species, but the aforementioned variability in N-desmethyl-venlafaxine exposure and the very high free fraction of O-desmethyl-venlafaxine in mice plasma suggest that these values should be interpreted with caution.
reasonable correlation can be established between nonspecific binding from homogenates and binding obtained using brain slices with a preserved cellular structure (Fridén et al., 2011).

By use of the same experimental methodology in rats as in mice, the validity of the mdr1a-deficient rat model was confirmed in the present study in which all selected P-gp substrates identified in mice were also recognized in rats. This model thus allows for a direct means to assess the potential influence of P-gp on pharmacokinetic and pharmacodynamic parameters in rats without the need for chemical P-gp inhibitors. Besides qualitative similarities in P-gp functionality between rats and mice, the data obtained here also indicate substantial quantitative similarities in the extent of P-gp-mediated efflux at the BBB between these species. This information might prove useful when existing knowledge on P-gp-mediated efflux obtained in mice is extrapolated to the rat species during pharmacokinetic and in vivo pharmacological evaluations.

Acknowledgments

Moni Elster is acknowledged for technical assistance.

Authorship Contributions

Participated in research design: Bundgaard and Jensen.

Conducted experiments: Jensen and Garmer.

Performed data analysis: Bundgaard and Garmer.

Wrote or contributed to the writing of the manuscript: Bundgaard.

References


Address correspondence to: Dr. Christoffer Bundgaard, H. Lundbeck A/S, Discovery DMPK, 9 Ottiliavej, DK-2500 Valby, Denmark. E-mail: cbu@lundbeck.com

Dr. Christoffer Bundgaard, H. Lundbeck A/S, Discovery DMPK, 9 Ottiliavej, DK-2500 Valby, Denmark. E-mail: cbu@lundbeck.com.