Olanzapine Penetration into Brain is Greater in Transgenic *Abcb1a* P-glycoprotein-Deficient Mice than FVB1 (Wild-Type) Animals

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The transmembrane energy-dependent efflux transporter P-glycoprotein (P-gp) limits a range of drugs from penetrating cells and deposits them into the extracellular space. P-gp is highly expressed in several normal tissues, including the luminal surface of capillary endothelial cells in the brain of humans. In this study, we tested whether olanzapine distribution to tissues highly expressing P-gp or devoid of this transporter was similar in *Abcb1a* (–/–) mice lacking P-gp and control animals. At 1 h following the intraperitoneal injection of 2.5 µg olanzapine/g mouse, olanzapine concentrations were statistically and significantly higher in brain (three-fold), liver (2.6-fold), and kidney (1.8-fold) of *Abcb1a* (–/–) mice than those of the control FVB *Abcb1a* (+/+) mice, and not statistically different in plasma, spleen, or penile tissue. Similar differences were also found for the ratios of organ:plasma and organ:spleen between the two groups. This is the first report that the presence of the *Abcb1a* gene is an important factor controlling brain access to olanzapine. The finding that the brain penetration of olanzapine is limited by P-gp implies that the highly prevalent functional polymorphisms of *ABCB1* in humans may be a factor contributing to variability in dose requirements for this antipsychotic drug.

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INTRODUCTION

The adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of efflux transport proteins contains subfamily B, often referred to as the multidrug-resistant subfamily (MDR), and subfamily C, also known as the multidrug-resistant related protein subfamily (MRP) (Germann, 1996; Carson *et al*, 2002). The most extensively studied ABC transporter is P-glycoprotein (P-gp). P-gp belongs to subfamily B and is a 170-kDa transmembrane protein encoded by the human gene *ABCB1*. Formerly known as *MDR1* or *PGY1*, *ABCB1* maps to chromosome 7q21.1 (Dean *et al*, 2001). It was the first ABC transporter cloned and characterized by its ability to confer a multidrug resistance phenotype to cancer cells that had become resistant to chemotherapy (Riordan *et al*, 1985; Roninson *et al*, 1986).

In addition to expression in tumor cells, the *ABCB1* protein (P-gp) is widely localized in normal tissues, including the apical membranes of the gastrointestinal tract, the biliary canalicular membranes of hepatocytes, the luminal membranes of proximal tubular epithelial cells in the kidney, and the luminal membranes of endothelial cells in cerebral capillaries forming the blood–brain barrier (Cordon-Cardo *et al*, 1989; Yu, 1999; Silverman, 2000).

The biological function of P-gp appears to be a protective role for major organs by limiting cellular uptake of xenobiotics by excreting these compounds into bile, urine, the intestinal lumen, and limiting accumulation in brain. This function as a drug efflux pump plays an important role in drug absorption, distribution, and elimination (Lin and Yamazaki, 2003). A large number of structurally diverse drugs from various pharmacologic classes are actively transported by P-gp (Schinkel *et al*, 1996; Schuetz *et al*, 1996; Mahar Doan *et al*, 2002; Weiss *et al*, 2003). P-gp is highly polymorphic and its expression in the endothelial cells of the blood–brain barrier limits substrate access to the central nervous system (CNS), thereby influencing the therapeutic consequences of psychoactive drugs (Thompson *et al*, 2000). A P-gp inhibitor was shown to increase the extracellular fluid concentration of morphine in the brain of

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rats, while chronic treatment with morphine leading to tolerance of the antinociceptive effect was shown to be mediated by a morphine-induced increase in the brain P-gp (Aquilante *et al*, 2000).

Several *in vitro* cell systems and a transgenic *Abcb1a/b*($-/-$) mouse model have been used to study the functional activity of the protein in the blood-brain barrier (Lin and Yamazaki, 2003; Schwab *et al*, 2003). Drug-stimulated ATPase activity can be determined by monitoring the release of inorganic phosphate (Sarkadi *et al*, 1992; Urbatsch *et al*, 1994). Alternatively, fluorescent indicators can be monitored where the inhibition of P-gp leads to cellular accumulation of rhodamine-123 or calcein AM (Eytan *et al*, 1997; Tiberghien and Loo, 1996). The *Abcb1a*($-/-$) 'knockout' mouse model exhibits functional deficiency of P-gp in the blood-brain barrier (Cordon-Cardo *et al*, 1989; Schinkel *et al*, 1996, 1997; Silverman, 2000), and therefore has provided considerable insight into the role and importance of P-gp in the brain entry of drugs (Kim *et al*, 1998; Schinkel *et al*, 1996, 1997; Letrent *et al*, 1999; Aquilante *et al*, 2000). Mice lacking the *Abcb1a* gene display no anatomical, biochemical, or other abnormalities (Schinkel *et al*, 1994, 1997).

Data documenting transport of antipsychotic drugs with P-gp are sparse. No previous reports could be found using the *Abcb1a*($-/-$) mouse to test antipsychotic drugs for transport by P-gp. Previous reports using *in vitro* methods have found that levomepromazine and risperidone may be substrates, while chlorpromazine, clozapine, haloperidol, chlorprothixene, and perphenazine may not (Mahar Doan *et al*, 2002; Schinkel *et al*, 1997). The data on chlorpromazine are conflicting with positive transport using the human CCRF-CEM leukemia cells (Syed *et al*, 1996) and low transport by multidrug resistance-transfected MDCK cells (Mahar Doan *et al*, 2002). Using ATPase activity as a marker for P-gp binding affinity, we previously found a rank order of activity from verapamil (positive control) > quetiapine > risperidone > olanzapine > chlorpromazine > haloperidol = clozapine (Boulton *et al*, 2002). These results suggested that P-gp may influence the access to the brain of the antipsychotics to various degrees.

Verapamil, quetiapine, and risperidone each are partially metabolized by CYP3A4 (Kroemer *et al*, 1993; DeVane and Nemeroff, 2001a, b). Olanzapine is predominantly biotransformed by CYP1A2 and glucuronyl transferases, yet a small contribution by CYP3A4 has been implicated (Ring *et al*, 1996; Markowitz *et al*, 2001). An inter-relationship in substrate specificity between cytochrome P450 (CYP) 3A4 and P-gp has been proposed (Kim *et al*, 1999). The *ABCB1* and *CYP3A* genes are located on the same chromosome with proximal gene loci in humans (Tsui *et al*, 1995); however, many drugs that are CYP3A4 substrates (midazolam, triazolam, alprazolam) are not transported by P-gp and vice versa (digoxin, fexofenadine; Perloff *et al*, 1999; Schuetz *et al*, 1996; von Moltke *et al*, 1999). Nevertheless, the overlap in CYP3A4 and P-gp substrates is consistent with our previous *in vitro* results based on ATPase activity, suggesting that quetiapine among the antipsychotics had the greatest degree of affinity for P-gp (Boulton *et al*, 2002). Olanzapine has the least evidence as a P-gp substrate. To further assess the role of P-gp in limiting brain entry of the antipsychotics, we compared the tissue distribution of

olanzapine in *Abcb1a*($-/-$) transgenic mice with that in the FVB control *Abcb1a*($+/+$) mice.

MATERIALS AND METHODS

Chemicals

Olanzapine and RS0244 (internal standard) were obtained from Eli Lilly Inc. (Indianapolis, ID, USA). Hank's balanced salt solution (HBSS) was purchased from Gibco BRL (Carlsbad, CA, USA). Other chemicals and reagents were the purest grade available and were obtained from Fisher Scientific Co. (Fairlawn, NJ, USA).

Experimental Animals

Male *Abcb1a*($-/-$) mice (FVB/TacBR-[KO]MDR1aN7) and genetically matched male *Abcb1a*($+/+$) mice (FVB/MTtacBR) 9–10 weeks of age weighing 20–30 g were obtained from Taconic (Germantown, NY, USA). The founding of this colony has been described previously (Schinkel *et al*, 1994). Animals were housed individually and maintained at a 12:12 h light/dark cycle. The animals were cared for in accordance with the US Public Health Service policy for the Care and Use of Laboratory Animals, and the experimental studies were approved by the Medical University of South Carolina Animal Care Committee.

Experimental Procedures

Olanzapine was dissolved in 0.9% saline with minimal use of 1 N HCL (1%, v/v). A dose of 2.5 μ g olanzapine/g mouse was administered intraperitoneally (i.p.). The entire volume injected was 10 μ l/g mouse. At 1 h after the injection, the mice were anesthetized with halothane and decapitated. Trunk blood was collected in EDTA-coated tubes and centrifuged at 3000g for 20 min to determine plasma concentrations of olanzapine.

The brain (brain tissue plus meninges), liver, kidney, spleen, and penis were dissected and weighed, and then homogenized in five-fold volume of an HBSS and 0.02 M HEPES buffer, pH 7.2. The homogenates were frozen at -70°C until high-performance liquid chromatography (HPLC) analysis.

HPLC Analysis of Olanzapine Plasma and Tissue Concentrations

The plasma and tissue samples obtained at 1 h following administration of olanzapine were extracted and determined using HPLC method previously developed and validated in our laboratory and modified for tissue samples (Boulton *et al*, 2001; Llorca *et al*, 2001). Briefly, olanzapine was extracted from 100 or 200 μ l of plasma or organ samples after the addition of 50 μ l of internal standard working solution (RS0244 10 μ g/ml), 0.2 ml of 1 M bicarbonate buffer (pH 11), and 5 ml of a mixture of hexane-isoamyl alcohol (98:2, v/v). The mixture was shaken for 15 min and centrifuged at 3000g for 5 min. The organic layer was transferred to another tube and back-extracted with 0.2 ml 0.2 M sulfuric acid. After shaking and centrifugation, the aqueous layer was re-extracted with 0.2 ml of 1 M bicarbonate

buffer. The organic phase was evaporated under nitrogen at 45°C. The residue was dissolved in 100 µl of mobile phase (65% methanol:35% water containing 0.2% triethylamine and was adjusted to pH 7.0 with 85% phosphoric acid), and 50 µl was injected into the chromatographic system. Olanzapine and internal standard were separated by a Luna 5u C18(2) column (250 × 4.6 mm; Phenomenex), and the effluent was monitored at a UV wavelength of 273 nm and at a flow rate of 1 ml/min at ambient temperature. The detection limit of olanzapine was 5 ng/ml. The intraday and interday coefficients of variation were lower than 7% at relevant concentrations ($n=7$). The plasma and tissue extraction recoveries ranged from 83 to 98%.

Data Analysis

Drug quantitation was performed by comparing peak height ratios of olanzapine to the internal standard with ratios derived from calibration curve of standards containing known amounts of drug extracted from plasma or tissues as described above. Final concentrations of olanzapine were expressed as either µg/ml for plasma or µg/g for tissues.

An unpaired *t*-test was used to compare the differences between the concentrations of olanzapine in the *Abcb1a*(-/-) mice and control mice and their tissue:plasma and tissue:spleen ratios. A two-tailed *P*-value was used and the level of statistical significance was $P<0.01$.

RESULTS

The plasma and organ concentrations of olanzapine 1 h after the i.p. injection of 2.5 µg olanzapine/g mouse are shown in Table 1 and Figure 1. Substantially and statistically higher olanzapine concentrations were found in the brain (three-fold), liver (2.6-fold), and kidney (1.8-fold) of *Abcb1a*(-/-) mice than those of the control mice (Table 1, Figure 1). Consistently higher ratios were also found for the organ:plasma and the organ:spleen concentrations of olanzapine in the *Abcb1a*(-/-) mice than in the control mice (Table 1, Figure 2). However, there was no statistically significant difference for concentrations of olanzapine in the plasma, spleen, and penis between the two groups, nor for the tissue:plasma ratios of spleen and penis (Table 1, Figures 1 and 2).

DISCUSSION

The current findings demonstrate that the brain penetration of olanzapine is limited by P-gp. The three-fold difference in the brain concentration of olanzapine between the P-gp-deficient *Abcb1a*(-/-) mice and the FVB control *Abcb1a*(+/+) mice were most likely due to the functional activity of P-gp residing in the blood-brain barrier. This conclusion is also in good agreement with the 2.6-fold difference observed in brain:plasma and brain:spleen ratios of olanzapine between the two groups, indicating that the difference in the brain olanzapine concentration between the two groups was not caused by differences of plasma olanzapine concentration that were identical. Similarly, differences in concentration in the spleen were absent, since the expression of P-gp in the spleen is very low (Cordon-

Table 1 Tissue Concentrations and Tissue:Plasma Ratios of Olanzapine in *Abcb1a*(-/-) ($n=6$) and Wild-Type Mice ($n=6$) after 1 h i.p. Injection of Olanzapine 2.5 µg/g Mouse

	<i>Abcb1a</i> (-/-) (mean ± SD)	Wild type (mean ± SD)	Ratio ^a	<i>P</i> -value (unpaired <i>t</i> -test)
Plasma	0.6 ± 0.1	0.6 ± 0.1	1.1	0.5
Brain	1.3 ± 0.3	0.5 ± 0.2	3.0	0.0006*
Liver	3.5 ± 0.6	1.5 ± 0.6	2.6	0.0002*
Kidney	2.8 ± 0.7	1.5 ± 0.2	1.8	0.002*
Spleen	3.1 ± 0.9	3.1 ± 0.3	1	0.9
Penis	0.5 ± 0.3	0.6 ± 0.3	0.9	0.5
Brain:plasma	2.0 ± 0.5	0.9 ± 0.4	2.6	0.002*
Liver:plasma	5.5 ± 1.2	2.5 ± 0.8	2.4	0.0005*
Kidney:plasma	4.4 ± 1.3	2.6 ± 0.1	1.7	0.006*
Spleen:plasma	4.7 ± 1.0	5.2 ± 1.0	0.9	0.5
Penis:plasma	0.8 ± 0.5	1.0 ± 0.3	0.9	0.4
Brain:spleen	0.5 ± 0.2	0.2 ± 0.1	2.6	0.009*
Liver:spleen	1.2 ± 0.5	0.5 ± 0.2	2.4	0.007*
Kidney:spleen	1.0 ± 0.4	0.5 ± 0.1	1.7	0.02*
Penis:spleen	0.2 ± 0.1	0.2 ± 0.05	0.9	0.9

^aCalculated as the average of the ratios from each individual animal.

*Statistical significance ($P<0.01$) by two-tailed unpaired *t*-test.

Figure 1 Plasma (µg/ml) and tissue (µg/g) concentrations of olanzapine in *Abcb1a*(-/-) (solid bars) and *Abcb1a*(+/+) mice (open bars) 1 h after the i.p. injection of 2.5 µg olanzapine/g mouse. Values are shown as mean ± SD ($n=6$). The *P*-values shown were calculated by two-tailed unpaired *t*-test.

Cardo et al, 1989; Schinkel et al, 1997). These findings of no drug concentration differences in organs with low P-gp expression (plasma and spleen) are consistent with the previous results of Uhr et al (2000) using amitriptyline.

Previously, the absence of P-gp in *Abcb1a*(-/-) mice has been shown as a pervasive influence on the tissue distribution and pharmacokinetics of drugs such as indinavir, nelfinavir, saquinavir, loperamide, ondansetron, ivermectin, vinblastin, cyclosporin A, amitriptyline, morphine, some endogenous steroid hormones, citalopram, trimipramine, carbamazepine, phenobarbital, lamotrigine,

Figure 2 Organ:plasma ratios of olanzapine in *Abcb1a*(-/-) (solid bars) and *Abcb1a*(+/+) mice (open bars) 1 h after the i.p. injection of 2.5 µg olanzapine/g mouse. Values are shown as mean $\pm$ SD ($n = 6$). The *P*-values shown were calculated by two-tailed unpaired *t*-test.

and felbamate (Kim *et al*, 1998; Schinkel *et al*, 1996, 1997; Letrent *et al*, 1999; Aquilante *et al*, 2000; Uhr *et al*, 2000, 2002; Fromm, 2000; Potschka *et al*, 2001, 2002; Uhr and Grauer, 2003). The brain concentrations of these drugs were remarkably higher in *Abcb1a*(-/-) mice than that of *Abcb1a*(+/+) mice. However, differences in the liver and kidney were smaller compared to those in the brain or not statistically significant. In the present study, similar ratios of olanzapine to plasma were found for the brain, liver, and kidney (3.0, 2.6, 1.8, respectively, Table 1) in *Abcb1a*(-/-) compared to *Abcb1a*(+/+) mice. When studies have found large differences in the brain concentration of drugs transported by P-gp in *Abcb1a*(-/-) animals compared to controls, the liver and kidney differences in drug concentration were frequently small, but for some drugs (experimental anticonvulsants and doxorubicin; Van Asperen *et al*, 1999; Cox *et al*, 2002) such differences have been substantial. The exact mechanism(s) for this inconsistency between study results is unclear, but it suggests that the tissue distribution of drugs transported by P-gp in the two animal groups is highly dependent upon the specific substrate involved. Nevertheless, there is consistency in our results that statistically significant differences in drug concentrations were found in tissues normally containing P-gp (brain, liver, kidney), while no differences in concentrations were found in those tissues devoid of P-glycoprotein (plasma, spleen, and penis).

Olanzapine is one of the most widely used antipsychotic agents that has been marketed post clozapine. It is a serotonin/dopamine antagonist that has proven efficacy against both the positive and negative symptoms of schizophrenia, and is widely used in other psychotic disorders (Bhana *et al*, 2001). It has a markedly better side-effect profile than older conventional agents such as haloperidol and chlorpromazine (Beasley *et al*, 1996). However, even with these improvements, not all patients respond adequately to olanzapine and dosage requirements vary widely. Approximately 20–30% of patients with schizophrenia show an inadequate efficacy and 5–10% are intolerant to olanzapine therapy (Bhana *et al*, 2001).

The exact reason for refractory symptoms in olanzapine-treated patients is unknown. However, recent findings of functional polymorphisms of *ABCB1* in humans may provide an insight into one mechanism (Hoffmeyer *et al*, 2000). Single-nucleotide polymorphisms (SNP) in exon 26 (C3435T) and exon 21 (G2677T) have been associated with the functional activity of P-gp. The homozygous T/T genotype mutant alleles were associated with more than two-fold lower intestinal P-glycoprotein expression levels and higher bioavailability of digoxin, a prototype P-gp probe (Kurata *et al*, 2002; Nakamura *et al*, 2002). High frequencies of the mutant alleles are present in the population (19% for the T/T 3435 allele and 28% for the T/T2677 allele; Lin and Yamazaki, 2003). These functional SNPs of P-gp in humans may represent a major source of interindividual variability in the therapeutic efficacy and toxicity of P-gp substrates. This effect may be even more remarkable for psychoactive drugs, which need to penetrate through the P-gp residing in the blood–brain barrier to exert their desirable pharmacological effects. Quite recently, Siddiqui *et al* (2003) reported that the *ABCB1* C3435T polymorphism was associated with drug responses of epilepsy. Patients with drug-resistant epilepsy were more likely to have the CC genotype at *ABCB1* 3435 than the TT genotype compared with patients with drug-responsive epilepsy. In addition, the homozygosity for 3435T alleles of *ABCB1* has been suggested as a risk factor for the occurrence of nortriptyline-induced postural hypotension, a CNS side effect related to the protective role of P-gp in the brain penetration of nortriptyline (Roberts *et al*, 2002). Accordingly, it is conceivable that patients who have a high P-gp expression in their blood–brain barrier may have low brain concentrations of olanzapine contributing to therapeutic resistance or failure, and patients who have a low P-gp expression in their blood–brain barrier may have a relatively high brain olanzapine concentration, resulting in adverse events, toxicity, or beneficial therapeutic effects with lower than usual daily drug doses.

Previous studies have indicated that the modulation of P-gp-mediated drug transport can dramatically increase or decrease drugs' penetration into the CNS and affect their therapeutic efficacy (Letrent *et al*, 1999; Aquilante *et al*, 2000). For example, the analgesic effect of morphine in rats was enhanced through the use of a specific P-gp inhibitor (Letrent *et al*, 1999). Accordingly, the current experimental findings provide a rationale for the potential use of P-gp modulators with olanzapine or other P-gp substrates, since a reversal of the functional activity of P-gp may result in improved therapeutic efficacy by increasing CNS drug concentration. However, the inhibition of P-gp activity in humans would likely increase both plasma and brain concentrations of olanzapine and other orally administered substrates, since P-gp is also present in the gastrointestinal tract (Fricker and Miller, 2002; Verstuyft *et al*, 2003; Becquemont *et al*, 2001; Hennessy *et al*, 2002). This is not necessarily a deterrent for the use of safe P-gp inhibitors. By artificially increasing the brain:plasma drug concentration ratio through the inhibition of P-gp, smaller oral substrate doses could be used resulting in lower plasma drug concentration, while maintaining an equivalent or higher concentration in the CNS. Adverse events related to systemic drug exposure should be decreased as a result of

using lower oral doses while promoting increased efficacy at the site of action as a result of increased brain drug concentration. Such an approach is currently being investigated to improve the therapeutic efficacy of the protease inhibitors (Choo *et al*, 2000). A requisite step to applying this approach in neuropsychopharmacology is the identification of relevant P-gp substrates.

By measuring P-gp ATPase activity, we previously reported that the antipsychotics have various degrees of binding affinity with P-gp *in vitro*, with rank order of affinity (by measuring $V_{\max}/K_m$) as: quetiapine (1.7) > risperidone (1.4) > olanzapine (0.8) > chlorpromazine (0.7) > haloperidol (0.3) = clozapine (0.3) (Boulton *et al*, 2002). Accordingly, olanzapine was identified as an intermediate affinity substrate among the antipsychotics (Boulton *et al*, 2002). Consistent with the present findings, a previous report using monolayer efflux in multidrug resistance-transfected cells identified that cellular uptake of risperidone is mediated by P-gp (Mahar Doan *et al*, 2002). In another study, the conventional antipsychotic chlorpromazine has been shown to be actively transported by P-gp in cancer-derived cells (Syed *et al*, 1996). Unfortunately, binding studies and *in vitro* transport measurements in monolayer cell systems under-represent the complexity of the *in vivo* situation. Inconsistencies have been noted (Polli *et al*, 2001). This is the first report using an *in vivo* model to identify that the brain entry of olanzapine is limited by P-gp. Unfortunately, no chemical, clinical, or pharmacological properties appear to allow accurate predictions within or between drug classifications of those compounds that are transported by P-gp. Even the widely used *in vitro* methods to evaluate transport have produced conflicting findings with approximately half of the studied compounds displaying significant variation in P-gp activity dependent on the specific assay used (Adachi *et al*, 2001; Yamazaki *et al*, 2001). The single and double knockout mouse model appears to produce the most unequivocal results (Schinkel *et al*, 1996).

In conclusion, the present results showed that the expression of P-gp in the blood-brain barrier significantly limits the penetration of olanzapine into the CNS. Functional polymorphisms of P-gp in humans may represent an unrecognized source of interindividual variability in the therapeutic efficacy of olanzapine. The recognition of brain entry of olanzapine is limited by P-gp offering intriguing possibilities for manipulating its therapeutic utility through the use of adjunctive P-gp modulators.

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