In Vitro Substrate Identification Studies for P-glycoprotein-Mediated Transport: Species Difference and Predictability of in Vivo Results

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ABSTRACT

Two different cellular assay models were assessed as in vitro systems for P-glycoprotein (P-gp) substrate identification: cellular accumulation studies with KB-V1, a human MDR1 P-gp-overexpressing multidrug-resistant human epidermoid carcinoma cell line; and transcellular transport studies with L-MDR1 (or L-mdr1a), a human MDR1 (or mouse mdr1a)-transfected porcine renal epithelial cell line. The in vitro-in vivo correlation for P-gp-mediated transport activity was also examined by comparing in vitro data obtained from L-mdr1a cell studies and in vivo data from mdr1a (−/−)/(+/+) CF-1 mice studies for several compounds. The results are summarized as follows: 1) two in vitro assay systems routinely identified the substrate for human MDR1 P-gp-mediated transport with similar quantitative results; 2) in vitro studies with L-MDR1 and L-mdr1a cells demonstrated that the P-gp substrate susceptibility is different between human and mouse for certain compounds (species difference); and 3) in vivo brain concentration ratios of mdr1a (−/−) to (+/+) CF-1 mice, either at a certain time point or up to 60 min, correlated well with the in vitro transcellular transport ratios from L-mdr1a cells ($r^2 = 0.968$ and 0.926, respectively). This indicates that, at least in mice, the in vitro data are valid predictors of the in vivo contribution of P-gp: the contribution of P-gp to the distribution of the compound to the brain up to 60 min post i.v. administration. These results provide a rationale for predicting in vivo relevance of P-gp in human from in vitro data using human P-gp-expressing cells.

Drug-transporting P-glycoproteins (P-gp), which play a major role in multidrug resistance in cancer cells, are known as MDR1 P-gp in human and mdr1a (and mdr1b) P-gp in mice. These P-gps can exclude/extrude a wide range of structurally diverse anticancer drugs from the cell (Ambudkar et al., 1999). P-gp is also known to govern pharmacokinetics/tissue distribution of many drugs, e.g., cyclosporin A, digoxin, vincristine, etc. (Schinkel et al., 1994, 1995, 1997; Van Asperen et al., 1996).

Recently, it has been emphasized that drug interaction at the membrane transport level, in particular, interaction with P-gp, should be considered to avoid unexpected changes in pharmacokinetics of the drugs as well as undesirable clinical outcomes (Fromm et al., 1998; Greiner et al., 1999; Yu, 1999; Kim, 2000). To evaluate the contribution of P-gp to the tissue distribution of drugs, gene knockout mice [mdr1a (−/−), mdr1b (−/−), and double knockout mice [mdr1a/1b (−/−)] have been used (Schinkel, 1999). Recently, a subpopulation of CF-1 mice [mdr1a (−/−)], deficient in mdr1a-P-gp expression, was characterized at Merck Research Laboratories (Lankas et al., 1997; Umbenhauer et al., 1997). This mutant mouse subpopulation is an alternative tool for examining the role of mdr1a and likely more suitable than gene knockout mice; the expression levels of other gene products in the mdr

ABBREVIATIONS: P-gp, P-glycoprotein; MDR, multidrug resistance; DG, digoxin; VBL, vincristine; FCS, fetal calf serum; B-to-A, basal-to-apical; A-to-B, apical-to-basal; AUC, area under the curve; KP,brain, brain-to-plasma concentration ratio; LC-MS, liquid chromatography-mass spectrometry.
family (mdr1b and mdr2) are not altered in mdr1a (−/−) CF-1 mice (Umbenhauer et al., 1997), whereas an increased expression of mdr1b in kidney and liver has been reported in mdr1a gene knockout mice (Schinkel et al., 1999). Thus far, extensive studies with the gene knockout mice and mutant mice have revealed that mdr1a P-gp is a major contributor to the maintenance of the blood-brain barrier (Kwei et al., 1999; Schinkel, 1999).

However, in vivo studies with these mice might not be very practical for a rapid and large-scale screening of compounds. Moreover, in vivo studies with mice cannot provide any definitive information on human P-gp. Thus, in vitro assays with human P-gp-overexpressing cell lines and human P-gp-transfected cell lines are necessary to evaluate the potential of compounds to act as P-gp substrates/inhibitors.

The purposes of the present studies were to 1) determine whether two different in vitro assay systems for human MDR1 P-gp could consistently identify human P-gp substrates, 2) identify the possible species difference in MDR1 (mdr1a) P-gp substrate susceptibility between human and mouse, and 3) examine the in vivo-in vitro correlation in mdr1a P-gp-mediated transport. The two different in vitro assay systems used in this study were 1) KB-V1, a human MDR1 P-gp-overexpressing multidrug-resistant human epidermoid carcinoma cell line, and its parental cell line KB-3-1 (Akiyama et al., 1985; Shen et al., 1986); and 2) L-MDR1 (L-mdr1a), a human MDR1 (or mouse mdr1a)-transfected porcine renal epithelial cell line, and its parental cell line LLC-PK1 (Schinkel et al., 1995). By comparing the results obtained from cellular accumulation studies with KB cells and transcellular transport studies with P-gp-transfected cells, we tried to determine whether the two different in vitro assays could identify human MDR1 P-gp substrate with similar quantitative results. By comparing the results between L-MDR1 and L-mdr1a, we examined the possible species difference in P-gp substrate susceptibility in vitro between human and mouse. The test compounds used for in vitro studies were indinavir and ritonavir (human immunodeficiency virus protease inhibitors); the three benzodiazepine derivatives diazepam, 3R(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-4-benzodiazepin-3-yl)-N′-[(3-methylphenyl)-urea (cholocystokinin₂ receptor antagonist, Chang et al., 1989), and N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-1H-indole-2-carboxamide (cholocystokinin₅ receptor antagonist, Chang et al., 1986); MK-0826 (a carbapenem antibiotic, Kohler et al., 1999); MK-0991 (an antifungal agent, Bartizal et al., 1997); and four miscellaneous Merck compounds (N-tertary-butyly-4-(benzofuran-2-ylmethyl)-1-[2(S)-hydroxy-4(R)-2(R)-hydroxy-indan-1(S)-yl]-[14C]carbamoy]-5-phenyl-pentyl-piperazine-2(1H)-carboxamide (compound A, 29.97 μCi/μmol); 4-[5-[(3-chlorophenyl)-3-oxopiperazin-1-ylmethyl]-imidazol-1-ylmethyl]-nitrile-14C] (compound B, 26.67 μCi/μmol); N-6-amino-2-methylpyridin-3-ylmethyl-2-(6-methyl-2-oxo-3-phenethylamino)-2H-pyrazin-[5,1-C]-1-y1acetamide dihydrochloride monohydrate (compound C, 15.04 μCi/μmol); and N-4-(1-methyl-2(4-piperidinyl)-4-[3-(trifluoromethyl)phenyl]-1H-1,2-[2-14C]imidazol-5-yl)-2-pyrindinyl-N-(1S)-1-phenylethylamine perchloric acid salt (compound D, 53.00 μCi/μmol) were synthesized at Merck Research Laboratories. Radiochemical purity of the compounds used in the present study was greater than 94.2%. Unlabeled VBL, DG, progestrone, diazepam, cyclosporin A, and vincristine were purchased from Sigma Chemical Co. (St. Louis, MO). Unlabeled indinavir and compounds A-D were synthesized at Merck Research Laboratories (Rahway, NJ, and West Point, PA). Unlabeled compound X and compound Y were synthesized at the Development Laboratories (Hoddesdon, UK). All other reagents were analytical grade.

Cell Lines and Cell Cultures

KB-V1 and KB-3-1. The drug-sensitive human epidermoid carcinoma cell line KB-3-1 and its VBL-selected MDR variant KB-V1 were kindly provided by Dr. Michael M. Gottseman (National Cancer Institute, Bethesda, MD) and used under license agreement. Both cell lines were maintained in Dulbecco’s modified Eagle’s medium (high glucose; Life Technologies, Gaithersburg, MD) containing 10% (v/v) fetal calf serum (FCS; BioWhittaker, Walkersville, MD), 50 units/ml penicillin (Life Technologies), 50 μg/ml streptomycin (Life Technologies), and 2 mM l-glutamine (Life Technologies) at 37°C in a humidified atmosphere of 5% CO₂, 95% air. KB-V1 cells were grown in the continuous presence of VBL (1 μg/ml) (Shen et al., 1986). Confluent monolayers were subcultured every 7 days by treatment with trypsin-EDTA (0.025% trypsin, 1 mM EDTA-4Na; Life Technologies).

L-MDR1, L-mdr1a, and LLC-PK1. Human MDR1 transfectants L-MDR1, mouse mdr1a transfectants L-mdr1a, and their parental cell line LLC-PK1 pig kidney epithelial cells were kindly provided by Dr. Alfred H. Schinkel (The Netherlands Cancer Institute, Amsterdam) and used under license agreement. Cells were cultured in medium 199 (Life Technologies) supplemented with 2 mM l-glutamine, penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% FCS (v/v) (Life Technologies) (Schinkel et al., 1995). For L-MDR1 and L-mdr1a, cells were maintained in the continuous presence of vincristine (640 nM) (Schinkel et al., 1995). Confluent monolayers were subcultured every 3 to 4 days by treatment with trypsin-EDTA. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂, 95% air.

Drug Accumulation/Transport Studies

To test whether certain compounds are P-gp substrates, we compared the cellular accumulation in KB-3-1 and -V1, as well as the data in predicting the relative contribution of P-gp to the distribution of the compound to the brain.

Materials and Methods

Chemicals

[3H]-Digoxin (DG; 19.0 Ci/mmol), [2,6,7,16,17-N-3H]progesterone (138.0 Ci/mmol), (R)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-4-benzodiazepin-3-yl)-N’-[3-methylphenyl-2,4,6-3H]urea (compound X, 75.6 Ci/mmol), (−)-(2,3-dihydro-1H-4-benzodiazepin-3-yl)-1H-indole-2-carboxamide (compound Y, 77.4 Ci/mmol), [methylnitric acid perchloric acid salt (compound D, 75.6 Ci/mmol), and [carboxoyle]-4-[3-(trifluoromethyl)phenyl]-1H-1,4-benzodiazepin-3-yl)-1H-indole-2-carboxamide (chosen as compound B, 26.67 μCi/μmol); [N-6-amino-2-methylpyridin-3-ylmethyl-2-(6-methyl-2-oxo-3-phenethylamino)phenyl]-1H-pyrazin-[5,1-C]-1-y1acetamide dihydrochloride monohydrate (chosen as compound C, 15.04 μCi/μmol); and N-4-(1-methyl-2(4-piperidinyl)-4-[3-(trifluoromethyl)phenyl]-1H-pyrazin-[5,1-C]-1-y1acetamide dihydrochloride monohydrate (chosen as compound D, 53.00 μCi/μmol) were synthesized at Merck Research Laboratories. Radiochemical purity of the compounds used in the present study was greater than 94.2%. Unlabeled VBL, DG, progestrone, diazepam, cyclosporin A, and vincristine were purchased from Sigma Chemical Co. (St. Louis, MO). Unlabeled indinavir and compounds A-D were synthesized at Merck Research Laboratories (Rahway, NJ, and West Point, PA). Unlabeled compound X and compound Y were synthesized at the Development Laboratories (Hoddesdon, UK). All other reagents were analytical grade.
unidirectional transeptal transport across the monolayers of L-MDR1, L-mdr1a, and LLC-PK1. To quantitatively compare a number of compounds for their susceptibility as a transport substrate of P-gp, we fixed the concentrations of all the compounds at 1 µM with the exception of MK-0991 and MK-0826 (10 µM, because of their low permeability across membranes and low specific activities). A serum/protein-free medium was used throughout the experiments to avoid any differences in protein binding among the compounds we tested. Experiments were conducted independently at least three times.

**Cellular Accumulation in KB Cells**

Measurement of the accumulation of test compounds in KB-3-1 and -V1 cell monolayers was carried out as described (Fojo et al., 1985) with minor modifications. In preparing the cell monolayers, 5 ml of a cell suspension containing 1 × 10^6 cells in Dulbecco’s modified Eagle’s medium (low glucose without phenol red; Life Technologies) with 10% FCS was plated in four wells of six-well tissue culture dishes (Nunc A/S, Roskilde, Denmark) the day before use. Blank wells were prepared simultaneously using the same media alone to assess background. After an overnight incubation at 37°C, cell monolayers were washed once with serum-free Hank’s balanced salt solution (Life Technologies) with 10 mM Hepes (pH 7.4). This medium is called “transport medium”. Three milliliters of transport medium containing test compound was then added to each well. Incubation in the drug-containing medium was carried out for 2 h, after which the reaction was terminated by removing the medium and rinsing twice with 5 ml of cold phosphate-buffered saline. Cells were lysed in 1 ml of 1 N NaOH at room temperature and transferred to scintillation vials containing 0.5 ml of 2 N HCl and 15 ml of scintillation cocktail (Ready Safe, Beckman, CA). The total radioactivity was measured by liquid scintillation counter (LS6500; Beckman, Fullerton, CA). Cellular accumulation was presented as mean ± S.D. after subtracting background.

**Transepithelial Transport across L-MDR1, L-mdr1a, and LLC-PK1 Cells**

Transepithelial transport study was carried out as described (Kim et al., 1998) with minor modifications. L-MDR1, L-mdr1a, and LLC-PK1 cells were plated at a density of 4 × 10^5 cells/12-mm well on porous (3.0-µm) polycarbonate membrane filters (Transwell; Costar Corp., Cambridge, MA). Cells were supplemented with fresh media on the second day and used for the transport studies on the fourth day after plating. Transepithelial resistance was measured in each well using a Millicell ohmmeter (model ERS; Millipore Corp., Bedford, MA); wells registering a resistance of 300 MΩ were used in this study. After an i.v. injection into the tail vein, the mice were sacrificed at 15, 30, and 60 min, and blood and brain were collected for analyses. Plasma samples were separated immediately by centrifugation. Brain samples were homogenized with 4 volumes of water. Samples were kept frozen at −20°C until analyzed.

**High Performance Liquid Chromatography Analysis of Indinavir, Ritonavir, Vinblastine, and Compounds A and D**

Plasma samples were extracted with 10 volumes of acetonitrile, and brain homogenates were added to 2 volumes of acetonitrile containing respective internal standards. After being vortex mixed and centrifuged, the supernatant was evaporated to dryness under nitrogen. The residue was reconstituted in high performance liquid chromatography mobile phase (75:25 acetonitrile and H_2O mixture containing 0.01% trifluoroacetic acid), and an aliquot was injected onto Vydac protein peptide C18 column (4.6 × 250 mm, 5 µm), with the mobile phase delivered isocratically at a flow rate of 1.0 ml/min. The eluate was monitored at 220 nm.

**Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of Compound X and Diazepam**

Plasma samples were extracted with 10 volumes of acetonitrile, and brain samples were extracted twice with 8 volumes of acetonitrile. All samples were dried under nitrogen, and the residue was reconstituted in 30% acetonitrile. Quantification was achieved by LC-MS, a PerkinElmer Sciex API 150EX mass spectrometer (Foster City, Canada). Chromatography was conducted using Betasil C18 column (3.0 × 50 mm, 5 µm; Keystone Scientific, Inc., Bellefonte, PA), with a mobile phase of 45:55 mixture of 5 mM ammonium acetate (pH 4.2) and acetonitrile (flow rate 0.6 ml/min). The nebulizer probe was set at 425°C. Detection of compound X and diazepam was carried out by selected ion monitoring, whereby the quadrupole transmitted the [M + H]^+ ion of compound X at m/z 399.2 and diazepam at m/z 285.2, respectively.

**Results**

**Cellular Accumulation in KB Cells.** The P-gp marker substrates showed greater cellular accumulation in KB-3-1 than in KB-V1 by factors of 4 for DG and 40 for VBL, whereas progesterone showed no difference (Fig. 1). Among the compounds tested, compounds A–D, indinavir, and ritonavir exhibited greater accumulation in KB-3-1 than in KB-V1 by more than a factor of 4 (Fig. 2). Cellular accumulations in KB-V1 cells were restored greatly in the presence of cyclosporin A (10 µM) for these six compounds (data not shown). In contrast, compound X demonstrated a relatively small inhibition of cyclosporin A (10 µM) for these six compounds (data not shown). In contrast, compound X demonstrated a relatively small increase (1.6–2-fold) in its accumulation in KB-3-1 in comparison with that in KB-V1 (Fig. 3). For compound Y, diazepam, MK-0826, and MK-0991, no significant difference was observed in their cellular accumulation in KB-3-1 cells in comparison with that in KB-V1 cells (Fig. 3). These results suggest that compounds A–D, indinavir, and ritonavir are substrates for human P-gp. Compound X appears to be a poor
substrate, whereas compound Y, diazepam, MK-0826, and MK-0991 are not likely to be substrates of human P-gp.

**Transcellular Transport across L-MDR1, L-mdrlα, and LLC-PK1.** The two marker substrates of P-gp, DG and VBL, exhibited basal-to-apical (B-to-A) directed vectorial transport in L-MDR1 and L-mdrlα cells (Fig. 4). In the parental LLC-PK1 cells, smaller but significant polarized transport was also observed. This is most likely due to the directional transport by the endogenous porcine P-gp in LLC-PK1 cells (Schinkel et al., 1995; Kim et al., 1998). Similar marked enhancement in B-to-A directed transport in LLC-PK1 has been reported for VBL by Smit et al. (1998). For the negative marker progesterone, no polarized transport was observed in these three cell lines (Fig. 4). These findings confirm that transcellular transport experiments using these three cell lines can be used as a powerful tool for P-gp substrate identification studies.

Merck compounds A–D showed typical transport characteristics of both human MDR1 and mouse mdr1α P-gp substrates (Fig. 5); that is, B-to-A directed vectorial transport was observed in both L-MDR1 and L-mdrlα, but no significant net flux was observed in LLC-PK1. There was no significant difference in the percentage of net transport amount between L-MDR1 and L-mdrlα for these four compounds. In contrast, no significant net flux was observed in the three cell lines for diazepam, MK-0826, and MK-0991 (Fig. 6).
Compound X was noticeably transported by the L-MDR1 cell line, but more substantially by the L-mdr1a cells (Fig. 7). Regarding the highly concentrated B-to-A directed transport observed for compound X in L-mdr1a, similar findings have also been reported by Schinkel et al. (1996). They also found that P-gp can accumulate substrates against a macroscopic concentration gradient, since at the end of the experiment (4 h) 4-fold more (80%) of the compounds was present on the apical side of the cell monolayer than on the basal side (20%), where the drugs were originally added.

Compound Y was not detectably transported by either L-MDR1 or LLC-PK1, whereas there was only a low but significant level of transport across L-mdr1a in a B-to-A direction (Fig. 7). For compound X and compound Y, vectorial transport in P-gp-transfected cells was completely inhibited in the presence of 100 \( \mu \text{M} \) verapamil (data not shown).

Human immunodeficiency virus protease inhibitors indinavir and ritonavir demonstrated B-to-A directed vectorial transport in both L-MDR1 and L-mdr1a cell lines (Fig. 8). In addition, the B-to-A directed vectorial transport was observed also in the parental cells for these two compounds. The present results for indinavir from L-MDR1 and LLC-PK1 were consistent with the previous report by Kim et al. (1998). Cyclosporin A (10 \( \mu \text{M} \)) inhibited vectorial transport of indinavir and ritonavir in L-MDR1, L-mdr1a, and LLC-PK1 (data not shown).

Species Difference in the Susceptibility of P-gp-Mediated Transport between Human MDR1 and Mouse mdr1a. Figure 9 illustrates a summary of the transcellular transport data obtained with the two P-gp transfectants L-MDR1 and L-mdr1a. There was a fairly good 1:1 correlation in the transcellular transport ratio [B-to-A versus apical-to-basal (A-to-B) at 3 h] between L-MDR1 and L-mdr1a, indicating that the susceptibility of P-gp between human MDR1 and mouse mdr1a was very consistent for most of the compounds. However, compound X exhibited a substantial (~3-fold) difference in its transported amount ratios between human and mouse (the values were higher for mdr1a than for L-MDR1). Furthermore, indinavir, DG, and compound C showed a higher (2–3-fold) transport ratio in L-MDR1 than in L-mdr1a. Indeed, in any of the experiments (n = 5–8), not only B-to-A-to-B ratios but also the permeability coefficients for L-MDR1 were always higher than those for L-mdr1a for indinavir, and vice versa for compound X. These results clearly demonstrate the existence of species difference in the susceptibility of P-gp-mediated transport for certain compounds between human and mouse.

Validation of in Vitro Assay Models for MDR1 P-gp Substrate Identification. Table 1 summarizes the in vitro results of MDR1 P-gp substrate identification obtained with two different assay models (cellular accumulation study with KB cells and transcellular transport study with P-gp-transfected LLC-PK1 cells). Both assay models routinely identified P-gp substrate with similar quantitative results, with a few exceptions. VBL exhibited an extremely high ratio in its cellular accumulation between KB-3-1 and V1 cells, which can likely be explained by the fact that KB-V1 was derived from the KB-3-1 through selection in increasing concentrations of VBL (Shen et al., 1986). Also, it would be conceivable that KB-V1 has another genetic alteration during selection, which causes such an exceedingly low accumulation of VBL.

In Vivo Study with mdr1a (+/+ and −/−) CF-1 Mouse Subpopulations. Seven compounds from those examined in vitro were administered i.v. to mdr1a (+/+ and
(−/−) CF-1 mice subpopulations, and plasma and brain concentrations of the unchanged (parent) compounds were measured at 15, 30, and 60 min time points. For all the compounds tested, there were no significant differences in the plasma concentration between mdr1a (1/1) and (2/2) subpopulations, irrespective of the time point (Table 2). However, brain concentrations (or AUCbrain values up to 60 min) showed a variety of differences between wild-type and mutant mice, depending on the compound. For example, compounds A and D displayed a 13- and 10-fold difference, respectively, whereas diazepam displayed no significant difference in its AUCbrain (0–60 min) values of mdr1a (+/+ ) and (−/−) CF-1 mice.

Comparison of in Vitro and in Vivo Results of Mouse mdr1a P-gp Substrate Identification: In Vitro-in Vivo Correlation. A plot of the in vivo Kp, brain ratio for mdr1a (−/−) and (+/+) CF-1 mice after i.v. administration versus in vitro B-to-A/A-to-B transport ratio in L-mdr1a cells showed a strong correlation ($r^2 = 0.968$, $p < 0.001$) for all the seven compounds (Fig. 10a). A positive correlation ($r^2 = 0.926$, $p <
Fig. 5. Transcellular transport of compounds A–D across L-MDR1, L-mdr1a, and LLC-PK1 cell monolayers. Concentration of each compound is 1 μM. Data shown are mean ± S.D.
0.01) is also observed if AUCbrain (0–60 min) is plotted as an ordinate for mouse in vitro-in vivo comparison (Fig. 10b). However, results from L-MDR1 and mouse in vivo did not correlate as strong (Fig. 11).

**Discussion**

The identification of compounds as substrates of P-gp-mediated transport is currently one of the key issues in the drug discovery and development process, in particular, for central nervous system-acting compounds. A quantitative characterization of drug candidates with regard to P-gp-mediated transport is crucial in predicting drug disposition and drug interactions. Presently, in vitro assay models with either P-gp-overexpressing cells or P-gp-transfected cells are most commonly used to assess the potential for a compound to act as a P-gp substrate (Kim, 2000). There are some limitations, however, in these two assays to obtain a definitive
Fig. 7. Transcellular transport of compound X and compound Y across L-MDR1, L-mdr1a, and LLC-PK1 cell monolayers. Concentration of each compound is 1 μM. Data shown are mean ± S.D.

Fig. 8. Transcellular transport of indinavir and ritonavir across L-MDR1, L-mdr1a, and LLC-PK1 cell monolayers. Concentration of each compound is 1 μM. Data shown are mean ± S.D.
answer. For instance, most of the P-gp substrates are lipophilic; thus, cellular accumulation results are not always reliable due to extensive nonspecific binding to surface membranes and intracellular components. Regarding transcellular transport studies, it is difficult to determine whether a compound is a P-gp substrate when it exhibits non-negligible B-to-A directed transport in LLC-PK1, as we observed for indinavir and ritonavir in this study (Fig. 8), cellular accumulation data are especially valuable since they can reinforce the results derived from transcellular transport studies alone. For in vitro P-gp substrate identification studies, it would be prudent to consider the results from at least two different assay models for any compounds of interest if we find the vectorial transport in the parental cells.

Our second objective was to examine species difference in the P-gp susceptibility of compounds between MDR1- and mdr1a-encoded P-gps. Currently, gene knockout mice and mdr1a-deficient CF-1 mice are the only practical models available for investigating the in vivo contribution of P-gp to the tissue distribution of compounds. If species difference does exist regarding P-gp susceptibility, it is inappropriate to predict in vivo relevance of P-gp in humans simply from that obtained from mice studies. Indeed, we have observed the difference in the susceptibility of P-gp for several compounds between human and mouse based on the in vitro results from P-gp transfectants, L-MDR1 and L-mdr1a (Fig. 9). The species difference in P-gp susceptibility we found was not due to the difference in the expression levels of respective P-gps in both cell lines because Western blotting using C219 monoclonal antibody showed similar amounts of expressed P-gp in these two cell lines (data not shown). Furthermore, we conducted the transcellular transport study with several compounds at a time, some of which demonstrated a higher B-to-A/A-to-B ratio in L-MDR1 than in L-mdr1a (e.g., indinavir) and vice versa (e.g., compound X).2 Regarding a simple comparison of B-to-A/A-to-B transport amount ratios obtained from two different cell lines (L-MDR1 and L-mdr1a) to discuss species difference in the susceptibility of P-gp, we must be aware of the caveat: these data could be affected by at least two other unknowns. One is a difference in total protein expression, which may affect the passive permeability properties of the membrane, and the other is a difference in the density of functional transporter in both cell lines. The former is likely not the case since 1) total protein concentration in the well was the same between L-MDR1 and L-mdr1a; and 2) both paracellular flux (assessed usingulin) and

2 We have confirmed that several compounds show mouse > human (one compound exhibited ~10-fold difference in the ratio between L-mdr1a versus L-MDR1) and/or human > mouse, and we have found that the results were consistent irrespective of the B-to-A/A-to-B ratio or the absolute difference in directional transport. Due to the restriction of the disclosure of their chemical structures, we cannot include the data for these compounds in this manuscript.
indinavir, ritonavir, diazepam, compound X, vinblastine, and compounds A and D after intravenous injection of each compound in the pooled samples of three mice were measured in µg/g of brain. Kp,brain represents the brain-to-plasma concentration ratio. AUCbrain (0–60 min) was calculated using trapezoidal rule and is shown in µg · min · ml−1.

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time (min)</th>
<th>Plasma (µg/ml)</th>
<th>Brain (µg/ml)</th>
<th>Kp,brain</th>
<th>AUCbrain (0–60 min) (µg · min · ml−1)</th>
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</thead>
<tbody>
<tr>
<td>Indinavir (10 mg/kg)</td>
<td>15 min</td>
<td>2.99 ± 0.15</td>
<td>3.28 ± 0.60</td>
<td>0.91</td>
<td>33.23</td>
</tr>
<tr>
<td>Brain</td>
<td>0.74</td>
<td>0.51</td>
<td>1.45</td>
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<tr>
<td>Kp,brain</td>
<td>0.25</td>
<td>0.16</td>
<td>1.59</td>
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<tr>
<td>30 min</td>
<td>0.78 ± 0.40</td>
<td>0.48 ± 0.15</td>
<td>2.50</td>
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<tr>
<td>Brain</td>
<td>0.75</td>
<td>0.30</td>
<td>2.05</td>
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<tr>
<td>Kp,brain</td>
<td>0.96</td>
<td>0.63</td>
<td>1.54</td>
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<tr>
<td>60 min</td>
<td>0.20 ± 0.35</td>
<td>0.09 ± 0.08</td>
<td>2.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>0.35</td>
<td>0.20</td>
<td>1.75</td>
<td></td>
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</tr>
<tr>
<td>Kp,brain</td>
<td>1.75</td>
<td>2.22</td>
<td>0.79</td>
<td></td>
<td></td>
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<tr>
<td>AUChbrain (0–60 min)</td>
<td>33.23</td>
<td>17.40</td>
<td>1.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ritonavir (10 mg/kg)</td>
<td>15 min</td>
<td>6.69 ± 1.32</td>
<td>6.27 ± 1.38</td>
<td>1.07</td>
<td>60 min</td>
</tr>
<tr>
<td>Plasma</td>
<td>1.62</td>
<td>0.24</td>
<td>6.68</td>
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</tr>
<tr>
<td>Kp,brain</td>
<td>0.24</td>
<td>0.04</td>
<td>6.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>3.29 ± 0.55</td>
<td>3.88 ± 0.19</td>
<td>0.85</td>
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<tr>
<td>Brain</td>
<td>1.84</td>
<td>0.24</td>
<td>7.74</td>
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<tr>
<td>Kp,brain</td>
<td>0.56</td>
<td>0.06</td>
<td>9.11</td>
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</tr>
<tr>
<td>60 min</td>
<td>0.77 ± 0.13</td>
<td>1.82 ± 0.13</td>
<td>0.42</td>
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<tr>
<td>Brain</td>
<td>1.77</td>
<td>0.30</td>
<td>5.86</td>
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<tr>
<td>Kp,brain</td>
<td>2.31</td>
<td>0.17</td>
<td>13.97</td>
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<td></td>
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<tr>
<td>AUChbrain (0–60 min)</td>
<td>92.20</td>
<td>13.50</td>
<td>6.83</td>
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<td></td>
</tr>
<tr>
<td>Diazepam (2 mg/kg)</td>
<td>15 min</td>
<td>0.27 ± 0.06</td>
<td>0.22 ± 0.02</td>
<td>1.18</td>
<td>60 min</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.26</td>
<td>0.34</td>
<td>0.96</td>
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<tr>
<td>Kp,brain</td>
<td>0.99</td>
<td>1.52</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>0.10 ± 0.02</td>
<td>0.11 ± 0.10</td>
<td>0.95</td>
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<td></td>
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<tr>
<td>Brain</td>
<td>0.10</td>
<td>0.10</td>
<td>0.93</td>
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<tr>
<td>Kp,brain</td>
<td>0.95</td>
<td>0.98</td>
<td>0.98</td>
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<tr>
<td>60 min</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.03</td>
<td>1.08</td>
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<td></td>
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<tr>
<td>Brain</td>
<td>0.04</td>
<td>0.04</td>
<td>1.13</td>
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<tr>
<td>Kp,brain</td>
<td>1.08</td>
<td>1.05</td>
<td>1.04</td>
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<tr>
<td>AUChbrain (0–60 min)</td>
<td>6.71</td>
<td>7.97</td>
<td>0.84</td>
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<tr>
<td>Compound X (2 mg/kg)</td>
<td>15 min</td>
<td>1.00 ± 0.27</td>
<td>0.80 ± 0.09</td>
<td>1.25</td>
<td>15 min</td>
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<tr>
<td>Plasma</td>
<td>0.46</td>
<td>0.09</td>
<td>5.14</td>
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<tr>
<td>Kp,brain</td>
<td>0.46</td>
<td>0.11</td>
<td>4.12</td>
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<tr>
<td>30 min</td>
<td>0.53 ± 0.03</td>
<td>0.38 ± 0.13</td>
<td>1.40</td>
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<tr>
<td>Brain</td>
<td>0.33</td>
<td>0.04</td>
<td>7.79</td>
<td></td>
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</tr>
<tr>
<td>Kp,brain</td>
<td>0.62</td>
<td>0.11</td>
<td>5.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td>0.12 ± 0.05</td>
<td>0.14 ± 0.08</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>0.08</td>
<td>0.02</td>
<td>3.86</td>
<td></td>
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<tr>
<td>Kp,brain</td>
<td>0.67</td>
<td>0.15</td>
<td>4.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUChbrain (0–60 min)</td>
<td>15.52</td>
<td>2.62</td>
<td>5.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinblastine (6 mg/kg)</td>
<td>15 min</td>
<td>0.31 ± 0.14</td>
<td>0.34 ± 0.04</td>
<td>0.91</td>
<td>15 min</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.33</td>
<td>0.06</td>
<td>5.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kp,brain</td>
<td>1.06</td>
<td>0.18</td>
<td>6.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>0.23 ± 0.03</td>
<td>0.29 ± 0.04</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>0.51</td>
<td>0.07</td>
<td>7.29</td>
<td></td>
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</tr>
<tr>
<td>Kp,brain</td>
<td>2.22</td>
<td>0.24</td>
<td>9.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td>0.21 ± 0.01</td>
<td>0.23 ± 0.04</td>
<td>0.88</td>
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</table>

Transcellular transport of diazepam, which were examined simultaneously with indinavir, compounds A and X, showed no significant difference between the two cell lines. The latter does not seem to be the case inasmuch as the species dependence is compound-specific. Although difference in the density of functional P-gp in the two cell lines would affect the relative transport ratios, this should be consistent for all of the P-gp substrates. Therefore, the disparity between indinavir and compound X indicates a species-dependent difference in substrate specificity.

In an attempt to find what causes the difference in the degree of the susceptibility between human and mouse P-gps, we conducted kinetic studies with compound A (which showed no significant difference in the percentage of net transport amount between L-MDR1 and L-mdrla; Figs. 5 and 9) and compound X (which exhibited 3-fold greater net transport in L-mdrla than in L-MDR1; Figs. 7 and 9) simultaneously. As a result, we obtained somewhat similar Vmax value for L-mdrla than for L-MDR1 with no difference in Km for compound X (M. Yamazaki, T. Ohe, W. E. Neway, J. H. Hochman, M. Chiba, and J. H. Lin, unpublished observations). This indicates that the difference in Vmax might cause the species difference in the susceptibility of P-gp for compound X. However, this is only one piece of information; it is unclear that this is the case for all other compounds that exhibit species difference in P-gp-mediated transport. It should also be noted that the resultant Km and Vmax are apparent parameters, not the intrinsic parameters for P-gp itself. Further investigation with more compounds will be required to determine conclusively what causes species difference in P-gp susceptibility.

Investigations on the differences among P-gps encoded by mdr1a and mdr1b genes regarding drug resistance profiles and sensitivity to known modulators have
the brain in humans, and/or 2) a “false-positive” prediction in drug-drug interactions in terms of P-gp-mediated transport in human. The opposite might be true for indinavir. Thus, when predicting the role of P-gp for a compound in human from that in mice, we should consider the possibility of the species difference in P-gp susceptibility, which we have demonstrated in the present study.

To what extent we can predict in vivo results from in vitro findings is a crucial question that continues to be a challenge. As shown in Fig. 10, in vitro transcellular transport ratio with L-mdr1a exhibits a fairly good correlation with in vivo brain concentration ratio between mdr1a (−/−) and (+/+) CF-1 mouse subpopulations. Based on the present results, it is likely that we can predict the in vivo contribution of P-gp to the brain distribution of the novel compound of our interest by using in vitro transport data relative to the P-gp contribution for known compounds having both in vitro and in vivo results.

In the present study, we concentrated on evaluating plasma and brain concentrations of the compounds up to 60 min to measure unchanged (parent) concentrations of each compound. Generally, most of the in vivo data obtained so far using knockout mice and mutant CF-1 mice are assessed at longer time points than those of the present studies. It is unclear for the moment whether the good correlation between in vitro and in vivo results will be evident after longer periods of administration and studies investigating this are in progress.

In conclusion, the present study has revealed the following: 1) two different in vitro experimental systems routinely identified the substrate for human MDR1 P-gp-mediated transport with similar quantitative results; 2) in vitro studies in L-MDR1 and L-mdr1a demonstrated that P-gp substrate susceptibility is different between human and mouse for several compounds; and 3) in vivo brain concentration ratio for mdr1a (−/−) and (+/+) CF-1 mice correlated well with in vitro B-to-A/A-to-B transport ratio in L-mdr1a cells. Thus, in vitro L-mdr1a transcellular transport data are valid predictors of the in vivo contribution of P-gp, the contribution of P-gp to the distribution of the compound to the brain in mice. Consequently, these results provide a rationale for predicting in vivo relevance of P-gp with respect to the distribution of compounds to the brain in human from in vitro data using human P-gp-expressing cells. Since we observed the species difference in the susceptibility in P-gp-mediated transport between human and mouse for certain compounds, it would be prudent to interpret any in vivo data obtained from mice along with in vitro results from both mouse and human P-gp transfectants.

Acknowledgments

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